LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Conjugated Linoleic Acid and Insulin Sensitivity: Insights from Metabolic and Molecular Markers

A thesis submitted to the University of Dublin, Trinity College for the degree of Doctor of Philosophy (Ph.D.)

by

Fiona Moloney

July 2005

Nutrigenomics Research Group,
Institute of Molecular Medicine,
Trinity College

TRINITY COLLEGE
12 MAY 2006
LIBRARY DUBLIN
THESIS
7871

Dedicated to my parents, Joan and Vincent

With Love

Declaration

I, the undersigned submit this thesis to the University of Dublin, Trinity College for examination for the degree of Ph.D. The work herein is entirely my own except where acknowledged and has not been previously submitted for any other degree at this or any other University. This thesis may be made available from the library for consultation or copying.

Fiona Moloney.

Fiona Moloney.

17th Dec 2005.

Date.

Summary

Insulin resistance signifies an impaired biological response to insulin, and usually implies resistance to the effects of insulin on glucose uptake, metabolism, or storage. This defect is commonly observed in type 2 diabetes mellitus (T2DM) and the metabolic syndrome and is often found in association with other cardiovascular disease (CVD) risk factors including obesity, dyslipidaemia, hypertension and atherosclerosis. Altering the composition of fat in the diet can have a profound effect on insulin sensitivity whereby saturated fatty acids attenuate insulin sensitivity, while monounsaturated fatty acids can reverse this effect. The objective of this thesis was to examine the relationship between the *n-6* polyunsaturated fatty acid conjugated linoleic acid (CLA) and insulin sensitivity, and its possible involvement in attenuating other risk factors associated with CVD. Several isoforms of CLA exist with *cis*-9, *trans*-11 CLA (c9, t11-CLA) which is the predominant dietary isoform and *trans*-10, *cis*-12 CLA (t10, c12-CLA) thought to have most biological activity.

In chapter three the effect of CLA supplementation on risk factors associated with CVD was investigated. Subjects with stable, diet-controlled T2DM were randomly allocated to receive 3.0g/day CLA (50:50 blend of c9, t11-CLA and t10, c12-CLA) or control (blend of fatty acids) for 8 wk. Fasting glucose concentrations were significantly increased ($P<0.05$) and insulin sensitivity was reduced as measured by indices derived from an oral glucose tolerance test namely homeostasis model assessment, oral glucose insulin sensitivity, and insulin sensitivity index (composite) ($P=0.05$). In contrast, positive effects were observed with respect to lipoprotein metabolism. Total HDL-cholesterol concentrations increased by 8% ($P<0.05$), which was due to a significant increase in HDL_2-cholesterol concentrations ($P<0.05$). The ratio of LDL to HDL cholesterol was significantly reduced ($P<0.01$). In addition, CLA supplementation reduced fibrinogen concentrations ($P<0.01$), but had no effect on the inflammatory markers of CVD, C-reactive protein and interleukin 6.

It has emerged that the isomers of CLA exert distinct physiological effects. Consequently, the *in vivo* effects of a c9, t11-CLA enriched diet on metabolic and molecular markers of glucose homeostasis was investigated in chapter 4. Particular attention was focused on adipose tissue biology by investigating the effect of the isomer on the insulin signalling pathway, adipokine expression and transcription factors known to regulate insulin sensitivity. The ob/ob mouse model was employed as it represents a suitable model for obesity, insulin resistance and related syndromes. Feeding a diet enriched with c9, t11-CLA significantly reduced fasting glucose ($P<0.05$), insulin ($P<0.05$) and triacylglycerol concentrations ($P<0.01$) compared to the control diet which was enriched with linoleic acid. Key targets of the insulin signalling cascade were significantly increased both at the level of mRNA and protein expression. While the alterations in adiponectin and resistin mRNA could not explain the

improvement in insulin sensitivity, the expression of several inflammatory mediators secreted from adipose tissue were decreased, in particular tumour necrosis factor (TNF)-α which was reduced by 50% ($P<0.05$). This was associated with an increase in mRNA expression in peroxisome proliferator activated receptor γ and liver X receptor ($P<0.05$) and a decrease in nuclear factor-κB DNA binding ($P<0.01$), a profile which would promote insulin sensitivity.

Although many factors may contribute to the development of insulin resistance, the insulin de-sensitising effects of TNFα are well characterised. In chapter 5 an *in vitro* model was employed to investigate whether the insulin sensitising effect of c9, t11-CLA observed *in vivo* could be replicated *in vitro*. Normal 3T3-L1 adipocytes or those made insulin resistant following TNFα treated were incubated with the individual isomers. Parallel experiments were set-up to examine acute and chronic treatment of the fatty acids. Results indicated that chronic treatment with c9, t11-CLA significantly increased IRS-1 and Glut4 mRNA in both normal and insulin resistant adipocytes. In contrast acute treatment with c9, t11-CLA did not increase the expression levels of these markers. Both acute and chronic treatments with t10, c12-CLA reduced the mRNA expression levels of both proteins. Glut4 protein expression, basal glucose uptake and insulin-stimulated glucose uptake following chronic treatment with c9, t11-CLA was comparable with cells treated with the control.

Acknowledgements

I would like to acknowledge and express my sincere gratitude to the many people who were so generous with their time, expertise and patience in guiding me to achieve this goal. A special mention is due to the following individuals:

Firstly I would like to thank my supervisor, Dr. Helen Roche for giving me the opportunity to carry out this research and for her guidance, encouragement and support during my time in the lab. I would also like to thank Prof. Mike Gibney for his guidance and direction and Prof. Dermot Kelleher for his support. I am grateful to Loders Crooklann for providing the supplements for the intervention trial, and both the Wellcome Trust, UK and the EU Sixth Framework Programme for funding this research.

For those who assisted me during the human intervention study, Thank you! A big thank you to Prof. John Nolan and Prof. Michael Cullen for supporting the study and allowing me to recruit volunteers from their clinical lists. Thanks to Dr. Toh Peng Yeow for her help with the clinical investigations and continued friendship long afterwards, Dr. Siobhan McQuaid for helping out whenever needed, Geraldine, Ciara and the other nurses in the Metabolic Research Unit, St. James's Hospital for assistance and advice, Declan Gasparro and all the staff in the Endocrinology Lab for performing the insulin and C-peptide assays. I am also indebted to Prof. Christine Williams in the University of Reading for allowing me to perform the lipoprotein analysis using the facilities in her lab and to Dr. Kim Jackson for her assistance with the assays. Thanks also Kim and Jan for rescuing me when I was stranded in Heathrow airport! Finally, to all my volunteers, thank you - I couldn't have done it without you!

A million thanks to all those who guided me as I took a giant leap into new waters and put on the white lab coat! Dr. Enda Noone for teaching me the lipoprotein assays, performing the animal feeding study and thereby providing me with lots of samples for chapter four, Dr. Sinead Weldon for making gene expression work look easy!, Dr. Christine Loscher for Western blot training and the EMSA, Dr. Henry Windle for showing me the Modified Lowry assay. I would also like to thank Dr. Lucy Norris, Dr. Sharon O'Toole and Jeanette in the Department of Obstetrics and Gynaecology, Dr. Paul Spiers, Dr. Pierce Kavanagh and Ken Scott in the Department of Pharmacology for help, equipment use and freezer space! In the Conway Institute, thanks to Dr. Bernard Allan for invaluable cell culture advise and assistance with the glucose uptake assay and Dr Graham Dixon for resolving technical difficulties with the scintillation counter! To everyone in the other labs in the IMM either across the corridor,

around the corner or down the stairs thanks for all the help, support and trouble-shooting advice over the past year and a half. A special thanks to Jodie for your friendship and chats over hot chocolate and croissants!

Thanks to all the staff in the Trinity Centre for the friendly faces and words of encouragement over the past number of years: Paul, Pauline, Olive and Eddie at reception, Eddie in the IMM, Virginia in the library, Mary and Catherine in the office in Dept. Clinical Medicine.

More recently to Jo and everyone in the IEFS and the IUNA, thanks for the banter and offers of support.

Sinead, Christine, Anne N, Catherine, Eve, Enda, John, Niamh, Audrey, Jolene, Lula, Benedicte and Cornelia in the lab for laughter, fun and frolics, understanding and reality checks. You made the problems of yesterday, the fond memories of today. A special word of thanks to Anne who's been there from the beginning, and shared all the highs and the lows of the past few years. I couldn't have chosen a better person to share the journey with!

To all my friends, especially Loretta, Eimear, Kate, Elaina and Helena for your patience and understanding, listening ears and unrelenting encouragement. Thanks, I owe you all! Loretta, I look forward to your graduation day!

To my family: Mam and Dad for the tippy-toes and cups of tea, endless patience, turning a blind eye whenever necessary and for constant support and encouragement in everything I do. To Paul and Emer for your friendship and emotional support. A special thanks to Paul for answering my damsel in distress calls, and Emer and Paul J for fine dining and charades! Finally to Dave for your shoulder, your sleeve and being my rock when most needed. Thanks for helping me retain my sense of humour and allowing me to dream!

I hope I make you all proud..... .

Table of Contents

Declaration		i
Summary		ii
Acknowledgements		iv
Table of Contents		vi
Abbreviations		xii
Brief synopsis of the reported effects of CLA		xv
	***Chapter One*: Introduction**	
1.1	**Insulin resistance**	**1**
1.1.1	Common disease states associated with insulin resistance	2
	(i) Type 2 diabetes mellitus (T2DM)	2
	(ii) Metabolic syndrome	3
1.2	***In vivo* measurement of insulin sensitivity**	**5**
1.2.1	Intravenous techniques	5
	(i) Euglycaemic hyperinsulinaemic clamp	5
	(ii) Intravenous glucose tolerance test	7
	(iii) Insulin suppression test	8
	(iv) Insulin tolerance test	8
1.2.2	Non intravenous techniques – fasting indices	8
	(i) Fasting Insulin	9
	(ii) Homeostasis model assessment-insulin resistance	10
	(iii) Quantitative insulin sensitivity check index	11
1.2.3	Non intravenous techniques - combined fasting and postprandial indices	11
	(i) Insulin sensitivity index (composite)	12
	(ii) Oral glucose insulin sensitivity index	12
1.2.4	Limitations in the use of measures derived from the oral glucose tolerance test	13
1.2.5	Synopsis of *in vivo* measurement of insulin sensitivity	13
1.3	**Lipoprotein metabolism**	**16**
	(i) Exogenous lipid transport	18
	(ii) Endogenous lipid transport	19
	(iii) Reverse cholesterol transport	20
1.3.1	Dyslipidaemia in T2DM and the metabolic syndrome	21
1.4	**Inflammation and prothrombotic risk in T2DM and the metabolic syndrome**	**23**
1.5	**Adipose tissue and insulin sensitivity**	**24**
1.5.1	Adipose tissue biology	24

1.5.2	The role of white adipose tissue in the pathogenesis of insulin resistance	25
	(i) Non-esterified fatty acids	25
	(ii) Secretory capacity of adipose tissue	26
1.5.3	Adipokines	27
	(i) Tumour necrosis factor α (TNF α)	29
	(ii) Interleukin-6 (IL-6)	30
	(iii) Adiponectin	32
	(iv) Resistin	34
	(v) Leptin	35
	(vi) Other cytokines	37
1.6	**Insulin**	**38**
1.6.1	Synthesis of insulin	40
1.6.2	Insulin secretion	40
1.7	**Insulin signalling in white adipose tissue**	**43**
1.7.1	Insulin receptor	43
1.7.2	Phosphoinositide (PI) 3-kinase dependent pathway	45
	(i) Insulin receptor substrate (IRS)	45
	(ii) Downstream effectors of IRS	47
1.7.3	Inhibition of the insulin signalling pathway	49
	(i) Protein tyrosine phosphatases	49
	(ii) Serine phosphorylation of IRS proteins	49
	(iii) Location of IRS-1	52
	(iv) Lipid phosphatases	53
	(v) Suppressors of cytokine signalling	53
	(vi) Feedback mechanism of insulin	53
1.7.4	PI-3 kinase independent pathway	54
1.7.5	Glucose transporter 4 (Glut4)	55
1.7.6	Effect of targeted deletions of components of the insulin signalling pathway	60
1.8	**Transcription factors involved in the regulation of insulin sensitivity**	**62**
1.8.1	Peroxisome proliferator-activate receptor (PPAR)	62
1.8.2	Liver X receptor	64
1.8.3	Sterol regulatory element binding protein	65
1.8.4	Nuclear Factor κB	66
1.9	**Nutrition and insulin sensitivity**	**67**
1.9.1	Role of fat in modulating insulin sensitivity-evidence to date	68
1.9.2	Conjugated Linoleic Acid (CLA)	69
1.9.3	CLA and insulin resistance	73
	(i) Studies using rat models	77
	(ii) Studies using murine models	78

(iii) Studies in man 80
(iv) CLA and insulin resistance - potential mechanisms of action 88
1.10 Research objectives 90
1.11 Hypothesis 90

Chapter Two: **Materials and Methods**
2.1 Collection and storage of blood samples 91
2.2 Calculation of insulin sensitivity 91
2.3 Biochemical assays 92
2.3.1 Quality control for colorimetric assays 92
2.3.2 Determination of cholesterol concentration 92
2.3.3 Determination of triacylglycerol (TAG) concentration 93
2.3.4 Determination of glucose concentration 93
2.4 Lipoprotein isolation 93
2.4.1 LDL isolation and analysis 93
2.4.2 HDL isolation and analysis 94
2.4.3 VLDL isolation and analysis 94
2.5 Determination of apolipoprotein B 95
2.6 Determination of fibrinogen 95
2.7 Determination of Insulin and C-Peptide 96
2.8 Determination of IL-6 and C-reactive protein (CRP) 96
2.9 Fatty Acid composition analysis 98
2.9.1 Extraction of total lipids 98
2.9.2 Transmethylation of lipid extracts 98
2.9.3 Gas chromatographic analysis of total plasma lipids 98
2.10 Cell culture 99
2.10.1 3T3-L1 cell line 99
2.10.2 3T3-L1 sub-culture 100
2.10.3 Cryopreservation and revival of cells 100
2.10.4 Fibroblast to adipocyte differentiation 100
2.10.5 Cell enumeration and viability assessment 101
2.10.6 Preparation of fatty acids 101
2.10.7 Experimental design 101
2.11 Mycoplasma Detection Assay 103
2.12 MTS viability assay 104
2.13 Oil Red O staining 104
2.14 RNA extraction and analysis 105
2.14.1 RNA extraction 105
2.14.2 Quantitation of RNA 106

2.14.3	RNA agarose gel electrophoresis	107
2.15	**Real-Time reverse transcription TaqMan® PCR**	**108**
2.15.1	Preparation of RNA prior to reverse transcription	108
2.15.2	Reverse transcription: synthesis of first strand cDNA	109
2.15.3	Real-time TaqMan® PCR	109
2.16	**Preparation of protein extracts for Western blotting**	**110**
2.16.1	Isolation of total protein extract from white adipose tissue	110
2.16.2	Isolation of plasma membrane extract from white adipose tissue	110
2.16.3	Isolation of membrane and cytosolic and nuclear fractions from adipose tissue	111
2.16.4	Isolation of total cell lysates from 3T3-L1s	111
2.16.5	Determination of protein concentrations using the Modified Lowry Assay	111
2.16.6	Determination of protein concentrations using the Bradford Assay	112
2.16.7	Preparation of samples for gel electrophoresis and Western blotting	112
2.17	**Principle of immunoblotting**	**113**
2.17.1	SDS polyacrylamide gel electrophoresis (SDS PAGE)	113
2.17.2	Western blotting	114
2.17.3	Detection of antigens	114
2.17.4	Reuse of Western blots	117
2.17.5	Quantification of protein bands	117
2.18	**Electrophoretic mobility shift assay**	117
2.18.1	Detection of antigens	117
2.19	**Immunohistochemistry**	118
2.19.1	Detection of antigens	118
2.20	**Glucose uptake assay**	**119**

Chapter Three: **Conjugated linoleic acid supplementation, insulin sensitivity and lipoprotein metabolism in patients with type 2 diabetes mellitus**

3.1	**Abstract**	**120**
3.2	**Introduction**	**121**
3.3	**Subjects and methods**	**123**
3.3.1	Study design	123
3.3.2	Dietary assessment	125
3.3.3	Clinical investigations	126
3.3.4	Collection of blood samples	126
3.3.5	Biochemical analysis	126
3.3.6	Fatty acid compositional analysis	127
3.3.7	Anthropometric measurements	128

3.3.8	Data preparation and statistical analysis	128
3.4	**Results**	**130**
3.4.1	Subject characteristics and anthropometric measurement details	130
3.4.2	Study compliance	131
3.4.3	Insulin and glucose metabolism	132
3.4.4	Lipoprotein metabolism	138
3.4.5	Markers of inflammation and coagulation	140
3.5	**Discussion**	**141**
3.6	**Appendices**	**145**
3.6.1	Appendix I: Dietary assessment information	145
3.6.2	Appendix II: Detailed description of methodology employed to record the anthropometric measures	146
3.6.3	Appendix III: Assessment of pancreatic β-cell function	148

***Chapter Four*: Anti-diabetic effects of *cis*-9, *trans*-11 conjugated linoleic acid may be mediated via anti-inflammatory effects in adipose tissue**

4.1	**Abstract**	**149**
4.2	**Introduction**	**151**
4.3	**Research design and methods**	**155**
4.3.1	Animal Experiment and intervention details	155
4.3.2	Sample preparation and handling	156
4.3.3	mRNA expression analysis	156
4.3.4	Protein analysis	158
4.3.5	Western Blotting	158
4.3.6	Electrophoretic mobility shift assay	158
4.3.7	Quantification of protein bands	158
4.3.8	Data preparation and statistical analysis	159
4.4	**Results**	**160**
4.4.1	Biochemical analysis	160
4.4.2	Gene expression analysis	161
	(i) Molecular markers of insulin sensitivity in white adipose tissue	161
	(ii) Molecular markers of glucose homeostatis in liver	164
	(iii) Molecular markers of lipid metabolism in white adipose tissue	166
	(iv) Molecular markers of lipid metabolism in Liver	167
4.4.3	Expression of proteins involved in the insulin signalling cascade in white adipose tissue	168
4.4.4	NF-κB p65 and IκBα expression, and NF-κB:DNA-binding in white adipose tissue	170
4.5	**Discussion**	**173**

Chapter Five: **The effect of *cis*-9, *trans*-11 conjugated linoleic acid and *trans*-10, *cis*-12 conjugated linoleic acid on insulin sensitivity in normal and insulin resistant 3T3-L1 adipocytes**

5.1 Abstract **183**

5.2 Introduction **185**

5.3 Material and methods **187**

5.3.1 Cell culture and experimental design 187

5.3.2 Oil Red O staining 188

5.3.3 MTS assay 189

5.3.4 mRNA expression analysis 189

5.3.5 Protein analysis and Western blotting 190

5.3.6 Glucose uptake assay 190

5.3.7 Investigation of the effect of TNFα in vivo 191

5.3.8 Data preparation and statistical analysis 191

5.4 Results **192**

5.4.1 MTS assay 192

5.4.2 Oil Red O staining 194

5.4.3 Gene Expression analysis 200

(i) Glut4 and IRS-1 mRNA following chronic treatment with fatty acids 201

(ii) Glut4 and IRS-1 mRNA following acute treatment with fatty acids 206

(iii) PPARγ1 and PPARγ2 mRNA following chronic treatment with fatty acids 211

(iv) In vivo effect of TNF α administration on the expression of genes regulating insulin sensitivity and glucose homeostasis in white adipose tissue 216

5.4.4 Western Blotting 217

(i) Glut4 expression following chronic treatment with fatty acids 217

(ii) IκBα expression following chronic treatment with fatty acids 219

5.4.5 Glucose Uptake Assay 221

5.5 Discussion **225**

Chapter Six: **General Discussion** **233**

Chapter Seven: **Bibliography** **239**

2-DOG	2-deoxyglucose
ABCA1	ATP-binding cassette A1
ACC	Acetyl-CoA carboxylase
Acrp-30	Adipocyte complement related protein of 30kDa/adiponectin
ADP	Adenosine triphosphate
ADSF	Adipose tissue-specific secretory factor
AMPK	AMP-activated kinases
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AP2	Adipose specific fatty acid-binding protein
ApM1	Adipose most abundant gene transcript
Apo	Apolipoprotein
ATP	Adenosine triphosphate
APS	Adaptor protein with a PH and SH2 domain
AUC	Area under the postprandial curve
BHT	Butylated hydroxy toluene
BMI	Body mass index
BSA	Bovine serum albumin
C3G	Cyanidin 3-glucoside
c9, t11-CLA	*cis*-9, *trans*-11 conjugated linoleic acid
CAD	Coronary artery disease
CAP	Cbl-associated protein
Cbl	Casitas b-lineage lymphoma
cDNA	Complementary deoxyribonucleic acid
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CM	Chylomicron
C_{max}	Maximum postprandial concentration
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotides
DTT	Dithiothreitol
EB/AO	Ethidium bromide/acridine orange
EDTA	Ethylene diamine tetra-acetic acid
EGIR	European group for the study of insulin resistance
ELISA	Enzyme linked-immuno-sorbent assay
EMSA	Electrophoretic mobility shift assay
EPA	Eicosapentaenoic Acid
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FFA	Free fatty acids
Fig.	Figure
FPI	Fasting plasma insulin
FSIGT	Frequently sampled intravenous glucose tolerance test
g	*g* force
G6Pase	Glucose-6-phosphatase
GAB	GTPase-activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBP28	Gelatin-binding protein of 28kDa
GDP	Guanosine diphosphate
GLC	Gas liquid chromatography
Glut	Glucose transporter
GSV	Glut4 storage vesicle
GTP	Guanosine triphosphate
HbA_{1c}	Glycated haemoglobin
HDL	High density lipoprotein
HGP	Hepatic glucose production
HL	Hepatic lipase
HMW	High molecular weight
HOMA-IR	Homeostasis model assessment-insulin resistance
Hr	Hour
HRP	Horseradish-peroxidase
HSL	Hormone sensitive lipase
HSP	High Speed Pellet
IAUC	Incremental area under the postprandial curve
IBMX	Isobutyl-1-methylxanthine
ICAM	Intercellular adhesion molecule

IDL	Intermediate density lipoprotein
Ig	Immunoglobulin
IGT	Impaired glucose tolerance
IHC	Immunohistochemistry
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAP	Insulin-responsive aminopeptidase
IRAS	Insulin resistance atherosclerosis study
IRS	Insulin receptor substrate
ISI	Insulin sensitivity index
IV	Intravenous
IVGTT	Intravenous glucose tolerance test
JNK	c-Jun N-terminal j-kinase
KCl	Potassium chloride
LA	Linoleic acid
LAR	Leucocyte antigen-related
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LMW	Low molecular weight
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein
MEK	MAPK Kinase
$MgCl_2$	Magnesium chloride
MI	Myocardial infarction
Min	Minute
MINMOD	Minimal model
MIP-1α	Macrophage inflammatory protein-1α
MODY	Maturity onset diabetes of the young
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian target of Rapamycin
MTP	Microsomal triglyceride transfer protein
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MUFA	Monounsaturated fatty acids
MUNC18c	Mammalian unc18c
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide (NAD)+
NADH	Reduced form of nicotinamide adenine
NaOH	Sodium hydroxide
NCEP	National Cholesterol Education Program
NEFA	Non esterified fatty acids
NF-κB	Nuclear factor-κB
NGT	Normal glucose tolerance
OD	Optical density
OGIS	Oral glucose insulin sensitivity
OGTT	Oral glucose tolerance test
ORO	Oil red O
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activated inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDK	Phosphoinositide-dependent kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology
PI	Phosphoinositide
PI(3)P	Phosphoinositide-3-phosphate
PI 3-kinase	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator activated receptor
PPRE	PPAR response element
PTB	Phosphotyrosine binding
PtdIns	Phosphatidlyinositol
$PtdIns(3,4,5)P_3$	PtdIns (3,4,5)-triphosphate
PTEN	Phosphatase and tesnin homolog gene
PTP	Protein tyrosine phosphatases
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene fluoride
QUICKI	Quantitative insulin sensitivity check index
RCT	Reverse cholesterol transport
RELM	Resistin like molecules
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference

RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
SDS	Sodium dodecylsulphate
Sec	Second
SEM	Standard error of the mean
Ser/Thr	Serine/threonine
SFA	Saturated fatty acids
SH2	Src homology 2
SHC	Src homology collagen
SHIP2	SH2 domain-containing inositol-5-phosphatase-2
SIRP	Signal-regulatory protein
SNAP-23	Soluble *N*-ethylmaleimide-sensitive factor attachment protein
SNP	Single nucleotide polymorphisms
SOCS	Suppressors of Cytokine Signalling
SoHo	Sorbin homology
SREPB	Sterol regulatory element binding protein
SSPG	Steady state plasma glucose
Syn4	Syntaxin 4
SYNIP	Syntaxin 4 interacting protein
t10, c12-CLA	*Trans*-10, *cis*-12 conjugated linoleic acid
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TBS	Tris buffered saline
T_{max}	Time to maximal postprandial concentration
TEMED	N,N,N'N'-tetramethylethylediene diamine
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor α
TNFR	Tumour necrosis factor receptor
TPL	Triacylglycerol poor lipoprotein
TRIG	Triglyceride
TRL	Triacylglycerol rich lipoprotein
t-SNARE	Target-soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TZD	Thiazolidinedione
UCP	Uncoupling protein
USFA	Unsaturated fatty acid
UTR	Untranslated region
UV	Ultraviolet
VAMP	Vesicle-associated membrane protein
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
V-SNARE	Vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor
WHO	World Health Organization
WHR	Waist-to-hip ratio
Wk	Week
yr	Year
ZDF	Zucker Diabetic Fatty fa/fa

Brief synopsis of the reported effects of CLA

Effect of CLA	Authors
Knowledge that c9, t11-CLA is an intermediate in the biohydrogenation of linoleic acid	Kepler *et al*, 1966
Seminal observation that CLA isolated from ground beef or produced by base-catalysed isomerisation of linoleic acid was an effective inhibitor of benzo(a)pyrene-initiated mouse epidermal neoplasia	Pariza *et al*, 1985; Ha *et al*, 1987
CLA inhibited chemically induced carcinogeneis in several rodent models	Ha *et al*, 1987; Ha *et al*, 1990; Ip *et al*, 1991; Liew *et al*, 1995
CLA enhanced immune function while reducing the catabolic effects of immune stimulation in rodents and chickens	Cook *et al*, 1993; Miller *et al*, 1994
CLA reduced total cholesterol, LDL-cholesterol and plasma TAG in rabbits	Lee *et al*, 1994
CLA reduced atherosclerosis in rabbits and hamsters	Lee *et al*, 1994; Nicolosi *et al*, 1997
CLA enhanced growth of rats, and pigs	Chin *et al*, 1994; Bee, 2000
CLA reduced body fat gain in mice, rats, hamsters, pigs, dogs and humans	Park *et al*, 1997; West *et al*, 1998; Azain *et al*, 2000; deDeckere *et al*, 1999; Ostrowska *et al*, 1999; Schoenherr & Jewell, 1999; Blankson *et al*, 2000; Halvorsen *et al*, 2000
CLA normalised impaired glucose tolerance	Houseknecht *et al*, 1998
CLA prevented the development of obesity-induced hypertension	Nagao *et al*, 2003

Chapter One
Introduction

1.1 INSULIN RESISTANCE

"Insulin resistance" signifies an impaired biological response to insulin, and usually implies resistance to the effects of insulin on glucose uptake, metabolism, or storage (Bailey, 1999; Kahn & Flier, 2000). Insulin resistance may exist during physiological states as occurs in pregnancy, puberty and in the elderly (Wallace & Matthews, 2002). Pathological states associated with insulin resistance include type 2 diabetes mellitus (T2DM), and as a result of a specific defect such as insulin receptor mutations or autoantibodies against the insulin receptor. Additionally insulin resistance may also occur secondary to other disorders including polycystic ovary syndrome, illness and cirrhosis (Wallace & Matthews, 2002). The two most common states associated with insulin resistance are T2DM and the metabolic syndrome. In association with insulin resistance, these two conditions are also linked to obesity, dyslipidaemia, hypertension and atherosclerosis.

Adipose tissue plays an important role in regulating glucose metabolism by releasing fatty acids into the circulation. In 1963 Randle *et al* hypothesised that the intracellular metabolism of fatty acids controls glucose metabolism. This mechanism was called the glucose-fatty acid cycle. They hypothesised that increased FFA oxidation enhances the generation of acetyl-CoA that leads up to increased citrate production through the Krebs cycle. Citrate, in association with an increase in the ATP/ADP ratio, inhibits phosphofructokinase, resulting in reduced glycolytic flux. As a consequence, there is an increase in the glucose 6-phosphate content, which inhibits hexokinase II activity, leading to an increase in intracellular glucose content and a reduction in glucose uptake (Randle *et al*, 1963; Randle *et al*, 1964). Although subsequent studies using nuclear magnetic resonance showed that free fatty acids decrease the glucose and glucose 6-phosphate intracellular content (Roden *et al*, 1996; Shulman, 2001), thereby suggesting that other mechanisms are involved in the free fatty acid induced insulin resistance, the realisation of the influence of fatty acids on glucose metabolism traces back many years.

There has been intense interest surrounding the role of additional secretory products from adipose tissue since the discovery of leptin secretion from adipose tissue (Zhang *et al*, 1994). Many proteins have been identified and have been shown to be important in allowing adipose tissue to maintain a complex communication with many other organs, and to modulate a number of whole body processes (Guerre-Millo, 2004; Rajala & Scherer, 2003). A number of these secreted proteins have been shown to have potent effects on whole-body insulin

sensitivity (Doria, 2003; Kahn & Flier, 2000), and on-going research provides fascinating insights into this area.

In recent years it has emerged that many non-communicable diseases e.g. cardiovascular disease (CVD) have an underlying inflammatory component associated with them, in addition to classical risk factors e.g. hyperlipidaemia and hypertension in CVD. A pro-inflammatory response has been found to be associated with insulin resistance both *in vivo* at a systemic level and also in governing some of the underlying molecular mechanisms in the pathogenesis of the condition.

Although a person's risk towards a disease is influenced by genetic predisposition, it is possible to attenuate modifiable risk factors through dietary and lifestyle factors. It is well established that dietary therapy can modify risk towards many disease including diabetes, hyperlipidaemia, cancer and bone health. Dietary therapy is at the cornerstone in the treatment of glycaemia in subjects with T2DM. In addition, dyslipidaemia and hypertension are modifiable through changes in dietary practices. The ability of dietary changes to modulate novel risk factors for T2DM and CVD is currently under investigation.

1.1.1 Common disease states associated with insulin resistance

(i) Type 2 Diabetes Mellitus

Diabetes mellitus, the disease characterised by the body's inability to metabolise glucose manifests as one of two types, type 1 or type 2 (Diabetes Care, Securing the Future, 2002). Type 1 diabetes mellitus results from insulin deficiency caused by cell-mediated autoimmune destruction of pancreatic β-cells, and generally develops in the young (Jun et al, 1999). T2DM accounts for approximately 90% of all reported cases of the disease. Here the pathogenesis of hyperglycaemia is rooted in a malfunction of the inter-relationship between insulin secretion and insulin action. Insulin resistance develops when target tissues fail to respond properly to normal levels of circulating insulin. Pancreatic β-cells first compensate for peripheral insulin resistance by increasing insulin secretion to maintain euglycaemia. Impaired glucose tolerance (IGT) manifests when sufficient hyperinsulinaemia cannot be sustained. An increasing mismatch between escalating insulin resistance and inadequate compensatory hyperinsulinaemia causes a progression to overt clinical T2DM (Ruan & Lodish, 2003). In muscle, insulin resistance is manifested by decreased glucose uptake as well and impaired utilisation of glucose by non-oxidative pathways, primarily glycogen formation, as well as a slight decrease in glucose oxidation. In liver, insulin resistance leads to impaired suppression of hepatic glucose production, which is fuelled by gluconeogenesis and to a lesser extent, glycogen breakdown (Reaven, 1995). Although T2DM predominately develops in adults it is becoming increasingly prevalent at a younger age (Jun *et al*, 1999).

Worldwide prevalence of T2DM is reaching epidemic proportions mainly as a consequence of lifestyle changes and an increased incidence of obesity (Nawrocki & Scherer, 2004). This is associated with enormous health and socio-economic burdens (Hu *et al*, 2001). It is estimated that CVD accounts for 40-50% of all deaths in people with T2DM (De Marco *et al*, 1999). Atherosclerotic CVD, including coronary artery disease (CAD), cerebrovascular and peripheral vascular disease are 2 to 5 times more common in people with diabetes. In addition, people with T2DM are 17 times more likely to develop renal disease (Diabetes Care, Securing the Future, 2002; Stamler *et al*, 1993; Manson *et al*, 1991). The CODE 2 study measured the total health-care costs for T2DM in eight European countries - Belgium, France, Germany, Italy, the Netherlands, Spain, Sweden and the UK (Jonsson, 1997). The total health-care cost for patients with T2DM in these eight countries amounted to €29 billion. The per capita cost varied from €1305 per patient in Spain to €3576 in Germany. Direct medical cost for patients with T2DM ranged from 1.6% of health-care expenditure in the Netherlands to 7.4% of health-care expenditure in Italy. In Ireland approximately €350 million is spent each year by the Irish health services on diabetes care, of which 59% is spent on treating complications (Diabetes Care, Securing the Future, 2002). It is estimated globally that the total number of persons with diabetes will rise from 151 million in the year 2000, to 221 million by the year 2010 and to 300 million by 2025 (Zimmet et al, 2003; Amos *et al*, 1997). This constitutes an increase in worldwide prevalence from 4.0% of the population in 1995 to 5.4% by the year 2025 (King *et al*, 1998). As a result there is extensive research investigating the molecular mechanisms governing T2DM with the aim of identifying novel targets, both dietary and pharmaceutical, which may help attenuate the consequences of the disease.

(ii) Metabolic syndrome

In recent years it has become increasingly apparent that insulin resistance is also linked to several important risk factors for coronary heart disease (CHD), such as dyslipidaemia, elevated blood pressure and propensity for thrombosis (Smith *et al*, 1999). Reaven proposed the term Syndrome X to describe the associated features of the syndrome. However this clustering of risk factors is also referred to as the Insulin Resistance Syndrome, Reaven's Syndrome, the Plurimetabolic Syndrome, the "Deadly Quartet" and the Metabolic Syndrome (Stears & Byrne, 2001; Bailey, 1999). Recent prospective studies have shown that the presence of the metabolic syndrome is associated with a 2-4 fold increased risk of developing cardiovascular complications (Hauner, 2005). It is also associated with increased risk of developing T2DM and premature death (Isomaa *et al*, 2001). In isolation each component of the cluster conveys increased CVD risk, but as a combination there is an additive or synergistic effect (Despres, 2003; Alberti & Zimmet, 1998). It is proposed that insulin resistance may be the common aetiological factor for the individual components of the syndrome (Alberti &

Zimmet, 1998). Thus, rigorous early management of the syndrome may have a significant impact on the prevention of both T2DM and CVD.

Both the World Health Organisation (WHO) (Alberti & Zimmet, 1998) and the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (2001) have proposed criteria for the metabolic syndrome. The criteria differ slightly, with the NCEP guidelines being slightly more conservative. However both sets of guidelines encompass the same constellation of metabolic abnormalities. *Table 1.1* highlights the differences between the two sets of criteria.

Table 1.1: Criteria for the metabolic syndrome according to the WHO and NCEP guidelines[1, 2]

	WHO guidelines[1]	**NCEP guidelines[2]**
Abdominal Obesity		
Waist-to-Hip ratio		
Men	> 0.9	NA
Women	> 0.85	NA
Waist girth (mm)		
Men	NA	> 1020
Women	NA	> 880
HDL-cholesterol (mmol/L)		
Men	≤ 0.9	< 1.1
Women	≤ 1.0	< 1.3
Triacylglycerol (mmol/L)	≥ 1.7	> 1.7
Blood pressure (mm Hg)	≥ 160/90	≥ 130/85
Fasting plasma glucose (mmol/L)	See [1]	> 6.1
Microalbuminuria		
urinary albumin excretion rate (µg/min) or	≥ 20	NA
albumin:creatinine ratio	≥ 20	NA

NA, not applicable for the criteria in question.

[1] Individuals should present with glucose intolerance, impaired glucose tolerance (fasting plasma glucose < 7.0mmol/L and 2 hr post glucose load ≥ 7.8 mmol/L and < 11.1mmol/L) or diabetes (fasting plasma glucose ≥ 7.0mmol/L and/or 2 hr post glucose load ≥ 11.1mmol/L) and/or insulin resistance (assessed using the hyperinsulinaemic euglycaemic clamp), together with two or more of the criteria listed (Alberti & Zimmet, 1998).

[2] Individuals should fulfil three or more of the five criteria listed (NCEP Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (2001).

Differences in the definition of the metabolic syndrome produce differences in the reported prevalence rates of the disorder. However a very consistent finding is that the prevalence of the metabolic syndrome is highly age-dependent, with increasing prevalence rates with increasing age (Eckel *et al*, 2005). Using the data from the European Group for the study of Insulin Resistance (EGIR) estimates that approximately 15% of Caucasians have the metabolic syndrome as defined by the WHO criteria (Beck-Nielson & EGIR, 1999). Villegas *et al* (2003) estimated that approximately one-fifth of Irish middle-aged men and women (50-69 yr) fulfilled one or both of the above criteria for the metabolic syndrome. Additionally by the year 2010, it is estimated that between 50 to 75 million people in the United States will have the Metabolic Syndrome (Das, 2002), with a current population in the United States estimated at approximately 297 million.

1.2 *IN VIVO* MEASUREMENT OF INSULIN SENSITIVITY

The insulin resistance atherosclerosis study (IRAS) demonstrated that reduced insulin sensitivity, or insulin resistance, is associated with an elevation in CVD risk factors, subclinical atherosclerosis, and prevalent cardiovascular events (Howard, 1999). Therefore the potential consequences of reduced insulin sensitivity pose a serious health risk. Insulin resistance signifies an impaired biological response to insulin, and usually implies resistance to the effects of insulin on glucose uptake, oxidation to produce energy, or storage as glycogen or TAG (Bailey, 1999; Kahn & Flier, 2000). As previously mentioned, the worldwide prevalence of T2DM is approximately 5% (King *et al*, 1998). Additionally, it is estimated that insulin resistance affects approximately 20-25% of normoglycaemic individuals (Matsuda & DeFronzo, 1999). Therefore it is critical to be able to make an accurate assessment of insulin sensitivity *in vivo*. The euglycaemic clamp is considered to be the "gold standard" in measuring insulin sensitivity *in vivo* (DeFronzo *et al*, 1979; Monzillo & Hamdy, 2003; Wallace & Matthews, 2002). However due to the time demands of the test and the number of trained personal required, other techniques have been proposed as alternatives. Each technique has favourable attributes, but demonstrates negative aspects also. Some of the most commonly used techniques will be discussed below.

1.2.1 Intravenous techniques

(i) Euglycaemic hyperinsulinaemic clamp

The euglycaemic hyperinsulinaemic clamp is deemed the "gold standard' for measuring insulin sensitivity (DeFronzo *et al*, 1979; Monzillo & Hamdy, 2003; Wallace & Matthews, 2002). Insulin sensitivity is assessed using clamps performed at fasting glucose concentration, termed euglycaemia in subjects with normal glucose tolerance (NGT) and isoglycaemia in

subjects with diabetes. Clamps may also be performed under hyperglycaemic conditions to determine β-cell function, assessing both first-phase and second-phase insulin responses. Both insulin sensitivity and β-cell function may be assessed simultaneously using the hyperglycaemic clamp. However the technique is criticised as the prevailing glucose is higher than that found in normal physiology and the glucose infusion may also be a consequence of glucose-mediated glucose disposal and renal glycosuria, in addition to insulin-mediated glucose disposal (Radziuk, 2000; Bergman *et al*, 1985; Cagnacci *et al*, 2001; Wallace & Matthews, 2002).

During the clamp a dorsal hand vein is cannulated in a retrograde fashion and kept in a warming devise (65°C) to facilitate venous sampling and provide arterialised venous blood (Matsuda & DeFronzo, 1999; Stumvoll *et al*, 2000). Insulin is infused to increase glucose disappearance and plasma glucose concentration is maintained by a calculated rate of exogenous glucose infusion (Bergman *et al*, 1985). For the initial 40 min of the clamp there is a quick rise in glucose infusion rates. When a steady state is reached the exogenous glucose infusion rate equals the amount of glucose disposed of by all the tissues in the body and thus provided an estimate of overall insulin sensitivity (Ferrannini & Mari, 1998). The more glucose infused per unit of time, the greater the insulin sensitivity. Endogenous glucose release should be suppressed by the insulin infusion, so that after 30-50 min all the glucose metabolised is of exogenous origin.

Glucose uptake can be normalised per kg body weight, fat-free mass or resting rate of energy expenditure. Values can be corrected by the steady state insulin concentration to account for minor differences in clamp insulin levels, insulin plateau and steady state plasma glucose, thereby calculating the metabolic clearance rate of plasma glucose (Ferrannini & Mari, 1998). The euglycaemic clamp measures whole-body insulin sensitivity (DeFronzo *et al*, 1979). However it is possible to quantify the separate contributions of peripheral tissues (predominantly muscle) and the liver to whole-body insulin sensitivity using a glucose tracer (DeFronzo *et al*, 1982; Matsuda & DeFronzo, 1999). This is essential in cases of insulin resistance or low insulin infusion rates (<2.5pmol/min/kg) to avoid erroneous results (Ferrannini & Mari, 1998). The clamp may also be employed with other techniques to gather further information on other aspects of glucose homeostasis including lipolysis and protein metabolism (using tracers of glycerol and amino acids) (Groop *et al*, 1989), muscle glycogen repletion (by taking muscle biopsies before and after the clamp) (Shulman *et al*, 1990) and assessing regional insulin-mediated glucose uptake (using positron emission tomography) (Paternostro *et al*, 1996).

(ii) Intravenous glucose tolerance test

The frequently sampled intravenous glucose tolerance test (FSIGT) involves the injection of glucose at baseline, and the measurement of plasma insulin and glucose at 30 time points over a 3 hr period (Pacini & Bergman, 1986). Beard *et al* (1986) proposed a modified protocol which requires an infusion of tolbutamide 20 min after the glucose infusion begins. This is to ensure an adequate endogenous plasma insulin response which is critical for determining insulin sensitivity using mathematical modelling. This modified protocol provided better correlation with the clamp. In individuals with impaired β-cell response to secretagogues, for example in type I diabetes and insulin-deficient T2DM, tolbutamide is replaced with insulin to augment second-phase insulin secretion (Osei *et al*, 1992; Taniguchi *et al*, 1995; Saad *et al*, 1994; Finewood *et al*, 1990; Welch *et al*, 1990). The recent IRAS performed a modified FSIGT in 1625 people, whereby an insulin injection was given rather than tolbutamide, and 12 sampling occasions were performed over the 3 hr period (Henkin *et al*, 2003). The correlation between insulin sensitivity obtained from 30 samples and 12 samples was shown to be strong (Cagnacci *et al*, 2001). Additionally this study showed a significant association between insulin sensitivity and CVD risk factors.

Since insulin causes glucose to fall and glucose causes insulin to rise, mathematical modelling is performed to partition these effects and provide an estimate of insulin sensitivity. Bergman and colleagues proposed the minimal model (MINMOD) which computes estimates of insulin sensitivity (insulin-enhanced glucose disappearance from the extracellular fluid), glucose effectiveness (glucose mediated glucose disposal) and β-cell function (both first phase and second phase responses) for each individual profile by curve fitting techniques (Bergman *et al*, 1979; Pacini & Bergman, 1986). More recently MINMOD Millennium has been launched, which has updated the modelling to provide more user-friendly software, a table of all minimal model parameters and their error estimates. Additionally, it also allows for the estimation of insulin sensitivity and glucose effectiveness for almost all profiles, which was a limiting factor of previous software (Boston *et al*, 2003). The approach recommended by Galvin *et al* (1992) requires an IV glucose tolerance test at 50 min with insulin infusion. Insulin sensitivity is determined as the decline in glucose over a 40 min period divided by the area under the insulin curve, a measure of the insulin load (Howard *et al*, 1998).

The IVGTT is unique as it provides an estimate of both insulin-dependent and insulin-independent glucose uptake. Additionally insulin sensitivity and β-cell secretion may be assessed simultaneously. However the minimal model technique does not permit the determination of the individual contributions of hepatic and peripheral insulin sensitivity to overall whole-body insulin sensitivity (Cagnacci *et al*, 2001). Nevertheless glucose tracer studies can be performed, as described above for the clamp, to yield an estimate of endogenous glucose production and hepatic insulin sensitivity (Caumo & Cobelli, 1993; Radziuk, 2000).

(iii) Insulin suppression test

The insulin suppression test is a reverse clamp in which the exogenous glucose infusion rate is kept constant while the plasma glucose concentration is allowed to vary (Ferrannini & Mari, 1998). Endogenous insulin release is suppressed by a constant infusion of somatostatin, glucose and insulin for 150-180 min (Wallace & Matthews, 2002). The somatostatin also suppresses endogenous secretion of glucagon and growth hormone, and inhibits gluconeogenesis (Harano *et al*, 1977). Steady state plasma glucose (SSPG) is calculated from blood sampling at 10 min intervals between 150-180 min (Laws *et al*, 1994). Since steady state plasma insulin are similar for all subjects, the SSPG concentrations provides a measure of the ability of insulin to mediate the disposal of an infused glucose load, and so provides a measure of insulin impedance or resistance. Higher SSPG levels indicate greater insulin resistance (Bergman *et al*, 1985; Yeni-Komshian *et al*, 2000).

(iv) Insulin tolerance test

The insulin tolerance was one of the first methods developed to assess insulin sensitivity *in vivo* (Horgaard *et al*, 1929). The test is based on the rate of decay of plasma glucose level after a bolus injection of regular insulin (0.1-0.5 U/kg BW). This model assumes that the glucose system is a single compartment from which insulin accelerates the net disappearance of the substrate both by promoting its uptake into target tissues and by shutting off endogenous glucose production (Ferrannini & Mari, 1998). Blood samples are collected at 2 min intervals for 15 min, and subsequently analysed for glucose and insulin. The test is stopped by the administration of intravenous glucose (Wallace & Matthews, 2002). Insulin sensitivity is determined by calculating the rate of decline of the log transformed glucose concentrations, estimated by linear regression (Wallace & Matthews, 2002). The rate constant K_{ITT} is derived from this, and is expressed as the percentage decline in glucose per min (Wallace & Matthews, 2002). The faster the decline in plasma glucose concentration indicates higher insulin sensitivity (Ferrannini & Mari, 1998). Normal K_{ITT} is >2.0%/min and values <1.5 are considered abnormal (Monzillo & Hamdy, 2003).

1.2.2 Non intravenous techniques – fasting indices

Both the clamp and IVGTT have been criticised as they assess insulin sensitivity under non-physiological conditions (Breda *et al*, 2001). Although the oral glucose tolerance test (OGTT) was originally used to classify carbohydrate tolerance, it represents a simple metabolic test which is more physiological than either of the above techniques. The time course of plasma glucose and insulin responses reflect the ability of the pancreatic β-cells to secrete insulin and the sensitivity of tissues to insulin. Consequently the OGTT has also been used to evaluate insulin sensitivity and β-cell function (Lerman *et al*, 2003; Stumvoll *et al*, 2001). Mathematical

modelling is required to disentangle the interaction of insulin secretion and insulin sensitivity. Nevertheless a plethorea of indices have been proposed from data which may be collected over the duration of the OGTT.

All measures offer an advantage in terms of ease of collection of the samples and analysis of the results. These measures are useful in epidemiological studies or longitudinal analysis within an individual. However, they are not the optimal choice for studying physiology since the OGTT does not provide a controlled stimulus (Phillips *et al*, 1994).

(i) Fasting insulin

It is not clear whether hyperinsulinaemia per sec contributes to the metabolic syndrome or is just a physiological consequence of diminished insulin-mediated glucose uptake in muscle and therefore only a marker of insulin resistance (Lerman *et al*, 2003). Prospective studies have shown that high insulin concentrations are associated with an increased risk of CVD and adverse changes in CVD risk factors (Despres *et al*, 1996; Gutt *et al*, 2000; Laakso, 1993). As a result fasting insulinaemia is often used as a surrogate marker of insulin resistance in epidemiological studies (Mannucci *et al*, 2003).

High fasting plasma insulin (FPI) concentrations are associated with insulin resistance as a reduction in tissue insulin sensitivity is matched by a proportionate rise in insulin secretion, until the β-cells fails (Ferrannini & Balkau, 2002). A pitfall of this concept is that insulin resistance is only one determinant of FPI concentrations. Systemic plasma insulin concentrations are also a result of pancreatic β-cell release, plasma removal of the hormone, fasting glucose concentration and degree of obesity (Ferrannini & Balkau, 2002). Ferrannini & Balkau (2002) reported that insulin sensitivity assessed using the euglycaemic clamp, explained only 4% of the variability in FPI concentrations using data collected from the EGIR study in 1308 subjects with NGT. Furthermore, elevated FPI concentrations and insulin sensitivity indices derived from the clamp identified partially different groups of individuals, after adjustment for age, sex and body mass index (BMI). *Table 1.2* illustrates the different phenotypes.

Table 1.2: Different phenotypes observed using fasting plasma insulin and clamp derived measures of insulin sensitivity (Ferrannini & Balkau, 2002).

"Pure Insulin Resistance" (from clamp derived measures)	"Pure Hyperinsulinaemia" (from fasting plasma insulin concentrations)
➢ ↑ central fat distribution	➢ Larger percent fat mass, but more peripheral distribution
➢ ↑ lipolysis	➢ ↓ lipolysis
➢ ↑ endogenous glucose production	➢ ↓ endogenous glucose production
➢ ↑ NEFA concentrations	➢ ↓ NEFA concentrations
	➢ ↑ systolic blood pressure
	➢ ↓ HDL-cholesterol concentration
➢ ↑ TAG concentrations	➢ ↑ TAG concentrations

HDL, high density lipoprotein; NEFA, non-esterified fatty acids; TAG, triacylglycerol.

In addition a number of limitations have been associated with the use of the insulin assay itself. These include test-to-test variability, rapid responsiveness of insulin to changes in hormonal and metabolic milieu and the biological variability of insulin arising from its short serum half-life and it's known cyclicity. Insulin is secreted in pulses with a periodicity of 10-15 min, and ultradian oscillations of 1-3 hr. This varies in the fasting state and in IGT and T2DM (Chevenne *et al*, 1999). The newer ultra-sensitive insulin assay can discriminate between insulin and partially metabolised proinsulin, which is biologically inactive. However the repeatability characteristics of the assay did not improve dramatically, probably due to the above reasons (Mather *et al*, 2001).

(ii) Homeostasis model assessment-insulin resistance

Homeostasis model assessment-insulin resistance (HOMA-IR) comprises mathematical modelling of the normal homeostasis balance of insulin and glucose providing equations for the estimation of insulin sensitivity and β-cell function, from simultaneous fasting measures of insulin and glucose (Matthews *et al*, 1985). HOMA-IR is based on the premise that circulating insulin and glucose levels are determined by a feedback loop between the liver and the pancreatic β-cell (Matthews *et al*, 1985). Increased levels of HOMA-IR indicate increased insulin resistance, or reduced insulin sensitivity. The original model allowed the calculation of HOMA-IR from an empirical formula. In recent years the model has been improved following a greater understanding of physiology e.g. insulin secretion, glucose metabolism, renal glucose losses in hyperglycaemic states (Rudenski *et al*, 1988; Rudenski *et al*, 1991; Levy *et al*, 1998). This more accurate model is now available as a computer programme.

Since measurements are taken in the fasting state, HOMA-IR provides a measure of basal or hepatic insulin sensitivity, providing little information about peripheral insulin

sensitivity. HOMA-IR has been shown to provide a better correlation with clamp measures of insulin sensitivity than fasting insulin. Additionally it is a good independent predictor of abnormalities associated with insulin resistance such as T2DM, risk of fatal and non-fatal CVD, stroke and CAD (Mannucci *et al*, 2003; Hanley *et al*, 2002; Bonora *et al*, 2002). A limitation of HOMA-IR and other indices derived solely from fasting measures is the assumption that hepatic and peripheral insulin sensitivity are equivalent, although they may differ greatly within an individual and between individuals.

(iii) Quantitative insulin sensitivity check index

QUICKI is another mathematical model derived from fasting insulin and glucose concentrations (Katz *et al*, 2000). HOMA-IR and QUICKI differ mathematically in that the latter uses the reciprocal of the logarithm of both plasma glucose and insulin to account for the skewed distribution of FPI (Katz *et al*, 2000). As expected, there is very good correlation between the two indices (Katz *et al*, 2000; Mather et al, 2001; Abbasi *et al*, 2002). Although some studies have shown that QUICKI offers no benefit compared to HOMA-IR, other studies report better correlations with the clamp and suggest that it has a higher discrimination capacity than HOMA-IR (Katz *et al*, 2000; Bastard *et al*, 2001; Hrebicek *et al*, 2002). A revised QUICKI has been proposed which includes fasting plasma non-esterified fatty acids (NEFA) concentrations in the calculations as a means for improving the validity of QUICKI in lean people. Revised QUICKI is calculated as 1/[log fasting insulin concentration + log fasting glucose concentration + log fasting NEFA concentration (Perseghin *et al*, 2001).

1.2.3 Non Intravenous techniques - combined fasting and postprandial indices

Variation in the diagnosis of T2DM can vary as much as 15-20% depending on whether fasting or 2 hr glucose concentrations are used in the diagnosis (Peters *et al*, 1996; McCance *et al*, 1994). Subjects who have a predominant defect in hepatic insulin sensitivity, present with excessive hepatic glucose production which manifests as fasting hyperglycaemia. However subjects with a more pronounced disturbance in peripheral insulin sensitivity present with postprandial glucose intolerance (Matsuda & DeFronzo, 1999). There is convincing epidemiological evidence (DECODE Study, Rancho Bernardo Study, Paris Prospective Study, HOORN Study and the Honolulu Heart Program) that an exaggerated post-load glucose response is associated with an increased risk of atherosclerosis and CVD, even in the presence of normal fasting glycaemia (The DECODE study group on behalf of the European Diabetes Epidemiology Group, 1999; Barrett-Connor & Ferrara, 1998; Balkau et al, 1999; DeVegt et al, 1999; Rodriguez et al, 1999; Bonora & Muggeo, 2001). Therefore it has been suggested that a combination of fasting and postprandial measures provides a more comprehensive assessment of insulin sensitivity than does either measure alone (Matsuda & DeFronzo, 1999).

Additionally it has been reported that more complex models tend to have a better correlation with clamp-derived measures of insulin sensitivity compared to fasting measures alone (Lerman *et al*, 2003). Although the ingestion of dextrose in the OGTT is not equivalent to the ingestion of a mixed meal providing different energy substrates (i.e. protein, carbohydrate and fat), a strong correlation ($r > 0.90$) has been found between glucose peaks following mixed meal and an oral glucose load (Wolever *et al*, 1998)

(i) Insulin sensitivity index (composite)

Assessment of insulin sensitivity following an oral load reflects both suppression of hepatic glucose production (HPG) and glucose disposal by all the tissues in the body (Matsuda & DeFronzo, 1999). Increased insulin resistance in the liver and/or in the peripheral tissues results in a greater rise in the mean plasma glucose concentration during the OGTT. Matsuda & DeFronzo (1999) hypothesised that whole-body insulin sensitivity during the OGTT is inversely proportional to the product of the mean plasma insulin and mean plasma glucose concentrations. Their model, termed insulin sensitivity index (ISI) composite, encompasses fasting values which represent hepatic insulin sensitivity, and the mean plasma insulin and glucose concentrations during the OGTT which reflects both hepatic and peripheral insulin sensitivity. Square root conversion was performed to correct the non-linear conversion of the values. The model was validated in 153 people (66 men and 87 women) with varying degrees of glucose tolerance (NGT = 62; IGT = 31; T2DM = 60), and BMI varying from 19.9 to 64.5kg/m^2 (Matsuda & DeFronzo, 1999).

(ii) Oral glucose insulin sensitivity index

Mari *et al* (2001) criticised ISI (composite) since it is based on an empirical formula, and HOMA-IR since the accuracy of it has not been fully demonstrated. They proposed oral glucose insulin sensitivity (OGIS) index, which is based on an equation that predicts glucose clearance during a hyperinsulinaemic euglycaemic clamp using the values of glucose and insulin concentrations obtained from an OGTT. The equation stems from established principles of glucose kinetics and insulin action but is simplified by introducing assumptions and determining unknown parameters, by matching the OGTT-predicted glucose clearance with the glucose clearance calculated from the clamp (Mari *et al*, 2001). The model was validated in a population of lean and obese subjects [104 subjects (93 males, 11 females; lean NGT = 15, obese NGT = 38, IGT = 13, T2DM = 38)].

The authors reported that OGIS has comparable discriminate power to the clamp in its ability to detect significant differences between the groups of subjects. In the subgroup with T2DM insulin sensitivity derived using OGIS correlated with the clamp measure of insulin sensitivity, whereas ISI (composite) did not. The authors suggest that the performance of

OGIS and the empirical indices may be equivalent in some situations, but in other circumstances the use of the model-based index OGIS is preferential (Mari *et al*, 2001).

1.2.4 Limitations in the use of measures derived from the OGTT

Indices derived from the OGTT show a correlation with the clamp in the region of 0.5-0.8. Therefore the variation of such an index can only explain between 25 to 64% of the variation in insulin sensitivity obtained from the clamp. The intrinsic limitation in the accuracy of the OGTT is due to:

- The influence of enteric hormones and neural responses to nutrient ingestion, which vary in concentration and magnitude among individuals
- The integrity of the pancreatic β-cells, such that insulin hypersecretion occurs in obesity while subjects with T2DM show a blunted response.
- Glucose homeostasis in the postprandial state depends on the suppression of glucagon secretion and the rate of entry of ingested glucose into the circulation. This rate of entry is dependent on gastrointestinal motility, gastric emptying and splanchnic glucose uptake which also show inter-subject and intra-subject variability (Fritsche *et al*, 2000; Stumvoll *et al*, 2001; Radziuk, 2000; Perseghin *et al*, 2001; Hrebicek *et al*, 2002).

As a result plasma glucose levels change continuously and variably between individuals after an OGTT, making strict matching of glucose stimulation among different subjects impossible (Stumvoll *et al*, 2001). During the OGTT suppression of HGP is minimal. Therefore it is impossible to differentiate between hepatic and peripheral insulin sensitivity using data from the OGTT. The OGTT has also been criticised in terms of poor reproducibility. However, 0 and 120 min time-points have been shown to be more reproducible than the intermediate time-points (Dix *et al*,1981; Gutt *et al*, 2000).

1.2.5 Synopsis of *in vivo* measurement of insulin sensitivity

Inherent methodological limitations mean there is no universally accepted model that defines insulin resistance (Lerman *et al*, 2003). Although the simplicity of the measures derived from the OGTT is an obvious advantage, their application is subject to numerous limitations. The choice of method to measure insulin sensitivity depends on the objectives and the facilities available. *Table 1.3a* and *Table 1.3b* compare the various methods of assessing insulin sensitivity. Regardless of the index chosen it is important to eludicate how well the measure correlates with the accepted standard, and also to determine whether this correlation is dependent on the glucose tolerance of the subjects, ethnicity, adiposity etc.

Table 1.3a: Comparison of intravenous techniques of measuring insulin sensitivity.

Test	Labour Intensity	Method	Calculations	Advantages	Disadvantages
Clamp	Blood glucose measurements every 3-5min, in addition to plasma samples over 150-180 min[1]	Constant rate IV insulin infusion calculated as a dose per unit of surface area and variable rate IV glucose infusion[1, 2]	Insulin resistance estimated from the ratio of the mean glucose infusion to the mean insulin concentration over the last 20-30 min[1]	Can compare subjects at similar glycaemia[3] Good intra-subject variability[1] Useful research tool for studying physiology[1] Can calculate hepatic and peripheral insulin sensitivity user a glucose tracer[7]	Time consuming, costly and labour intensive[1] Non-physiological conditions[4] Inter-subject variability 21% in NGT rising to 46% in T2DM[1]
IVGTT - Minimal Model	12-35 sampling occasions over 3 hr[1, 5]	IV glucose bolus, IV insulin/ tolbutamide at 20 min[1]	Analysis of results using computer programme[1, 2]	Good validity and reliability across the spectrum of glucose tolerance when compared to the clamp[1] Less labour intensive than clamp[1] Useful research tool for studying physiology[1]	Comparisons between subjects are not made at the same glucose level[6] Frequent sampling during the IVGTT[1] Labour intensive data analysis with the minimal model, some profiles can be difficult to model[6, 1] Non-physiological conditions[4]
Insulin Suppression Test	Six samples at 0, 60, 120, 150, 160, 170, 180 min[1]	Constant rate infusion of somatostatin, glucose and insulin for 150-180 min[1]	SSPG calculated from the mean of the glucose concentrations over the last 30 min[1]	Less labour intensive than IVGTT or clamp[1] Useful research tool for studying physiology[1]	Time consuming[1] Requirement to infuse pharmacological agents, as opposed to the clamp whereby euglycaemia can be attained with IV glucose and insulin alone[3]
Insulin Tolerance Test	9 samples over 15 min[1]	IV bolus of insulin[1, 2]	Logarithm of glucose concentrations plotted against time. Insulin sensitivity estimated from the slope of the regression line[1]	Useful research tool for studying physiology[1] Short duration[1]	A potential problem is hypoglycaemia[2] Hypoglycaemia triggers counter-regulatory hormonal responses, which may interfere with insulin sensitivity[2] Supraphysiological dose of insulin[2] Does not differentiate between peripheral and hepatic insulin resistance[2]

IGT, impaired glucose tolerance; IVGTT, intravenous glucose tolerance test; NGT, normal glucose tolerance; SSPG, steady state plasma glucose; T2DM, type 2 diabetes mellitus.
[1]Wallace & Matthews, 2002; [2]Monzillo & Hamdy, 2003; [3]Bergman et al, 1985; [4]Breda *et al*, 2001; [5]Howard et al, 1998; [6]Stumvoll et al, 2001; [7]Matsuda & DeFronzo, 1999.

Table 1.3b (contd.): Comparison of non-intravenous techniques of measuring insulin sensitivity - Indices derived from data collected during OGTT data.

Test	Labour Intensity	Calculations	Advantages	Disadvantages
Fasting Insulin	2 fasting samples (average taken)	Logarithm usually best transformation[2, 13]	Only requires assessment of one parameter[2] Useful measure in normoglycaemic individuals[2]	Unsuitable marker in T2DM since FPI low when β-cell decompensation occurs, yet subjects markedly insulin resistant[3] Considerable overlap between insulin resistant and normal subjects[4,5] FPI has also been shown to correlate with β-cell function[6]
HOMA-IR	3 basal samples at 5 min intervals (one sample often used)[2]	HOMA-IR = fasting glucose (mmol/L) x fasting insulin (μU/ml)]/22.5 or use computer programme[7,8]	Better correlation with clamp than FPI Good predictor of abnormalities associated with IR such as DM[9] Better discriminate power between NGT, IGT and T2DM than FPI[11] Repeatability characteristics better than LOGinsulin[13]	Assumes that hepatic and peripheral insulin sensitivity are equivalent, although they may differ greatly within an individuals and between individuals[14] May not be accurate in subjects with severe hyperglycaemia[2]
QUICKI	1 sampling occasion	QUICKI = [1/(log fasting insulin + log fasting glucose)][12]	Correlates well with clamp[12, 11] Repeatability characteristics better than LOGinsulin[13] Has been shown to have better discrimination power than HOMA-IR, especially in obese and diabetic individuals with relatively preserved β-cell function[11]	Limited applicability when endogenous insulin secretion is reduced e.g. in Type 1 diabetes, or in non-obese insulin-sensitive subjects and diabetic subjects with diminished insulin production, due to increased variability in determined insulin concentrations[2]
ISI (composite)	5 sampling occasions (at 0, 30, 60, 90 and 120 min)[14]	ISI = 10000/√ [(fasting glucose x fasting insulin) x ($glucose_{mean}$ OGTT x $insulin_{mean}$ OGTT)][14]	Uses both fasting and postprandial measures[14] Authors show a reasonably good correlation with clamp for whole group (r = 0.73), which was higher than HOMA-IR (r = 0.69)[14]	More frequent sampling required compared to fasting measures, resulting in greater cost[15] More time consuming[14] Correlation with clamp more robust in NGT and IGT, than DM, presumably reflecting the decline in insulin secretion in this group[14]
OGIS	3 sampling periods (at 0, 10 and 180 min). Weight and height also required[16]	Analysis of results using computer programme (freely available)[16]	Uses both fasting and postprandial measures[16] Authors show good correlation with clamp for whole group (r = 0.77), which was higher (both overall and in subgroups) compared to HOMA and ISI (composite)[16]	More frequent sampling required compared to fasting measures, resulting in greater cost[15] Test 1 hr longer than ISI (composite)[16] Correlation with clamp lower in lean people and in T2DM[16]

FPI, fasting plasma insulin; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus.
[1]Wallace & Matthews, 2002; [2]Monzillo & Hamdy, 2003; [3]Ferrannini & Balkau, 2002; [4]Lerman et al, 2003; [5]Hanley et al, 2002; [6]Stumvoll, 2000; [7]Matthews et al, 1985; [8]Levy et al, 1998; [9]Mannucci et al, 2003; [10]Ferrannini & Mari, 1998; [11]Hrebicek et al, 2002; [12]Katz et al, 2000; [13]Mather et al, 2001; [14]Matsuda & DeFronzo, 1999; [15]Mannucci et al, 2000; [16]Mari et al, 2001.

1.3 LIPOPROTEIN METABOLISM

A key feature of both T2DM and the metabolic syndrome is an abnormal lipid profile. The general pattern of dyslipidaemia observed in the metabolic syndrome is very similar to that seen in T2DM and is characterised by increased TAG, decreased high density lipoprotein (HDL)-cholesterol, increased small dense low density lipoprotein (LDL), and elevated NEFAs, all of which have been correlated with insulin resistance and an increased risk of CVD (Rett, 1999). Total cholesterol and LDL-cholesterol are usually comparable to control subjects if glycaemic control is adequate (Papadakis *et al*, 2001). This section will provide an overview of lipoprotein metabolism, prior to explaining the specific defects observed in insulin resistant states.

Since free (unesterified) cholesterol, esterified cholesterol and TAG are hydrophobic, they are transported in the plasma in macromolecular complexes called lipoproteins. Lipoproteins are spherical particles comprised of a central core of hydrophobic neutral lipid (TAG and cholesterol esters) encased in a more hydrophilic shell of phospholipids, free cholesterol and associated proteins called apolipoproteins (apo) or apoproteins. Apolipoproteins serves to stabilise the lipoprotein, and also confer specificity on the lipoprotein complexes, allowing them to be recognised by specific receptors on cell surfaces, and stimulate enzyme activity. Plasma lipids and lipoproteins exist in a dynamic state and there is constant flux between different lipoprotein particles as well as with tissues and cells (Groff *et al*, 1995a). *Table 1.4a* illustrates the different classes of lipoproteins, their associated apolipoproteins and their key function. *Table 1.4b* illustrates the key functions of the apolipoproteins.

Table 1.4a: Classes of lipoproteins, their associated apolipoproteins and key function (Adapted from Groff *et al*, 1995a).

Lipoprotein	Source	Main Function	Apolipoprotein
Chylomicrons	Intestine	Transport of exogenous TAG	B48, A1, AII, AIV,
Chylomicron remnants	Chylomicron	Transport of exogenous cholesterol	E, CI, CII, CIII, B48, E
VLDL	Liver	Transport of endogenous TAG	B100, E, AI, CI,
IDL (VLDL remnants)	VLDL	Transport of endogenous TAG and cholesterol	CII, CIII, B100, E
LDL	VLDL	Cholesterol transport to all tissues	B100
HDL	Liver/ Intestine	Reverse cholesterol transport Reservoir of apolipoproteins	AI, AII, CI, CII, CIII

HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; TAG, triacylglycerol; VLDL, very low density lipoprotein.

Table 1.4b: Apolipoproteins and their key functions (Adapted from Groff *et al*, 1995a; Rifai *et al*, 2001).

Apolipoprotein	Key function
AI	Activator of LCAT; ligand for HDL receptor
AII	Enhances hepatic lipase activity
AIV	Associated with the formation of TAG-rich lipoproteins
B100	Ligand for LDL receptor
B48	Secretion of TAG from intestine
CI	Possible activator of LCAT
CII	activation of extra-hepatic LPL
CIII	inhibition of ApoCII
E	ligand for chylomicron remnant receptor in the liver and LDL receptor.
(a)	Forms a complex with LDL by covalently linking to apoB100 to produce lipoprotein (a). Apo(a) associated with high risk of premature CVD, may deliver cholesterol to sites of vascular injury

CVD, cardiovascular disease; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; LPL, lipoprotein lipase; TAG, triacylglycerol; VLDL, very low density lipoprotein.

Lipoprotein metabolism may be divided into three inter-related and inter-dependent pathways, which are depicted in *Fig. 1.1.*

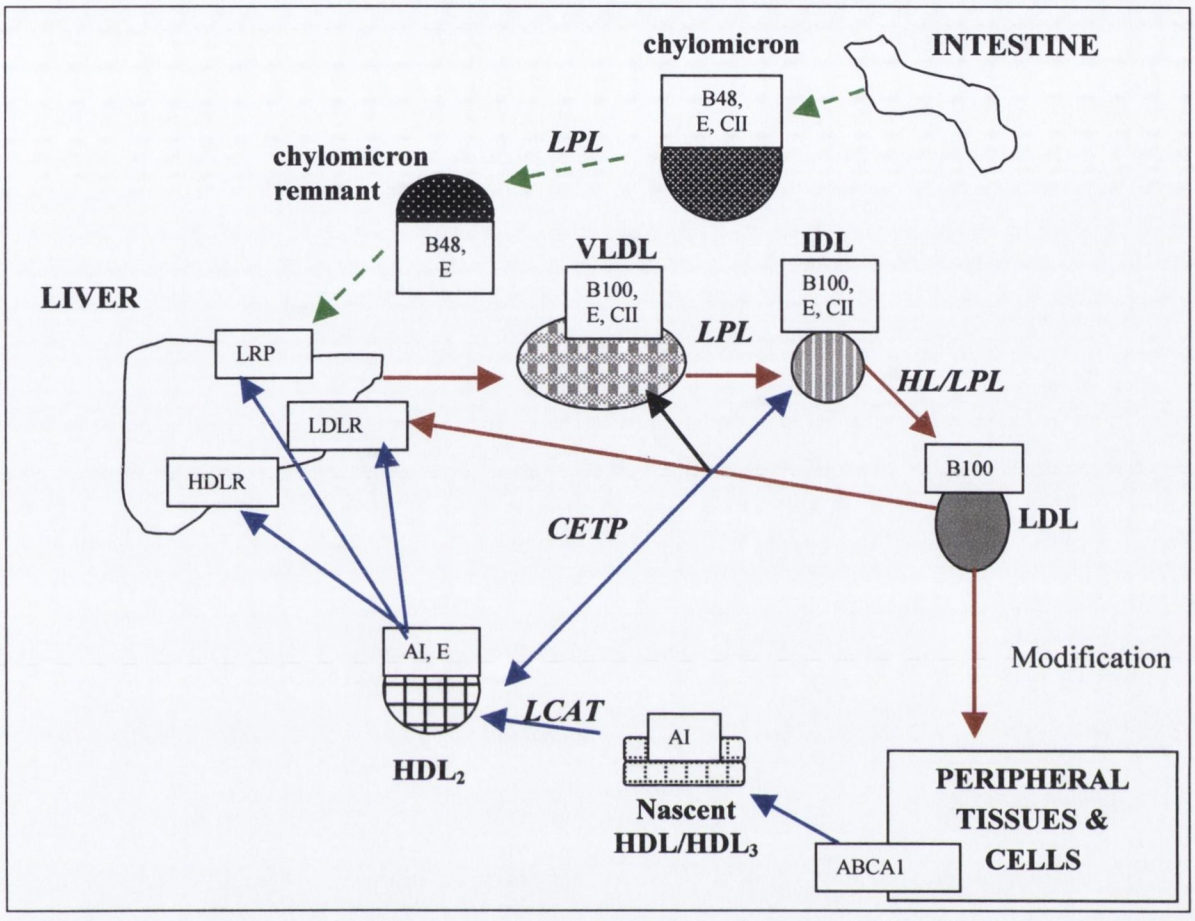


Fig. 1.1: Overview of lipoprotein metabolism (Adapted from Kwiterovich, 2000).

ABCA1 = adenosine triphosphate binding cassette transporter A1; AI = apolipoprotein AI; B48 = apolipoprotein B48; B100 = apolipoprotein B100; CII = apolipoprotein CII; CETP = cholesteryl ester transfer protein; E = apolipoprotein E; HDL = high density lipoprotein; HDLR = HDL receptor; HL = hepatic lipase; IDL = intermediate density lipoprotein; LCAT = lecithin:cholesterol acyltransferase; LDL = low density lipoprotein; LDLR = LDL receptor; LPL = lipoprotein lipase; LRP = LDL receptor like protein; VLDL = very low density lipoprotein.

⇢ Indicates exogenous lipid transport pathway
→ Indicates endogenous lipid transport pathway
→ Indicates reverse cholesterol transport pathway

(i) Exogenous lipid transport

This pathway describes the fate of lipoproteins of dietary origin after they are absorbed from the intestine, and therefore is of most relevance in the postprandial period. Nascent chylomicrons are assembled in the Golgi and comprise dietary TAG (90% by mass) and cholesterol. They are introduced into the circulation through the intestinal villi where they acquire apoC and apoE from circulating HDL. ApoCII activates lipoprotein lipase (LPL) attached to the luminal surfaces of the endothelial cells. LPL rapidly hydrolyses the TAG

yielding free fatty acids which may be used as a source of energy in muscle or stored in adipose tissue. Additionally, phospholipids and apoA are transferred to HDL. The chylomicron remnant, comprising 80-90% of the original TAG content, is recognised by specific hepatic remnant receptors due to the surface expression of ApoE and apoB48 on the lipoprotein, and is subsequently internalised by endocytosis, whereby the individual components may be hydrolysed.

(ii) Endogenous lipid transport

Lipoproteins of this pathway are of hepatic origin, due to the ability of hepatocytes to synthesise TAG from carbohydrates and fatty acids (Rifai *et al*, 2001). TAG-rich VLDL particles are synthesised and secreted by the liver for delivery to various tissues, in particular muscle and adipose tissue, for storage or production of energy through oxidation. This pathway is of particular relevance during periods of fasting. Structurally TAG is composed of a trihydroxy alcohol known as glycerol, to which three fatty acids are attached by ester bonds. The first committed step of *de novo* fatty acid synthesis involves the synthesis of malonyl-CoA, catalysed by acetyl-CoA carboxylase (ACC). Multiple enzymatic activities of fatty acid synthase (FAS) system catalyses the conversion of acetyl-CoA and malonyl CoA to form palmitate (Sul *et al*, 2000). Palmitate can then undergo separate elongation and/or desaturation to yield other fatty acid molecules. Alternatively fatty acids may be taken up from plasma and incorporated into VLDL. Other important enzymes in endogenous TAG synthesis include diacylglycerol acyltransferase (DGAT) and microsomal triglyceride transfer protein (MTP). DGAT is a key regulatory enzyme involved in TAG assembly as it catalyses the final reaction in the glycerol phosphate pathway (converting diacylglycerol into triacylglycerol), which is considered the main pathway of TAG synthesis in mammals (Chen & Farese, 2000). MTP catalyses the assembly of VLDL by promoting the fusion of apolipoprotein B with TAG (Gibbons *et al*, 2004). This TAG-rich particle (55% by mass) contains apoB100, and small amounts of apoC on its surface, in addition to some cholesterol and cholesterol esters. Additional apoC is acquired from circulating HDL.

ApoCII present on the surface of VLDL activates LPL on endothelial cells, which leads to the hydrolysis of VLDL TAG and the release of fatty acids. During the hydrolysis of VLDL apoC are transferred back to HDL, some VLDL remnants are taken up the liver and the rest are converted to smaller, denser particles called intermediate density lipoproteins (IDL). IDL consists of several molecules of apoE and may be taken up by the liver and removed from the circulation, or undergo further hydrolysis by LPL or hepatic lipase (HL). During hydrolysis the remaining TAG is removed and all apolipoproteins except apoB100 are transferred to other lipoproteins, ultimately leading to the formation of LDL. LDL may be removed from the plasma via LDL receptors in the liver which accounts for ~75% of the

removal of LDL from the circulation. Alternatively LDL may be removed by extrahepatic tissues through scavenger receptors (e.g. in macrophages) or by non-receptor mediated pinocytosis (Rifai *et al*, 2001).

(iii) Reverse cholesterol transport

This pathway describes the transport of cholesterol from the periphery to the liver for excretion. Nascent HDLs secreted from the liver or intestine, consist primarily of phospholipids and apoAI. HDL is converted to a spherical particle through the extracellular addition of surface components of TAG-rich particles, such as phospholipids, certain apolipoproteins and cholesterol esters (Rifai *et al*, 2001). In addition free cholesterol is acquired from the cell membranes of several tissues by the action of adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) (Brewer *et al*, 2004). Free cholesterol is esterified by the action of LCAT, in the presence of its cofactor apoA1, yielding a hydrophobic core of cholesterol esters. Cholesterol esters may then be transferred to the liver by the following mechanisms:

- Cholesterol esters are transferred from HDL to LDL by cholesteryl ester transfer protein (CETP), in exchange for TAG, and enter the liver through the specific LDL receptor pathway.
- Cholesterol esters are selectively taken from HDL by hepatic HDL receptors, and HDL particles are returned to plasma for further transport.
- Since HDL has accumulated apoE they may enter the liver through remnant receptors.

Cholesterol is essential as it serves as a structural element in the walls of cells, and is necessary for the synthesis of bile acids, steroid hormones, vitamin D, and lipoproteins (Rifai *et al*, 2001). Although most cells are capable of synthesising cholesterol, catabolism is very limited and most cells need to export cholesterol by the aforementioned processes in order to maintain homeostasis. Delivery of cholesterol to the liver is an important source of biliary secretion of cholesterol from the body (Rifai *et al*, 2001).

Hydrolysis of VLDL results in the production of LDL and also the conversion of HDL subfractions due to the accumulation of surface materials. LCAT esterifies free cholesterol, thereby producing the larger HDL_2 from HDL_3. HDL_2 contains twice as many cholesterol molecules per unit of apolipoproteins as does HDL_3 and therefore this lipoprotein is of particular importance in mediating RCT (Rifai *et al*, 2001).

The classic concept of atherosclerosis as a disorder of lipid metabolism has gained wide acceptance following evidence that cholesterol-rich diets induce atherosclerosis in rabbits (Libby, 2003). Accumulation of LDL-cholesterol in the artery is considered a key instigating

event in the pathogenesis of atherosclerosis, and cholesterol accumulation increases with elevated circulating levels of LDL (Lusis, 2000). LDL-cholesterol adheres tightly to arterial wall proteoglycans and diffuses passively through endothelial cell junctions (Camejo *et al*, 1998). Cholesterol retention in the vessel wall seems to involve interactions between LDL-associated apoB and matrix glycoproteins. In addition, other apoB containing lipoproteins namely VLDL, IDL and lipoprotein (a) accumulate in the arterial wall and promote atherosclerosis (Lusis, 2000). In contrast HDL represents a crucial step in the prevention or reversal of atherosclerosis, by serving as an acceptor particle of cholesterol efflux from macrophage (Linsel-Nitschke & Tall, 2005). In this role HDL is inducing the excretion of cholesterol from the arterial wall cells and is facilitating reverse cholesterol transport. HDL also exerts an anti-inflammatory effect by suppressing the induction of cell-adhesion molecules by tumour necrosis factor (TNF)-α in endothelial cells (Cockerill *et al*, 1995). HDL is also proposed to exert antioxidative and anticoagulant effects, although the *in vivo* significance of these mechanisms in explaining the protective properties of HDL is yet to be determined.

1.3.1 Dyslipidaemia in T2DM and the metabolic syndrome

The pathogenesis of dyslipidaemia in T2DM is not fully understood. It has been suggested that in insulin resistant states impaired suppression of lipolysis, due to impaired suppression of hormone sensitive lipase or HSL, results in increased flow of NEFAs from adipose tissue to the liver. This increased substrate delivery stimulates increased TAG synthesis and production of VLDL, which compete with chylomicrons for clearance. Consequently LPL becomes saturated, leading to an increased half-life of TAG-rich particles. Stimulation of CETP and lipid exchange, results in VLDL remnants which are more cholesterol enriched and LDL particles and HDL particles which become enriched in TAG. These are hydrolysed by hepatic lipase, leading to the formation of small, dense LDL and HDL particles (Betteridge, 2000). *Table 1.5* summaries the key lipid abnormalities which are present in both T2DM and the metabolic syndrome.

Table 1.5: Fasting lipid abnormalities in T2DM and the metabolic syndrome (Adapted from Coppack, 1997; Betteridge, 2000, Howard, 1999).

Lipoprotein Fraction	Change	Consequence
VLDL	➢ ↑ particle numbers due to increased TAG synthesis, ➢ Change in particle size towards larger, TAG-richer particles	➢ Contributes to fasting and postprandial hypertriglyceridaemia ➢ ↑ TAG exchange for cholesterol with LDL and HDL particles via CETP promoting TAG enrichment of these particles
LDL	➢ ↔ number of LDL particles ➢ Change in particle size with relative ↑ in small, dense LDL due to TAG enrichment ➢ ↑ LDL oxidation and glycosylation	➢ Both glycated apoB and small, dense LDL are more atherogenic due to ↑susceptibility to oxidation ➢ Oxidised LDL central to early mechanisms of atherosclerosis, including foam cell formation, stimulation of cell adhesion and endothelial dysfunction ➢ Clearance from plasma slower due to ↓ efficiency in ligand binding
HDL	➢ ↓ plasma HDL and ApoA-1 concentrations ➢ Change in particle size towards smaller TAG-enriched particles, resulting in a relative ↑ in HDL_3 and a ↓ decrease in HDL_2	➢ Small, dense HDL rapidly cleared from circulation ➢ ↓ capacity for RCT and removal of cholesterol from the periphery ➢ ↓ capacity to exert anti-inflammatory and other protective effects in endothelium
NEFA	➢ ↑ plasma NEFA concentrations ➢ ↓ suppressibility of NEFA concentrations by postprandial insulin	➢ ↑ substrate supply for VLDL production ➢ Proposed to be involved in the pathogenesis of T2DM (Section 1.5.2)

CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LDL, low density lipoprotein; NEFA, non-esterified fatty acids; RCT, reverse cholesterol transport; TAG, triacylglycerol; VLDL, very low density lipoprotein.

The dyslipidaemic profile observed in subjects with T2DM and/or the metabolic syndrome is associated with increased CVD risk. In addition postprandial hyperlipidaemia exists, in the form of elevated TAG. This is a consequence of excess production of VLDL and defective postprandial clearance chylomicrons (Coppack, 1997). Epidemiological and mechanistic studies have identified that perturbations of the postprandial state are involved in CVD (Stampfer et al, 1996; Karpe et al, 1994; Tushuizen et al, 2005). Identification and correction of the abnormalities of the postprandial state must form part of the strategy for the prevention and management of CVD, particularly those that are associated with diabetes mellitus and the metabolic syndrome.

1.4 INFLAMMATION AND PROTHROMBOTIC RISK IN T2DM AND THE METABOLIC SYNDROME

CVD is the leading cause of mortality in subjects with T2DM, accounting for 40-50% of all deaths (De Marco *et al*, 1999). Subjects with T2DM have a 2-10 fold increased mortality risk for coronary, cerebrovascular and peripheral vascular disease compared to healthy subjects (Stamler *et al*, 1993; Manson *et al*, 1991). Although classical risk factors, namely plasma lipoprotein concentrations, identify individuals at increased risk for CVD, one-half of all myocardial infarctions occur in persons in whom plasma lipid levels are normal (Das, 2002). In addition, it is estimated that the relative risk reduction achieved by lipid-lowering therapy is only approximately 30% (Depres, 2003).

Obesity, T2DM and atherosclerosis are now all recognised to be associated with a state of abnormal inflammatory response (Hotamisligil, 2003; Linton & Fazio, 2003). Atherosclerosis is the main cause of CHD. It is a progressive condition characterised by deposition of complex lipids in the innermost layer of the arterial wall (intima), principally in the large and medium sized elastic and muscular arteries, and can lead to ischaemia of the heart, brain or extremities, resulting in infarction (Ross, 1999). In addition to the lipid disorder, a chronic inflammatory condition is initiated and progressed in the context of hypercholesterolaemia (Steinberg, 2002). Macrophages contribute to the local inflammatory responses through production of cytokines, free oxygen radicals, nitric oxide, proteases and complement factors. The uptake of modified lipoproteins by macrophages leads to the accumulation of cholesterol esters and formation of macrophage-derived foam cells, the hallmark of fatty streak, and the advancement of atherosclerotic lesion development (Linton & Fazio, 2003). A number of systemic markers have been proposed to identify this additional risk presented by the exacerbated inflammatory response.

C-reactive protein (CRP) is an unspecific acute phase reactant that serves as an excellent indicator of systemic inflammation (Rajala & Scherer, 2003). CRP is synthesised predominantly in the liver largely under the regulation of the pro-inflammatory cytokine interleukin (IL)-6, although TNFα and IL-1 also contribute (Yudkin *et al*, 2000). Circulating levels of CRP are increased in obesity, insulin resistance, T2DM and CHD (Sonnenberg *et al*, 2004; Trayhurn & Wood, 2004). Conversely concentrations fall with weight loss (Trayhurn & Wood, 2004). CRP enhances the uptake of LDL by macrophage thereby promoting foam cell formation, and also stimulates macrophages to express cytokines (Tracy, 2003). Measurement of CRP has been viewed as particularly useful in the assessment of CVD risk, as elevated CRP concentrations are additive to total cholesterol:HDL-cholesterol in determining future risk of myocardial infarction (MI) (Ridker, 1999).

Levels of adhesion molecules, namely vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and E-selectin, are closely related to measures of

obesity and visceral fat accumulation, abnormalities in lipid metabolism, essential hypertension, insulin resistance and T2DM (Sonnenberg *et al*, 2004). Secretion of adhesion molecules is stimulated by cytokines through nuclear factor-κB (NF-κB) activation (Sonnenberg *et al*, 2004). Adhesion molecules contribute to endothelial dysfunction and aggravates intimal-medial thickening (Sonnenberg *et al*, 2004).

Disturbances of the haemostatic system may favour the development of vascular damage and consequently a pro-thrombotic risk profile is associated with an increased risk of CVD. Fibrinogen has also been proposed as a novel cardiovascular risk factor to aid in predicting cardiovascular events (Ridker, 1999). Fibrinogen promotes a hypercoagulable state by increasing platelet cross-linking and aggregation, thereby enhancing clot formation. Increased fibrinogen concentrations relative to a given amount of thrombin, also confers resistance to fibrinolysis (Tracy, 2003). Alterations in serum fibrinogen, plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator and von Willebrand factor, promote a pro-thrombotic risk profile, and have been shown to contribute to the increased risk of vascular events in subjects with insulin resistance and T2DM (Frohlich & Steiner, 2000; Juhan-Vague *et al*, 1996).

Inflammatory mediators have also been shown to directly influence insulin sensitivity. This will be discussed in Section 1.5.3.

1.5 ADIPOSE TISSUE AND INSULIN SENSITIVITY

1.5.1 Adipose tissue biology

Adipose tissue comprises two tissue types, white and brown adipose tissues, which have distinct structural features, as well as function. Both tissues collaborate in partioning the energy contained in lipids between thermogenesis and metabolic functions (Cinti, 2001). Brown adipose tissue consists of multilocular adipocytes which are rich in mitochondria expressing uncoupling protein (UCP)-1, which is responsible for the thermogenic activity of the tissue (Cinti, 2001). In humans, brown adipose tissue is present in neonates and rapidly diminishes with age (Sell et al, 2004). Consequently it is thought to have less relevance in whole body physiology than white adipose tissue. Both brown adipose tissue and white adipose tissue has its own discrete vascular and nerve supply, although that in brown adipose tissue is richer.

White adipose tissue is unique in that it is the only tissue in the body that can dramatically change its mass after adulthood is reached. Consequently, fat mass may be as low as 2-3% in elite athletes, or may increase to 60-70% in morbid obesity. Typical fat mass for a male is between 9-18% body weight, and for a female between 14-28% body weight (Hausman *et al*, 2001). The cellular composition of adipose tissue can vary substantially

depending on anatomical location and body weight (Hauner, 2005). White adipose tissue depots comprise unilocular adipocytes, but also stromal-vascular cells, blood vessels, lymph nodes and nerves (*Table 1.6*) (Cinti, 2001; Hausman *et al*, 2001). Regional differences have been reported to be most pronounced between omental and subcutaneous depots (Hauner, 2005).

Table 1.6: Cellular components of human adipose tissue (Adapted from Hauner, 2005).

	%
Mature adipocytes	50-70
Stromal 'preadipocytes'	20-40
Endothelial cells	1-10
Macrophages	1-30
Other cell types	?

1.5.2 The role of adipose tissue in the pathogenesis of insulin resistance

(i) Non-esterified fatty acids

Insulin resistance is a fundamental aspect of the aetiology of T2DM and the metabolic syndrome (Wallace & Matthews, 2002). There are at least two hypotheses proposed to explain how adipose tissue may influence glucose homeostasis and act as a key aetiological factor in the development of insulin resistance and the metabolic syndrome. Firstly excessive adipose tissue storage in obesity leads to increased NEFA flux to peripheral tissues (Kahn & Flier, 2000). The distribution of body fat is critical, with central or visceral adiposity associated with chronic disease, even in the absence of obesity (Boyko *et al*, 2000). Visceral adiposity is deemed more atherogenic as it is more metabolically active than subcutaneous fat, and therefore releases more NEFAs. In addition, NEFAs released from visceral depots have direct access to the liver via the portal circulation, whereby it can modulate hepatic glucose metabolism (Fujimoto, 2000).

Both fasting and postprandial NEFA concentrations are elevated in T2DM and those at risk of the disease (Coppack, 1997; Bergman & Ader, 2000). Elevated plasma NEFAs influences key organs involved in regulating glucose homeostatis, namely the pancreas, liver and skeletal muscle. In skeletal muscle NEFAs impair insulin-stimulated glucose uptake e.g. a 3 fold increase in NEFA (above basal) was shown to suppress glucose uptake by 50% in individuals with NGT (Bergmen & Ader, 2000; Boden, 2002). Evidence gathered from animal studies indicates that chronic exposure to high levels of NEFA in plasma impairs insulin secretion from the pancreatic β-cell (Bergman & Ader, 2000). Longitudinal data in humans indicate that elevated plasma NEFA precedes a demonstrated β-cell defect (Bergman & Ader,

2000). Additionally in the liver, elevated NEFA's impairs the ability of insulin to suppress hepatic glucose output (Kahn & Flier, 2000). While saturated NEFAs have been shown to have a detrimental effect, studies are inconclusive regarding the effect of monounsaturated and polyunsaturated NEFAs.

Elevated plasma NEFAs promotes TAG storage in the peripheral tissues, as well as increasing intracellular concentrations of NEFAs (Saltiel, 2001; Nawrocki & Scherer, 2004; Kahn & Flier, 2000). Several studies have demonstrated a significant correlation between intramyocellular TAG and insulin resistance in humans (Borkman *et al*, 1993; Szczepaniak *et al*, 1999; Krssak *et al,* 1999; Perseghin *et al*, 1999). Insulin resistance is also associated with high levels of TAG in liver and pancreatic islets (Frayn, 2001). This hypothesis of lipid accumulation in non-adipose tissues attenuating insulin sensitivity is supported by evidence from transgenic mouse models of lipoatrophy. These mice present with reduced or absent fat, a 2 fold increase in intramuscular and intrahepatic TAG, and severe insulin resistance. Transplantation of adipose tissue ameliorates the condition (Colombo *et al*, 2002). However there is controversy whether the observed improvement in insulin sensitivity is a function of the storage capacity of adipose tissue, or whether it is a consequence of the secretory products derived from adipose tissue influencing insulin sensitivity.

(ii) Secretory capacity of adipose tissue

A second hypothesis proposed to explain how adipose tissue may act as an aetiological factor in the development of insulin resistance, reflects on the secretory capacity of adipose tissue. Traditionally white adipose tissue was seen as a lipid-storing organ that mobilises fatty acids in case of increased energy demand or shortage of energy supply, in addition to providing thermal and mechanical insulation (Trayhurn & Wood, 2004; Hauner, 2005). However it is now evident that white adipose tissue is a secretory organ with potent autocrine, paracrine and endocrine functions, allowing it to maintain a complex communication with many other organs, far beyond what it's role in fuel storage would imply (Berg *et al*, 2002; Hauner, 2005). Some of these secretory factors, termed adipokines, have a direct effect on insulin sensitivity (Kahn & Flier, 2000). It has been suggested that due to the close spatial proximity, adipocytes dispersed within skeletal muscle may exert a strong influence on the surrounding muscle fibres through paracrine secretion of adipokines (Dietze *et al*, 2002). However these secretory products may also exert systemic effects (Hauner, 2005). This realisation raises many possibilities for potential links between adipose tissue and insulin resistance, independent of the adipocytes' role in energy storage and release. This thesis will focus on the second hypothesis and Section 1.5.3 will discuss adipokines in more detail.

1.5.3 Adipokines

The term "adipokine" had been coined to describe a protein that is synthesised by and secreted from adipocytes (Trayhurn & Wood, 2004). To date > 100 products, covering a broad range of protein families as well as many fatty acids and prostaglandins, have been reported to be secreted from white adipose tissue (Hauner, 2005). These products exert multiple effects at both local and systemic levels, thereby playing a central role in whole body homeostasis. *Fig. 1.2* illustrates the diversity in secretory products from white adipose tissue.

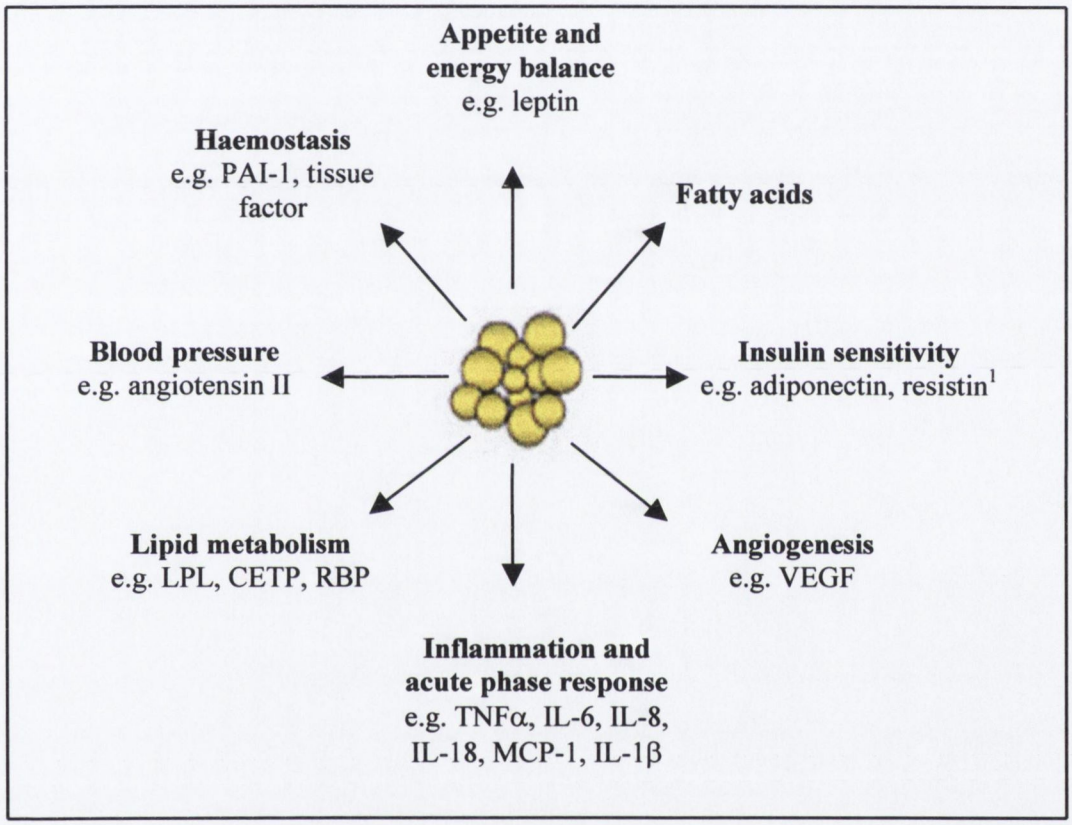


Fig. 1.2: Adipokines classified by functional role (Adapted from Trayhurn & Wood, 2004; Hauner, 2005; Doria, 2003).

CETP, cholesteryl ester transfer protein; IL, interleukin; LPL, lipoprotein lipase; MCP-1, monocyte chemoattractant protein 1; PAI-1, plasminogen activator inhibitor-1; RBP, retinol binding protein; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor.

[1] Main source of resistin in humans is macrophage, and mRNA expression levels have been reported to be very low in human adipose tissue (Nawrocki & Scherer, 2004; Banerjee & Lazar, 2003).

Since adipokines have been shown to exert such profound effects on metabolism, variability in adipokine secretion has been proposed to play an important role in the pathophysiology of insulin resistance and other components of the metabolic syndrome (Doria, 2003; Kahn & Flier, 2000). Therefore it is critical to identify and maintain an awareness of the cellular composition within an adipose tissue depot, as any cell type could potentially

contribute to the secretory function of the tissue (Hauner, 2005). Recent evidence showing that white adipose tissue is infiltrated by macrophages in obesity, clearly suggests that the non-adipocyte fraction may constitute a significant component of the inflammatory state within adipose tissue (Weisberg *et al*, 2003; Xu *et al*, 2003). In addition endothelial cells and sympathetic nerve fibres contribute to adipose tissue function (Hauner, 2005). However it should be emphasised that quantitatively fatty acids are the major secretory product of white adipose tissue, reflecting the role of the tissue as a fuel reserve (Trayhurn & Beattie, 2001). The secretory products which are implicated in insulin resistance are illustrated in *Fig. 1.3*, and will be discussed in the following sections.

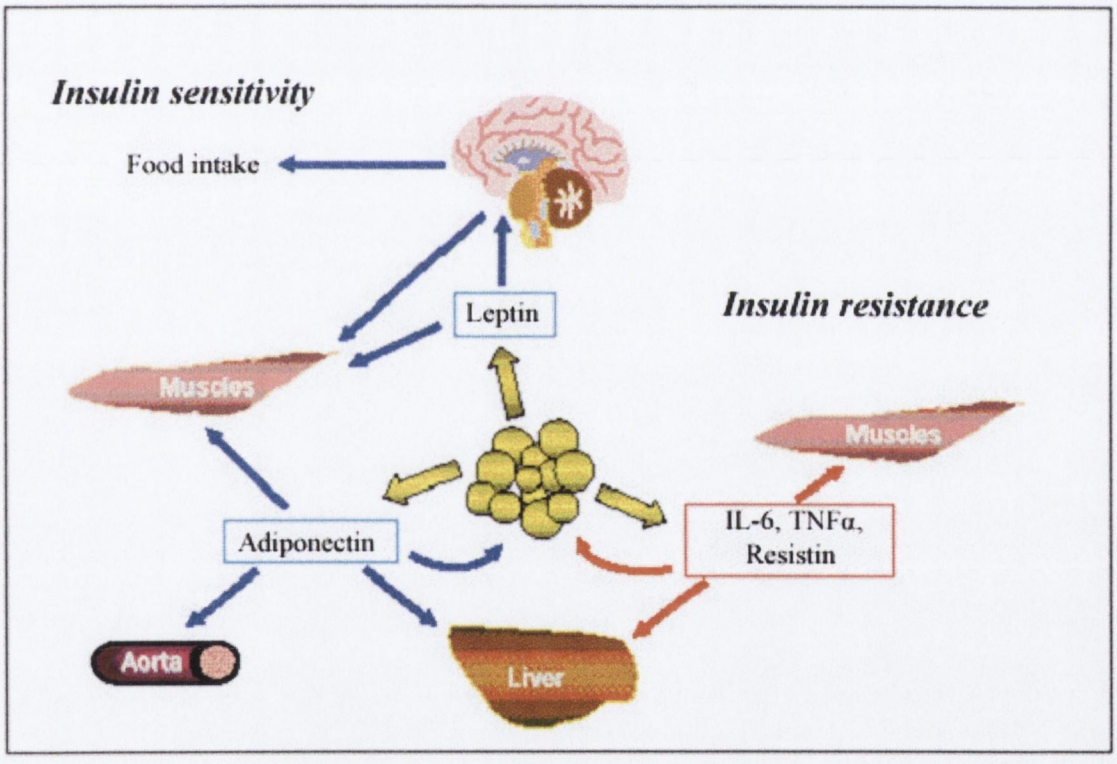


Fig. 1.3: Secretory factors from adipose tissue which modulate insulin sensitivity (Adapted from Guerre-Millo, 2003; Guerre-Millo, 2004).

IL, interleukin; TNFα, tumour necrosis factor α.
← Blue arrows indicates an increase in insulin sensitivity.
← Red arrows indicate a decrease in insulin sensitivity or conversely an increase in insulin resistance.

(i) TNFα

TNFα was the first cytokine discovered to be expressed in and secreted by white adipose tissue (Hotamisligil *et al*, 1993). Evidence suggests that there is a hierarchy of cytokines within white adipose tissue, so that some of the "higher order" cytokines such as TNFα may play a pivotal role in relation to the synthesis, secretion and activity of others (Coppack, 2001). TNFα is secreted as a membrane-bound precursor which is subsequently acted upon by TNFα converting enzyme, a matrix metalloproteinase, which cleaves off soluble TNFα comprising 157 amino acids (Gearing *et al*, 1994; McDermott, 2001). Membrane-bound and soluble TNFα can interact with receptors, tumour necrosis factor receptor (TNFR)-1 and TNFR-2. In humans these are known as gp60/TNFR60 and gp80/TNFR80 respectively, and in mice the p55 receptor and the p75 receptor respectively (Coppack, 2001). A clear consensus of the biological importance of each receptor remains to be resolved (Coppack, 2001).

Hotamisligil *et al* (1993) first reported elevated levels of TNFα in serum and adipose tissue in rodent models of obesity. They also demonstrated an association between elevated TNFα and impaired insulin action in these animals, as an infusion of a soluble TNFα-antibody ameliorated insulin sensitivity. Subsequently an up-regulation of the TNFα system was reported in obese humans with studies demonstrating increased adipose expression of TNFα and the two receptor subtypes, gp60/TNFR60 and gp80/TNFR80, with increased adiposity (Hotamisligil *et al*, 1995; Kern *et al*, 1995; Hube *et al*, 1999). White adipose tissue TNFα messanger ribonucleic acid (mRNA) is increased with weight gain and obesity and decreases with weight loss and improvement of insulin sensitivity (Guerre-Millo, 2003; Hotmaisligil *et al*, 1993; Moller *et al*, 2000). Regional differences have been observed with higher mRNA expression of both TNFα and it's receptors reported in subcutaneous white adipose tissue compared to omental tissue (Hube *et al*, 1999). Several lines of evidence suggest that TNFα mediates its effects in an autocrine-paracrine manner rather than exerting systemic effects:

- Normal circulating levels of the cytokine are at the limit of detection making quantitative analysis difficult (Kahn & Flier, 2000)
- TNFα concentrations are not increased in venous blood draining subcutaneous adipose tissue (Mohamed-Ali *et al*, 1997)
- Treatment of obese and diabetic individuals with TNFα neutralising antibodies over a 4 wk period did not improve insulin sensitivity, suggesting that systemic inhibition of TNFα might not effect insulin sensitivity (Ofei *et al*, 1996).

It was originally thought that most of the mRNA for TNFα was found within adipocytes. However recent evidence suggests that in humans the majority of TNFα secretion may come from the cells of the stromal vascular and matrix fractions, including the macrophages (Weisberg *et al*, 2003, Xu *et al*, 2003; Fain *et al*, 2004; Trayhurn & Wood, 2004).

TNFα knockout animals have lower insulin and NEFA concentrations, and increased insulin sensitivity compared to their wild type littermates when placed on an equivalent diet (Uysal *et al*, 1997; Ventre *et al*, 1997). Rodents with TNFα receptor knocked out also show improved insulin sensitivity compared to control animals, even when obese (Hotamisligil *et al*, 1993). It has been hypothesised that the function of TNFα is to limit white adipose tissue expansion, and that insulin resistance and the concomitant disturbances in glucose and lipid metabolism is a consequence of that. *In vitro* TNFα has been shown to induce apotosis both in mature adipocytes and in preadipocytes (Prins *et al*, 1997), with omental preadipocytes more sensitive to this effect than subcutaneous preadipocytes (Niesler *et al*, 1998). Additionally TNFα blocks the differentiation of new adipocytes and induces the de-differentiation of both preadipocytes and mature adipocytes (Pekala et al, 1983; Torti et al, 1989; Petruschke & Hauner, 1993). However rodents with either TNFα or the TNFα receptor knocked out, showed improvements in insulin sensitivity while maintaining a similar body weight to their control (Uysal *et al*, 1997; Ventre *et al*, 1997). Nevertheless, the insulin de-sensitising effects of the TNFα are well characterised. *Table 1.7* summarises these effects.

Table 1.7: Effects of TNFα in adipocytes (Adapted from Coppack, 2001).

Effect exerted by TNFα	Mechanism	References
Increases Insulin resistance	↓ tyrosine autophosphorylation of the insulin receptor	Hotamisligil *et al*, 1994
	↑ serine phosphorylation of IRS-1	Hotamisligil *et al*, 1996
	↓ Glut4 mRNA	Stephens & Pekala, 1991
	↑ IL-6 mRNA[1], ↑ PAI-1 mRNA[1]	Sonnenberg *et al*, 2004
	↓ adiponectin mRNA and secretion[1]	Guerre-Millo, 2004
Increases lipolysis	↑ HSL mRNA	Sumida *et al*, 1997
Inhibits lipogenesis	↓ LPL mRNA, ↓ FAS mRNA	Feingold & Grunfeld, 1992

FAS, fatty acid synthase; Glut4, glucose transporter 4; HSL, hormone sensitive lipase; IL, interleukin; IRS, insulin receptor substrate; LPL, lipoprotein lipase lipase; PAI-1, plasminogen activator inhibitor-1; TNFα, tumour necrosis factor α.

[1] By influencing the expression of these adipokines, TNFα can influence insulin sensitivity in tissues remote from adipose tissue.

(ii) IL-6

IL-6 is secreted by activated macrophage and lymphocytes, but evidence now exists that white adipose tissue also secretes it (Yudkin *et al*, 2000). Human white adipose tissue secretion of IL-6 represents approx 10-30% of circulating levels (Mohamed-Ali *et al*, 1998). In contrast to TNFα, omental white adipose tissue secretes substantially more IL-6 than subcutaneous white adipose tissue (Coppack, 2001). Isolated fat cells secrete substantially less IL-6 than whole

white adipose tissue, indicating that cells other than adipocytes are the major source of the cytokine in white adipose tissue (Hauner, 2005). IL-6 expression and secretion is induced by IL-1 and TNFα (Pang *et al*, 1994). IL-6 in turn regulates these cytokines, by directly influencing TNFα, and promoting the release of IL-1 from IL-1 receptor antagonist, and inducing activity (Yudkin *et al*, 2000). IL-6 is unusual among cytokines as most function via autocrine-paracrine mechanisms. Instead the major effects of IL-6 take place at sites distinct from its origin and are consequent upon circulating concentrations (Yudkin *et al*, 2000). The biological activities of IL-6 are initiated by binding to a high-affinity receptor complex, consisting of 2 membrane glycoproteins. The 80kDa ligand binding component binds IL-6 with low affinity, while a second 130kDa signal-transducing component, although not binding free IL-6, is required for affinity binding of gp80-bound IL-6. It has been suggested that elevated levels of IL-6 are associated with increased production of IL-6 receptors. (Mohamed-Ali *et al*, 1998).

IL-6 may play a central role in the pathophysiology of CVD, as this adipokine is believed to be the major regulator of the hepatic acute phase response (Yudkin *et al*, 2000). Clinical studies have shown that circulating concentrations of IL-6 are elevated in obesity (Hauner, 2005). Furthermore prospective studies have indicated that elevated serum concentrations of IL-6 are predictive for the development of both T2DM and CVD (Ridker *et al*, 2000; Spanger *et al*, 2003). *Table 1.8* highlights some of the molecular effects of this cytokine.

Table 1.8: Effects of IL-6 on insulin resistance and atherothrombotic risk.

Effect exerted by IL-6	Mechanism	References
Induces Insulin Resistance	↑ expression of SOCS-3 which inhibits insulin receptor autophosphorylation	Senn *et al*, 2003
	↓ glycogen synthesis following insulin stimulation of isolated hepatocytes	Kanemaki *et al*, 1998
	↓ IRS-1, Glut4 and PPARγ mRNA expression in adipocytes	Rotter *et al*, 2003
	↓ insulin-stimulated glucose uptake in skeletal muscle, which was associated with defects in insulin-stimulated IRS-1-associated PI 3-kinase activity	Kim *et al*, 2004
Promotes Atherothrombotic risk	↑ fibrinogen and CRP production by the liver	Woods *et al*, 2000
	↑ aggregability of thrombocytes	Yudkin *et al*, 2000
	↑ expression of adhesion molecules by endothelial cells	Hardardóttir *et al*, 1994
	↑ uptake of lipids by macrophages	Coppack , 2001
	↑ lipolysis	
	↓ HDL-cholesterol	
	↓ LPL	

CRP, C-reactive protein; Glut4, glucose transporter 4; IL, interleukin; IRS, insulin receptor substrate; PI, phosphoinositide; PPAR, peroxisome proliferator-activated receptor; SOCS, suppressors of cytokine signalling.

However paradoxically IL-6 deficient mice developed mature-onset obesity associated with glucose intolerance, elevated TAG and leptin insensitivity (Wallenius *et al*, 2002). These data suggest that elevated IL-6 is not the underlying cause for metabolic disturbances in insulin resistance but rather, that the balance of IL-6 is important (Nawrocki & Scherer, 2004). The challenge will be to design interventions that attenuate the deleterious effects of IL-6 on insulin resistance and atherothrombotic risk, without compromising its beneficial role in the innate immune response (Yudkin *et al*, 2000).

(iii) Adiponectin

Adiponectin is a 30kDa protein secreted exclusively from adipocytes. Adiponectin is also called acrp30 (adipocyte complement related protein of 30kDa), adipoQ, ApM1 (adipose most abundant gene transcript) and GBP28 (gelatin-binding protein of 28kDa) due to cloning or isolation by different groups (Guerre-Millo, 2004). Adiponectin consists of an N-terminal collagenous domain, followed by a C-terminal globular trimerisation domain. It is found in serum in two higher order structures, a dimer of trimers which is termed a low molecular

weight (LMW) complex, and a larger complex of 12-18 subunits which is termed a high molecular weight (HMW) complex (Pajvani *et al*, 2003). These oligomerisations influence its biological activity (Miner, 2004). Pajvani *et al* (2004) proposed that the ratio between the two oligomeric forms (HMW to LMW) is important in determining insulin sensitivity, rather than the absolute amount of each form, and suggested that the HMW complex constitute the active form of adiponectin. Two murine receptors have recently been cloned, Adipo R1 and Adipo R2, which are expressed predominantly in muscle and liver, respectively (Yamauchi et al, 2003; Guerre-Millo, 2004).

Adiponectin is relatively abundant in serum representing 0.05% of circulating proteins (Doria, 2003). Serum concentrations are 2-3 times higher in females than in males (Combs *et al*, 2003; Scherer *et al*, 1995). This adipokine is unique in that expression and circulating levels are decreased with increasing adiposity, especially in the visceral depot (Cancello *et al*, 2004). It is negatively correlated with insulin resistance and T2DM, independent of body weight (Hauner, 2005; Rajala & Scherer, 2003). Interestingly baseline plasma levels of adiponectin in healthy individuals are an independent risk factor for the development of T2DM, and are also associated with the development of metabolic syndrome and atherosclerosis i.e. a negative relationship exists (Spranger *et al*, 2003; Hauner, 2005). Nutritional and therapeutic manipulations including weight loss, calorie restriction and thiazolidinedione (TZD) treatment, which are known to ameliorate insulin sensitivity, also increase adiponectin gene expression in white adipose tissue and plasma concentrations (Bruun *et al*, 2003; Combs *et al*, 2003; Yu *et al*, 2002b). Both TNFα and IL-6 are potent inhibitors of adiponectin expression and secretion in human white adipose tissue biopsies and cultured adipose cells, suggesting that TNFα and IL-6 induced insulin resistance might rely in part on an autocrine-paracrine inhibition of adiponectin release (Fasshauer *et al*, 2002; Fasshauer *et al*, 2003; Bruun *et al*, 2003). Evidence suggests that adiponectin also regulates TNFα metabolism, as elevated TNFα levels have been reported in the adiponectin knockout mouse, and adenoviral infection of adiponectin normalises serum TNFα concentrations (Yamauchi *et al*, 2002).

Animal studies have indicated that administration of recombinant adiponectin, either full length or in the form of its isolated globular head, ameliorates insulin resistance in murine models of obesity and diabetes by decreasing gluconeogenesis in the liver and decreasing lipid accumulation in non-adipose tissues (Tsao *et al*, 2002; Berg *et al*, 2002). The insulin-sensitising effect of adiponectin is mediated, at least in part, by an increase in fatty-acid oxidation through activation of AMP-activated kinases (AMPK) in skeletal muscle, resulting in reduced accumulation of TAG (Tomas *et al*, 2002). The reduction in gluconeogenesis was due to down-regulation of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate (PEPCK), two key gluconeogenic enzymes, inhibition of which would lead to an attenuation in

hepatic glucose production (Combs *et al*, 2001). Additionally liver AMPK has been shown to be activated, resulting in reduced rate of hepatic glucose production (Yamauchi *et al*, 2002; Combs *et al*, 2001). These glucose-lowering effects were independent of any change in insulin secretion, peripheral glucose uptake, glycolysis, or glycogen synthesis suggesting that the liver is the primary site of adiponectin bioactivity (Nawrocki & Scherer, 2004; Trujillo & Scherer, 2005). The ability of adiponectin to suppress glucose production was reproduced *in vitro* in isolated primary hepatocytes (Rajala & Scherer, 2003).

In addition adiponectin has anti-atherogenic properties, as shown by its capacity to inhibit the expression of adhesion molecules on aortic endothelial cells, inhibit monocyte adhesion to endothelial cells, inhibit macrophage-to-foam-cell transformation *in vitro* and to suppress human aortic smooth muscle cell proliferation (Ouchi *et al*, 1999; Tsao *et al*, 2002; Berg *et al*, 2002; Arita *et al*, 2002). Adiponectin-null mice presented with severe insulin resistance and impaired glucose metabolism when fed a high-fat, high-sucrose diet. Knockouts mice also developed intimal thickening in response to endothelial injuiry, thus confirming the protective role of the protein against atherosclerosis and diet-induced insulin resistance (Maeda *et al*, 2002; Kubota *et al*, 2002).

(iv) Resistin

Resistin is a 12.5kDa cysteine rich protein that was identified by Steppan *et al* (2001) as being acutely up-regulated during the differentiation of preadipocyte to adipocytes. Resistin belongs to a family of molecules named FIZZ proteins (found in inflammatory zone). It is also known as adipose tissue-specific secretory factor (ADSF). In mice, resistin mRNA is predominantly expressed in white adipose tissue, with lower levels in brown adipose tissue and mammary tissues, and no detectable expression in any other tissues (Steppan *et al*, 2001). It is secreted by adipocytes, and is detectable in serum (Doria, 2003). Resistin like molecules (RELM)-α and β, are also expressed by adipose (RELMα) and various tissues (RELM β) and demonstrate 29% and 37% sequence identity to resistin respectively (Blagoev *et al*, 2002; Miner *et al*, 2004). Resistin can dimerise with itself or with RELMα, an oligerimersation which is requisite to biological activity (Miner, 2004).

Expression of resistin is reduced on fasting, with a parallel fall in the circulating level of the protein, and is increased during refeeding. Both gene expression and circulating levels of the molecule are markedly increased in obese, insulin resistant mice (Way *et al*, 2001; Le Lay *et al*, 2001) and during adipogenesis (Banerjee & Lazar, 2003). Surprisingly TNFα consistently decreases resistin expression, and IL-6 seems to have no effect (Banerjee & Lazar, 2003). Although resistin was originally cloned on the criteria of its expression being reduced by TZDs, treatment of animals with these insulin-sensitising drugs has produced an inconsistent pattern regarding resistin regulation (Guerre-Millo, 2004). However it has been

suggested that the length of treatment is critical in determining the regulation of resistin by TZD's, whereby a couple weeks is successful in lowering resistin concentrations, whereas a couple day's treatment is ineffective (Banerjee & Lazar, 2003).

Resistin has been shown to induce insulin resistance. Administration of recombinant protein to wild-type mice impaired glucose tolerance and insulin action. Correspondingly treatment with antibodies for resistin improved glycaemia and reversed insulin resistance (Steppan *et al*, 2001). In adipocytes, resistin has been shown to antagonise both basal and insulin-stimulated glucose uptake in cultured 3T3-L1 adipocytes (Steppan *et al*, 2001) and to inhibit 3T3-L1 differentiation into mature adipocytes (Kim *et al*, 2001). Additionally chronic exposure of resistin in L6 skeletal muscle cells inhibited glucose uptake (Moon *et al*, 2003), and elsewhere it inhibited C2C12 myoblast differentiation into myotubes (Miner, 2004). Nawrocki & Scherer (2004) demonstrated that the infusion of resistin in rats induced severe hepatic insulin resistance, which was entirely attributable to an increased rate of glucose production. They confirmed these results in mice with a genetic deletion of the resistin gene, further high-lighting the liver as the primary site of action for this protein. Resistin knockout mice display increased phosphorylation of hepatic AMPK, suggesting that resistin inhibits AMPK activation under normal conditions (Banerjee *et al*, 2004).

A human homolog of murine resistin has been identified, however its sequence and expression in white adipose tissue are quite different from that in rodent. The amino acid sequence is only 59% identical to the murine counterpart, compared to adiponectin which is 83% identical to its murine counterpart (Steppan & Lazar, 2004; Berg *et al*, 2002). As a result it is not clear whether this protein plays a significant role in the development of insulin resistance in humans (Savage *et al*, 2001). In contrast to murine models, resistin is preferentially expressed in humans in macrophage and several groups have reported very low mRNA expression levels in adipose tissue (Patel *et al*, 2003; Savage *et al*, 2001; Nawrocki & Scherer, 2004; Banerjee & Lazar, 2003). Identification of the receptor, the signalling pathways and analysis of the phenotypes resulting from deletion or over-expression of resistin will help our understanding in defining the biological relevance of resistin in obesity and insulin resistance (Guerre-Millo, 2004).

(v) Leptin

Leptin is produced specifically by adipocytes, and is secreted and transported in the blood to receptors in numerous tissues (Miner, 2004). Leptin's main site of action is the hypothalamus (Flier, 1998). The discovery of leptin (Zhang *et al*, 1994) as an essential factor involved in long-term regulation of food intake, body weight, energy expenditure and neuroendocrine function has increased our understanding of the mechanisms underlying the development of obesity and its complications. Either a deficiency of leptin, as occurs in the ob/ob mouse

strain, or its receptor, as occurs in the db/db mouse strain, results in a severe insulin resistant phenotype (Kahn & Flier, 2000). This condition is rapidly ameliorated by leptin administration in the ob/ob mouse, even before reduction in body weight is observed. Furthermore the insulin-sensitising effect of leptin exceeds that seen in pair-fed animals (Halaas *et al*, 1995).

Accumulating evidence suggests that leptin improves insulin sensitivity by promoting fatty acid oxidation and reducing ectopic fat accumulation in non-adipose tissue (Muoio *et al*, 1997; Shimabukuro *et al*, 1997). This effect is mediated by activation of AMPK by an early direct effect of leptin on certain skeletal muscles, followed by a later indirect effect of AMPK activation by leptin through the hypothalamic-sympathetic nervous axis (Minokoshi *et al*, 2002). When activated AMPK phosphorylates Ser-221 of acetyl-CoA carboxylase (ACC) which suppresses the activity of the enzyme (Rutter *et al*, 2003). ACC catalyses the synthesis of malonyl-CoA (Rudderman *et al*, 2003). Consequently there is a decrease in the concentration of malonyl CoA. Malonyl-CoA is an allosteric inhibitor of carnitine palmitoyltransferase 1, the enzyme which regulates the rate at which long-chain fatty acyl-CoA are transferred from the cytosol into mitochondria where they are oxidized (Rudderman *et al*, 2003). Thus, phosphorylation of ACC by AMPK leads to inhibition of ACC activity, a fall in malonyl-CoA content, and a subsequent increase in fatty-acid oxidation of long-chain acyl-CoAs by mitochondria via dis-inhibition of carnitine palmitoyltransferase in skeletal muscle (Neilsen *et al*, 2003).

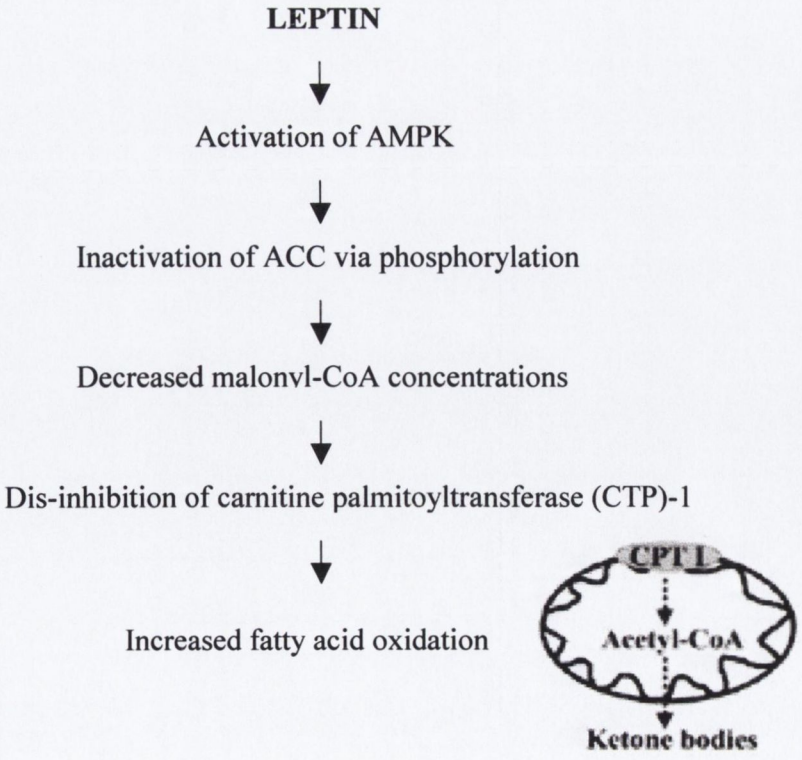


Fig. 1.4: AMPK activation by leptin. (McGarry, 2002; Rudderman *et al*, 1999)

In addition, AMPK decreases fatty acid incorporation into glycerolipids, either secondary to its effect on fatty acid oxidation or by virtue of the fact that in some tissues it phosphorylates and inhibits *sn*-glycerophosphate acyltransferase, the first committed enzyme in diacylglycerol and triglyceride synthesis (Rudderman *et al*, 2003).

Although recombinant leptin administration can produce profound effects on energy expenditure and feed intake when accompanied with the leptin-deficient state, administration of leptin to normal or obese hyperleptinaemic mice or humans produces unremarkable effects. Additionally humans with mutations of leptin or the leptin receptor present with obesity but do not appear to have insulin resistance, as assessed by hyperinsulinaemia, and no reports of T2DM have been described (Kahn & Flier, 2000). However leptin administration may offer potential as a new treatment to ameliorate insulin sensitivity in lipoatrophic diabetes, where low leptin prevails (Shimomura *et al*, 1999; Ebihara *et al*, 2002). Insulin resistance was fully reversed in lipoatrophic mice following a combination of physiological doses of adiponectin and leptin, whereas only partial restoration of insulin sensitivity was observed when either adipokine was given alone (Yamauchi *et al*, 2001). In addition, over-expression of globular adiponectin in transgenic mice in the background of the ob/ob mutation reverses insulin resistance without affecting body weight (Pajvani *et al*, 2003). This suggests that although adipokines have distinct functions, there may be synergy also (Guerre-Millo, 2004).

(vi) Other cytokines

Several other cytokines are also synthesised within white adipose tissue but their role in insulin sensitivity is not as well established as that of TNFα and IL-6. Adipocyte production, modulation and secretion of IL-8, has been described *in vitro*, and a relationship has been observed between increased IL-8 levels and obesity (Cancello *et al*, 2004; Straczkowski *et al*, 2003). Elevated levels of IL-18 have also been reported in obese individuals, and conversely levels were reduced with weight loss (Esposito *et al*, 2002). In addition IL-17 mRNA has recently been described in adipocytes, although its release from adipocytes has not been documented yet (Trayhurn & Wood, 2004). Interestingly circulating levels of the anti-inflammatory cytokine IL-10 are raised in obese women (Cancello *et al*, 2004). The role these cytokines play, if any, in modulating insulin sensitivity and glucose tolerance remains to be determined.

Fukuhara *et al* (2005) recently identified a novel adipokine which is preferentially produced in visceral adipose tissue, which they named visfatin. Visfatin is highly enriched in the visceral fat of both humans and mice, and plasma concentrations increase during the development of obesity. The authors indicate that visfatin exerted insulin-mimetic effects in cultured adipocytes, myocytes and hepatocytes, and lowered plasma glucose concentrations in

both insulin-resistant and insulin-deficient mice. Heterozygous mice displayed modestly higher levels of plasma glucose relative to wild-type littermates. Visfatin represents another adipokine which may aid our understanding of glucose homeostasis, and potentially be a useful target for ameliorating insulin resistance.

Altered expression and activity of adipokines may predispose to insulin resistance and other components of the metabolic syndrome. It is likely that all adipokines discussed contribute to insulin resistance, but other factors must also exist. Research is on-going worldwide aimed at identifying single nucleotide polymorphisms (SNPs) of adipokines, which may predispose to insulin resistance. In particular several groups have identified a variety of SNPs associated with the adiponectin and resistin loci, but to date there is no clear consensus of an association with T2DM or obesity (Doria, 2003; Banerjee & Lazar, 2003).

1.6 INSULIN

In healthy individuals plasma glucose concentrations remain in a narrow range, typically between 4 and 7mmol/L, despite periods of feeding and fasting. This tight control is governed by the balance of glucose absorption from the intestine, endogenous production by the liver and uptake and metabolism by peripheral tissues (Saltiel & Pessin, 2002). Insulin directs energy metabolism during the postprandial phase, and in doing so is the only hormone committed to the prevention of hyperglycaemia (Bailey, 1999). Insulin is a pleiotrophic hormone exerting an anabolic effect (Pessin & Saltiel, 2000).

Elevated insulin concentrations in the postprandial state promotes the uptake of glucose in skeletal muscle and adipose tissue, and inhibits hepatic glucose production, thereby serving as the primary regulator of plasma glucose concentrations (Saltiel & Pessin, 2002). Insulin also promotes the storage of substrates in adipose tissue, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and by inhibiting opposing catabolic effects namely, lipolysis, glycogenolysis and protein breakdown (Groff *et al*, 1995b). Insulin also activates LPL in adipose tissue resulting in increased clearance of TAG-rich lipoproteins from the plasma (Ruan & Lodish, 2003). In addition to its metabolic functions, insulin stimulates cell growth and differentiation (Le Marchand-Brustel *et al*, 2003; Groff *et al*, 1995b). Dysregulation of insulin, either through resistance or deficiency results in a profound disruption of these processes, and typically manifests with elevated concentrations in fasting and postprandial glucose and lipid profiles (Saltiel & Pessin, 2002). Failure of adipose tissue to fulfil its role in energy storage, exposes other organs to high levels of plasma glucose and NEFA, and promotes the storage of these fuel molecules in the form of TAG in peripheral organs e.g. liver, muscle, pancreatic β-cell. Intramyocellular TAG accumulation is associated

with increased insulin resistance in humans (Szczepaniak *et al*, 1999; Krssak *et al*, 1999; Perseghin *et al*, 1999). It is thought that intramyocellular TAG is not the cause of insulin resistance, but rather that it is a marker of other lipid intermediates present in muscle such as fatty acyl-Co A, ceramides or diacylglycerides, which attenuate various aspects of the insulin signalling cascade (Schmitz-Peiffer, 2002). Additionally insulin resistance is also associated with high levels of TAG in the liver and the pancreatic islets. In the liver this increased TAG accumulation is associated with an impaired ability of insulin to suppress endogenous glucose production (Kim *et al*, 2001b). Meanwhile TAG overload in the pancreas results in dysregulated insulin secretion (Shimabukuro *et al*, 1998). Thus, accumulation of TAG in non-adipose tissue is associated with a reduction in insulin sensitivity.

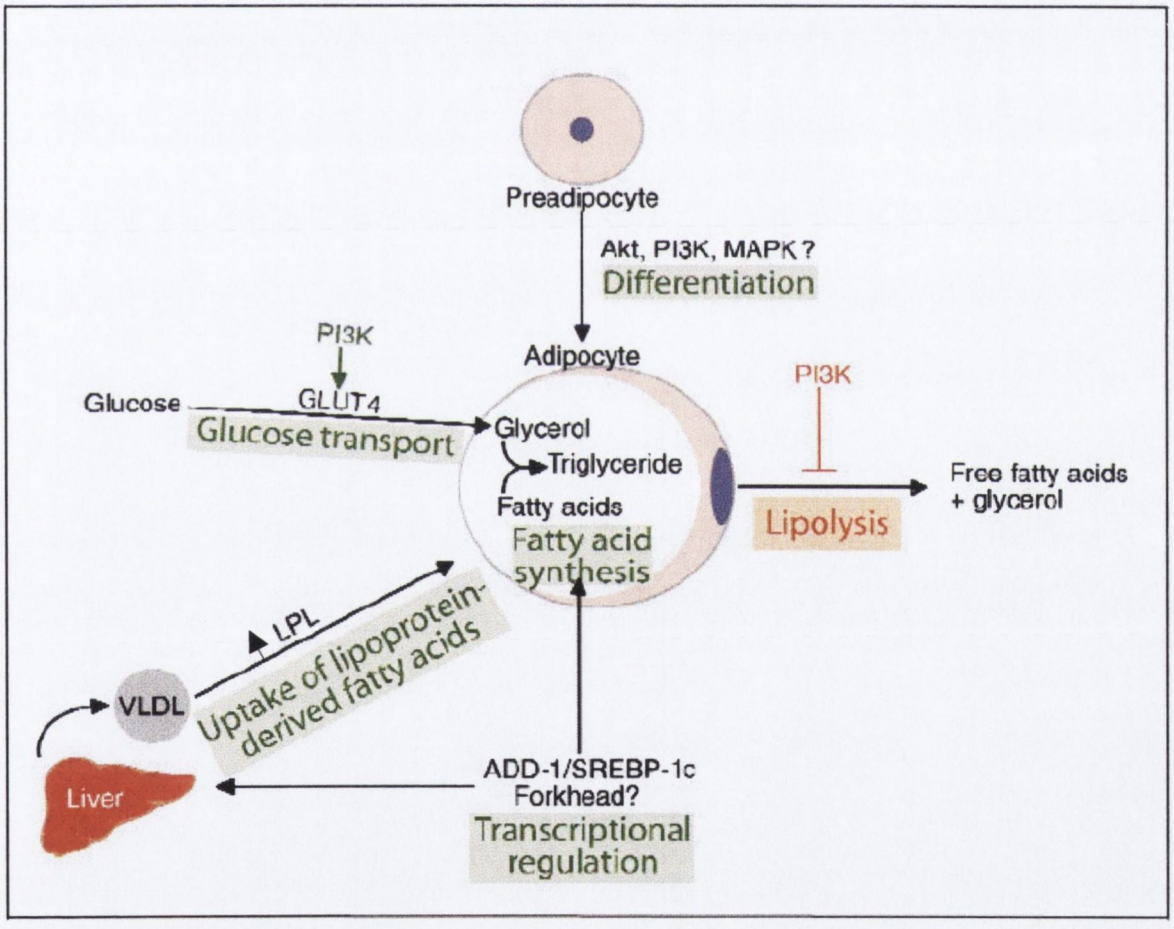


Fig. 1.5: Regulation of metabolism by insulin. Pleitrophic effects of insulin includes promoting the differentiation of preadipocytes to adipocytes, lipogenesis mediated by increased glucose and fatty acid uptake and inhibition of lipolysis (Adapted from Kahn & Flier, 2000).

Akt, also known as protein kinase; Glut4, glucose transporter 4; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; VLDL, very low density lipoprotein.
← Green arrows indicate a stimulatory effect.
← Red arrows indicate an inhibitory effect.

During the fasting state, the reduction in plasma insulin concentrations together with the rise in the levels of the counter-regulatory hormones such as glucagon, the catecholamines (epinephrine/adrenaline and norepinephrine/noradrenaline) and increased sympathetic nervous system activity stimulates lipolysis and free fatty acid release from adipose tissue. Fatty acid oxidation becomes a major source of energy, and also serves as a substrate for the induction in gluconeogenesis. In addition hepatic glycogenolysis is initiated (Ruan & Lodiah, 2003; Groff *et al*, 1995b). The different pathways which mediate insulin's effects show differential sensitivity to varying levels of insulin. The antilipolytic effect of insulin for example requires much lower concentrations of insulin than stimulation of glucose transport (Kahn & Flier, 2000). Consequently "insulin resistance" usually implies resistance to the effects of insulin on glucose uptake, metabolism, or storage (Bailey, 1999; Kahn & Flier, 2000).

1.6.1 Synthesis of insulin

Insulin is synthesised in and secreted from the β-cells within the islets of Langerhans in the pancreas. It is formed from a single amino acid chain precursor molecule called proinsulin. Synthesis begins with the formation of preproinsulin, which is cleaved by protease activity to form proinsulin. Proinsulin is packaged into vesicles in the Golgi apparatus of the cell, and in the maturing secretory granules it is converted by enzymes into split (32-33) proinsulin, Des (31,32) proinsulin and finally insulin. The insulin molecule consists of two polypeptide chains linked by disulphide bridges, the A chain contains 21 amino acids and the B chain contains 30 amino acids. C-peptide also produced from proinsulin, and is secreted in equimolar concentrations to insulin (Weir *et al*, 2000).

Glucagon, which opposes the action of insulin is produced in the α cells of the islets of Langerhans. Somatostatin is produced in the δ cells of the islets of Langerhan (Weir *et al*, 2000). It suppresses endogenous secretion of glucagon and growth hormone, and inhibits gluconeogenesis (Harano *et al*, 1977; Wallace & Matthews, 2002).

1.6.2 Insulin secretion

The tight regulation of normal blood glucose concentrations is largely achieved by the minute-to-minute regulation of insulin production by the pancreatic β-cells. Plasma glucose is the most important regulator of insulin secretion. Some neural stimuli e.g. sight and taste of food and increased blood concentrations of other fuel molecules, including amino acids and fatty acids, also promote insulin secretion. Glut 2, which is expressed in the β-cells, has a high K_m and therefore high capacity. This allows the facilitated diffusion of glucose at a rate proportional to plasma glucose concentrations. Following glucose equilibration across the plasma membrane, it is phosphorylated by glucokinase, the first enzyme of the glycolytic pathway (Weir *et al*, 2000). Glucokinase acts as a "glucose sensor" coupling insulin secretion

to the prevailing glucose level. Glycolysis produces pyruvate which subsequently enters the mitochondria and is a substrate for pyruvate dehydrogenase and pyruvate carboxylase. The TCA cycle or citric acid cycle generates reducing equivalents (red. equ.), which are transferred to the electron transport chain, leading to hyperpolarisation of the mitochondrial membrane and the generation of ATP (Newsholme *et al*, 2005). This production of ATP by the mitochondria is the main coupling factor in insulin secretion. ATP is then transferred to the cytosol, leading to an increase in the ratio of ATP to adenosine diphosphate (ADP) which consequently inhibits ATP-sensitive potassium channels. This inhibition of K^+ efflux induces depolarisation of the plasma membrane and consequently the voltage-gated Ca^{2+} channels open. The increase in the intracellular/cytosolic calcium concentration and energy-dependent cellular events such as the activation of ATPases, triggers the translocation and exocytosis of insulin-containing granules (Weir *et al*, 2000).

More recent work has indicated an important role of amino acids in regulating insulin secretion. Metabolism of L-leucine generates ATP, thus causing closure of the ATP-sensitive potassium channels in the membrane, depolarisation and subsequent increase in intracellular Ca^{2+} concentrations. Metabolism of L-glutamine also generates ATP (Flatt, 2003). Meanwhile the inward transport of positive charge carried by L-arginine, L-lysine and L-histidine, or by the co-transport of L-alanine with Na^+ is thought to directly depolarise the β-cell membrane, leading to calcium influx and the stimulation of insulin secretion (Persaud & Howell, 2003). *Fig. 1.6* summarises the meachanism by which nutrients regulate insulin secretion from the pancreatic β-cell.

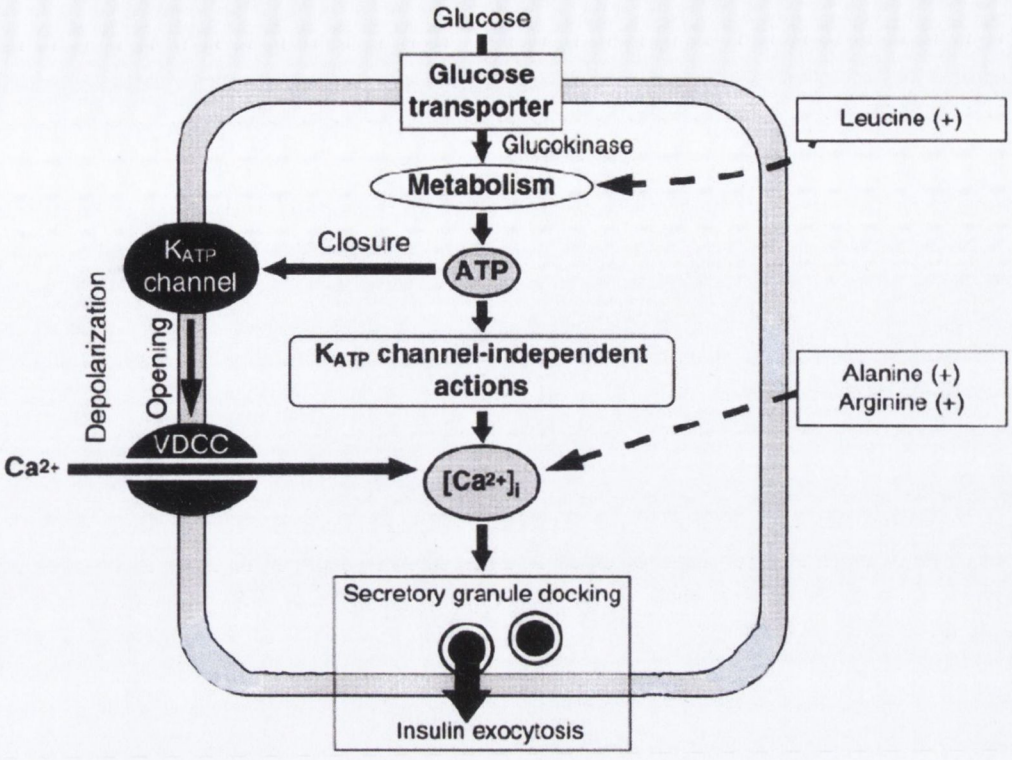


Fig. 1.6: Insulin secretion from the pancreatic β-cell (Adapted from McClenaghan & Flatt, 1999).

ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; VDCC, voltage-dependent Ca^{2+} channel.

In vitro the exposure of β-cells to glucose results in an immediate increase in insulin secretion, termed first-phase insulin secretion, followed by a decrease to basal rates. If exposure to glucose is prolonged, a slower second- or late-phase insulin secretion is observed, termed second-phase insulin secretion. Further exposure to glucose results in a reduction in insulin secretion, due to the desensitisation of β-cells to glucose. In non-diabetic animals and humans, the infusion of glucose elicits an insulin secretory response that follows the same diphasic pattern as seen *in vitro* (Fig. 1.7). First-phase insulin secretion occurs rapidly, resulting in a sharp peak in plasma insulin concentrations within 3 to 5 min of the start of the glucose infusion. This first-phase insulin secretion is completed within 10 min. Second-phase insulin secretion occurs gradually, beginning 10 to 20 min after the start of the glucose infusion, and can last for as long as exposure to glucose lasts. First-phase insulin secretion represents the release of mature secretory granules present close to the β-cell plasma membrane. Second-phase insulin secretion corresponds to the release of stored secretory granules and to *de novo* synthesis of insulin (Weir *et al*, 2000). Progression from NGT to T2DM is characterised by a loss of first-phase insulin response and may be observed even

before fasting glucose concentrations rise above the levels required for diagnosis (Weir *et al*, 2000).

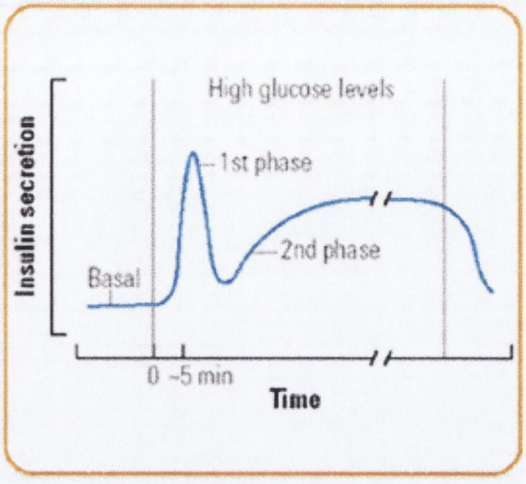


Fig. 1.7: The diphasic pattern of glucose stimulated insulin secretion from the ß-cell (Persaud & Howell, 2003).

1.7 INSULIN SIGNALLING IN WHITE ADIPOSE TISSUE

Insulin resistance is a fundamental defect in T2DM, and aberrations in the insulin signalling pathways underpin this resistance. Circulating insulin rapidly reaches its target where it interacts with its receptor. At the molecular level, insulin signalling in white adipose tissue begins at the cell surface when insulin binds to its transmembrane tyrosine kinase receptor, the insulin receptor, resulting in autophosphorylation and activation. The activated insulin receptor subsequently phosphorylates selective tyrosine residues of target proteins e.g. IRS-1, which then serves as a docking site for downstream effector molecules including phosphoinositide (PI)-3 kinase. The end product, translocation of the Glut4 vesicle from an intracellular compartment to the plasma membrane facilitates increased glucose transport into the cell.

Defects in both the expression of key proteins and the transduction of the signal have been reported in T2DM. The individual components of the pathway will be discussed in more detail.

1.7.1 Insulin Receptor

The insulin receptor belongs to the family of cell-surface receptors possessing intrinsic tyrosine kinase activity (van Obberghen *et al*, 2001). The receptor is composed of two extracellular α-subunits and two transmembrane β-subunits linked together by disulphide bonds (Saltiel & Kahn, 2001). In the basal state the subunits function as allosteric enzymes in

which the α-subunit inhibits the tyrosine activity of the β-subunit. Insulin signalling is initiated by binding of the ligand to the α-subunit, which transmits a signal across the plasma membrane that activates the intracellular tyrosine kinase domain of the β-subunit (Patti & Kahn, 1998). Consequently the receptor undergoes a series of intramolecular transphosphorylation reactions in which one β-subunit phosphorylates its adjacent partner on specific tyrosine residues. Phosphorylation of different tyrosine residues account for distinct functions including substrate binding, catalytic activity and mitogenic actions of insulin (Pessin *et al*, 1999). Phosphorylation of the tyrosine residue Y960, which is located in the juxtramembrane domain, is part of the recognition motif for several IRS proteins. Meanwhile adaptor protein with a pleckstrin homology (PH) and Src homology 2 (SH2) domain, known as APS, interacts with the tyrosine residue Y1158/1162 (Le Marchand-Brustel *et al*, 2003).

Gene knockout studies in mice have established that the insulin receptor is essential for survival. Insulin receptor deficient homozygous pups ($IR^{-/-}$) appear normal at birth, but die within a week due to severe diabetic ketoacidosis. This is not seen in the human form of insulin receptor deficiency, called leprechaunism (Bruning *et al*, 1997; Pessin & Saltiel, 2000). Heterozygous mutants ($IR^{+/-}$) show much more moderate hyperinsulinaemia and 10% of them develop mild diabetes (Mauvais-Jarvis *et al*, 2002). The structure of the insulin receptor is normal in T2DM. Additionally due to the excess of receptors it is thought unlikely that a reduced population of insulin receptors makes a major contribution to insulin resistance in most patients (Bailey, 1999). Although there does not appear to be a defect in the insulin receptor affinity, several studies have shown that the insulin receptor autophosphorylation and tyrosine kinase activity of the β-subunit are reduced in T2DM (Kahn, 1994; Nyomba *et al*, 1990). However this is reversible and normalises as glycaemia improves (Smith et al, 1999). Consequently it is thought that defects in the insulin receptor are too rare in the general population to account for the increased incidence of insulin resistance. Instead it is thought that post-insulin receptor defects are the primary site leading to peripheral insulin resistance (Pessin & Saltiel, 2000).

Once activated, the insulin receptor phosphorylates a number of important proximal substrates including members of the IRS family, the Src homology collagen (Shc) adapter protein isoforms, signal-regulatory protein (SIRP) family members, GTPase-activating protein (GAB)-1, Casitas b-lineage lymphoma (Cbl) and APS, which mediate various aspects of insulin action (Saltiel & Kahn, 2001). The mitogen-activated protein kinase (MAPK) pathway mediates the growth-promoting function of insulin. The PI 3-kinase pathway leads to enhanced glucose transport, glycogen synthesis and protein synthesis. The TC10 pathway mediates glucose transport in a PI 3-kinase independent manner (Le Marchand-Brustel *et al*, 2003). The PI 3-kinase signalling pathway has been shown to be unequivocally essential for insulin-stimulated Glut4 translocation (Pessin & Saltiel, 2000) and will be discussed in most

detail. More recently attention has been drawn to the TC10 pathway and a summary of this pathway will be provided. *Fig. 1.8* illustrates the insulin signalling pathways.

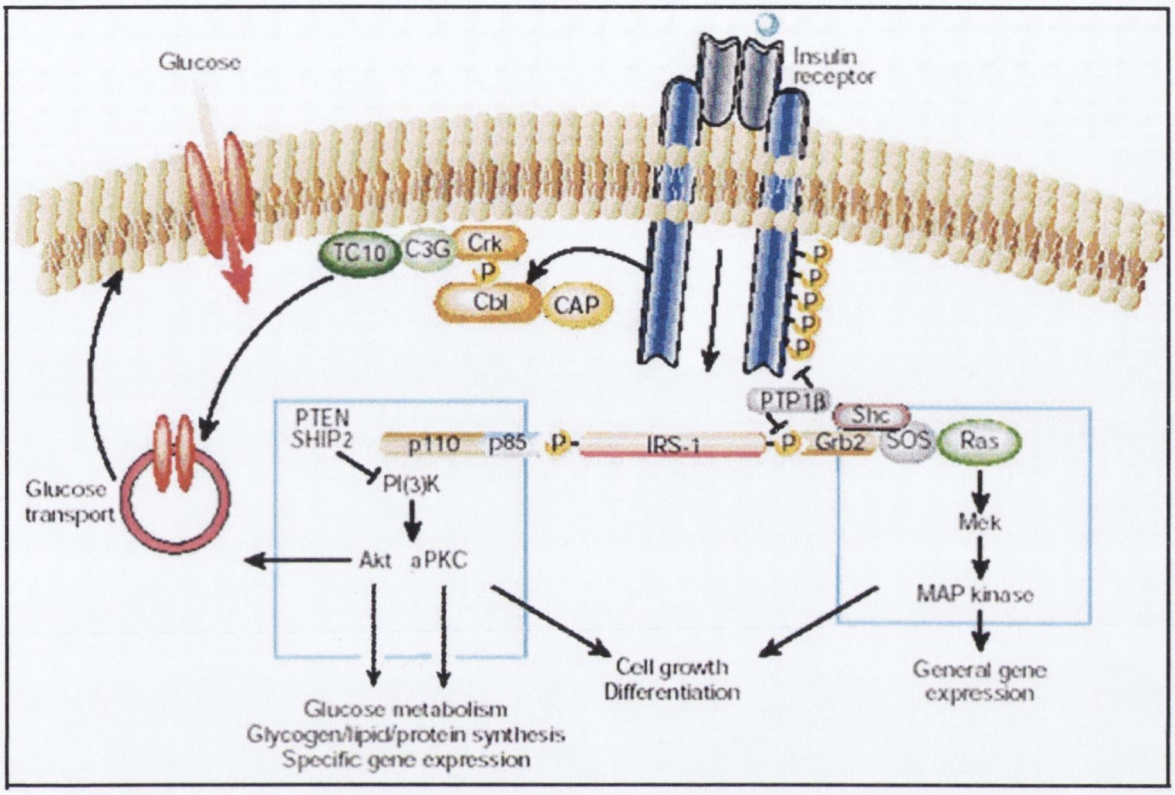


Fig. 1.8: Insulin signalling pathways (Adapted from Saltiel & Pessin, 2001). Phosphorylation of proximal substrates by the insulin receptor results in the activation of different pathways. The PI(3)K pathway and the Cbl pathway stimulate glucose transport, while the MAP kinase pathway promotes growth.

Akt, also known as protein kinase B; aPKC, atypical protein kinase C; CAP, Cbl-associated protein; Cbl, Casitas b-lineage lymphoma; Crk, adaptor protein; IRS-1, insulin receptor substrate-1; MAP, mitogen-activated protein; Mek, MAPK kinase; p110, catalytic subunit of phosphoinositide-3 kinase; p85, regulatory subunit of phosphoinositide-3 kinase; PI3K, phosphoinositide-3 kinase; PTEN, phosphatase and tesnin homolog gene; PTP1β, protein tyrosine phosphatases β; Shc, Src homology collagen; SHIP2, SH2 domain-containing inositol-5-phosphatase-2; TC10, lipid raft associated protein.

1.7.2 Phosphoinositide-3 kinase dependent pathway

(i) Insulin receptor substrate

The IRS family plays a central role in insulin signalling and are considered the major substrate of the insulin receptor (Zick, 2004). Four members of this scaffold molecule have been identified in mammalian cells namely IRS-1, IRS-2, IRS-3 and IRS-4 (Sesti et al, 2001). All members share in common an NH2-terminal PH domain that serves to anchor the IRS proteins to membrane phosphoinositides, which helps to localise the IRS proteins in close proximity to the insulin receptor (Zick, 2004). The PH domain is flanked by a phosphotyrosine binding

(PTB) domain, which binds IRS to the juxta-membrane region of the insulin receptor (Zick, 2004). Both the PH and the PTB domains are highly conserved in each protein (Van Obberghen *et al*, 2001). In contrast the C-terminal region is poorly conserved. It contains a domain of variable length containing several tyrosine phosphorylation motifs, that are phosphorylated directly by the insulin receptor and serve as docking sites for SH2 domain containing proteins (Zick, 2004; Van Obberghen *et al*, 2001). The different IRS proteins appear to have different functions at the cellular level, probably due to differences in tissue distribution, subcellular localisation and intrinsic activity of the proteins (Saltiel & Kahn, 2001). Evidence suggests that IRS-1 functions primarily in adipose tissue and skeletal muscle, whilst IRS-2 plays the dominant role in the liver and pancreas. IRS-3 is expressed in rodents, mainly in adipose tissue. However it is uncertain whether the protein is expressed in humans. IRS-4 is expressed in the thymus, brain, kidney and possibly pancreatic β-cells (White, 2002; Sesti *et al*, 2001). Whilst there may be some degrees of redundancy, specific phosphotyrosines are involved in the recruitment of certain effector, by virtue of their SH2 domains. For example, PI-3 kinase binds to phosphotyrosines 608 and 941, whilst the protein tyrosine phosphatase SHP2 binds at phosphotyrosines 1172 and 1222 which activates the MAPK pathway (Schmitz-Peiffer & Whitehead, 2003).

The majority of IRS-1 is found in an ill-defined fraction termed the High Speed Pellet (HSP) along with low-density microsomes, Glut4 vesicles and cytoskeletal elements (Clark *et al*, 1998). This HSP represents the "active pool" of IRS-1 as PI-3 kinase is recruited and activated specifically by IRS-1 in the HSP in response to insulin (Nave *et al*, 1996).

Mice with homozygous disruption of the IRS-1 gene present with generalised pre- and post-natal growth retardation, as well as insulin resistance in peripheral tissues and impaired glucose tolerance. They do not develop frank diabetes, presumably owing to pancreatic β-cell compensation (Araki *et al*, 1994; Tamemoto *et al*, 1994). In contrast, mice with homozygous disruption of the IRS-2 gene present with decreased β-cell mass and impaired insulin secretion, in addition to insulin resistance in both peripheral tissues and liver, leading to the development of diabetes (Withers *et al*, 1998). Interestingly mice which are heterozygous for both the insulin receptor and IRS-1 ($IR^{-/+}$ IRS-$1^{-/+}$) develop both insulin resistance and diabetes, indicating that the development of diabetes may be polygenic, multihit process (Bruning *et al*, 1997).

Smith *et al* (1999) reported that IRS-1 protein expression in adipocytes were approximately 30% lower in subjects with T2DM. In addition IRS-1 associated PI 3-kinase activity was also markedly reduced. Elsewhere IRS-2 protein expression in adipocytes from subjects with T2DM has been reported to be normal, but the dose-response curve for insulin was markedly shifted to the right for IRS-2-associated PI-3 kinase activity, indicating insulin resistance (Rondinone *et al*, 1997).

(ii) Downstream effectors of IRS

Tyrosine phosphorylation of the IRS family members generates docking sites for several SH2-containing proteins, which plays a pivotal role in the metabolic and mitogenic actions of insulin (Saltiel & Kahn, 2001). The predominant partner seems to be the p85 regulatory subunit of the type1A PI-3 kinase (Saltiel & Pessin, 2002). IRS-1 recruits PI-3 kinase to the plasma membrane of cells whereby the catalytic subunit of PI-3 kinase p110 phosphorylates phosphatidlyinositol (PtdIns) (4,5)-bisphosphate, generating PtdIns (3,4,5)-triphosphate, which is known as PtdIns(3,4,5)P_3. These events happen within the first minute of insulin binding to its receptor (Lizcano & Alessi, 2002). Three isofoms of the p110 catalytic subunit have been identified p110α, p110β and p110δ, and these are collectively termed the class-1a PI 3-kinases (Shepherd, 2005). Evidence suggests that p110β is the most important isoform for insulin signalling (Asano *et al*, 2000; Roche *et al*, 1998). However since p110α and p110β gene knockouts both results in embryonic lethality, tissue specific knockouts or isoform specific inhibitors will be required to identify the precise role of each isoform (Bi *et al*, 1999; Bi *et al*, 2002; Shepherd, 2005). The importance of PI 3-kinase as a second messenger in the insulin signalling pathway is evident from studies in which the p110 PtdIns 3-kinase catalytic activity is inhibited. Use of pharmacological inhibitors such as wortmannin, the production of dominant-interfering mutants, or microinjection of blocking antibodies can completely inhibit most of the cellular responses to insulin, including insulin-induced glucose transport (Okada *et al*, 1994; Cheatham *et al*, 1994).

PtdIns(3,4,5)P_3 serves as an allosteric regulator of phosphoinositide-dependent kinase (PDK) (Alessi *et al*, 1997). PDK mediates the translocation of protein kinase B (PKB), also known as Akt, from the cytosol to the plasma membrane, whereby it is phosphorylated by kinases at Thr308 and Ser473. Once activated, PKB is released from the plasma membrane and phosphorylates other substrates (Lizcano & Alessi, 2002). The importance of PKB is evident as stable expression of a constitutively active, membrane-bound form in 3T3-L1 adipocytes results in increased glucose transport and persistent localisation of Glut4 to the plasma membrane (Kohn *et al*, 1996; Kohn *et al*, 1998). Conversely, expression of a dominant-interfering PKB mutant inhibits insulin-stimulated Glut4 translocation (Cong *et al*, 1997; Wang *et al*, 1999). Results from ribonucleic acid interference (RNAi)-based gene silencing indicate that the PKBβ isoform, also known as Akt2, is of most significance for insulin-stimulated glucose transport as 70% depletion of the protein reduced glucose uptake by 60%, whereas 95% inhibition of PKBα, which is also known as Akt1, resulted in only 10-20% inhibition of insulin action on glucose transport (Zhou *et al*, 2004). Interestingly PKBβ is the major isoform in adipose tissue accounting for around 70% of PKB, while PKBα accounts for around 30% (Smith *et al*, 1999). In addition to stimulating glucose uptake, PKB also activates glycogen synthesis. PKB phosphorylates and consequently inactivates glycogen synthase

kinase-3. A key substrate of glycogen synthase kinase-3 is glycogen synthase, the enzyme which catalyses the final step in glycogen synthesis, converting UDP-glucose into glycogen. Phosphorylation of glycogen synthase by glycogen synthase kinase-3 inhibits its action, thereby reducing glycogen synthesis. Therefore, inactivation of glycogen synthase kinase-3 by PKB results in the dephosphorylation of glycogen synthase via activation of a glycogen-associated form of protein phosphatase-1, and stimulation of glycogen synthesis (Lizcano & Alessi, 2002).

PDK1 also phosphorylates and activates protein kinase C (PKC) isoforms, PKCζ and PKCλ which have also been proposed to play a role in regulating glucose transport (Lizcano & Alessi, 2002). Overexpression of a dominant-negative mutant of PKCζ or PKCλ abrogates insulin-stimulated glucose transport and Glut4 translocation in adipose and muscle cells (Kotani *et al*, 1998; Bandyopadhyay *et al*, 2000). Conversely, overexpression of constitutively active PKCλ in adipocytes or wild-type PKCζ in muscle *in vivo* enhances both basal and insulin-stimulated glucose transport (Kotani *et al*, 1998; Etgen *et al*, 1999). Kim *et al* (2003) reported impaired insulin stimulated PKCλ and PKCζ activity in obese subjects and those with T2DM, which was associated with impaired glucose disposal in both populations. However controversy exists in defining the role of PKC in insulin signalling. PKCθ has been shown to induce serine phosphorylation of IRS protein in rats, thereby reducing insulin stimulation of PI 3-kinase (Yu *et al*, 2002b. In addition PKCζ has been shown to phosphorylate a residue in the PH domain of PKB which blocks its ability to interact with $PI(3,4,5)P_3$ (Powell *et al*, 2003). Since acute treatment of PKC by phorbol esters inhibits glucose transport and Glut4 translocation, while chronic treatment often fails to impair insulin action, it has been suggested that PKC is part of normal insulin signalling (Frevert & Kahn, 1996).

In addition to the class-1a PI 3-kinases, class-II PI 3-kinase, comprising PI-3 kinase-C2α, PI-3 kinase-C2β, PI-3 kinase-C2γ, use PI 3-kinase as a substrate, producing PI(3)phosphate [PI(3)P]. Evidence has emerged that PI(3)P is sufficient to facilitate a step in the trafficking of Glut4 to the plasma membrane, and also that insulin causes an increase in the cellular levels of PI(3)P suggesting that it could play a role in insulin's ability to stimulate glucose transport (Maffucci *et al*, 2003). However the mechanism of how PI(3)P may contribute to increasing glucose transport remains to be elucidated.

Reports indicate that there is a defect in insulin stimulation of class-1a PI 3-kinase in muscle and adipocytes from obese subjects and those with T2DM (Bjornholm *et al*, 2002; Smith, 2002; Krook *et al*, 2000). However, no difference in the level of expression of the components of class 1a PI 3-kinases has been found. To date no studies have examined the regulation of class-II PI 3-kinases in diabetes or insulin resistance (Shepherd, 2005). Both insulin-stimulated serine phosphorylation of PKB, and insulin-stimulated PKB activity is

reduced by around 50% in cells from subjects with T2DM. This probably reflects impaired upstream signalling, as the protein expression was found to be normal (Rondinone *et al*, 1999).

1.7.3 Inhibition of the insulin signalling pathway

The insulin signalling cascade can be modulated in several ways ranging from insulin receptor association and tyrosine phosphorylation, to localisation and degradation of key components of the pathway. Recent studies reveal that agents that induce insulin resistance exploit phosphorylation-based negative feedback control mechanisms otherwise utilised by insulin itself to uncouple the insulin receptor from its downstream effectors and thereby terminate insulin signal transduction. Inhibitors of the insulin signalling cascade are discussed in turn.

(i) Protein tyrosine phosphatases

A number of protein tyrosine phosphatases (PTP) have been identified that can catalyse the dephosphorylation of the insulin receptor, reducing its kinase activity and thereby attenuating insulin action (Saltiel & Kahn, 2001). Elevated expression of PTP-1B and leucocyte antigen-related (LAR) have been reported in subjects with insulin resistance (Goldstein *et al*, 1998; Drake & Posner, 1998). In addition PTP1B$^{-/-}$ mice display increased tyrosine phosphorylation of the insulin receptor and IRS protein in muscle, leading to improved insulin sensitivity (Elchebly *et al*, 1999). It therefore could represent an important target for insulin-sensitising agents.

(ii) Serine Phosphorylation of IRS proteins

In addition to tyrosine phosphorylation, both the insulin receptor and IRS proteins can undergo serine/threonine (Ser/Thr) phosphorylation and > than 70 putative Ser/Thr phosphorylation sites have been identified (Zick, 2004). Ser/Thr phosphorylation has a dual function in serving as either a positive or a negative modulator of insulin signalling. Phosphorylation of Ser residues within the PTB domain of IRS-1 by PKB in response to insulin is considered a positive modulator of insulin signalling. This protects IRS-1 from the action of PTPases, and enables them to maintain their tyrosine phosphorylated "active" conformation (Paz *et al*, 1999). In addition, removal of basal Ser/Thr phosphatases reduces the ability of IRS-1 to undergo insulin receptor-mediated tyrosine phosphorylation, indicating that Ser/Thr phosphorylation is necessary (Greene & Garofalo, 2002). However, Ser/Thr phosphorylation of IRS proteins may inhibit insulin-stimulated tyrosine phosphorylation of IRS proteins, thereby attenuating the insulin signal (Saltiel & Kahn, 2001). *Table 1.9* highlights the pleiotrophic effects explaining how IRS-1 Ser/Thr phosphorylation can attenuate insulin signal transduction.

Table 1.9: Mechanisms explaining how IRS-1 Ser/Thr phosphorylation can attenuate insulin signal transduction.

IRS, insulin receptor substrate; Ser/Thr, serine/threonine.

Effect	Reference
Inducing the dissociation of IRS proteins from the insulin receptor	Paz *et al*, 1997 ; Liu *et al*, 2001
Promoting the release of IRS proteins from intracellular complexes that maintain them in close proximity to the receptor	Tirosh *et al*, 1999
Inducing IRS protein degradation	Pederson et *al*, 2001
Turning IRS proteins into inhibitors of the insulin receptor kinase activity	Hotamisligil *et al*, 1996a
Hindering tyrosine phosphorylation sites of IRS proteins	Mothe & Van Obberghen, 1996

Prolonged stimulation of cells with insulin induces Ser phosphorylation at Ser307, Ser612 and Ser632, suggesting that it could be a negative-feedback mechanism that uncouples the IRS-1 proteins from their upstream and downsteam effectors, thereby blocking insulin signal transduction under physiological conditions (Zick, 2001). Interestingly inducers of insulin resistance including the pro-inflammatory cytokine, TNFα, NEFA and oxidative stress induces phosphorylation of these same residues, thereby taking advantage of the negative-feedback control system. Consequently IRS-1 function is inhibited and the insulin signal terminated *(Fig. 1.9)* (Zick, 2005). Some of these effects may be mediated by activating the same Ser/Thr kinases as insulin, including PKCζ, c-Jun N-terminal j-kinase (JNK) and inhibitor of κB kinase (IKKβ). However the question remains whether insulin, TNFα and NEFA activate these kinases along the same or alternate pathways (Zick, 2004).

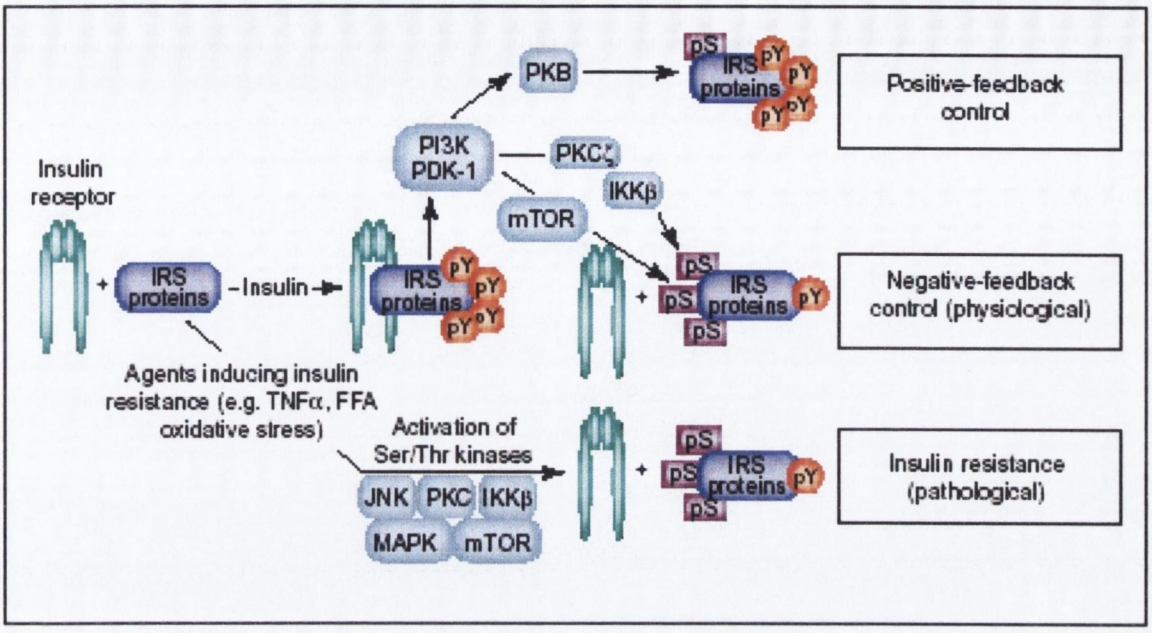


Fig. 1.9: Ser/Thr-phosphoryled IRS-1 proteins as modulators of insulin action and insulin resistance (Zick, 2001).

FFA, non-esterified fatty acids; IKK, inhibitor of κB kinase; IRS, insulin receptor substrate; JNK, c-Jun N-terminal j-kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PDK, phosphoinositide-dependent kinase; PKB, protein kinase B; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; pS, Serine/Thr phosphorylated; pY, tyrosine phosphorylated; TNF, tumour necrosis factor α.

The best characterised of the serine phosphorylation sites of IRS-1 is Ser307, equivalent to Ser312 in humans. Phosphorylation at Ser307 disrupts the interaction between the insulin receptor and the PTB domain of IRS-1 (Aguirre *et al*, 2000). The importance of this site emerged from studies in JNK deficient mice which exhibited enhanced insulin-stimulated IRS-1 tyrosine phosphorylation and diminished Ser307 phosphorylation following treatment with TNFα (Hirosumi *et al*, 2002). In addition when fed a high fat diet, mice showed a partial improvement in glucose tolerance in comparison with wild type mice (Hirosumi *et al*, 2002). IKKβ have also been shown to phosphorylate this site in response to TNFα. Recently the beneficial effects of salicyclates on glucose tolerance has been shown to be mediated, at least in part, through attenuation of Ser307 phosphorylation via reduced IKKβ expression and/or activation (Yuan *et al*, 2001). Conversely it is thought that elevated NEFA concentrations may exert part of their negative effect through enhanced Ser307 phosphorylation, following activation of IKKβ by PKC (Le Marchand-Brustel *et al*, 2003). Chronic hyperinsulinaemia promotes Ser307 phosphorylation in a PI-3 kinase/mammalian target of rapamycin (mTOR)-dependent manner (Greene *et al*, 2003). Additionally phosphorylation of this residue also occurs following relatively short-term insulin treatment in

healthy individuals, most likely contributing to attenuation of the insulin signal, highlighting the feedback control mechanism of insulin (Greene *et al*, 2003). A mutation of Ser307 to Ala provided partial protection against IRS-1 degradation during chronic insulin treatment, suggesting that phosphorylation at this site may also serve to target IRS-1 for proteasomal degradation (Greene *et al*, 2003).

Other residues of IRS-1 which undergo serine phosphorylation are highlighted in *Fig. 1.10.*

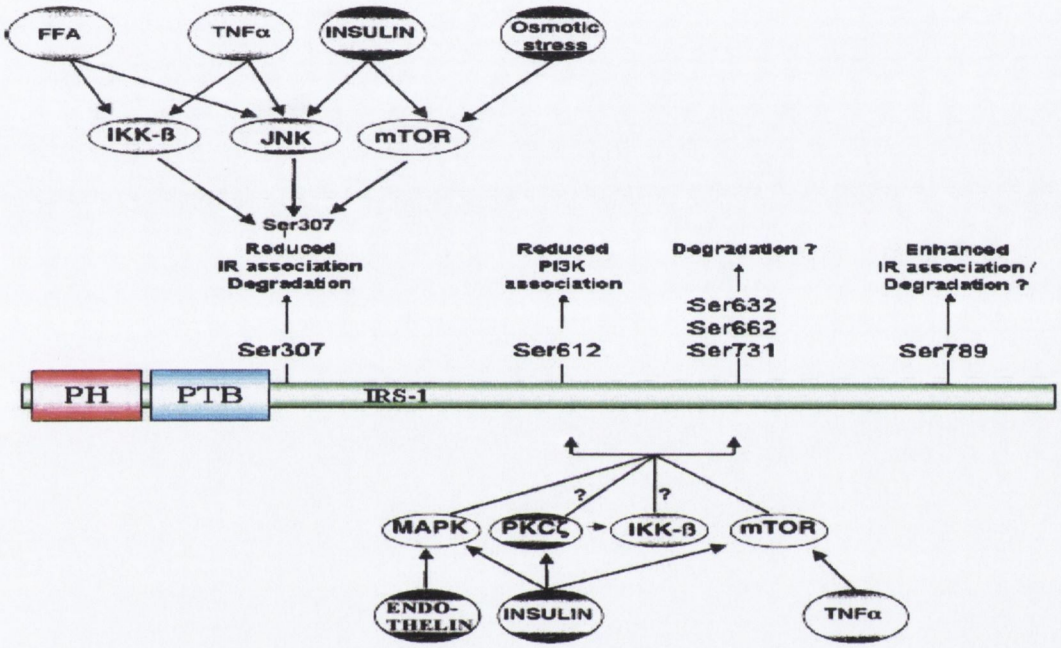


Fig. 1.10: Potential pathways and consequences of IRS-1 serine phosphorylation (Adapted from Schmitz-Peiffer *et al*, 2003; Le Marchand-Brustel *et al*, 2003).

IKK, inhibitor of κB kinase; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal j-kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PH; pleckstrin homology; PKB, protein kinase B; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; Ser, Serine/Thr phosphorylion; PTB, phosphotyrosine binding domain; TNF, tumour necrosis factor α.

(iii) Location of IRS-1

Translocation of IRS-1 may represent another potential mechanism of how the insulin signal may be attenuated in white adipose tissue. Increased interaction of IRS-1 with 14-3-3, a phosphoserine binding protein, triggers the translocation of IRS-1 into the cytosol. As previously mentioned, the HSP fraction represents the "active" pool of IRS-1 (Nave *et al*, 1996). Additionally it has been suggested that once in the cytosol, IRS-1 is targeted for ubiquitination and subsequent degradation by the proteasome (Zhande *et al*, 2002).

(iv) Lipid phosphatases

Insulin action can also be temporally controlled by lipid phosphatases that dephosphorylate PtdIns(3,4,5)P_3. Overexpression of either the SH2 domain-containing inositol-5-phosphatase (SHIP)-2 or PtdIns(3c)phosphate phosphatase and tesnin homolog gene (PTEN) attenuates insulin stimulation of Glut4 translocation, by reducing PtdIns(3,4,5)P_3 levels, with SHIP2 converting PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 and PTEN converting it to PtdIns(4,5)P_2 (Wada et *al*, 2001; Nakashima *et al*, 2000). Conversely disruption of SHIP2 genes yields mice with increased insulin sensitivity (Clement *et al*, 2001). In addition, 50% depletion of PTEN expression by RNAi-based gene silencing caused a 2-4 fold increase in glucose transport (Zhou *et al*, 2004).

(v) Suppressors of cytokine signalling

The SOCS proteins have also been implicated in down-regulating the insulin signal (Emanuelli *et al*, 2000). The SOCS proteins compete for crucial receptor binding sites e.g. SOCS-3 has been shown to bind to the Y960 residue of the insulin receptor, an important docking site for IRS-1. This association with the insulin receptor may inhibit the association between IRS and the insulin receptor, thereby reducing the phosphorylation of IRS-1 (Emanuelli *et al*, 2000). In addition SOCS-1 and SOCS-3 have been implicated in promoting the ubiquitination and subsequent degradation of IRS-1 by the proteosome (Rui *et al*, 2002). Cytokines are the classical regulators of SOCS proteins, thus illustrating another possible mechanism by which these inflammatory agents may contribute to insulin resistance (Mooney *et al*, 2001; Senn *et al*, 2003).

(vi) Feedback mechanism of insulin

It should be emphasised that some aspects of the signalling pathway are regulated by insulin, thereby providing a feedback mechanism. In addition to Ser/Thr phosphorylation of the IRS-1 proteins, insulin can also up-regulate SOCS-1 and SOCS-3 which also impairs insulin signalling and increases the rate of proteosomal degradation of IRS-1 and IRS-2 (Shepherd, 2005). Chronic insulin treatment promotes the redistribution of IRS-1 from the HSP to the cytosol, resulting in a reduction in insulin-stimulated tyrosine phosphorylation of IRS-1, and also increased ubiquitination and degradation of IRS-1 (Zhande *et al*, 2002). Additionally it has been postulated that insulin can regulate its own signalling by directly down-regulating PTP-1B activity and PTEN activity via stimulation of H_2O_2 production, thereby promoting insulin signalling via PI 3-kinase (Mahadev *et al*, 2001).

1.7.4 Phosphoinositide-3 kinase independent pathway

A second pathway has been identified which regulates insulin-stimulated glucose uptake. Evidence of an additional pathway emerged from experiments using a cell-permeable analog of PtdIns(3,4,5)P_3. In these experiments, addition of the analog had no effect on Glut4 translocation. However, treatment of adipocytes with wortmannin, insulin and the analog, resulted in enhanced glucose uptake. This indicated that there is at least one additional pathway which can stimulate Glut4 translocation, independent of PI 3-kinase activation (Jiang *et al*, 1998b). Thus although there is an absolute requirement for PI 3-kinase mediated PtdIns(3,4,5)P_3 generation in insulin-stimulated glucose uptake, activation of PI 3-kinase alone is not sufficient. In addition exercise, hypoxia, and skeletal muscle contraction have all been shown to induce Glut4 translocation independently of PI 3-kinase activation (Cortright & Dohm, 1997; Lund *et al*, 1995). This alternative pathway appears to involve tyrosine phosphorylation of the Cbl proto-oncogene (Ribon & Saltiel, 1997).

The adaptor protein APS has an SH2 domain that interacts directly with the phosphorylated insulin receptor, and subsequently undergoes tyrosine phosphorylation. Upon phosphorylation APS recruits Cbl which is then phosphorylated. In most insulin-responsive cells Cbl is associated with the adapter protein, Cbl-associated protein (CAP) which binds to proline rich sequences in Cbl through its carboxyl-terminal SH3 domain (Ribon *et al*, 1998a). CAP also interacts with APS through its N- and C-terminal SH3 domains. Upon phosphorylation the Cbl-CAP complex translocates to lipid raft domains in the plasma membrane, mediated by the interaction of the sorbin homology (SoHo) domain of CAP with the protein flotillin (Baumann *et al*, 2000). An adapter protein CrkII is recruited to the lipid raft via interaction of the SH2 domain of CrkII with phospho-Cbl. CrkII also forms a constitutive complex with the guanyl nucleotide-exchange protein cyanidin 3-glucoside (C3G) (Chiang *et al*, 2001). Once translocated into lipid rafts C3G comes into proximity with the G protein TC10, and catalyses the conversion of guanosine diphosphate (GDP)-bound TC10 to its guanosine triphosphate (GTP)-bound state, resulting in the activation of the protein (Chiang *et al*, 2001). TC10 is produced in adipose tissue and muscle, and is acutely activated by insulin upon localisation of TC10 to the lipid rafts (Saltiel & Pessin, 2002; Chiang *et al*, 2001). Mis-targeting of TC10 into non-lipid raft regions of the plasma membrane impedes the ability of TC10 to modulate insulin stimulated Glut4 translocation (Saltiel & Pessin, 2002). The precise effectors acting downstream of TC10 remain unknown (Saltiel & Pessin, 2002). However once activated, TC10 seems to provide a second signal to the Glut4 protein which functions in parallel with the activation of the PI 3-kinase pathway. This may involve the stabilisation of cortical actin, which seems to be important in insulin-stimulated Glut4 translocation to the plasma membrane and glucose uptake (Saltiel & Kahn, 2001). Support for the importance of this pathway is also evident from results showing that the expression of a

dominant-interfering CAP mutant completely inhibited insulin-stimulated glucose uptake and Glut4 translocation. This was due to a marked reduction in the localisation of tyrosine-phosphorylated Cbl in the plasma membrane lipid raft domain (Pessin & Saltiel, 2000). *Fig. 1.11* illustrates the PI-3 kinase independent pathway.

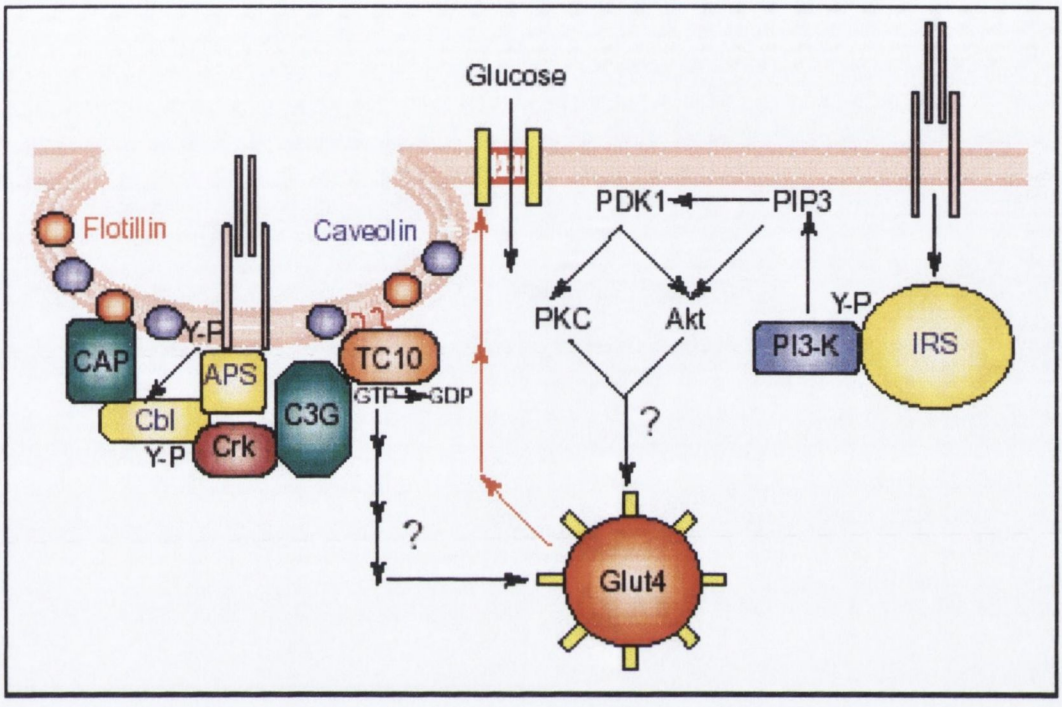


Fig. 1.11: PI-3 kinase independent pathway mediating glucose transport (Saltiel & Pessin, 2002).

Akt, also known as protein kinase B; APS, adaptor protein with a PH and SH2 domain; CAP, Cbl-associated protein; Cbl, Casitas b-lineage lymphoma; Crk, adaptor protein; Glut4, glucose transporter 4; GDP, guanosine diphosphate; GTP, guanosine triphosphate; IRS-1, insulin receptor substrate-1; PDK, phosphoinositide-dependent kinase; PI3K, phosphoinositide-3 kinase; PIP3, phosphatidlyinositol (3,4,5)-triphosphate; PKC, atypical protein kinase C; TC10, lipid raft associated protein; Y-P, tyrosine phosphorylated.

1.7.5 Glucose transporter 4

Insulin-stimulated glucose transport is primarily mediated by the facilitative transporter Glut4. Glut4 is a member of a family of related transporters which have distinct substrate specificities, kinetic properties, and tissue distributions that dictate their functional role. Glut4 is different from the other members in that approximately 90% of it is sequestered intracellularly, and is activated by insulin (Shepherd & Kahn, 1999). All the other members actively transport glucose in the absence of insulin down a glucose-concentration gradient. *Table 1.10* highlights some of the characteristics of the individual members of the family of facilitative glucose transporters.

Table 1.10: Characteristics of the family of facilitative glucose transporters (Adapted from Ducluzeau *et al*, 2002).

Name	Tissue Distribution	Functions
Glut1	Widely expressed, abundant in red blood cells, endothelial cells and tissue culture cell lines	Basal glucose uptake in many cells including insulin sensitive cells, transport in growing cells and across blood brain barrier
Glut2	Limited to pancreatic β-cells, hepatocytes, intestine, kidney	High Km glucose transporter which allows high capacity transport, trans-epithelial transport, major transporter in pancreatic β-cells and the liver
Glut3	Wide distribution in humans, limited to brain in some species	Basal transport, uptake from cerebral fluid
Glut4	Largely expressed in insulin-responsive tissues, skeletal and cardiac muscle and adipose tissue	Insulin-sensitive glucose uptake, vital in postprandial glucose disposal
Glut5	Primarily intestine, small amounts in adipose, muscle, brain, kidney	Absorption of fructose in intestine
Glut7	Gluconeogenic tissues, hepatocytes	Release of glucose from gluconeogenesis from ER lumen
Glut8	Blastocyst, possibly other tissues	Insulin-stimulated glucose uptake into blastocyst and possible other tissues lacking Glut4
GlutX1	High in testes, moderate in central nervous system, low in insulin responsive tissues	? sequestered intracellularly may play a role in regulatable glucose uptake

ER, endoplasmic reticulum; Glut, glucose transporter.

In the basal state Glut4 slowly recycles between the plasma membrane and vesicular compartments within the cell, where most of the Glut4 resides (Pessin *et al*, 1999). Insulin stimulates the translocation of a pool of Glut4 to the plasma membrane through a process of targeted exocytosis. At the same time the rate of internalisation (endocytosis) is slightly attenuated. The large increase in exocytosis (10-20 fold increase) coupled with a smaller decrease in the rate of plasma membrane internalisation (2-3 fold decrease) results in a dramatic accumulation of plasma membrane localised Glut4 protein (Czech, 1995). The rate of glucose transport into adipose tissue and muscle cells is primarily governed by the concentration of Glut4 at the cell surface, and the time for which the protein is maintained at this site (Saltiel & Pessin, 2002).

Glut4 appears to reside in at least two distinct but inter-related vesicular pools, both in terms of protein and sensitivity to insulin stimulation (Ducluzeau *et al*, 2002). Morphological analysis by immunofluorescence and subcellular fractionation, suggests that some Glut4 vesicles in adipocytes reside in an endosomal pool that is continuously recycling between the plasma membrane and the cytosol (Rodnick *et al*, 1992). In addition Glut4 also resides in a compartment termed the "Glut4 storage vesicle", or GSV, a pool which may be more efficiently mobilised following insulin stimulation (Rea & James, 1997). This pool appears to lack endosomal markers e.g. vesicle-associated membrane protein (VAMP)-3. The small GTP-binding proteins of the Rab family play an important role in endosomal recycling, sorting and exocytic movement of Glut4. Rab5 for example, facilitates endocytosis of Glut4. In the basal state, Rab5 is activated. However, it is inactivated during the process of insulin-stimulated Glut4 translocation, thereby increasing Glut4 expression at the plasma membrane. Rab5 becomes reactivated when insulin's effects dissipate thereby allowing Glut4 endocytosis to proceed normally (Elmendorf, 2002).

Insulin-responsive aminopeptidase (IRAP) localises in Glut4 vesicles, and the C-terminus of Glut4 and the N-terminus of IRAP contribute to the sequestration of Glut4 (Waters *et al*, 1997). However the cytoplasmic targets of IRAP and Glut4 are unknown. One potential site for Glut4 tethering is the actin cytoskeleton, which undergoes considerable rearrangement following insulin stimulation, possibly through the activation of proteins known to regulate actin polymerisation, such as effectors of the Rho family of small GTPases (Tsakiridis *et al*, 1999). The C-terminal of Glut4 can interact with the glycolytic enzyme aldolase. Aldolase can also bind to actin, and substrates of aldolase can mediate the dissociation of the Glut4-aldolase complex, potentially providing a negative-feedback signal for glucose transport (Kao *et al*, 1999).

Evidence suggests that target (t)-SNARE Syn4, or soluble N-ethylmaleimide-sensitive factor attachment protein receptor syntaxin 4, is the membrane target for the Glut4 vesicle. Glut4 contains a vesicle (v)-SNARE protein termed VAMP2, or vesicle-associated membrane protein 2, which may interact with t-SNARE Syn4 *(Fig. 1.12)* (Volchuk *et al*, 1996; Cheatham *et al*, 1996).

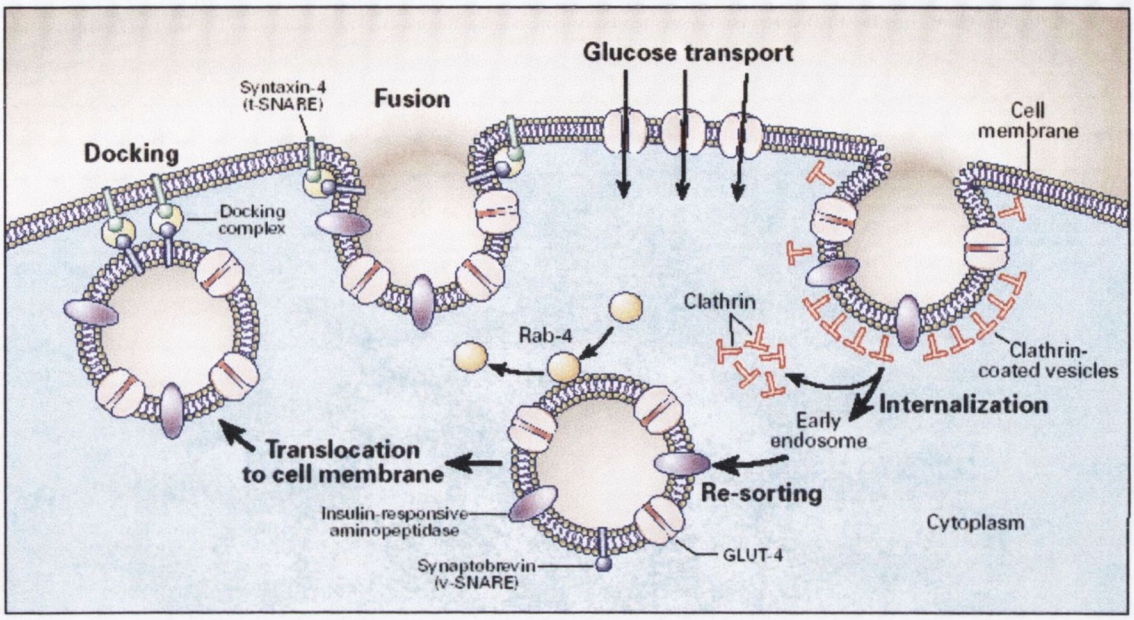


Fig. 1.12: Mechanisms involved in the translocation of the Glut4 vesicle (Shepherd & Kahn, 1999).

Glut-4, glucose transporter-4; t-SNARE, Target-soluble N-ethylmaleimide-sensitive factor attachment protein receptors; v-SNARE, vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor.

Although these SNARE interactions are essential, neither protein is a direct target of insulin. Instead the SNARE accessory proteins soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP)-23, syntaxin 4 interacting protein (Synip) and Munc18c may be involved in regulating the docking and fusion of VAMP2-containing Glut4 vesicles (Rea *et al*, 1998; Thurmond *et al*, 1998; Min *et al*, 1999). Synip has been shown to interact with Syn4 under basal conditions and to dissociate from it in response to insulin. Since Synip competes for VAMP2 binding, the dissociation of Synip following insulin stimulation is thought to allow VAMP2 to bind Syn4, and might provide a signal for the docking of the Glut4 vesicle *(Fig. 1.13)* (Watson & Pessin, 2001). Munc18c may mediate its effect in a similar way by inhibiting Glut4 vesicle fusion in the basal state, and then undergoing a conformational change so that interactions between Syn4 and VAMP2 are facilitated (Watson & Pessin, 2001).

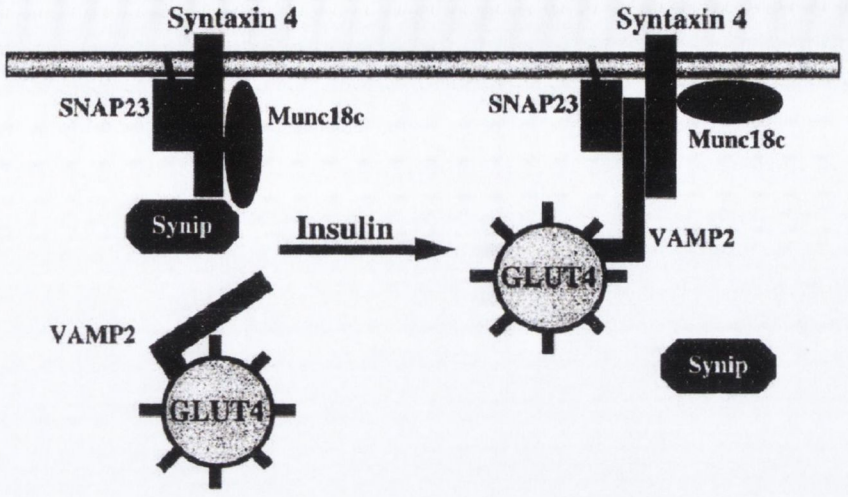


Fig. 1.13: Proposed model of Glut4 vesicle docking with the plasma membrane (Watson & Pessin, 2001).

Glut, glucose transporter; VAMP, vesicle-associated membrane protein; Synip, syntaxin 4 interacting protein; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; Munc18c, Mammalian unc18c.

Glut4 mRNA and protein in adipose tissue have been shown to be severely reduced in subjects with obesity, IGT or T2DM. In addition the ability of insulin to stimulate Glut4 translocation is also impaired (Shepherd & Kahn, 1999). Originally this was not considered to be physiologically important as skeletal muscle accounts for up to 85% of glucose disposal, and adipose tissue accounts for less than 10% of whole-body glucose uptake (DeFronzo *et al*, 1981). However tissue specific gene targeting of the Glut4 transporter provided an insight into the importance of adipose tissue in whole-body insulin sensitivity. Interestingly adipose tissue specific ablation of Glut4 caused a similar degree of insulin resistance as muscle specific Glut4 ablation. Following adipose tissue specific ablation of Glut4, glucose uptake in skeletal muscle was impaired despite preserved Glut4 expression. In addition, insulin-stimulated suppression of hepatic glucose production was also impaired. Insulin-stimulated activation of PI 3-kinase was impaired in both these tissues, which could contribute to the defective insulin responses observed (Abel, 2001; Minokoshi *et al*, 2003). It was postulated that an adipokine, or lack thereof, was a causative factor for the insulin resistance in muscle and liver as basal and insulin-stimulated glucose uptake in muscle were normal from these mice *ex vivo*. Serum levels of leptin, adiponectin and TNFα were normal, therefore suggesting that an unidentified adipocyte-derived molecule could be mediating the effect (Minokoshi *et al*, 2003). Nevertheless these results highlight the importance of inter-tissue communication in regulating glucose homeostasis.

Insulin sensitivity is determined by the ability of insulin to promote glucose uptake and also to utilise glucose effectively. Once transported into the cell glucose is rapidly phosphorylated to glucose 6-phosphate by hexokinases. Subsequently there are three major pathways governing glucose utilisation:

- Oxidation to pyruvate, which may undergo further oxidation in the citric acid cycle (also called Krebs cycle and the tricarboxylic acid cycle), thereby generating energy. This occurs in virtually all living-cells in the body. Non-essential amino acids may be generated in the liver following the entry of acetyl Coenzyme A (derived from pyruvate) into the citric acid cycle.
- Storage as the polysaccharide glycogen for rapid utilisation at a later time. This occurs predominantly in liver and muscle
- Conversion to other sugars and other intermediates essential for other biosynthetic pathways e.g. generation of glycerol 3-phosphate used in TAG and phospholipid synthesis. This is the major cellular fate of glucose in adipose tissue, muscle and liver (Bouche *et al*, 2004)

The proportion of glucose entering into each of the pathways depends on physiological conditions. If energy is immediately required, oxidation of glucose provides a source of energy. When liver glycogen stores are depleted, most of the glucose carbon taken up by the liver is used for glycogen synthesis. Once liver glycogen is replenished, more of the ingested glucose and more of the lactate is used for fatty acid synthesis. With increases in the size of the glucose load, more glucose is stored as muscle glycogen (Kruszynske, 2003). Pertubations in each of the aforementioned pathways occurs in T2DM (Bouche *et al*, 2004).

1.7.6 Effect of targeted deletions of components of the insulin signalling pathway

Evidence suggests that there are multiple genes which are associated with an increased risk of T2DM and insulin resistance. However in addition, variation due to racial background and modulation by environmental factors contribute to the heterogeneity and overall complexity of these conditions. Targeted deletions of components of the insulin signalling have yielded some insights into the functional relevance of individual components. *Table 1.11* provides a summary of the phenotypes observed in selected monogenic, polygenic and tissue-specific murine knockout models of insulin resistance.

Table 1.11: Monogenic, polygenic and tissue-specific murine knockout models of insulin resistance (Adapted from Saltial & Kahn, 2001; Mauvais-Jarvis *et al*, 2002; Nandi *et al*, 2004)[1].

Gene	Phenotype	Reference
Monogenic knockout models		
Insulin receptor $^{-/-}$	Normal intrauterine growth; die of diabetic ketoacidosis at 3-7 days	Accili *et al*, 1996; Joshi *et al*, 1996
IRS-1 $^{-/-}$	Insulin resistance, IGT, growth retardation	Araki *et al*, 1994; Tamemoto *et al*, 1994
IRS-2 $^{-/-}$	Insulin resistance, decreased β-cell development, T2DM	Withers *et al*, 1998
IRS-3 $^{-/-}$	Normal growth, NGT	Fantin *et al*, 2000
IRS-4 $^{-/-}$	Normal growth, NGT	Fantin *et al*, 2000
Akt2 (PKBβ) $^{-/-}$	Insulin resistance in liver and muscle	Cho *et al*, 2001
Glut 4 $^{-/-}$	Cardiac hypertrophy and/or failure, NGT but possible insulin resistance (evident by hyperinsulinaemia)	Katz *et al*, 1995
P85a $^{-/-}$	Increased insulin sensitivity, hypoglycaemia	Terauchi *et al*, 1999
PTP1B $^{-/-}$	Increased insulin sensitivity, resistance to diet-induced obesity	Elchebly *et al*, 1999
SHIP2 $^{-/-}$	Increased insulin sensitivity	Clement *et al*, 2001
Syn4 $^{-/-}$	Insulin resistance	Yang *et al*, 2001
JNK $^{-/-}$	Increased insulin sensitivity	Hirosumi *et al*, 2002
IKKβ $^{+/-}$	Increased insulin sensitivity	Yuan *et al*, 2001
Polygenic knockout models		
IR/IRS-1 $^{+/-}$	Severe muscle and liver insulin resistance, selective β-cell hyperplasia, development of T2DM in 50% of adult mice	Bruning *et al*, 1997
Tissue specific knockouts - Glut4 $^{-/-}$		
Adipose tissue	IGT, hyperinsulinaemia, secondary insulin resistance in muscle and liver	Abel *et al*, 2001
Skeletal Muscle	Reduced basal, insulin and contraction-stimulated glucose transport; severe insulin resistance; glucose intolerance, T2DM	Zisman *et al*, 2000

Glut, glucose transporter; IGT, impaired glucose tolerance; IKK, inhibitor of κB kinase; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal j-kinase; NGT, normal glucose tolerance; p85α, p85 regulatory subunit of the phosphoinositide-3 kinase; PKB, protein kinase B; PTB-1B, protein tyrosine phosphatases-1B; SHIP2, SH2 domain-containing inositol-5-phosphatase-2; Syn4, syntaxin 4.
[1] All knockout were generated in murine models.

To finalise Pessin & Saltiel (2000) observe that it is possible that there may be no single defect that underlies peripheral insulin resistance. They consider two options. Firstly that insulin resistance is a complex phenomenon in which several genetic defects combine with environmental stresses, such as obesity or infections, to generate the phenotype. Alternatively it is possible that there are no molecular defects in any signalling or effector system, but that

several key molecules function at the lower range of normal. In combination these contribute to reduced signal transduction, which is insufficient to generate the full response to glucose (Pessin *et al*, 1999).

1.8 TRANSCRIPTION FACTORS AND INSULIN SENSITIVITY

Regulation of metabolic pathways involves the rapid modulation of the activity of specific proteins including enzymes and transporters but also, on a longer-term basis, changes in the quantity of the protein. This can be achieved by modulating their transcription rate through the action of specific transcription factors (Ferre, 2004). Peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), sterol regulatory element binding protein (SREPB) and nuclear Factor kB (NF-κB) are all transcription factors which are involved in the regulation of glucose homeostasis either through direct and indirect mechanisms. Each will be discussed in turn below. Nuclear receptors are one of the largest groups of transcription factors (Francis *et al*, 2003).

1.8.1 Peroxisome proliferator-activated receptor

Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor and member of the nuclear receptor superfamily (Chinetti *et al*, 2003). Once bound to a ligand, the PPAR-ligand complex forms a heterodimer with another nuclear hormone receptor, retinoid X receptor (RXR), which is bound to its' ligand, 9-cis-retinoic acid. The PPAR-RXR heterodimer binds to a specific DNA response elements known as PPAR response elements or PPRE. PPAR-RXR can modulates the transcription of target genes either through direct promoter-binding and transcriptional modulation, termed "transactivation". Alternatively PPAR-RXR can modulate the transcription of target genes by indirect interference with other transcription factor pathways, leading in most cases to transcription inhibition, termed "transrepression" (Sewter & Vial-Puig, 2002; Chinetti *et al*, 2003).

Three subtypes of PPAR have been identified in amphibians, rodents, fish and humans, termed PPARα, PPARβ (or PPARδ in mice) and PPARγ. Each receptor subtype displays distinct patterns of expression and overlapping but distinct biological activities (Li & Glass, 2004). PPARα is predominantly expressed in liver, kidney, and the heart and activation of this isoform induces the expression of the fatty acid transporters and enzymes including LPL, fatty acid acetyl-CoA synthase, acyl-CoA oxidase, and carnitine palmitolytransferase I, which contribute to fatty acid catabolism (Moller & Berger, 2003). PPARα is also involved in the regulation of oxidation. Activation of PPARα results in a decrease in serum TAG levels and an increase in HDL-cholesterol concentrations (Choi *et al*, 2004). Insulin sensitising effects have also been observed with PPARα ligands, most likely due to a decrease in hepatic and/or

muscle lipid content (Moller & Berger, 2003). PPARβ is expressed ubiquitously and is thought to modulate skin biology, lipid metabolism and energy homeostasis. The role it plays in atherosclerosis is unclear at present (Li & Glass, 2004). The PPARγ gene gives rise to three isoforms, PPARγ1, PPARγ2 and PPARγ3. Proteins produced from PPARγ1 and PPARγ3 mRNAs are identical, whereas the PPARγ2 protein contains an additional 30 amino acids at the NH_2-terminus (Kintscher & Law, 2005). Both PPARγ isoforms are most highly expressed in adipose tissue, with PPARγ2 expression specific to this tissue (Moller and Berger, 2003). PPARγ1 is also detectable in other insulin target tissues including the liver and heart (Smith, 2003). PPARγ is the master regulator of adipogenesis (Kintscher & Law, 2005). The expression of both PPARγ1 and PPARγ2 are induced upon differentiation, with a marked induction of PPARγ2 (Chawla *et al*, 1994). The ligand-dependent activation domain of PPARγ2 has been reported to be 5-10 fold more effective than PPARγ1 (Wang & Tafuri, 2003). PPARγ2 expression is increased in adipocytes from morbidly obese individuals compared with lean individuals, while PPARγ1 expression is similar or slightly decreased (Vidal-Puig *et al*, 1997; Hotta *et al*, 1998). PPARγ2 expression was found to be 20 fold more abundant than PPARγ1 mRNA in both omental and subcutaneous fat depots of obese women (Giusti *et al*, 2003). It is thought that increased PPARγ mRNA may be a consequence of the expansion of fat mass observed in these subjects. The PPARs are activated by natural ligands such as fatty acids, eicosanoids and oxidised fatty acids and by pharmacological compounds (Chinetti *et al*, 2003). The fibrates and the TZD's are synthetic ligands for PPARα and PPARγ, respectively (Chinetti *et al*, 2003).

Much attention has focused on the TZD's as they are high affinity ligands for PPARγ and markedly improve whole body insulin sensitivity (Sewter & Vidal-Puig, 2002). TZD's differ from other anti-hyperglycaemic medication in that they enhance insulin sensitivity. In contrast, sulphonylureas and related insulin secretagogues increase insulin release from the pancreatic islets, metformin acts to reduce hepatic glucose production, α-glucosidase inhibitors interfers with gut glucose absorption, and insulin itself suppresses glucose production and augments glucose utilisation (Moller, 2001). TZD's directly increase the expression of a number proteins involved in the insulin signalling cascade including IRS-2, CAP and Glut4 (Ribon *et al*, 1998b; Smith *et al*, 2001; Young *et al*, 1995). TZD's also decrease the expression of TNFα and increase the expression of adiponectin (Combs *et al*, 2002). TZD treatment increased insulin-stimulated glucose uptake in L6 myocytes *in vitro* (Ciaraldi *et al*, 1990). Several potential mechanisms have been proposed to explain the insulin sensitising effects of the TZD's:

- TZD's are thought to function by sequestering NEFAs from the circulation, thereby reducing the deleterious effects of NEFA on muscle, hepatic and pancreatic insulin sensitivity.

- By promoting adipocyte differentiation they lead to the formation of small insulin-sensitive adipocytes (Okuno *et al*, 1998).
- TZD treatment has been shown to change body fat distribution from the omental to the subcutaneous depot (Sewter & Vidal-Puig, 2002; Adams *et al*, 1997).
- In addition, activation of PPARγ has been shown to inhibit the production of several pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 through inhibition of NF-κB activity (i.e. transrepression), thereby offering another potential mechanism for the amelioration in insulin sensitivity (Jiang *et al*, 1998a). However evidence also suggests that glitazones and natural PPARγ ligands exert anti-inflammatory activities in a PPARγ-independent manner (Chinetti *et al*, 2003).
- TZD's may also reduce the risk of atherosclerosis by increasing HDL-cholesterol and decreasing both intima and media thickness in carotid arteries (Minamikawa *et al*, 1998).

In mice PPARγ knockout is embryonically lethal due to a defect in placental development (Barak *et al*, 1999). A surprising result was found in heterozygous mice, as they were expected to develop insulin resistance and diabetes. However when PPARγ$^{+/-}$ mice were fed a normal diet, insulin sensitivity was similar to that of wild-type controls. When mice were fed a high-fat diet, wild-type mice developed insulin resistance as was expected. Meanwhile PPARγ$^{+/-}$ mice remained insulin sensitive. Therefore it appears that a reduction in PPARγ expression could protect against insulin resistance (Kubota *et al*, 1999). It has been suggested that the normal role of the endogenous receptor and ligands may be to attenuate insulin action, thereby offsetting exaggerated effects of glucose metabolism caused by unrestrained activation of the differentiation program induced by PPARγ stimulation. Consequently by displacing an endogenous ligand an increase in insulin sensitivity ensues (Miles *et al*, 2000). Alternatively in the absence of a ligand, the binding of co-repressor proteins may lead to transcriptional repression of key glucoregulatory genes. This may lead to a reduction in insulin action with insulin sensitivity being restored by genetically reducing the level of PPARγ or reversing the repression with a partially agonistic TZD (Miles *et al*, 2000; Sewter & Vidal-Puig, 2002). Nonetheless, activation of PPARγ is associated with an improvement in insulin sensitivity as mentioned above.

1.8.2 Liver X receptor

LXRα and LXRβ form obligate heterodimers with RXR and control transcription by binding to a direct repeat type 4 LXR response element located in the promoter of their target genes (Willy *et al*, 1995; Willy & Mangelsdorf, 1997). LXRβ is ubiquitously expressed, whereas the expression of LXRα is predominantly restricted to liver, skeletal muscle, adipose tissue, kidney

and the small intestine (Dalen *et al*, 2003). Naturally occurring oxysterols, mainly derivatives of cholesterol, function as high affinity ligands for LXRs (Janowski *et al*, 1996; Lehmann *et al*, 1997). LXR regulates the expression of a number of proteins involved in cholesterol and fatty acid metabolism including SREBP, FAS, LPL, CETP and phospholipid transfer protein (DeBose-Boyd *et al*, 2001; Joseph *et al*, 2002; Schultz *et al*, 2000). In macrophage and other peripheral cells, LXRs have been implicated in reverse transport by regulating genes involved in cellular cholesterol efflux including ABCA1, ABCG1 and apolipoprotein E (Chawla *et al*, 2001; Laffitte *et al*, 2001).

Recent evidence supports a role for LXR in regulating glucose homeostasis. Laffitte *et al* (2003) reported that activation of LXR in the liver led to the suppression of the gluconeogenic programme including down-regulation of peroxisome proliferator-activated receptor y coactivator-1α, PEPCK and G6Pase. Inhibition of gluconeogenic genes was accompanied by an induction in expression of glucokinase, which promotes hepatic glucose utilisation. In adipose tissue, activation of LXR resulted in the transcriptional induction of Glut 4. Therefore activation of LXR limits hepatic glucose output and improves peripheral glucose uptake (Laffitte *et al*, 2003). In addition LXR agonists have been shown to inhibit the expression of key enzymes involved in glycolysis in WAT (Steffensen & Gustafsson, 2004). Dalen *et al* (2003) demonstrated that adipose tissue expression of Glut4 is directly regulated by both LXRα and LXRβ upon ligand stimulation, but that basal expression of Glut4 is selectively dependent on the LXRα isoform. LXRα is down-regulated in obesity and diabetes, and therefore has been advocated as a novel antidiabetic drug (Garvey *et al*, 1991; Garvey *et al*, 1992; Dalen *et al*, 2003). However selective LXRα agonists will be required as administration of current LXR agonists increase plasma and liver TAG levels (Schultz *et al*, 2000; Cao *et al*, 2003). Nutritional conditions do not markedly change the expression of hepatic LXRα (Yoshikawa *et al*, 2001).

1.8.3 Sterol regulatory element binding protein

SREBPs are membrane-bound transcription factors that belong to the basic helix-loop-helix leucine zipper family (Brown & Goldstein, 1997). There are three forms of SREBP, SREBP-1a, SREBP-1c and SREBP-2 (Yoshikawa *et al*, 2003). SREBP-2 regulates genes encoding proteins involved in cholesterol metabolism. SREPB-1 exists in two forms, -1a and -1c, which are derived from the same gene through the use of alternate transcription promoters, resulting in different forms of exon 1 (Shimomura *et al*, 1997). SREBP-1a is the dominant form in cell lines and regulates genes encoding proteins involved in both lipogenesis and cholesterogenesis. SREBP-1c constitutes 90% of the SREBP-1 found *in vivo* and is a determinant of lipogenic gene transcription (Osborne, 2000; Brown & Goldstein, 1997). Recent *in vivo* studies have demonstrated that SREBP-1c plays a crucial role in the dietary

regulation of the majority of lipogenic genes (Yoshikawa *et al*, 2003). SREBP-1 is synthesised as a 125kDa precursor protein which is anchored in the endoplasmic reticulum membrane. Proteolytic release of the 68kDa mature SREBP-1 occurs in the Golgi system, and movement of SREBP-1 from the endoplasmic reticulum to the Golgi requires the trafficking protein SREPB cleavage-activating protein. Once released, mature SREPB-1 translocates to the nucleus where it binds to the enhancer promoter region of target genes (Osborne, 2000; Brown & Goldstein, 1997). Over-expression of mature SREBP-1a in the liver is associate with high rates of fatty acid biosynthesis, and ablation of the SREBP-1 gene results in low expression of lipogenic genes (Osborne, 2000; Brown & Goldstein, 1997). Shimomura *et al* (2000) showed that insulin promotes hepatic lipogenesis via SREBP-1c. Hepatic SREBP-1c is nutritionally regulated, such that levels are induced by feeding and suppressed by fasting (Yoshikawa *et al*, 2003). Polyunsaturated fatty acids (PUFA) have been shown to inhibit the lipogenic pathway by competitive inhibition with physiological LXR agonists (oxysterols) for LXR ligand binding domain. This reduces SREBP-1c induction by LXR's which is a crucial step in lipogenesis (Clement *et al*, 2002). Diets rich in linoleic acid (18:2n-6) or eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were found to reduce both the hepatic nuclear and precursor content of mature SREBP-1. Hepatic nuclear SREBP-1 was reduced by 65% by linoleic acid and by 95% by EPA/DHA. Mature SREBP-1 was reduced by 60% by linoleic acid and by 75% by EPA/DHA (Xu *et al*, 1999). Unlike PUFA saturated and monounsaturated fatty acids had no effect on the nuclear content or precursor content of SREBP-1 or on lipogenic gene expression (Xu *et al*, 1999; Worgall *et al*, 1998; Osborne, 2000; Brown & Goldstein, 1997).

1.8.4 Nuclear Factor-κB

NK-κB is a heterodimeric transcription factor that plays a key role in inflammatory and immune responses (Karin & Delhase, 2000). In non-stimulated cells NF-κB is maintained in an inactive form in the cytoplasm through interaction with inhibitory proteins of NF-κB (IκBs) (Schmitz *et al*, 2001). In response to cell stimulation by pro-inflammatory cytokines including IL-1 and TNFα, physical and chemical stresses, viruses or bacteria, the IκB kinase (IKK) complex, comprising IKKα, IKKβ, IKKγ/NFκB essential modulator, is rapidly activated and phosphorylates two critical serine residues in the N-terminal regulatory domain of the IκBs (Karin & Delhase, 2000; Schmitz *et al*, 2001). This promotes the release of the NF-κB dimer and IκBs are targeted for ubiquitination and proteolytic degradation (Schmitz et al, 2001). Released NF-κB then translocates to the nucleus, binds to its cognate DNA element and activates transcription of numerous target genes encoding proteins involved with immune or inflammatatory responses (Schmitz *et al*, 2001; Baldwin, 1996). These include IL-2, IL-6, IL-8, ICAM, VCAM and E-selectin (Baldwin, 1996). Many of these protein are elevated in

obesity and insulin resistance, as discussed earlier. The DNA-binding form of NF-κB is dimeric, with the p50-p65 heterodimer the most frequently observed form (Schmitz et al, 2001). In addition to regulation at the level of IκB, modulatory phosphorylations of the DNA-binding subunits can control several functions of NF-κB including DNA-binding and transactivation properties, as well as interactions between the transcription factor and regulatory proteins (Schmitz *et al*, 2001).

In vivo salicylate treatment, a known inhibitor of IKK-β, prevented fat-induced insulin resistance in rats (Kim *et al*, 2001). In addition, lipid infusion failed to reduce insulin-stimulated glucose uptake and IRS-1 activation in skeletal muscle of heterozygous IKK-β mice (Kim *et al*, 2001). These studies suggest that attenuation or lack of key inflammatory components may offer substantial protection from obesity induced insulin resistance. Ruan *et al* (2002a) showed that NF-κB is an obligatory mediator of the TNFα induced changes in gene expression which could lead to insulin resistance in 3T3-L1 adipocytes. Therefore components of the NF-κB system are potential targets for pharmaceutical interventions aimed at ameliorating obesity induced insulin resistance

1.9 NUTRITION AND INSULIN SENSITIVITY

In view of the significant health and socio-economic burdens associated with T2DM and the metabolic syndrome, there a need to identify effective dietary strategies which are capable of attenuating the impact of T2DM either by reducing risk of the disease, or minimising it's complications. Dietary manipulation has yielded positive results. Intensive lifestyle changes significantly improve insulin sensitivity. McAuley *et al* (2002) reported a 23% increase in insulin sensitivity following a 4 month diet and exercise intervention programe in normoglycaemic insulin-resistant individuals [Dietary intervention comprised <26% of total energy from fat, <6% energy from saturated fatty acids (SFA), 13% energy from monounsaturated fatty acids (MUFA), 7% energy from PUFA, 55% energy from carbohydrate and 18% energy from protein, cholesterol target <140mg/day and dietary fibre >35g/day; Exercise intervention comprised 5 training sessions per week for at least 20 min each at an intensity of 80-90% of age-predicted maximum heart rate]. As little as 5% weight loss has been shown to significantly improve insulin action, decrease fasting blood glucose concentrations, decrease blood pressure, improve serum lipid concentrations, and reduce the need for diabetes medication (Klein *et al*, 2004). Data from the Diabetes Prevention Program indicated that lifestyle changes were nearly twice as effective as the pharaceutical agent metformin in the prevention of T2DM in subjects with IGT. Lifestyle changes resulted in a 58% reduction in the incidence of T2DM, compared to a 31% reduction following treatment

with metformin (Knowler *et al*, 2002). However despite the benefits associated with reducing adiposity, the ability to maintain this weight loss has had little success, with reports indicating that less than 10% of subjects are able to maintain the weight loss (Garner, 1991; Goodrick & Foreyt, 1991).

Insulin sensitivity may also be influenced by diet composition. Altering the quality of fat or carbohydrate in the diet offers potential ways of modulating insulin sensitivity. The source and quality of dietary carbohydrates differentially influence insulin action such that higher intakes of whole-grain foods, dietary fibre, cereal and fruit fibre, and diets with a lower glycaemic index are associated with higher insulin sensitivity (McKeown *et al*, 2004). Altering the quality of fat in the diet may offer another potential route for improving insulin sensitivity through dietary therapy. This is the focus of the current post-graduate doctorate.

1.9.1 Role of fat in modulating insulin sensitivity

Several cross-sectional studies have shown that there is a positive relationship between saturated fat intake and hyperinsulinaemia, independent of body fat (Maron *et al*, 1991; Parker *et al*, 1993; Mayer *et al*, 1993; Feskens *et al*, 1994; Marshall *et al*, 1997). The KANWU study showed that at a total fat intake of <37%, isoenergetic substitution of MUFA for SFA improved insulin sensitivity (Vessby *et al*, 2001). A reduction of 12.5% in insulin sensitivity was observed following the high SFA diet, while insulin sensitivity increased by 8.8% following the high MUFA diet. In individuals with a fat intake >37%, this beneficial effect was not observed. Similarily Perez-Jimenez *et al* (2001) reported that isoenergetic substitution of MUFA or carbohydrates for SFA markedly improved insulin sensitivity in healthy young subjects. In contrast Lovejoy *et al* (2002) failed to see any major effect on insulin sensitivity when they compared MUFA, SFA and trans-fatty acid diets in healthy men and women. However this may be due to the small number of subjects recruited (n=25). Closer analysis indicated that insulin sensitivity was 24% lower in overweight individuals (BMI 25-30kg/m^2) following the SFA diet compared to the MUFA diet. These diets were quite low in fat, comprising 28% total energy, which may suggest that the effects of altering dietary fat composition may be more easily detected without the background of a low-fat diet (Roche, 2005).

Stefan *et al* (2001) showed the benefit of PUFA in an acute study in which NEFA with two different compositions were infused in healthy humans. Results indicated a significant decrease in insulin sensitivity after both infusions, as expected. However the infusion of NEFA with a low level of PUFA lowered insulin sensitivity approximately twice as much as the NEFA infusion with a high level of PUFA.

Prospective cohort studies suggest that in an elderly population, the habitual consumption of small amounts of fish may protect against the development of IGT and T2DM

(Feskens *et al*, 1991). Animal studies have demonstrated protective effects of long-chain n-3 PUFA, EPA (20:5n-3) and DHA (22:6n-3) on insulin sensitivity. Rats fed a high fat diet developed insulin resistance, while rats fed a high-fat diet which was also high in n-3 PUFA remained insulin sensitive. Marked effects were observed in both the liver and skeletal muscle (Storlien *et al*, 1987). In addition, Aguilera *et al* (2004) reported improvements in plasma insulin concentrations following 6 wk fish oil administration in a rat model of the metabolic syndrome. However human studies have provided mixed results. Both Fasching *et al* (1991) and Popp-Snijders *et al* (1987) reported improvements in insulin sensitivity following fish oil supplementation in subjects with IGT and T2DM respectively. However Brady *et al* (2004) failed to show an improvement in insulin sensitivity following fish oil supplementation in Indian Asian men with a background of a high dietary intake of n-6 PUFA, despite observing improvements in fasting and postprandial plasma TAG concentrations. In addition the inclusion of n-3 PUFA to either the high MUFA diet or the high SFA diet had no effect on insulin sensitivity in the KANWU study. Similarly insulin-mediated glucose utilisation was unchanged following 6 month fish oil supplementation in subjects with T2DM (Rivellese *et al*, 1996).

In summary, both observational and intervention studies underlie the importance of the quality of fat in the diet. However using this strategy to improve insulin sensitivity is hindered in view of the poor success in individuals and populations reaching nutritional targets e.g. reducing saturated fat to 10% total calories, and with the ever-increasing availability of snack-type foods on supermarket shelves. Unequivocal evidence of the benefits of long chain n-3 PUFA remains to be established. However using natural food constituents with bioactive properties, with a view to forming functional foods, may represent an effective strategy to attenuate the clinical consequences of insulin resistance.

1.9.2 Conjugated linoleic acid

Conjugated dieonic derivatives of linoleic acid are a mixture of geometric and positional isomers of linoleic acid (18:2n-6) (Pariza *et al*, 2000). Conjugated linoleic acid (CLA) isomers differ from linoleic acid, in that the double bonds of CLA are separated by a single carbon-carbon bond, and not by a methylene group (MacDonald *et al*, 2000; Roche *et al*, 2001). Structurally the double bonds may be present on carbons 7 and 9, 8 and 10, 9 and 11, 10 and 12, 11 and 13, in either a *cis* or *trans* configurations (Moya-Camarena & Belury, 1999). CLA is a natural food component found in the lipid fraction of ruminant meat, milk and other dairy products (Roche *et al*, 2001). *Cis*-9, *trans*-11 CLA (c9, t11-CLA or rumenic acid) is the predominant dietary isoform, followed by *trans*-7, *cis*-9 CLA, *cis*-11, *trans*-13 CLA and *cis*-8, *trans*-10 CLA and *trans*-10, *cis*-12 CLA (t10, c12-CLA) (Belury, 2002: Griinari *et al*, 1998; Kramer *et al*, 1998; Corl *et al*, 2002). c9, t11-CLA and t10, c12-CLA are thought to have most

biological activity, and most research has investigated these two isoforms. *Fig. 1.14* illustrates the structural differences of the two isoforms compared to linoleic acid.

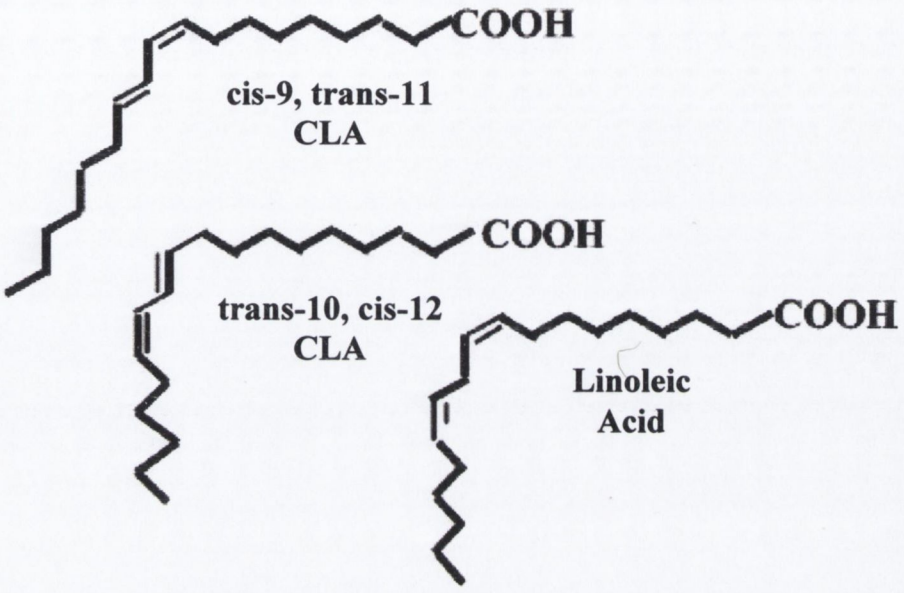


Fig. 1.14: Structure of cis-9, trans-11 CLA and trans-10, cis-12 CLA and linoleic acid (Belury, 2002)

Dietary intakes of CLA are estimated to be in the range of 15 to 430mg/day, with c9, t11-CLA contributing approximately 85-95% total CLA content (Ens *et al*, 2001; Ritzenthaler *et al*, 2001). Dietary sources of CLA are listed in *Table 1.12*.

Table 1.12: CLA content of various foods (Data from Chin *et al*, 1992; Evans *et al*, 2002a).

Food Source	g/100g fat	Food Source	g/100g fat
Dairy Products		***Meat/Poultry/Fish***	
Whole Milk	0.55	Lamb	0.58
Low fat milk	0.41	Fresh Ground Beef	0.43
Condensed Milk	0.70	Veal	0.27
Cultured buttermilk	0.54	Fresh Ground Turkey	0.26
		Chicken	0.09
Butter	0.47	Pork	0.06
		Egg Yolk	0.06
Plain Yoghurt	0.48	Salmon	0.03
Low-fat yoghurt	0.44		
Custard style yoghurt	0.48	***Vegetable Oils***	
Frozen yoghurt	0.28	Safflower Oil	0.07
		Sunflower Oil	0.04
Cheddar Cheese	0.41	Peanut	0.02
Processed Cheese	0.50	Olive	0.00
Ricotta	0.56		
Mozarella	0.49		
Cottage	0.45		
Parmesan	0.30		
Ice-cream	0.36		
Sour cream	0.46		

Initially it was thought that c9, t11-CLA was primarily produced during microbial biohydrogenation of linoleic and linolenic acids by Butyrovibrio fibrisolvens in the rumen of cattle and other ruminant animals (Kepler *et al*, 1966). However Griinari and Bauman (1999) reported that approximately 70% of c9, t11-CLA in milk fat is derived from endogenous synthesis in the mammary gland by the action of Δ9 desaturase on vaccenic acid or *trans*-11 octadecenoic acid. Vaccenic acid itself is formed by rumen metabolism of c9, t11-CLA, which then passes from the rumen to the mammary gland. The amount of CLA present in food is influenced by rumen microbial population, frequency of lactation, feeding regimens, animal breed and the season (Sebedio *et al*, 1999; MacDonald, 2000). CLA levels are higher in milk from cows fed on pasture in the summer than cows fed concentrate diets (MacDonald, 2000). Aged cheeses have lower amounts of CLA, while high temperatures, the addition of iron, whey powder or non-fat dry milk result in higher concentrations of CLA in processed cheeses (MacDonald, 2000). CLA in foodstuffs is stable, and is not destroyed by cooking or storage (Shantha *et al*, 1994). Herbel *et al* (1998) found that feeding 16g/day linoleic acid for 6 wk did not increase plasma CLA concentrations, despite the presence of Butyrivbrio fibrisolvens in the digestive tract of man. More recently however, several strains of bifidobacterium, found in either adult or infant intestine, have been shown to produce CLA and in particular the

c9, t11-CLA isoform from free linoleic acid *ex vivo*. This may offer novel opportunities for producing health-promoting functional foods, with the benefits of enriched CLA and probiotic bacteria (Coakley *et al*, 2003). CLA may be biosynthesised in man by the conversion of trans fatty acids into CLA (Salminen *et al*, 1998; Adlof *et al*, 2000). However, it appears that in man the predominant source of CLA is through dietary intake (Herbel *et al*, 1998; Roche *et al*, 2001).

Industrially CLA is produced via alkaline-catalysed isomerisation of linoleic acid. Synthetically prepared oils of CLA are composed of an isomeric composition that differs from isomers found naturally in food. Typically industrial production yields a CLA oil of 90% purity composed of approx 42% c9, t11-CLA, 43% t10, c12, with minor amounts of other CLA isomers (including *cis*-9, *cis*-11 CLA, *trans*-9, *trans*-11 CLA), 0.5% residual linoleate, 5.5% oleate and 4% unidentified fatty acid) (Belury, 2002). This is designated a 50:50 product. However the chemical method of synthesis allows a variety of ratios in the final mixture (Wahle *et al*, 2004). The high cost and/or lack of availability of highly purified isomers or naturally extracted CLA oil, initially limited the investigation of the specific effects of different isomers. Consequently the majority of physiologically effects of CLA determined to date are due to synthetic mixtures of CLA isomers (Belury, 2002).

The CLA isomers in commercial products additionally differ from dietary sources of CLA, as they are usually present as free fatty acids whereas dietary sources of CLA isomers are largely in the form of triglycerides (Taylor & Zahradka, 2004). Ip *et al* (1995) demonstrated that the major outcomes for mammary gland morphogenesis and cancer risk were similar in rats fed CLA-enriched butter with CLA in the form of triglycerides compared to rats fed a commercial mixture of CLA isomers as free fatty acids. However Rahman *et al* (2001) reported elevated plasma glucose concentrations in male OLETF rats following feeding 1% CLA in the form of FFA, whereas plasma glucose concentrations were comparable with the control group when CLA was provided as triglycerides. In theory providing fatty acids in the form of FFA or triglyceride should give similar results. However, potential differences in the biological activity of CLA may stem from differences in digestion, absorption and transport of CLA isomers present as free fatty acids versus triglyceride (Taylor & Zahradka, 2004). The majority of published studies have used CLA in the form of free fatty acid. However the provision of CLA in the form of triglyceride may be more acceptable (Sededio *et al*, 1999) in the event of functional foods being produced, as this is the natural form of the fatty acid.

c9, t11-CLA was found to comprise between 0.12 to 0.5% by weight (mean 0.25% by weight) of fatty acids in total serum lipids from fasted adult human blood and approximately 0.5% by weight of total fatty acids in human adipose tissue (Jiang *et al*, 1999). Supplementation with c9, t11-CLA or t10, c12-CLA increases the incorporation of these

isomers into human plasma lipids (total lipid fraction, phospholipid fraction, TAG and cholesterol ester fraction) and peripheral blood mononuclear cells in a dose-dependent manner (Benito *et al*, 2001: Kelley *et al*, 2001; Burdge *et al*, 2004). Similarly serum levels of CLA are increased in humans consuming foodstuffs which are rich in CLA (Britton *et al*, 1992; Huang et al, 1994). White adipose tissue levels of c9, t11-CLA correlate with milk fat intake (Jiang *et al*, 1999).

Isomers of CLA are readily metabolised *in vivo* and metabolites of CLA including conjugated gamma linoleic acid, conjugated dihomogamma linoleic acid and conjugated arachionic acid have been found in plasma, liver, mammary and adipose tissue of rats (Ip *et al*, 1997; Banni *et al*, 2002), and in adipose tissue and sera of humans (Banni *et al*, 1999; Lucchi *et al*, 2000; Belury, 2002). The physiological roles of such CLA metabolites remains to be determined.

Although initially identified as a component in fried ground beef with potential anti-carcinogenic properties against chemically induced tumour formation, an array of health benefits has subsequently been attributed to CLA. CLA is reported to decrease the initiation, promotion and progression of carcinogenesis in both *in vitro* and *in vivo* models of skin, mammary, forestomach, prostate and colon cancer (Ip *et al*, 1991; Liew *et al*, 1995; Belury *et al*, 1996). Anti-diabetic, anti-obesity, anti-atherosclerotic, lipid lowering, immunomoulatory and effects on bone formation have also been attributed to CLA (Kelly, 2001; Roche *et al*, 2001; Belury, 2002). The effect of CLA on insulin resistance will be discussed in detail.

1.9.3 CLA and insulin resistance

Studies to date have indicated that the effect of CLA on insulin resistance and glucose homeostasis is variable and dependent on species and the type of isomer present. These studies are decribed below. A summary of the results from the animal feeding studies is provided in *Table 1.13a* and a synopsis of the phenotypes of the animal models is highlighted in *Table 1.13b*.

Table 1.13a: Results from animal feeding studies assessing the effect of CLA on indices of insulin sensitivity.

Reference	Model	Number per Group	Intervention details	Study period (d or wk)	Glucose Tolerance	Fasting Glucose conc.	Fasting Insulin conc.
Houseknecht *et al*, 1998	6 wk old male Zucker Diabetic Fatty rats	8	5% corn oil + 1.5% CLA (50:50 blend) 5% corn oil + 1.5% lard + 0.2% troglitazone	14 d	↑ ↑	↓ ↓	↓ ↓↓
Stangl *et al*, 1999	Male pigs	8	1% CLA per weight diet (34.6% c9, t11-CLA, 18.4% t10, c12-CLA, 5.4% t9, t11-CLA, 2.1% c9, c11-CLA)	6 wk	NA	↔	↔
DeLany *et al*, 1999	6 wk old male AKR/J mice	12	High-fat diet (45% energy) 0.25% CLA (50:50 product) 0.5% CLA (50:50 product) 0.75% CLA (50:50 product) 1% CLA (50:50 product)	39 d	 NA NA NA NA	 ↔ ↔ ↔ ↔	 ↑ NS ↑ NS ↑ NS ↑
Tsuboyama-Kasaoka *et al*, 2000	8wk old female C57BL/6J mice	4-5	Low-fat diet (11% energy) 1% CLA (50:50 product)	17 wk	↔	↔	↑
West *et al*, 2000	4 wk old male AKR/J mice	8	High fat diet (45% energy) 1% CLA (50 :50 blend)	5 wk	NA	↔	↑NS
Ryder *et al*, 2001	6 week old male Zucker Diabetic Fatty rats	8 for ctrl 16 for CLA	High-fat diet (40% energy) 1.5% CLA (50:50 isomer blend) 1.5% CLA (90% c9, t11-CLA, 0.8% t10, c12-CLA)-from butter fat	14 d	 ↑ ↔	 ↓ ↔	 ↓ ↔
Rahman *et al*, 2001	4 wk old male OLETF rats	5-6	5.5% safflower oil + 1.0% CLA as triglyceride 5.5% safflower oil + 1.0% CLA as free fatty acids (CLA 50:50 blend in both)	4 wk	NA NA	↔ ↑	↔ ↔
Roche *et al*, 2002[1]	6 wk old male ob/ob mice	8	High fat diet (30% energy) c9, t10-CLA [0.43% CLA (82.3% c9, 15.6% t10)] t10, c12-CLA [0.47% CLA (77.5% t10, 17.4% c9)]	4 wk	 NA[1] NA[1]	 ↑ NS[1] ↑[1]	 ↑ NS[1] ↑[1]
Hamura *et al*, 2002	7 wk old Diabetic C57BLKS-lepr^{db}/lepr^{db} mice	Not provided	Medium fat diet 1.2% CLA as triglyceride (33.7% cis, trans/trans cis-9, 11; 35.2% trans, cis-10, 12, 2.8% other isomers)	5 wk 11 wk	↑ ↑	↔ ↔	↑ ↓

50:50 isomer blend indicates equal quantities of c9, t11-CLA and t10, c12-CLA

↓ = decrease compared to placebo; ↑ = increase compared to placebo; ↓NS = decreased but not statistically significant compared to placebo; ↑ NS = increased but not statistically significant compared to placebo; ↔ = no statistically significant change; NA = not measured

[1] non-fasting samples.

Table 1.13a (contd.): Results from animal feeding studies assessing the effect of CLA on indices of insulin sensitivity.

Reference	Model	Number per Group	Intervention details	Study period (d or wk)	Glucose Tolerance	Fasting Glucose conc.	Fasting Insulin conc.
Clement *et al*, 2002	Female C57BL/J mice	8	2.4% energy form sunflower oil 0.4% c9, t11-CLA (92% purity) 0.4% t10, c12-CLA (96% purity)	4 wk	NA NA	↔ ↔	↔ ↑
Henriksen *et al*, 2003	8-9 wk female obese Zucker rats	5	1.5g total CLA/kg body weight 50:50 blend c9, t11-CLA (76% purity) t10, c12-CLA (90% purity)	21 d	↑ ↔ ↑	↔ ↔ ↓	↓ ↔ ↓
Nagao *et al*, 2003	6 wk old male ZDF rats	6	5% corn oil + 1.0% CLA (50 :50 blend)	8 wk	NA	↓	↓
Faulconnier *et al*, 2004	17 wk old male Wistar rats	6	*Sedentary Rats* 1% c9, t11-CLA 1% t10, c12-CLA 1% c9, t11-CLA + 1% t10, c12-CLA *Trained Rats* 1% c9, t11-CLA 1% t10, c12-CLA 1% c9, t11-CLA + 1% t10, c12-CLA	6 wk	NA NA NA NA NA NA	↔ ↔ ↔ ↔ ↔ ↔	↔ ↔ ↔ ↔ ↓ NS ↔
Choi *et al*,2004	6 wk old male Sprague-Dawley rats	7	High fat diet (45% energy) 1% c9, t11-CLA 1% t10, c12-CLA 1% CLA mix (31.3% c9, t11-CLA, 36.6% t10, c12-CLA, 17.3% other isomers, 14.8% other fatty acids)	8 wk	NS ↓ (at 30 min) NS	↔ ↔ ↔	↓ ↓ NS ↓ NS
Wargent *et al*, 2005	Female C57BI/6 ob/ob mice	6	(Fat reported as g CLA/kg diet) CLA Blend (50:50) 25g/kg t10, c12-CLA (90% purity) 25g/kg c9, t11-CLA (90% purity) 25g/kg CLA Blend (50:50) 10g/kg CLA Blend (50:50) 25g/kg CLA Blend (50:50) 10g/kg CLA Blend (50:50) 25g/kg	2 wk 5 wk 10 wk 	↓ ↓ ↔ ↓ ↓ NS ↑ NS ↑	↑ ↑ ↔ ↑ ↑ ↔ ↔	↑ ↑ ↑ NS ↑ ↑ ↓ ↓

50:50 isomer blend indicates equal quantities of c9, t11-CLA and t10, c12-CLA

↓ = decrease compared to placebo; ↑ = increase compared to placebo; ↓NS = decreased but not statistically significant compared to placebo; ↑ NS = increased but not statistically significant compared to placebo; ↔ = no statistically significant change; NA = not measured

Table 1.13b: Animal models used in feeding studies assessing the effect of CLA on insulin sensitivity.

Animal	*Study Model was used in*	*Phenotype of animal*
Rodent Models		
Male Zucker Diabetic Fatty rats	1, 2, 3	Spontaneously develop diabetes at 7-12 wk due to β-cell decompensation; the development of hyperglycaemia is secondary to obesity, since food restriction can prevent hyperglycaemia in this model; phenotype due to a mutation in the leptin receptor[17, 18]
Female Zucker fa/fa rat model	4	Hyperlipemic, hypercholesterolemic, hyperinsulinemic, but not hyperglycaemic[19, 20, 21]
Male adult Wistar rats	5	Normal rats
Male Sprague-Dawley rats	6	Normal rats, disease resistant species[6]
Male OLETF rats	7	Displays hyperglycaemia secondary to mild obesity, hyperplastic pancreatic islets and insulin resistance; calorie restriction and exercise training are effective in causing weight reduction and in preventing the development of diabetes[14]
Murine Models		
Male AKR/J mice	8, 9	Sensitive to dietary obesity[9], moderate hyperglycaemia, diet-induced diabetes[15]
Female C57BL/6J	10, 11	Susceptible to diet-induced obesity, diabetes and atherosclerosis if fed a high fat diet. Both CLA studies which used this model fed the mice low-fat diets
Male C57BL/6 ob/ob mice	12	Exhibits marked obesity, hyperphagia, transient hyperglycaemia (present at around 1month of age and subsides by around 4-5 months), hyperinsulinaemia, insulin resistance, pancreatic β-cell hypertrophy and hyperplasia due to deficiency of leptin; hepatic glucose production is excessive; model for obesity, insulin resistance and related syndromes[16, 17]
Female C57BL/6 ob/ob mice	13	Similar to male ob/ob mice

[1]Houseknecht *et al*, 1998; [2]Ryder *et al*, 2001; [3]Nagao *et al*, 2003; [4]Henriksen *et al*, 2003; [5]Faulconnier *et al*, 2004; [6]Choi *et al*, 2004; [7]Rahman *et al*, 2001; [8]DeLany *et al*, 1999; [9]West *et al*, 2000; [10]Tsuboyama-Kasaoka *et al*, 2000; [11]Clement *et al*, 2002; [12]Roche *et al*, 2002; [13]Wargent *et al*, 2005; [14]Sone *et al*, 2001; [15]The Jackson Laboratory; [16]Bray & York, 1979; [17]Nagao *et al*, 2003; [18]Ryder *et al*, 2001; [19]Zucker & Zucker, 1962; [20]Zucker & Zucker, 1963; [21]Zucker & Antoniades, 1972; [22]Leiter (1992).

(i) Studies using rat models

The anti-diabetic effects of dietary CLA were first examined in studies with the Zucker Diabetic Fatty fa/fa (ZDF) rat model of obesity and T2DM. The ZDF rat exhibits an obese and hyperglycaemic phenotype at an early age (7-8 wk) due to a mutation in the leptin receptor. Feeding 1.5% CLA (50:50 product) to 6 wk old ZDF rats for 14 days normalised impaired glucose tolerance and attenuated fasting hyperinsulinaemia and NEFA concentrations in male ZDF rats (Houseknecht *et al*, 1998). Similar results were obtained when these rats were treated with troglitazone, a TZD. Although feed intakes were comparable, rats fed the control diet or the CLA-enriched diet weighed less than rats treated with TZD.

In a subsequent study it was found that a 1.5% CLA mixture (50:50 product) for 14 days reduced adiposity, fasting glucose, insulin and NEFA concentrations (Ryder *et al*, 2001). In addition glucose tolerance and insulin sensitivity were improved in skeletal muscle following the CLA blend compared with control ZDF rats. The pair-fed rats had a glucose tolerance response that was intermediate between the CLA mixture and control groups. Therefore the lower body weight of the CLA mixture group contributed in part to their improved response. These effects were not observed in rats fed a diet enriched with the c9, t11-CLA isomer (91% c9, t11-CLA derived from butter fat). These results imply that the anti-diabetic effects of CLA was mediated by the t10, c12-CLA isomer, as no improvement in the parameters were observed following the c9, t11-CLA enriched diet. A limitation of both these studies is that they were of short duration (2 wk) during the growing stage of the rats. However Nagao *et al* (2003) also demonstrated improved insulin and glucose concentrations in male ZDF rats following a 50:50 blend of CLA contributing 1% total energy. This study was of 8 wk duration.

Further studies also alluded to a beneficial role of feeding the t10, c12-CLA isomer to rats. Henriksen *et al* (2003) utilised the female Zucker fa/fa rat model, a model for obesity and the early stages of diabetes, whose phenotype also stems from a mutation of the leptin receptor. The fa/fa Zucker rat has impaired oral glucose tolerance and is hyperinsulinaemic but not hyperglycaemic. A 50:50 blend of CLA isomers, and preparations enriched with either c9, t11-CLA or t10, c12-CLA were fed to female obese Zucker rats for 21 days. Both glucose and insulin responses during an OGTT were reduced in the groups fed the 50:50 isomer blend or the t10, c12-CLA enriched diet, thus signifying improved insulin sensitivity. Glucose response was reduced by 10% and insulin responses were reduced by 21% following the 50:50 isomer blend. Similarly glucose response was reduced by 16% and insulin responses were reduced by 19% following the t10, c12-CLA enriched diet. In addition, insulin-mediated glucose transport activity was enhanced by both diets. Insulin-mediated glucose uptake was increased by 32% in type I soleus muscle and by 58% in type IIb epitrochlearis muscles following the 50:50 isomer blend. Meanwhile glucose-uptake was increased by 36% in type I

soleus muscle and by 48% in type IIb epitrochlearis muscles following the t10, c12-CLA enriched diet. These parameters were unchanged following the c9, t11-CLA enriched diet.

Faulconnier *et al* (2004) investigated the effects of CLA enriched diets (providing 1% c9, t11-CLA or 1% t10, c12-CLA or 1% c9, t11-CLA + 1% t10, c12-CLA) in adult male sedentary or trained Wistar rats. Exercise significantly lowered plasma insulin concentrations, but had no effect on plasma glucose concentrations. There was a trend towards reduced plasma insulin concentrations following the t10, c12-CLA diet. The other dietary treatments had no effect on plasma insulin or glucose concentrations.

17 wk old male Sprague-Dawley rats are adult rats with normal glucose tolerance, and have no genetic predisposition to obesity or T2DM. Following 8 wk supplementation with 1% c9, t11-CLA, 1% t10, c12-CLA or 1% CLA mix, Choi *et al* (2004) indicated that insulin resistance, measured by HOMA, was attenuated in all groups fed a CLA-enriched diet compared to a control diet. In addition glucose concentrations were significantly lower in the c9, t11-CLA at 7 wk, and significantly lower in the t10, c12-CLA at 5 wk and 7 wk. However, no significant differences were observed in plasma concentrations at the end of the study, at 8 wk. However plasma insulin concentrations were reduced by 48% in the group receiving the c9, t11-CLA enriched diet.

The study by Rahman *et al* (2001) highlighted a difference in glucose response dependent upon the form of CLA being provided. 4 wk old Male OLETF rats were feed 1% CLA, either as triglyceride or free fatty acids, for 4 wk. Plasma glucose concentrations were found to be significantly increased in the group which received CLA as free fatty acids compared to the control group. No difference was observed in the group who received CLA as triglyceride.

To summarise, feeding studies in rat models of obesity and T2DM would suggest a positive effect of CLA in attenuating plasma glucose and insulin concentrations. A t10, c12-CLA enriched diet in particular would seem to be advantageous. Rat models with IGT or T2DM appear to be more responsive to CLA-induced changes, than models with normoglycaemia.

(ii) Studies using murine models

In contrast to improved glucose tolerance in ZDF rats and fa/fa Zucker rats, both male AKR/J mice and female C57BL/6J mice developed hepatomegaly as a result of fatty liver (DeLany *et al*, 1999; Tsuboyama-Kasaoka *et al*, 2000). DeLany *et al* (1999) first reported increased insulin resistance, due to elevated plasma insulin concentrations following a 3 hr fast, in AKR/J mice fed a high-fat diet (45% kcal from fat) for 5, 8 or 12 wk. Subsequently Tsuboyama-Kasaoka *et al* (2000) reported lipodystrophy (insulin resistance, hepatomegaly and reduction of white and brown adipose tissue) in female C57BL/6J mice fed 1% CLA (50:50

product) as part of a low-fat diet (11% kcal from fat). CLA-fed mice had normal glucose concentrations during an oral glucose tolerance test but significantly elevated blood glucose concentrations during an insulin tolerance test. CLA-fed mice also exhibited elevated fed and fasting plasma insulin concentrations which were increased by 4 fold and 8 fold respectively. Later Tsuboyama-Kasaoka *et al* (2003) provided evidence that the percentage of CLA in dietary fat may be important, whereby feeding the same blend of isomers at a lower concentration lessened the negative effect observed with a high concentration of CLA. However, West *et al* (2000) indicated that plasma insulin concentrations were elevated (8.6 fold) in male AKR/J mice following feeding 1% CLA (50:50 blend) as part of a high fat diet (45% energy) for 5 wk.

Roche *et al* (2002) reported divergent metabolic effects of the c9, t11-CLA isomer and the t10, c12-CLA isomer in ob/ob mice, a model of obesity and hypoleptinaemia due to a mutation in the ob gene. Feeding c9, t11-CLA for 4 wk had no effect on body weight, serum glucose, or insulin concentrations, although serum TAG and NEFA concentrations were reduced by 27% and 22% respectively, compared to controls. In contrast, feeding the t10, c12-CLA enriched diet promoted insulin resistance, with increases in both plasma glucose and insulin concentrations observed (1.5 fold and 7.5 fold increase respectively). t10, c12-CLA also induced profound weight loss.

Clement *et al* (2002) also indicated exacerbated insulin resistance in C57BL/J mice following a t10, c12-CLA enriched diet, with a 10 fold increase in plasma insulin concentrations, while glucose concentrations remained normal. No alteration in plasma glucose or insulin concentrations were observed in the group receiving the c9, t11-CLA enriched diet.

In contrast Hamura et al (2001) fed 1.2% CLA as triglyceride to Zucker diabetic C57BLKS-lepr^{db}/lepr^{db} mice. An OGTT was performed at 5 wk and at 11 wk and revealed that CLA had suppressed the increase in plasma glucose concentrations following the glucose load. Insulin concentrations during the OGTT were increased at 5 wk, but were suppressed at 11 wk.

More recently Wargent *et al* (2005) observed increased plasma glucose and insulin concentrations and exacerbated glucose tolerance following feeding a 50:50 CLA blend or t10, c12-CLA enriched diet to female C57Bl/6 ob/ob mice for 2 wk. Interestingly following 10 wk feeding of the 50:50 blend, beneficial effects were observed on plasma glucose and insulin concentrations, and insulin resistance, assessed as the product of fasting plasma glucose and fasting plasma insulin concentrations.

The differences observed between murine models and rat models raises a critical question regarding the effects of CLA. Differences in responses may reflect species differences or differences due to metabolic state (normal glucose tolerance and impaired

glucose tolerance compared to overt T2DM). In contrast to rats, feeding a t10, c12-CLA enriched diet to mice exacerbated insulin resistance.

Stangl *et al* (1999) fed 1% CLA per weight diet (providing 34.6% c9, t11-CLA; 18.4% t10, c12-CLA, 5.4% t9, t11-CLA and 2.1% c9, c11-CLA) to male pigs for 6 wk. No significant differences in plasma glucose and insulin concentrations were observed compared to the control group. However, a 37% increase in plasma insulin concentration was observed, demonstrating a metabolic response which was more similar to murine models than the rat models.

(iii) Studies in man

A limited number of human studies have examined the effects of CLA on insulin sensitivity. *Table 1.14a* summarises these results. The first studies in humans were completed using CLA preparations that contained various CLA isomers and were produced by Pharmanutrients Inc (Lake Bluff, IL). Later studies used more-defined CLA preparations that were comprised predominantly of t10, c12-CLA and c9, t11-CLA isomers in a 1:1 ratio, and were manufactured by Natural Ltd. (Hovdebygda, Norway) and Loders Croklaan (Wormerveer, Netherlands). More recently isomers with greater purity are been used (>80%) which are also supplied by Natural Ltd. and Loders Croklaan. *Table 1.14b* illustrates the fatty acid composition and isomer content of the CLA preparations use in human intervention studies investigating the effect of CLA on insulin sensitivity in man.

Table 1.14a: Results from human intervention studies assessing the effect of CLA on indices of insulin sensitivity.

Reference	Model	Intervention details	Compliance (%) [a]	Study period (wk)	Insulin Sensitivity	Fasting Glucose conc.	Fasting Insulin conc.
Medina *et al*, 2000	17 healthy women (age 20-41yr)	3.0g/day (mixture of isomers) or placebo (sunflower oil)	100	9 wk	NA	↔	↔
Smedman & Vessby, 2001	53 healthy subjects (27 men & 26 women, age 23-63yr	4.2g/day (50:50 isomer blend) or placebo (olive oil)	> 80	12 wk	NA	↔	↔
Riserus *et al*, 2001	25 abdominally obese men (age 39-64yr)	4.2g/day CLA (50:50 isomer blend) or placebo (olive oil)	96	4 wk	NA	↔	↔
Riserus *et al*, 2002a	60 Caucasian obese men with the metabolic syndrome (age 35-65yr)	3.4g/day CLA (50:50 isomer mixture), 3.4g/day purified t10, c12-CLA or placebo (olive oil)	90	12 wk	↔ ↓ [b]	↔ ↑	↔ ↑
Noone *et al*, 2002	51 healthy men and women (18 males, 33 females, mean age 21.3 yr, SD 9.9).	3g/day CLA (50:50 isomer mixture) or 3.0g/day CLA (80:20 isomer mixture) or placebo (linoleic acid)	90 87	8 wk	NA NA	↔ ↔	↔ ↔
Kamphuis *et al*, 2003	26 male & 28 female overweight subjects (age 20-50yrs)	1.8g or 3.6g CLA (mixture of isomers) or 1.8g or 3.6g placebo (oleic acid)	Not disclosed	13 wk	NA	↔	↔
Tricon *et al*, 2004	49 healthy male volunteers (age 20-47yr)	0.59g, 1.19g, 2.38g/day c9, t11-CLA or 0.63g, 1.26g,2.52g/day t10, c12-CLA [f]	92%	8 wk per dose with 6 wk washout between different isomers	↔ [c, d]	Mean plasma glucose ↑ with t10, c12-CLA	↔

50:50 isomer blend indicates equal quantities of c9, t11-CLA and t10, c12-CLA; 80:20 isomer blend indicates c9, t11-CLA and t10, c12-CLA present in a ratio of 80:20
↓ = decrease compared to placebo; ↑ = increase compared to placebo; ↔ = no statistically significant change; N/A = not measured
[a] compliance assessed by capsule or fatty acid compositional analysis
[b] measured using euglycaemic hyperinsulinaemic clamp; [c] measured using HOMA-IR; [d] measured using revised QUICKI; [e] measured using insulin sensitivity index;
[f] cross-over design, therefore each person served as their own control and no placebo was required

Table 1.14a (contd.): Results from human intervention studies assessing the effect of CLA on indices of insulin sensitivity.

Reference	Model	Intervention details	Compliance (%) [a]	Study period (wk)	Insulin Sensitivity	Fasting Glucose conc.	Fasting Insulin conc.
Riserus *et al*, 2004	25 Caucasian abdominally obese men (35-65yr)	3g/day c9, t11-CLA or placebo (olive oil)	Not disclosed	12 wk	↓ [b]	↔	↔
Whigham *et al*, 2004	15 male and female overweight subjects (age 18-50 yr)	6g/day (50:50 isomer blend) or placebo (high oleic sunflower oil)	Not disclosed	52 wk	↔ [c]	↑ at wk 2 but ↔ at all other time points (n = 9)	↔
Eyjolfson *et al*, 2004	16 young sedentary individuals (mean age 21.5yr, SEM 0.4)	4g/day (50:50 isomer blend) or placebo (safflower oil)	Unknown	8 wk	↑ [e]	↔	↓
Gaullier *et al*, 2004	157 male and female overweight subjects (age 18-65 yr)	4.5g/day CLA as free fatty acids or 4.5g/day CLA as triglyceride (both 50:50 blend) or placebo (olive oil)	88.1% 90.8%	52 wk	NA	↔ ↔	↔ ↔
Malpuech-Brugere *et al*, 2004	81 middle-aged, overweight, healthy men and women (age 35-65 yr)	1.5g/day c9 t11-CLA or 3.0g/day c9, t11-CLA or 1.5g/day t10, c12-CLA or 3.0g/day t1-, c12-CLA or 3.0g/day placebo (high oleic sunflower oil) all as drinkable dairy product	Not disclosed but isomers incorporated into plasma phosolipids	18 wk	NA	↔ ↔ ↔ ↔	↔ ↔ ↔ ↔

50:50 isomer blend indicates equal quantities of c9, t11-CLA and t10, c12-CLA; 80:20 isomer blend indicates c9, t11-CLA and t10, c12-CLA present in a ratio of 80:20

↓ = decrease compared to placebo; ↑ = increase compared to placebo; ↔ = no statistically significant change; NA = not measured

[a] compliance assessed by capsule or fatty acid compositional analysis

[b] measured using euglycaemic hyperinsulinaemic clamp; [c] measured using HOMA-IR; [d] measured using revised QUICKI; [e] measured using insulin sensitivity index;

[f] cross-over design, therefore each person served as their own control and no placebo was required

Table 1.14b: Fatty acid composition and isomer content of CLA preparation used in human intervention studies examining insulin sensitivity.

	Medina *et al*, 2000	Smedman & Vessby, 2001	Riserus *et al*, 2001	Riserus *et al*, 2002a	Riserus *et al*, 2002a	Noone *et al*, 2002	Noone *et al*, 2002	Kamphuis *et al*, 2003
Isomer blend	Mixture of isomers	50:50 blend	50:50 blend	50:50 blend	Purified t10, c12-CLA	50:50 blend	80:20 blend	Mixture of isomers
Form of CLA	NA	NA	NA	FFA	FFA	FFA	FFA	75%
% purity	65%	75.9%	77.5%	80%	75%	67.2%	58.8%	TRIG
Manufacturer	Pharmanutrients	Natural	Natural	Natural	Natural	Loders	Loders	Natural
Fatty Acid (%)								
c16:0	NA	4.4	4.3	7.2	3.4	ND	ND	NA
c18:0	NA	1.5	1.4	2.4	0.8	ND	ND	NA
c18:1	NA	14.0	13.9	13.1	11.4	22.7	28.8	NA
c18:2n-6	NA	0.4	NA	1.3	2.0	ND	ND	NA
CLA isomers								
c9, t11-CLA	17.6	37.9	36.9	35.4	2.9	31.0	44.9	
t10, c12-CLA	22.6	37.9	37.0	35.9	76.5	31.5	11.0	[b]
t10, t12-CLA	[a]	-	[a]	-	-	2.4	1.1	
c11, t13-CLA	23.6	-	-	-	-	-	-	
t8, c10-CLA	16.6	-	-	-	-	-	-	
t9, t11-CLA & t10, t12-CLA	7.7	-	1.4	-	-	-	-	
Other isomers	11.9	-	2.2	-	-	-	-	
Other fatty acids	NA	NA	NA	NA	NA	NA	NA	

FFA, free fatty acids; TRIG, triglyceride; SFA, saturated fatty acid; USFA, unsaturated fatty acid. NA - not given. ND - not detected.
50:50 isomer blend indicates equal quantities of c9, t11-CLA and t10, c12-CLA; 80:20 isomer blend indicates c9, t11-CLA and t10, c12-CLA present in a ratio of 80:20
Pharmanutrients, Pharmanutrients Inc , Lake Bluff, IL; Natural, Natural Lipids Ltd, Hovebygda, Norway; Loders, Loders Croklann, B.V., Wormeveer, The Netherlands
[a] combined percentage of t9, t11-CLA & t10, t12-CLA given.,
[b]information not provided by author, but isomer blend comprises equal quantities of c9, t11-CLA and t10, c12-CLA along with several other isomers.

Table 1.14b (contd.): Fatty acid composition and isomer content of CLA preparation used in human intervention studies examining insulin sensitivity.

	Tricon *et al*, 2004	**Tricon *et al*, 2004**	**Riserus *et al*, 2004**	**Whigham *et al*, 2004**	**Eyjolfson *et al*, 2004**	**Gaullier *et al*, 2004**	**Gaullier *et al*, 2004**	**Malpuech-Brugère *et al*, 2004**	**Malpuech-Brugère *et al*, 2004**
Isomer blend	Enriched c9, t11-CLA	Enriched t10, c12-CLA	Enriched c9, t11-CLA	50:50 blend	50:50 blend	50:50 blend	50:50 blend	Purified c9, t11-CLA	Purified t10, c12-CLA
Form of CLA	TRIG	TRIG	TRIG	NA	FFA	FFA	TRIG	TRIG	TRIG
% purity	80-85%	80-85%	83%	79.4%	75%	80%	76%	> 80%	> 80%
Manufacturer	Natural	Natural	Natural	Loders	Pharmanutrients	Natural	Natural	Natural	Natural
Fatty Acid (%)									
c16:0	NA	NA	NA	NA	NA	1.3	2.7	NA	NA
c18:0	NA	NA	NA	NA	NA	2.3	2.6	NA	NA
c18:1	5.8	1.7	5.5	NA	NA	9.4	10.6	NA	NA
c18:2n-6	NA	NA	NA	NA	NA	0.7	0.9	NA	NA
CLA isomers									
c9, t11-CLA	79.3	10.6	83.3	37.3	35.5	39.0	38	> 80	> 80
t10, c12-CLA	7.8	84.1	7.3	37.6	36.8	41.0	38	-	-
t10, t12-CLA	-	-	-	-	-	-	-	-	-
c11, t13-CLA	-	-	-	-	-	-	-	-	-
t8, c10-CLA	-	-	-	-	-	-	-	-	-
t9, t11-CLA & t10, t12-CLA	-	-	1.4	1.3	-	-	-	-	-
Other isomers	-	-	0.66	-	-	4.0	4.0	-	-
Other fatty acids	7.1	5.3	NA	SFA - 6.9% USFA – 92.4%	NA	2.3	3.3	NA	NA

FFA, free fatty acids; TRIG, triaglyceride; SFA, saturated fatty acid; USFA, unsaturated fatty acid. NA - not given. ND - not detected.
50:50 isomer blend indicates equal quantities of c9, t11-CLA and t10, c12-CLA; 80:20 isomer blend indicates c9, t11-CLA and t10, c12-CLA present in a ratio of 80:20
Pharmanutrients, Pharmanutrients Inc , Lake Bluff, IL; Natural, Natural Lipids Ltd, Hovebygda, Norway; Loders, Loders Croklann, B.V., Wormeveer, The Netherlands
[a] combined percentage of t9, t11-CLA & t10, t12-CLA given,
[b]information not provided by author, but isomer blend comprises equal quantities of c9, t11-CLA and t10, c12-CLA along with several other isomers.

The studies carried out by Medina *et al* (2000) and Kamphuis *et al* (2003) used a supplement which contained a variety of CLA isomers (Tonalin® capsules composition: t10, c12-CLA = c11, t13-CLA > c9, t11-CLA > t8, c10-CLA). Medina *et al* (2000) conducted their study in a metabolic suite and gave 17 healthy women 3.0g CLA per day for 9 wk. Neither plasma glucose or plasma insulin concentrations were significantly altered following supplementation. All other human intervention studies investigating the effect of CLA on insulin sensitivity have been conducted on a free-living outpatient basis. Kamphuis *et al* (2003) recruited 54 overweight subjects (26 men & 28 women; age 20-50 yr), submitted them to a very low calorie diet for 3 wk followed by supplementation with either CLA or placebo to test the effect of CLA following weight loss. At the end of the 13 wk intervention neither plasma glucose or insulin concentrations were significantly altered.

Similar results were observed in studies in which subjects were given CLA supplements which consisted of a 50:50 blend of c9, t11-CLA and t10, c12-CLA. Smedman and Vessby (2001) gave 53 healthy subjects (27 men & 26 women; age, 23-63 yr), 4.2g per day of a 50:50 CLA blend. No changes in plasma insulin or glucose concentrations were observed. Riserus et al (2001) gave 25 abdominally obese men (age 39-64 yr) supplements containing the same dose and blend as Smedman and Veesby (2001). An increase was observed in glucose concentrations after both the CLA and placebo supplements, but no difference was observed between the groups. Plasma insulin concentrations also remained unchanged.

Noone *et al* (2002) investigated the effect of 3g per day of a 50:50 isomer blend and a 80:20 isomer blend (c9, t11-CLA: t10, c12-CLA) in 51 healthy men and women (18 males, 33 females; mean age 21.3 yr, SD 9.9 yr). Similarly no changes were observed with respect to plasma insulin or glucose concentrations.

Riserus *et al* (2002a) recruited 60 Caucasian obese men with the metabolic syndrome (age 35-65 yr) and treated them with 3.4g per day 50:50 CLA or purified t10, c12-CLA for 12 wk. In agreement with the aforementioned studies, no effect was observed following the 50:50 isomer blend. However, purified t10, c12-CLA resulted in a significant increase in insulin resistance and fasting plasma glucose concentrations. Insulin resistance was increased by 19%, measured using the eugylcaemic-hyerinsulinaemic clamp, and fasting plasma glucose concentrations were increased by 4% compared to placebo. Significant increases were also observed in fasting insulin and HbA_{1C} concentrations within the t10, c12-CLA group compared to the baseline values, but these were not statistically significant compared to the placebo. This result is in agreement with the effects observed in mice, indicating a pro-diabetic effect of the t10, c12-CLA isomer.

Tricon *et al* (2004) investigated the effects of 3 doses of highly enriched c9, t11-CLA (0.59g, 1.19g or 2.38g/day; 79.3% c9, t11-CLA) or t10, c12-CLA (0.63g, 1.26g or 2.52g/day;

84.1% t10, c12-CLA) in 49 healthy male volunteers (age 20-47 yr) for consecutive 8 wk periods, with a 6 wk wash-out period before crossover to the other isomer. Mean plasma glucose concentrations were significantly higher following supplementation with t10, c12-CLA compared to c9, t11-CLA supplementation. CLA supplementation had no significant effect on plasma insulin concentration or insulin sensitivity, determined by HOMA-IR and revised QUICKI.

Malpuech-Brugere *et a*l (2004) completed a human intervention study in 81 middle-aged (age 35-65 yr) overweight healthy men and women. Following a 6 wk run-in period in which all subjects were given 3.0g/day high oleic acid sunflower oil, volunteers were randomly allocated to receive 1.5g or 3.0g of either purified c9, t11-CLA or purified t10, c12-CLA, or 3.0g high oleic acid in a drinkable dairy product for 18 wk. No differences were observed between the groups with respect to either plasma glucose or insulin concentrations at any of the time points measured (after the run-in period, wk 9, wk 14, wk 19, wk 23 or wk 24).

In contrast Eyjolfson *et al* (2004) reported an improvement in insulin sensitivity index, derived from data collected during an OGTT, and a decrease in fasting insulin in young sedentary subjects (mean age 21.5 yr, SEM 0.4 yr) given 4g/day of a 50:50 blend of CLA for 8 wk. However the number of volunteers recruited was small (n = 18), and the authors noted considerable variability in the response, with some people demonstrating an improvement in insulin sensitivity, while others showed a reduction or no change.

Gaullier et al (2004) assessed the safety and efficacy of CLA. This study comprised a large cohort of 157 overweight individuals who were randomly assigned to receive a 50:50 blend of CLA, either in the form of FFA or TRIG, or placebo for 1 yr, while consuming an ad libitum diet. HbA_{1c} concentrations increased in all groups compared to baseline values. However neither fasting insulin nor glucose concentrations were significantly different between the groups at the end of the supplementation period, leading the authors to conclude that CLA supplementation was not diabetogenic in this population of healthy subjects.

Whigham et al (2004) also assessed the safety profile of a 50:50 isomer blend, called Clarinol, following 1 yr supplementation in overweight men and women (age 18-65 yr). This was a randomised, double-blind study consisting of 3 phases in which subjects were given 6g/day CLA or placebo. Phase I comprised a low-calorie diet (13kcal/kg desirable weight) for 12 wk or until 10-20% of initial body weight was lost. Phase II (wk 12-28) was a weight maintenance/weight-regain phase, in which subjects were fed a diet providing 25-30kcal/kg desirable body weight. Phase III was open label, with subjects from both groups taking CLA from wk 28-52. Results indicated a small, but significantly higher plasma glucose concentration in the CLA group after 2 wk on the low-calorie diet, but these differences disappeared, and neither plasma glucose or insulin concentrations were different thereafter.

Assessment of insulin resistance using HOMA-IR, indicated no differences between the groups throughout the study. Liver function tests, serum lipids, blood counts and general chemistry were also assessed. The authors concluded that CLA in the form of Clarinol is safe for use in obese humans for at least 1yr.

More recently Riserus *et al* (2004) investigated the effects of c9, t11-CLA on insulin sensitivity in 25 Caucasian men with abdominal obesity (age 35-65 yr). Surprisingly c9, t11-CLA decreased insulin sensitivity by 15%, measured using the eugylcaemic-hyerinsulinaemic clamp. Mean insulin-mediated glucose uptake was also significantly lower after c9, t11-CLA supplementation compared to the control supplement. c9, t11-CLA had no significant effect on either fasting plasma glucose or insulin concentrations. This is the first study demonstrating a negative effect of the predominant dietary isoform on insulin sensitivity.

However this study and a number of the other studies (Smedman & Vessby, 2001; Riserus *et al*, 2001; Riserus *et al*, 2002a; Riserus *et al*, 2004; Gaullier *et al*, 2004; Malpuech-Brugere *et al*, 2004) may be criticised due to their choice of placebo. In these studies olive oil was chosen as the control supplement. Olive oil is rich in monounsaturated fatty acids, and has been associated with beneficial health effects, including reducing CVD risk factors, preventing cancer, and modifying immune and inflammatory responses (Ozyilkan *et al*, 2005). As mentioned previously, isoenergetic substitution of MUFA for SFA improves insulin sensitivity (Vessby *et al*, 2001; Perez-Jimenez *et al*, 2001; Lovejoy *et al*, 2002). The choice of placebo for a fatty acid intervention is a difficult decision. It may be argued that an ideal placebo should have no proven intervention effects. Alternatively one may argue that a placebo should represent the optimum treatment available. In either case, a placebo should not be associated with increased risk. Olive oil may not represent the optimum placebo since it has been shown to improve insulin sensitivity and other CVD risk factors. Consequently a beneficial effect of another treatment, in this case CLA, may be masked. Alternatively an excessive negative association may be attributed to the treatment if it fails to yield demonstrate effects. In contrast, if one deems that a placebo should represent the best available option, olive oil represents an ideal choice. Uniform guidelines on the choice of placebo for fatty acid intervention trials would allow comparisons to be made across studies, and enable differences in outcome measures to be interpreted more effectively.

Nevertheless, evidence to date would suggest that the reported adverse effects of CLA in humans, murine models and pigs appear to be largely due to the presence of the t10, c12-CLA isomer. This is in contrast with the reported beneficial effects of a 50:50 blend of CLA isomers and the t10, c12-CLA isomer, in particular, in enhancing insulin sensitivity in rats.

(iv) CLA and insulin resistance - potential mechanisms of action

A number of mechanisms explaining how CLA could potentially influence insulin sensitivity have been proposed. Firstly, studies in humans have shown that that insulin action is positively correlated with the degree of unsaturation and the proportion of long-chain PUFA, in particular arachidonic acid, in muscle phospholipids (Borkman *et al*, 1993; Pan *et al*, 1995). Animal feeding studies have shown that the degree of unsaturation of the fatty acids in muscle lipids and the proportion of arachidonic acid decreases consistently after CLA consumption (Tischendorf *et al*, 2002; Demaree *et al*, 2002; Alasnier *et al*, 2002). The desaturase enzymes insert double bonds at specific positions in a fatty acid chain, thereby increasing the degree of unsaturation of the fatty acid. Δ^9-desaturase plays a role in the desaturation of fatty acids belonging to the n-9 series. Both Choi *et al* (2000) and Lee *et al* (1998) have shown that t10, c12-CLA, but not c9, t11-CLA attenuates the activity of hepatic Δ^9-desaturase (or stearoyl Co-A desaturase). Consequently the conversion of stearic acid and palmitic acid into oleic acid and palmitoleic acid respectively is attenuated. Δ^5- and Δ^6-desaturases play a role in the desaturation process of fatty acids derived from the n-6 and n-3 series, resulting in the generation of long-chain PUFAs (Terpstra, 2004). Production of arachionic acid (20:4n-6) is derived from the n-6 series series of fatty acids. One hypothesis is that reduced insulin sensitivity following the consumption of t10, c12-CLA in mice and humans may be due to changes in fatty acid composition, possibly mediated by altered activity of the desaturase enzymes (Terpstra, 2004).

Insulin resistance is also associated with elevated concentrations of TAG in the peripheral tissues. Brown & McIntosh (2003) reported that the t10, c12-CLA isomer reduced insulin-stimulated glucose uptake, oxidation and incorporation into cellular lipid in human preadipocytes. In addition fatty acid uptake and utilisation was attenuated. This provides a mechanism explaining how CLA could induce weight loss in rodents. However failure to store TAG in adipose tissue can have negative implications *in vivo*, as NEFA concentrations remain elevated in plasma and excess TAG are deposited into peripheral tissues, thereby attenuating whole-body insulin sensitivity. Both Clement *et al* (2002) and Tsuboyama-Kasaoka *et al* (2000) reported lipodystrophy together with the accumulation of TAG in the liver following feeding a CLA-enriched diet to mice. To date, the lipid content of muscle tissues have not been examined following CLA consumption in either humans or murine models (Terpstra, 2004). However Tischenorf *et al* (2002) reported a trend towards an increase in intra-muscular fat in pigs following a CLA enriched diet (blend of isomers), along with a trend towards increased plasma insulin concentrations. Conversely Henriksen *et al* (2003) reported that intramuscular fat was reduced in obese Zucker rats following CLA consumption (either a 50:50 isomer blend or a t10, c12-CLA enriched diet), with a concomitant improvement in insulin action.

CLA-induced insulin resistance may be related to alterations in plasma leptin concentrations. Studies in humans, mice and rats have all reported reductions in plasma leptin concentrations following CLA supplementation or feeding a CLA-enriched diet (Medina *et al*, 2000; Riserus *et al*, 2002a; DeLany *et al*, 1999; Tsuboyama-Kasaoka *et al*, 2000; Takahashi *et al*, 2002). As discussed in Section 1.5.3, leptin is an important hormone involved in maintaining blood glucose concentrations. Therefore it is reasonable to consider that reductions of plasma leptin concentration following CLA could alter insulin sensitivity.

Evidence also exists that fatty acid-induced oxidative stress is closely related to CLA-induced insulin resistance (Riserus *et al*, 2002b; Riserus *et al*, 2004). Carantoni *et al* (1998) suggest that lipid peroxidation is an early sign of insulin resistance. Levels of 8-iso-prostaglandin $F_{2\alpha}$ and 15-oxo-dihydro- prostaglandin $F_{2\alpha}$ which are indicators of non-enzymatic and enzymatic lipid peroxidation respectively, have been shown to be elevated following CLA supplementation both in healthy subjects and obese subjects with the metabolic syndrome (Basu *et al*, 2000a; Basu *et al*, 2000b; Riserus *et al*, 2002b; Riserus *et al*, 2004). In addition, these lipid oxidation parameters returned to baseline levels 2 wk after the cessation of CLA supplementation (Basu *et al*, 2000a). CLA-induced cytotoxicity in cancer cell lines has been shown to be associated with increased lipid peroxidation (Schonberg & Krokan, 1995; O'Shea *et al*, 1999). It remains to be determined whether CLA-induced lipid peroxidation is proatherogenic in humans (Riserus *et al*, 2004).

CLA has been shown to affect gene expression and may exert such effects by modulating the activity of trancription factors. Reports have suggested that CLA represents a natural PPAR ligand capable of activating both PPARα and PPARγ (Moya-Camerena & Belury, 1999; Belury, 2002; Yu *et al*, 2002b). PPARγ regulates insulin sensitivity by activating the transcription of genes which are involved in insulin signalling, glucose uptake, and fatty acid uptake and storage (Brown & McIntosh, 2003). Houseknecht *et al* (1998) reported that CLA stimulated PPARγ-mediated reporter gene activity, and CLA has also been shown to modulate PPARγ mRNA levels (Takahashi *et al*, 2002; Tsuboyama-Kasaoka *et al*, 2000). Brown & McIntosh (2003) found that the t10, c12-CLA isomer decreased PPARγ reporter activity and mRNA levels in human preadipocytes. They suggest that t10, c12-CLA may attenuate insulin sensitivity by antagonising PPARγ signalling.

It is unlikely that changes in adiposity underlie the effect of CLA on insulin sensitivity. Mice are the most responsive to CLA and diets containing 0.5% CLA elicit between 40-80% reduction in body fat (Keim *et al*, 2003). Accumulating evidence suggests that t10, c12-CLA is responsible for the observed reductions in body fat. This may be mediated by increased lipolysis and fat oxidation (Parodi, 1997; McGuire *et al*, 1999; Parodi, 2003). However feeding a t10, c12-CLA enriched diet to mice also resulted in elevated plasma insulin and glucose concentrations (Roche *et al*, 2002; Clement *et al*, 2002). In addition, the effects of

CLA on body weight and body fat in humans are considerably less than those seen in mice (Terpstra, 2004).

1.10 RESEARCH OBJECTIVES

Previous work has demonstrated that CLA possesses the ability to modulate insulin sensitivity and risk factors associated with it. At the initiation of this post-graduate doctorate a limited number of human intervention studies had been conducted, and were limited to healthy populations, except for one study which investigated the effect of CLA supplementation in men with abdominal obesity. Furthermore the primary focus of many *in vivo* and *in vitro* experiments was to investigate the ability of CLA to modulate adiposity and lipid metabolism. A gap existed in determining whether CLA could modulate insulin sensitivity in subjects with a metabolic derangement. In addition research has indicated that both atherosclerotic CVD and insulin resistance have an underlying inflammatory component associated with them. The role of adipose tissue as a secretory organ has become evident, and several adipokines have been shown to modulate insulin sensitivity. The ability of CLA to modify these factors had not been investigated, either in a normal metabolic milieu or in an insulin resistant environment.

The aims of this PhD. thesis are as follows:

- To investigate the impact of CLA supplementation on risk factors associated with T2DM namely insulin resistance, dyslipidaemia, adiposity and inflammation, by conducting a human intervention study in subjects with T2DM.
- To determine the isomer specific effect of feeding a CLA-enriched diet to ob/ob mice on metabolic and molecular markers of glucose homeostasis, focusing in particular on altered adipose tissue biology.
- To validate the results in an *in vitro* adipocyte model.
- To investigate whether fatty acids can modulate the inflammatory, insulin de-sensitising effect of the pro-inflammatory cytokine, TNFα.

1.11 HYPOTHESIS

Dietary fatty acids can modulate insulin sensitivity and other risk factors related to CVD risk at a systemic as well as a molecular level. Different fatty acids have a differential effect.

Chapter Two

Materials and Methods

2.1 COLLECTION AND STORAGE OF BLOOD SAMPLES

Phlebotomy training was completed in phlebotomy department in St. James's Hospital. Blood samples were obtained by venupuncture, following minimal compression with a tourniquet. This is required as excess compression can lead to changes in the concentration of coagulant proteins in the plasma as well as platelet activation, leading to erroneous coagulation results. In addition, grossly haemolysed specimens are unsuitable for use in many assays including biochemical assays, enzyme linked-immuno-sorbent assays (ELISAs) and fluoroimmunometric assays. Blood collected for the future determination of cholesterol, triacylglycerol (TAG), low density lipoprotein (LDL), interleukin-6 (IL)-6, insulin and C-peptide concentrations were collected in serum tubes (Becton & Dickinson, Oxford, UK). Blood for high density lipoprotein (HDL) and very low density lipoprotein (VLDL) analysis was collected in EDTA-coated evacuated tubes (Becton & Dickinson, Oxford, UK). Samples for glucose determination were collected in tubes containing sodium fluoride, and blood for fibrinogen, C-reactive protein (CRP) and fatty acid analyses were collected in citrate tubes (Becton & Dickinson, Oxford, UK). Tubes were inverted eight to ten times to ensure that the coagulant was mixed throughout.

Serum samples were allowed to clot for 1 hr prior to centrifugation. Samples for glucose determination, insulin, C-peptide, IL-6 and lipid analysis were centrifuged immediately at 1400 g for 10 min, at room temperature. Blood samples collected for fatty acid analysis were centrifuged at 200 x *g* for 20 min at room temperature to harvest platelet rich plasma. Citrated samples for CRP and fibrinogen determination were centrifuged after collection at 2000 x *g* for 20 min at room temperature. Plasma was immediately harvested and snap frozen to preserve coagulation factor activity. All samples were immediately frozen and stored at -70°C until subsequent analysis

2.2 CALCULATION OF INSULIN SENSITIVITY

Volunteers who participated in the human intervention study (Chapter 3) completed an oral glucose tolerance test (OGTT) before and after the intervention i.e. conjugated linoleic acid (CLA) or placebo supplementation. On the morning of each investigation, a fasting blood sample was drawn and a standard 3 hr, 75g OGTT was performed. The 75g anhydrous glucose equivalent (Polycal; Nutricia Clinical, Trowbridge, UK) was consumed in 300 ml water within 5 min. Blood samples were collected at 0, 30, 60, 90, 120, and 180 min for the measurement of plasma glucose and serum insulin and C-peptide concentrations.

The postprandial data were expressed in summary form i.e. area under the postprandial curve (AUC), incremental area under the postprandial curve (IAUC), maximum postprandial concentration (C_{max}), and time to maximal postprandial concentration (T_{max}). These data were used to investigate postprandial variations between the supplement groups. AUC was calculated using the trapezium rule (Matthews *et al*, 1990), and IAUC was calculated as the total incremental AUC according to Le Floch (Allison *et al*, 1995). In addition, a number of indices derived from data collected over the duration of the OGTT were utilised. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). Quantitative insulin sensitivity check index (QUICKI) was calculated as 1/(log fasting insulin concentration + log fasting glucose concentration) (Katz *et al*, 2000). The insulin sensitivity index (ISI) composite was calculated as proposed by Matsuda & De Fronzo (1999). Oral glucose insulin sensitivity (OGIS) was calculated using the published formula (Mari *et al*, 2001).

2.3 BIOCHEMICAL ASSAYS

Colorimetric analyses described in Section 2.2.2, Section 2.2.3 and Section 2.2.4 were performed on an ILAB 600 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). In addition all reagents used in these assays, including calibrators and control sera were supplied by Instrumentation Laboratory, Warrington, UK.

2.3.1 Quality control for colorimetric assays

Daily maintenance checks were performed. ReferrIL G Calibrator was used to calibrate the cholesterol, TAG and glucose assays prior to sample analysis. Normal and abnormal control sera (Serachem Control Level 1 and Serachem Control Level 2 respectively) spanning the appropriate range of analyte concentrations were used to test the performance of each assay. Control samples were measured in duplicate, at the beginning and the end of each batch of 30-40 samples. Results were accepted if the quality control values were within the range specified by the manufacturers. Samples were analysed at the end of the study period to minimise inter-assay variability. In addition, for each marker both pre-intervention and post-intervention samples for each subject were analyzed within a single batch. The inter-assay coefficient of variation coefficient of variation (CV) for total cholesterol, TAG and glucose was 1.25%, 3.15% and 1%, respectively.

2.3.2 Determination of cholesterol concentration

The concentration of cholesterol in plasma and in the lipoprotein fractions was determined by endpoint biochromatic (deleted enzymatic) analysis using a colorimetric assay. The assay is based on the principle that cholesterol ester is converted to free cholesterol and fatty acids by

the action of cholesterol esterase. The free cholesterol is then oxidised by the action of cholesterol oxidase forming cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide, in combination with 4-aminoantipyrine and phenol and in the presence of peroxidase, yields the chromagen quinoneimine and water. Quinoneimine production is proportional to the cholesterol concentration in the sample, and is quantified spectrophotometrically at 510 nm, with a blanking wavelength of 700 nm.

2.3.3 Determination of TAG concentration

The concentration of TAG in plasma, in the VLDL fraction and in the TAG-poor lipoproteins (TPL) was measured by enzymatic, endpoint analysis using a colorimetric assay. In this assay TAG is hydrolysed by lipoprotein lipase forming glycerol and fatty acids. In the presence of adenosine triphosphate (ATP), glycerol is converted to glycerol-3-phosphate and adenosine diphosphate (ADP) by glycerol kinase. Glycerol-3-phosphate is then oxidised by glycerophosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase, yielding quinoneimine and water. Quinoneimine production is proportional to the concentration of TAG in the sample, and is quantified spectrophotometrically at 510 nm, with a blanking wavelength of 700 nm.

2.3.4 Determination of glucose concentration

Plasma glucose was determined by endpoint, bichromatic analysis with coupled hexokinase methodology using a colorimetric assay. In the presence of ATP, glucose is converted to glucose-6-phosphate by the action of hexokinase. Glucose-6-phosphate dehydrogenase converts glucose-6-phosphate and nicotinamide adenine dinucleotide (NAD)+ to 6-phosphogluconate and the reduced form of nicotinamide adenine, NADH. Although hexokinase catalyses the phosphorylation of hexoses other than glucose, the glucose-6-phosphate dehydrogenase is specific for glucose-6-phosphate, and therefore glucose. The increased absorbance due to the conversion of NAD^+ to NADH is directly proportional to the glucose present in the sample. Absorbance is measured at a primary wavelength of 340 nm and a blanking wavelength of 375 nm.

2.4 LIPOPROTEIN ISOLATION

2.4.1 LDL isolation and analysis

LDL was isolated using a LDL Precipitation Reagent (Randox, Antrim, Ireland). The reagent contains heparin, which causes LDL to be precipitated at its isolelectric point (pH 5.04). LDL precipitating reagent was added to fresh serum (1 ml/100 µl serum) vortexed, and incubated at room temperature for 10 min. Samples were then centrifuged for 15 min at 2500 x *g*. After

centrifugation, HDL and VLDL remain in the supernatant while LDL forms a pellet at the bottom of the tube. The supernatant was removed and stored at -20°C until cholesterol analysis was preformed (Section 2.2.2). The concentration of plasma LDL-cholesterol was calculated as the difference between the total cholesterol concentration of the plasma sample and the cholesterol concentration of the supernatant. An imunolip control sample (Immuno Ag, Vienna, Austria) was precipitated with every batch of samples and analysed to ensure that the LDL precipitation was successful. Results were only accepted when the control value was within two standard deviations of the manufacturers expected LDL-cholesterol concentration.

2.4.2 HDL isolation and analysis

Plasma total HDL and HDL_3 were isolated using the Quantolip HDL (HDL_2/HDL_3) Cholesterol Precipitation Reagents (Immuno Ag, Vienna, Austria). These reagents contain polyethylene glycol (PEG) of different concentration and pH, dissolved in 0.1 M phosphate buffer, and 0.9g/L sodium azide. These PEG solutions enable the selective precipitation of the different lipoprotein fractions. Reagent A precipitates the VLDL and LDL fractions, leaving HDL in the supernatant. Reagent B precipitates the HDL_2 subclass in addition to the VLDL and LDL fractions, leaving only the HDL_3 subfraction in the supernatant. The precipation reagents were added to fresh plasma samples in a ratio of 2:1. The mixtures were vortexed, incubated at room temperature for 10 min, and centrifuged for 15 min at 2500 x *g*. The supernatants were removed and frozen at -20°C for subsequent analysis. An immunolip control sample (Immuno Ag, Vienna, Austria) was precipitated with every batch of samples and assayed to ensure that the precipitation step was successful. Results were only accepted when the control value was within two standard deviations of the manufacturers expected HDL-cholesterol concentration.

The supernatant from the HDL fractions were assayed for cholesterol as described in Section 2.2.2. The concentration of cholesterol in the HDL_2 sub-fraction was determined by calculating the difference between total HDL-cholesterol and HDL_3-cholesterol.

2.4.3 VLDL isolation and analysis

VLDL was isolated using ultracentrifugation techniques (Noone *et al*, 2002). Two stock solutions were prepared:

1. Stock solution of density = 1.34 g/ml (153 g/L NaCl, 354 g/L KBr)
2. Stock solution of density = 1.005 g/ml (0.15 M NaCl). This second stock solution was used to prepare a density solution = 1.02 g/ml according to the following calculation:

$$(A \times 1.005) + (B \times 1.34) = (A + B)\ X$$

A = volume of saline (d = 1.005 g/ml)
B = volume of stock solution
X = desired density of solution

VLDL was isolated at a density of 1.02 g/ml. The density of plasma was adjusted to 1.02 g/ml by the addition of an appropriate quantity of density solution (d=1.34 g/ml) to plasma using the following equation:

$$(A \times Y) + (B \times Z) = (A + B)\ X$$

A = volume of serum
B = volume of stock solution (d = 1.34 g/ml)
Y = density of serum (1.006 g/ml)
Z = density of salt solution i.e. 1.34 g/ml
X = desired density of solution

1.6 ml plasma was carefully overlaid with 1.6 ml of saline solution of density 1.02g/ml and centrifuged at 250,000 x *g* for 3 hr at 4°C. The supernatant, which contains the VLDL fraction, was removed within 0.5 ml using a glass Pasteur pipette. The infranatant containing TPL was mixed to ensure a homogenous solution. Both supernatant and infranatant were stored at -70°C until subsequent analysis. Cholesterol and TAG concentration were determined in both VLDL and TPL fractions, according to the methods described in Section 2.2.2 and Section 2.2.3. VLDL apolipoprotein B (apo B) was analysed as described in Section 2.4.

2.5 DETERMINATION OF APOLIPOPROTEIN B

ApoB analysis was preformed using an Apolipoprotein B Assay Kit (Wako, Neuss, Germany) on the ILAB 600 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). This assay is based on turbimetric immunoassay methodology. When a sample contains apoB, antigen-antibody complexes form yielding an insoluble aggregate that causes increased turbidity. The degree of turbidity is directly proportional to the apo B concentration in the sample, and is quantified by measuring the increase in absorbance at 700 nm.

An apolipoprotein calibrator (Wako, Neuss, Germany) was used to calibrate the assay. Normal and abnormal controls (Control serum, Wako, Neuss, Germany) were used to verify that the assay was performing satisfactorily. Sample analysis was performed in duplicate. The inter-assay CV for apoB was 2.26%.

2.6 DETERMINATION OF FIBRINOGEN

Quantitative determination of fibrinogen was performed using an automated clotting assay (Fibriquik, Organon Teknika Corporation, NC, USA) using a Coag-A-Mate MTX (Organon Teknika Corporation, NC, USA). The assay is based on the principle that when thrombin is

added to a sample of plasma, fibrinogen is converted enzymatically to fibrin. Fibrin, in turn, undergoes polymerisation to form a fibrin network. When activated by thrombin, Factor XIII catalyses the formation of stabilising cross links to produce a visible clot. The elapsed time from the addition of thrombin to the formation of a clot is inversely proportional to fibrinogen level.

Verify Reference Plasma (Bio Mérieux, NC, USA) was used to calibrate the assay prior to sample analysis. Two sets of coagulation reference plasma, a normal and an abnormal control sera (Universal Coagulation Reference Plasma and Abnormal Coagulation Reference Plasma, respectively, Pacific Haemostasis, Fischer Diagnostics, Middleton, VA, USA) were used to test assay performance. Duplicate measurements were recorded and readings averaged. The inter-assay CV for fibrinogen was 3.25%.

2.7 DETERMINATION OF INSULIN AND C-PEPTIDE

Serum insulin and C-peptide concentrations were measured using solid phase, two-site fluoroimmunometric assays (AutoDELFIA Insulin kit and AutoDELFIA C-Peptide kit respectively, Wallac Oy, Turku, Finland) on a 1235 AutoDELFIA automatic immunoassay system (Wallac Oy, Turku, Finland). The assays are based on a direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants of the protein under investigation i.e. either the insulin or the C-peptide molecule. Samples are simultaneously reacted with immobilised monoclonal antibodies directed against the protein of interest and with europium-labelled monoclonal antibodies directed against a different specific antigenic sites on the insulin or C-peptide molecule. Enhancement solution dissociates europium ions from the europium-labelled monoclonal antibody into solution, where they form highly fluorescent chelates with components of the enhancement solution. The fluorescence in each well is measured, the concentration being directly proportional to the concentration of insulin/C-peptide in the sample.

2.8 DETERMINATION OF IL-6 AND CRP

Both plasma IL-6 and CRP were measured using human, high sensitivity, solid phase sandwich ELISA test kits (Biosource, Camarillo, CA, USA and BioCheck Inc, Burlingame, CA, USA respectively). These kits provide a monoclonal capture antibody directed against a distinct antigenic determinant on the target of interest, pre-coated onto the wells of microtitre strips (capture antibody). Specific conditions and details of each assay are outlined in *Table 2.1*. Samples, controls and standard were pipetted into the wellls and incubated, in order to allow the antigen to bind to the immobilised antibody on each site. During a second incubation, a biotinylated detection monoclonal antibody specific for the target of interest was

added to the wells. This antibody binds to the immobilised antigen captured during the first incubation. Excess second antibody was removed by washing and streptavidin conjugated to horseradish-peroxidase (HRP) was added to the wells. This binds to the biotinylated antibody to complete the four-member sandwich for IL-6. In the case of the CRP assay the detection antibody is conjugated to HRP and is added in parallel with the sample. After further wash steps to remove unbound enzyme, stabilised chromagen (tetramethylbenzidine, TMB, Sigma-Aldrich, Poole, Dorset, UK) was added to each well. The reaction was stopped by the addition of stop solution. The intensity of the colour produced is measured spectrophotometrically at 450 nm and is directly proportional to the concentration of the antigen present in the original specimen.

A standard curve was constructed by plotting the absorbance values, or optical density (OD) of the standards versus standard concentrations. All samples were run in duplicate and readings averaged. Concentrations of IL-6 and CRP for each sample were extrapolated from the appropriate standard curve. Samples with a concentration greater than the standard curve were diluted and re-assayed to obtain a precise concentration. Control wells containing substrate and stop solution alone were included.

Table 2.1: Details of IL-6 and CRP ELISAs

STEP	IL-6 [1]	CRP [2]
Sample - Incubation	100 µl sample, control or standard per well 3hr at 37°C	10 µl sample, control or standard + 100 µl CRP enzyme conjugate reagent per well 45 min at RT
Wash Step	4 x wash buffer	NA
Detection antibody - Incubation	100 µl/well 45 min at 37°C	NA
Wash Step	6 x wash buffer	NA
Streptavidin-HRP - Incubation	100 µl/well 45 min at RT protected from light	NA
Wash Step	6 x wash buffer	5 x distilled water
TMB - Incubation	100 µl/well 30 min at RT protected from light	100µl, 20min at RT protected from light
Stop solution	100 µl/well (provided in the kit)	100 µl/well 1N HCl

NA, not applicable for the assay; HRP, horseradish-peroxidase; RT, room temperature; TMB, tetramethylbenzidine.

[1] Biosource, Camarillo, CA, USA

[2] BioCheck Inc, Burlingame, CA, USA

2.9 FATTY ACID COMPOSITION ANALYSIS

2.9.1 Extraction of total lipids

Total plasma lipids were isolated as described by Noone *et al* (2002). Blood was collected in citrate tubes and immediately centrifuged at 200 x *g* for 20 min. Platelet rich plasma was harvested and transferred into a 10 ml conical polypropylene centrifuge tube and centrifuged at 670 x *g* for 15 min yielding platelet poor plasma and a platelet pellet. 400 µl of platelet poor plasma was transferred to a 16 x 100 mm boroscillate glass tube. Lipid extraction was achieved by the addition of 2.5 ml cholorform:methanol (v/v 2:1) with 0.01% w/v butylated hydroxy toluene (BHT; Sigma-Aldrich, Poole, Dorset, UK). Samples were vortexed for 1 min and then centrifuged for 10 min at 2500 rpm to separate the aqueous and organic phases. The infranatant containing the organic phase was removed using a glass pasteur pipette and transferred to a 12 x 75 mm boroscillate glass tube. A further lipid extraction of the sample was performed by the addition of 2 ml cholorform with 0.01% w/v BHT to the remaining plasma, vortexing for 30 sec and centrifuging for 5 min at 2500 rpm. The infranatant was removed and pooled with the first extract, and evaporated to dryness in a vortex evaporator (AGB Scientific Ltd., Dublin, Ireland). The dried samples were flushed with nitrogen (N_2), sealed to prevent lipid oxidation and stored at -20°C.

2.9.2 Transmethylation of lipid extracts

Methyl esters were prepared by adding 0.5 ml 0.01 M NaOH in dry methanol according to the method of Jiang *et al* (1999). Samples were vortexed for 30 sec, flushed with N_2, sealed with a small marble, and incubated at for 15 min at 60°C. 0.5 ml of 14% boron trifluoride methanol (Sigma-Aldrich, Poole, Dorset, UK) was added to the mixture. Samples were vortexed for 30 sec, flushed with N_2, sealed with a marble and incubated a further 15 min at 60°C. Methyl esters were extracted by the addition of 0.5 ml hexane to samples and vortexing for 1 min. The supernatant was removed using a glass pasteur pipette and transferred to a 12 x 75 ml boroscillate glass tube. This extraction procedure was repeated twice to ensure maximum extraction. Pooled extracts were dried in the vortex evaporator, flushed with N_2 and stored at -20°C.

2.9.3 Gas chromatographic analysis of total plasma lipids

The fatty acid methyl ester (FAME) composition of total plasma lipids were identified using a Shimadzu GC-14A Gas Lipid Chromatograph (Mason Technologies, Dublin, Ireland), which was fitted with a Shimadzu C16-A integrator and a CP Sil 88 fused column (50 m x 0.22 mm, 0.2 µm file thickness; Chrompack Ltd., Middleburg, Netherlands). The CP-Sil 88 colum contains a highly substituted cyanopropyl phase that has been stabilised. The extremely high

polarity of this column offers maximum resolution in separations where the boiling point and polarity of the analytes are nearly equal, for example during the separation of positional and geometric isomers. Analytical conditions were as follows:

Carrier gas:	
Oxygen free nitrogen	0.4 kg/cm^3
Column gas:	
Oxygen free nitrogen	0.8 kg/cm^3
Hydrogen	0.6 kg/cm^3
Dry air	0.5 kg/cm^3

Temperature Programme:
Column initial temperature: 180°C
Increase by 5°C/min for 3 min to 195°C
Hold at 195°C for 40 min
Increase by 2°C/min for 12.5 min to 220°C
Hold at 220°C for 20 min

Samples were reconstituted in 50 µl of hexane and 8 µl was injected onto the column. The FAMEs were identified with a flame ionisation detector. Peak identification was performed by comparison of retention times against a FAME standard which was spiked with a known concentration of transmethylated isomers of conjugated linoleic acid, *cis*-9, *trans*-11 CLA (c9, t11-CLA) and *trans*-10, *cis*-12 CLA (t10, c12-CLA). The standard was run on a daily basis. Fatty acid compositions were calculated as a percentage of the total fatty acids.

2.10 CELL CULTURE

2.10.1 3T3-L1 cell line

The 3T3-L1 murine unipotent preadipocyte cell line was purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The cell line L1 is a continuous substrain of the 3T3 cell line (Swiss albino) developed through clonal isolation. 3T3-L1s propagated under normal conditions have a fibroblastic phenotype. Differentiation to an adipocyte phenotype is hallmarked by the accumulation of lipid and expression of adipose specific fatty acid-binding protein. Differentiation is induced by treating the cells with:

1. Dexamethasone, which stimulates the glucocorticoid receptor pathway,
2. Isobutyl-1-methylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor which is used to stimulate the camp-dependent protein kinase pathway, and
3. Insulin

Upon treatment 3T3L1s adopt a rounded phenotype a rounded phenotype and within 4-5 days begin to accumulate lipids intracellularly in the form of lipid droplets. The acquisition of insulin responsiveness occurs at day 6-8. The 3T3-L1 cell line represents the first and best characterised model of adipogenesis (Green & Kehinde, 1975).

2.10.2 3T3-L1 sub-culture

3T3-L1s were routinely sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM) which was high in glucose (Cambrex, Belgium) supplemented with 10% foetal bovine serum (FBS; Invitrogen Corporation, Paisley, UK), 50 mg streptomycin/ml and 50 μg penicillin/ml (Cambrex, Belgium) (Haruta et al, 1995). The same batch of FBS was utilised in all of the *in vitro* work, thereby minimising the effect of variation with respect to the content and composition of growth factors, nutrients and antibiotics noticeable between commercial sera. Cells were cultured in 150 mm petri dishes (Greiner Bio-One, Frickenhausen, Germany), at 37°C, 8% $C0_2$ and 92% air in a humidified incubator (Thermo Forma Steri-Cycle incubator, Thermo Electron Corporation, OH, USA) (Fawcett et al, 2004).

2.10.3 Cryopreservation and revival of cells

In order to obtain a healthy viable stock of 3T3-L1s, a cell bank was prepared. This ensured that all experiments were performed on cells from the same passage. Cells were grown to approximately 40% confluency, washed twice with sterile phosphate buffered saline (PBS), and removed from the plate using 0.25 typsin (without ethylene diamine tetra-acetic acid, EDTA) (Cambrex, Belgium). All cells were pooled and then split 1:2-4. When sufficient plates were prepared, cells were allowed to grow to approximately 70% confluency. Cells were removed from the plates using 0.25X trypsin (without EDTA) centrifuged for 5 min at 900 x *g*, and the pellets were resuspended in cryopreservation solution [90% FBS, 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Poole, Dorset UK)]. 1 ml aliquots of the cell suspension were immediately transferred into sterile cyrotubes (Nunc plasticware, Life Technologies, Roskilde, Denmark), slowly frozen to -80°C for overnight storage, and subsequently transferred to liquid nitrogen for long-term storage.

To revive cells, cryotubes were quickly thawed to minimise damage to the cell membranes. Under sterile conditions, the cells were added to pre-warmed media, and split into four 150 mm cell culture plates (Greiner Bio-One, Frickenhausen, Germany). Following 1 wk of culture, these fibroblasts reached approximately 70% confluency.

2.10.4 Fibroblast to adipocyte differentiation

When the fibroblasts had reached approximately 70% confluency, they were stimulated to induce differentiation to adipocyte. Cells were incubated with primary differentiation media [DMEM, 10% FBS, 50 mg streptomycin/ml and 50 μg penicillin/ml, 0.6 μM insulin (Sigma-Aldrich, Poole, Dorset, UK), 0.25 μM dexamethasone (Sigma-Aldrich, Poole, Dorset, UK) and 517 μM IBMX (Sigma-Aldrich, Poole, Dorset, UK)] for 4 days. This was replaced with a secondary differentiation media (DMEM, 10% FBS) for a further 3 days, according to a

protocol established by Metabolex Inc., Hayward, CA, USA, which is a modified version of Haruta et al (1995). Under routine culture conditions, both fibroblasts and adipocytes adhere to the plastic surface of the culture plate. Following addition of the differentiation media, fibroblasts stop proliferating.

2.10.5 Cell enumeration and viability assessment

Cell viability and yield were assessed prior to experiment set-up using 0.1% w/v ethidium bromide/acridine orange (EB/AO) (Lee *et al,* 1975). Cells were counted on a Neubaeur haemocytometer using a modification of the protocol of Hudson and Hay (1976). Under ultraviolet (UV) light, viable cells fluoresce green while nonviable cells stain red/orange. Following enumerations cells were resuspended at a concentration of 0.25 x 10^6 adipocytes/ml media.

2.10.6 Preparation of fatty acids

CLA isomers, c9, t11-CLA and t10, c12-CLA were obtained from Cayman Chemical, MI, USA. Linoleic acid was obtained from Sigma-Aldrich (Poole, Dorset, UK). At the start of the *in vitro* experiments a stock of fatty acids was prepared in sterile DMSO. 100 mM of the fatty acids were prepared in sterile DMSO, aliquoted, and stored at -20°C until use. Cells were treated with 50 μM linoleic acid, c9, t11-CLA or t10, c12-CLA as described in Section 2.9.7. A dose of 50 μM fatty acids was chosen as previous *in vitro* work in 3T3-L1s have used this concentration of CLA isomers (Evans et al, 2002b) without reporting toxicological effects. Linoleic acid was included in the experiments as a control for CLA as it is the unconjugated form of the fatty acid. In all experiments cells which were treated with DMSO alone were included. For all treatments, the final concentration of DMSO in the culture medium was ≤0.1%.

2.10.7 Experimental design

Following differentiation adipocytes were gently washed twice with PBS and detached from the plates using 0.5X trypsin (without EDTA, Cambrex, Belgium). When cells began to detach, trypsin was inactivated using fresh media. Non-adherent cells were removed following 8-10 washes with media. Following centrifugation, the pellets were re-suspended in pre-warmed fresh media. Cells were counted as described in Section 2.9.5 and seeded at the appropriate concentration.

Experiments were performed in normal adipocytes and adipocytes made insulin-resistant following treatment with recombinant murine tumour necrosis factor α (TNFα; R&D Systems, MN, USA). Stock solutions of TNFα were prepared at a concentration of 1 μg/ml in sterile PBS containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, Poole, Dorset, UK).

Aliquots were frozen at -20°C until required. Adipocytes were allowed to settle for 24 hr after replating. Mature adipocytes were then incubated for 3 days in the presence of 3 ng/ml TNFα, with fresh media changes each day. This TNFα treatment has previously been shown to significantly reduce the rate of insulin-stimulated glucose uptake by approximately 60% without inducing morphological changes in mature adipocytes (Sartipy & Loskutoff, 2003a). Adipocytes treated in parallel but without TNFα were considered normal adipocytes.

Fatty acids were added in parallel with the differentiation media to test whether chronic treatment with the fatty acids is effective in altering the metabolic and molecular markers. In addition fatty acids were added following full differentiation of the cells to an adipocyte phenotype, to test whether acute treatment of fatty acids is effective. *Fig. 2.1* provides an illustration of the cell culture experimental conditions.

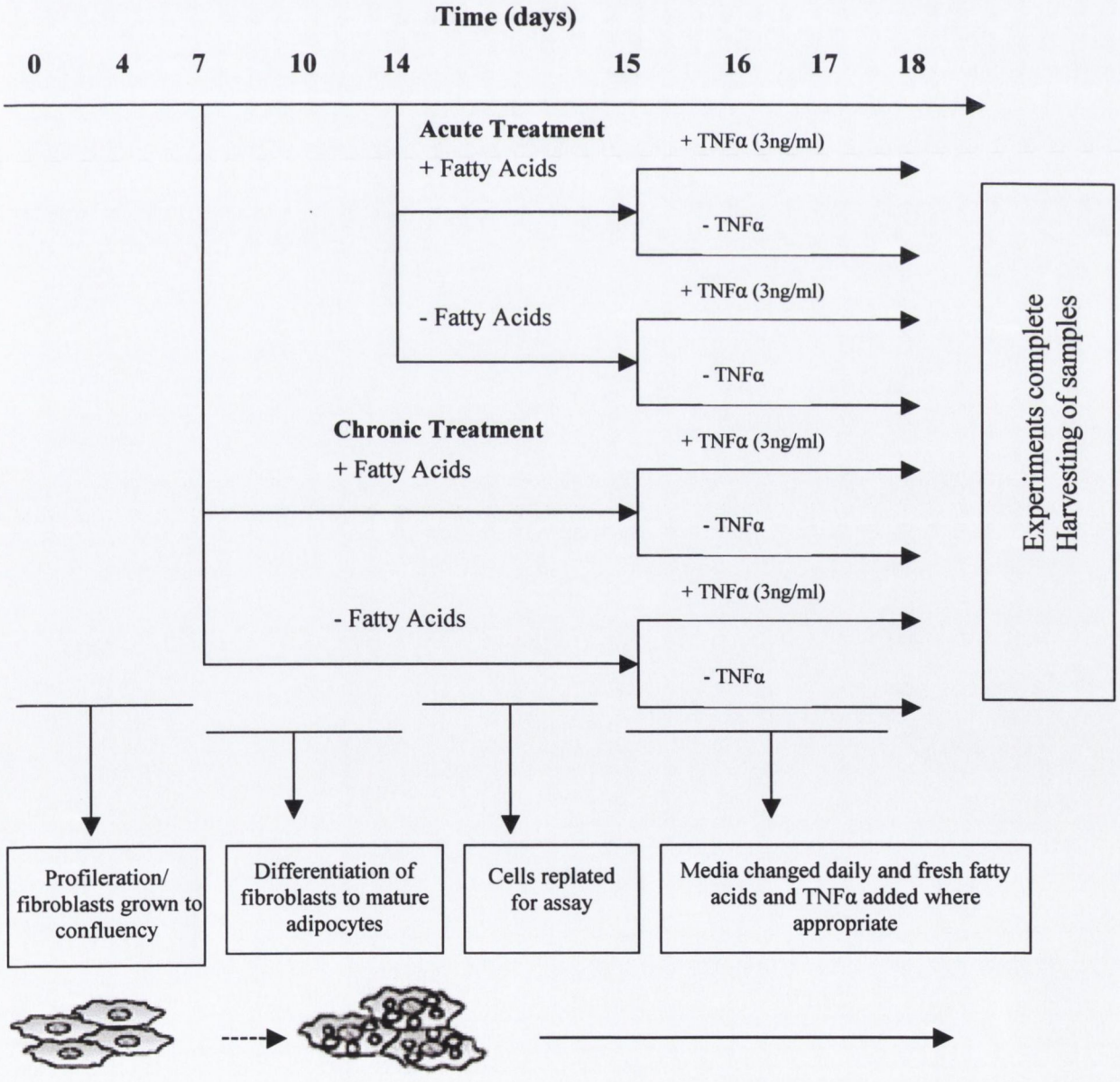


Fig. 2.1: Outline of cell culture experiments

2.11 MYCOPLASMA DETECTION ASSAY

Mycoplasmas are the smallest and simplest prokaryotes, and can cause a wide variety of disease including acute respiratory diseases, genitourinary tract and joint disease and autoimmune disorders (Zeng *et al,* 2004). They depend on their hosts for many nutrients due to their limited biosynthetic capabilities. Mycoplasmas have been shown to be potent modifiers of biological response by activating macrophages and other immune cells. This stimulated release of pro-inflammatory cytokines is mediated in part by nuclear factor-κB (NF-κB) (Zeng *et al*, 2004; Rawadi *et al*, 1999). Mycoplasmas are recognised as common contaminants in cell lines in continuous culture but their presence may go undetected for months thereby causing erroneous results.

3T3-L1s were tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Assay (Cambrex, UK). Viable mycoplasma are lysed and specific mycoplasmal enzymes enzymes react with MycoAlert™ substrate catalysing the conversion of ADP to ATP. The level of ATP in a sample is measured, via a bioluminescent reaction, both before and after the addition of the MycoAlert™ substrate. In the presence of ATP, oxygen and magnesium, luciferin is catalysed by luciferase to form oxyluciferin, adenosine monophosphate, pyrophosphate, CO_2 and light. The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The ratio of ATP following substrate addition:ATP prior to substrate addition is calculated. A value greater than 1 indicates mycoplasma infection.

Cells were grown for 2 passages in the absence of antibiotics to prevent masking of potential mycoplamsa contamination. Cell culture supernatant was centrifuged for 5 min at 200 x *g* to pellet any cells. 100 µl of culture supernatant was added to 100 µl MycoAlert™ Reagent, incubated for 5 min and luminescence measured (measurement A) on a Wallac 1420 Multi-Label Counter (Perkin Elmer, MA, USA). 100 µl MycoAlert™ Substrate was added to each sample, and incubated for a further 10 min before luminescence was measured again (measurement B). The ratio of B:A was calculated. A positive and negative control was included with each experiment to ensure the assay was valid.

Cells were tested at regular intervals and results indicated no contamination with mycoplasma as detailed in *Table 2.2*.

Table 2.2: Results of the MycoAlert™ assay for 3T3-L1 fibroblasts

Date of MycoAlert™ Testing	MycoAlert™ Ratio	Conclusions
Oct 2004	0.7841	Negative
December 2004	0.6759	Negative
Feb 2005	0.9405	Negative
April 2005	0.5916	Negative

2.12 MTS VIABILITY ASSAY

The viability of cells that received medium alone, 50 μM fatty acids, or an equivalent volume of DMSO was quantified spectrophotometrically using an MTS-based *in vitro* toxicology assay kit (Promega, Madison, WI, USA). The MTS system contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) which is incorporated into the mitochondria of living cells and phenazine ethosulfate, an electron coupling reagent, which combines with MTS to form a stable solution. Solutions of MTS dissolved in phenol red-free cell culture medium are yellowish. However, in metabolically active cells mitochondrial dehydrogenases bioreduce the MTS tetrazolium compound yielding a soluble coloured formazan product. The number of viable cells is directly proportional to the quantity of formazan product, measured at 490 nm.

Fibroblasts were grown to confluency as described in section 2.9.2. Cells were removed from the 150 mm plates using 0.25X trypsin (without EDTA) resuspended in media and 100 μl was added to each well of a 96-well microtitre plate. Cells were allowed to settle for 24 hr, checked for confluency, and then differentiated into adipocytes as described in Section 2.9.4. Fatty acid and TNFα treatments were performed as outlined in Section 2.9.7. Control cells were left untreated. Wells containing medium alone were included as blanks. At the end of the experimental protocol, i.e. day 18 on the outline of cell experiment *(Fig. 2.1)*, 20 μl of MTS reagent was added to each well and the plate was incubated for 1 hr at 37°C in a humidified, 5% CO_2 atmosphere. Absorbance was measured at 490 nm, with a reference wavelength of 650 nm. The OD of the blank was subtracted from the sample values to account for spontaneous absorbance which occurs in culture media incubated with the MTS reagent. Cytotoxicity was expressed as a percentage of untreated control cells.

2.13 OIL RED O STAINING

The Oil Red O (ORO; Sigma-Aldrich, Poole, Dorset, UK) stain is used for the demonstration of neutral fat, mainly TAG. It is a physical method of staining fat as the dye is more soluble in the lipoid substances than in the hydroalcoholic dye solvents (Lillie & Ashburn, 1943). Using ORO neutral lipids are stained with an orange/red tint (Koopman *et al*, 2001). ORO is used to test for differentiation of fibroblasts to mature adipocytes, since the differentiation process involves the accumulation of lipids intracellularly in the form of lipid droplets (Brown *et al*, 2001a).

In order to confirm that the fibroblasts and fibroblasts treated with fatty acids differentiated into mature adipocytes, ORO staining was performed. In addition it is recognised that treatment with TNFα can induce the de-differentiation of mature adipocytes (Coppack, 2001). Therefore, it was investigated whether treatment of 3 ng/ml TNFα for 3 days had any impact on the differentation status of the cells.

Fibroblasts were grown to confluency, removed from the 150 mm culture plate using 0.25X trypsin (without EDTA) and resuspended in media. Cells were replated in 24-well plates for future quantification and 6-well plates for future micrographs. Cells were allowed to settle for 24 hr, checked for confluency and then were differentiated to adipocytes as described in Section 2.9.4. Fatty acid and TNFα treatments were performed as outlined in Section 2.9.7. Control cells were left untreated. To test that the untreated and fatty acid-treated fibroblasts differentiated into mature adipocytes, ORO staining was performed on day 15 *(Fig. 2.1)*. The effect of TNFα treatment was assessed on day 18 *(Fig. 2.1)*.

Cells were washed with PBS and fixed with neutral buffered formalin (Sigma-Aldrich, Poole, Dorset, UK) for 1 hr. An ORO stock solution was prepared by dissolving ORO stain (Sigma-Aldrich, Poole, Dorset, UK) in isopropanol (0.5% solution), and then diluting to 60% in distilled water. The solution was allowed to stand for 20 min, and was subsequently filtered through Whatman 3mm filter paper (Whatman International Ltd., Maidstone, UK) to remove the precipitate. The formalin was removed from the cells and ORO stain was added to the cells for 1 hr. Cells were washed 5 times with distilled water. Adipocytes grown in the 6-well plates were maintained in water to prevent lipid droplets exploding, and micrographs were taken using an Olympus CKX41 (Olympus Corporation, Japan). In order to quantify the staining, adipocytes grown in the 24-well plates were allowed to dry for 30 min. Cells were then dissolved in 250 µl isopropanol, and 100 µl solution was transferred to a 96-well microtitre plate. Absorbance was measured at 490 nm. After micrographs were recorded, cells grown in the 6-well plates were dissolved in isopropanol as above. Absorbance values were comparable to cells grown in 24-well plates.

2.14 RNA EXTRACTION AND ANALYSIS

An animal experiment was performed in the Nutrigenomics Research Group to investigate the isomer specific effects of c9, t11-CLA and t10, c12-CLA on lipid and glucose metabolism. Sixteen 6 wk old male ob/ob mice were randomly allocated to receive a high-fat diet supplemented with c9, t11-CLA, t10, c12-CLA or linoleic acid for 6 wk. Plasma was collected for biochemical analysis, and adipose tissue and liver were collected for gene expression and protein analysis. The animal feeding study was completed by Dr. Enda Noone, Nutrition Laboratory, Department of Clinical Medicine, Trinity College Dublin. Analysis of the tissue samples forms the basis of the research in Chapter 4 of this thesis.

2.14.1 RNA extraction

Total ribonucleic acid (RNA) was isolated from adipose tissue and cultured cells using TRI Reagent® (Molecular Research Centre Inc., OH, USA). TRI Reagent® consists of a monophasic solution of phenol and guanidine thiocyanate and represents an improved version

of the single-step method of Chomczynski & Sacchi (1987). TRI Reagent® maintains the integrity of RNA by inhibiting ribonuclease (RNase) activity, while disrupting cells and dissolving cell components.

Cells:

Cells were washed twice with sterile PBS to remove non-adherent cells. Cells were lysed directly in 6-well plates by adding 1 ml TRI Reagent® per well, followed by repetitive scraping and pipetting using a sterile pasteur pipette.

Tissue:

50 mg sections of adipose tissue and 20 mg of liver were homogenised in 1ml TRI Reagent® for approximately 30 sec at room temperature using a T18 basic Ultra Turrax® homogeniser (IKA® Works Inc, NC, USA).

Homogenates from cells, adipose tissue and liver were subsequently transferred to ribonuclease (RNase)-free microfuge tubes and incubated at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. Following incubation, an additional centrifugation step was performed in tissue samples by centrifuging the homogenate at 12,000 x *g* for 10 min at 4°C. The resulting pellet contains extracellular membranes, polysaccharides and high molecular weight deoxyribonucleic acid (DNA). In adipose tissue samples an excess of fat collects as a top layer. The clear supernatant which contains RNA, was transferred to a fresh microtube. All other steps of the RNA extraction were equivalent for both tissue and cell samples. Samples were supplemented with 0.2 ml of chloroform. The tubes were shaken vigorously for 15 sec, incubated at room temperature for 15 min, and centrifuged at 12,000 x *g* for 15 min at 4°C to separate the aqueous and organic phases. RNA was precipitated from the aqueous phase by incubating with 0.5 ml 100% isopropanol for 10 min at room temperature. After centrifugation at 12,000 x *g* for 10 min at 4°C, the RNA pellet was washed with 1ml 75% ethanol in RNase free water (Sigma-Aldrich, Poole, Dorset, UK). The RNA was air-dried for 5-10 min, resuspended in 20-50 µl RNAse-free water, and solubilised by heating at 55-60°C for 10 min. The samples were snap frozen and stored at -80°C until further analysis.

2.14.2 Quantitation of RNA

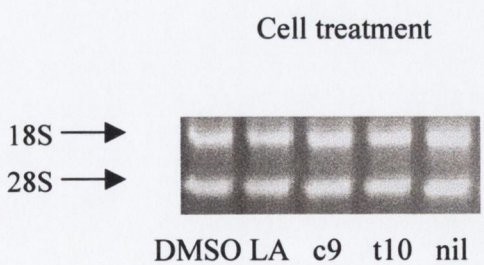
The quantity and purity of the eluted RNA was determined spectrophotometrically by measuring absorbance at 230 nm, 260 nm and 280 nm (Eppendorff Biophotometer, Hamburg, Germany) in a UVette® (Eppendorff AG, Hamburg, Germany). RNase-free water was used as

the diluent control. An $OD_{260/280}$ less than 1.6 implies partially dissolved RNA samples, 1.7-2.0 indicates good purity while an $OD_{260/280}$ of greater than 2 implies purity. An $OD_{260/230}$ greater than 2 indicates no residual guanidinium contamination.

2.14.3 RNA agarose gel electrophoresis

RNA integrity was assessed electroporetically using a formaldehyde denaturing agarose gel. The gel was prepared by dissolving 1.2 g agarose in 87 ml RNase-free water in a microwave oven. 10 ml MOPS [3-(N-morpholino) propanesulfonic acid] running buffer, pH 7.0 [0.2 M MOPS, 10 mM EDTA (pH 8), 10 mM sodium acetate (pH 7)] and 3 ml of 37% (w/v) formaldehyde were added when the agarose solution had cooled to approximately 55°C. Following mixing, the gel was poured and allowed to set in a fume hood for 1 hr prior to electrophoresis.

The volume of each sample required to yield approximately 1 μg total RNA was calculated. This volume to added to 5 times the volume of mastermix (1 μl 10X MOPS running buffer, 5 μl formamide, 2 μl 37% (w/v) formaldehyde, 1 μl ethidium bromide (400 μg/ml in RNase free water water) and 1 μl RNA loading dye [50% glycerol, 1mM EDTA (pH 8.0), 0.1% bromophenol blue, 0.1% xylene cyanol FF]. Samples were incubated at 65°C for 10 min, vortexed, centrifuged briefly and stored on ice until loading. Residual formaldehyde was removed from the wells of the gel by pre-running the gel for 10 min at constant voltage (70V). The samples were loaded and the gel was run until the bromophenol blue tracing dye had run three-quarters of the length of the gel. RNA was visualised using a UV trans-illuminator (Kodak Image Station 440 CF, Perkin Elmer, MA, USA). Intact RNA is represented by two clear bands representing 18S and 28S RNA, respectively as illustrated in *Fig. 2.2*.



DMSO	dimethyl sulfoxide
LA	linoleic acid
c9	c9, t11-CLA
t10	t10, c12-CLA
nil	untreated cells

Fig. 2.2: Representative gel illustrating RNA bands obtained during experiments performed in Chapter 5.

2.15 REAL-TIME REVERSE TRANSCRIPTION TAQMAN® PCR

Real-time reverse transcription Polymerase Chain Reaction (PCR) allows for sensitive detection and quantification of PCR amplification while the reaction is occurring. Data is collected throughout the PCR process and reactions are characterised by the time when amplification of a target is first detected, rather than the amount of target accumulated at the final phase end of the PCR reaction.

TaqMan® chemistry uses a fluorogenic probe to enable the detection of a specific PCR product. When the probe is intact, the proximity of the reporter dye (attached to the 5' end of the probe) and quencher dye (attached to the 3' end of the probe) results in suppression of reporter fluorescence. During the PCR reaction, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. Taq DNA Polymerase cleaves the probe if it is hybridised to the target, displacing the probe and separating the reporter and quencher dyes, thus increasing the flouresence of the reporter dye (Livak *et al*, 1995; Holland *et al*, 1991). This process continues during every amplification cycle. An increase in fluorescence signal emitted is detected only if the target sequence is complimentary to the probe and amplified during PCR hence non-specific amplification is not detected. Accumulation of PCR product is detected directly by examining the increase in fluorescence emitted by the reporter dye. Thus, samples that contain fewer starting copies of the target gene require a greater number of amplification cycles to generate a detectable signal.

In order to account for small differences in the amount of template added to each reaction, target gene levels were normalised to an endogenous control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference/housekeeping gene in both tissue and cell experiments. Relative quantification was used to analyse changes in gene expression in a given sample. Standard curves for both the target and endogenous reference were generated from pooled RNA from the experimental set of samples. The Ct is the fractional cycle number at which fluorescence signal exceeds a fixed threshold level in the exponential phase of amplification (Higuchi *et al,* 1991) and was plotted against log standard cDNA concentration (ng). For each sample the amount of target and endogenous control gene expression were extrapolated from the appropriate standard curve using the Ct readings for each sample. The target amount was then divided by the endogenous reference (GAPDH) amount to obtain the normalised target value. Each of the normalised target values were divided by the experiment's control normalised value (animals fed linoleic enriched diet in Chapter 4, cells treated with DMSO in Chapter 5) to generate relative expression levels.

2.15.1 Preparation of RNA prior to reverse transcription

To eliminate any possible contamination of the RNA by genomic DNA, all RNA samples were treated with DNase I amplification grade, (GIBCO BRL, Life Technologies™, Gaitherburg,

MD, USA). 1 µg of total RNA was incubated with 1 µl of 10X DNase I Reaction buffer [200 mM Tris-HCL (pH 8.4), 20 mM $MgCl_2$, 500 mM KCL], and 1 µl of DNase I, Amp Grade (1 U/µl), made up to a final volume of 10 µl with RNase-free water. This reaction was incubated at room temperature for 15 min. DNase I was then inactivated by adding 1 µl 25 mM EDTA and heating samples at 65°C for 10 min.

2.15.2 Reverse transcription: synthesis of first strand cDNA

DNase-treated RNA was reverse transcribed in a 25 µl reaction. Firstly, 500ng random primers (Promega Corporation, Madison, US) were added to 1 µg of DNase-treated RNA and samples heated to 70°C for 5 min to melt secondary structure within the template. Samples were immediately placed on ice to prevent secondary structure reforming. 1X RT buffer [50 mM Tris-HCL, 75 mM KCL, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT)], 10 mM deoxynucleotides (dNTPs) and 200U of Moloney-Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT; Promega Corporation, Madison, WI, USA) were added to each sample. Samples were gently mixed and incubated for 60 min at 37°C followed by 70°C for 10 min to inactivate the enzyme. Reactions in which M-MLV RT was omitted served as a negative control. Samples were diluted with RNase-free water to give a final concentration of 10 ng/µl. Additional RNA was prepared simultaneously and 1:4 dilutions performed to create a standard curve (ranging from 80 ng/µl to 0.3125 ng/µl) for the Taqman PCR analysis. 2 µl of both samples and standard curve samples were used in subsequent RT-PCR reactions. All samples were stored at -20°C.

2.15.3 Real-time TaqMan® PCR

RNA expression was quantified by real-time Taqman (RT-PCR) using an ABI 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). Each 25 µl reaction contained 20 ng cDNA, forward and reverse primers, probe and a mastermix solution containing dNTPs, reaction buffer and Taq polymerase. For gene expression analysis outlined in Chapter 4, 2X TaqMan® Universal PCR Mastermix (Applied Biosystems, Warrington, UK) was used. In the cell culture experiments (Chapter 5) JumpStart™ Taq ReadyMix (Sigma, Poole, Dorset, UK) was employed as it produced similar results to TaqMan® Universal PCR Mastermix and was more cost effective. The majority of target genes were investigated using pre-developed primer and probe kits (Taqman® Gene Expression Assays, Applied Biosystems, Warrington, UK). When pre-developed primer and probe kits were unavailable for the target of interest, custom made primer and probe sequences were developed. Whereas the Taqman® gene expression assay kits provide a pre-optimised primer/probe mix for the detection and quantification of specific genetic sequences, optimisation of custom made primers and probes was performed on an individual assay basis. Reactions in which cDNA was omitted served as

a no template control, and cDNA in which the M-MLV RT was omitted served as a no amplication control. All reactions were performed in duplicate. PCR reaction thermal cycle conditions are illustrated below:

Table 2.3: PCR reaction thermal cycle conditions

	TaqMan® Universal PCR Mastermix	JumpStart™ Taq ReadyMix
	50°C for 2 min	50°C for 2 min
Initial denaturation	95°C for 10 min	94°C for 2 min
40 cycles:		
Denaturation	95°C for 15 sec	94°C for 15 sec
Annealing	60°C for 1 min	60°C for 1 min
Extension	NA	72°C for 1min
Hold	4°C	4°C

NA, not applicable for the assay

2.16 PREPARATION OF PROTEIN EXTRACTS FOR WESTERN BLOTTING

For all protein extractions, either from white adipose tissue or 3T3-L1s, all buffers were prepared immediately prior to use and were kept ice-cold. In addition, all steps were carried out on ice. Adipose tissue was homogenised using a T18 basic Ultra Turrax® homogeniser (IKA® Works Inc, NC, USA).

2.16.1 Isolation of total protein extract from white adipose tissue

Total protein was isolated from adipose tissue using the lysis buffer (50 mM Tris-HCL (pH 8.0), 300 mM NaCl, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 50 mM ß-glycerol phosphate, 50 mM NaF, 1mM $NaV0_4$, 0.5% Triton X-100, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, and 14 µg/ml aprotinin) as previously described (Takemori *et al*, 2002). 0.3-0.5 g adipose tissue was homogenised in 2 ml lysis buffer. The homogenate was allowed to sit on ice for 30 min at 4°C, with regular vortexing, followed by centrifugation at 12,000 x *g* for 30 min at 4°C to remove insoluble material. The supernatant was collected as the total protein fraction, vortexed and stored at -20°C for future protein estimation.

2.16.2 Isolation of plasma membrane extract from white adipose tissue

The plasma membrane fraction of white adipose tissue was prepared from the pooled fat of two animals according to dietary intervention to gather 300 mg of tissue. Tissues were

homogenised for 30 sec in 3 ml buffer (10 M Tris-HCl, 1 M EDTA, 250 mM sucrose, 0.1 mM PMSF, pH 7.4) followed by centrifugation at 3000 x *g* at 4°C for 15 min, as previously described (de Carvalho Papa *et al*, 2002). Fat cakes were discarded, and the infranatant (a fat-free extract) was centrifuged at 12000 x *g* for 15 min at 4°C. The pellet was resuspended as the plasma membrane fraction in 1 ml buffer, vortexed and stored at -20°C for future protein estimation.

2.16.3 Isolation of membrane and cytosolic and nuclear fractions from adipose tissue

All buffers were prepared (as described by Osborn *et al*, 1989) immediately prior to use and were kept ice-cold. The cytosolic and membrane fraction of adipose tissue was isolated by swelling on ice for 20 min, homogenising the sample in 3 ml ice-cold buffer (10 mM Hepes pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT and 0.5% v/v Nonidet P-40), lysing on ice for a further 20 min, and centrifuging at 9,300 x *g* for 10 min at 4°C. The supernatant was collected as the cytosolic and membrane fraction.

To prepare the nuclear extract, the pellet was resuspended in 15 µl buffer (20 mM Hepes pH7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF), samples were incubated on ice for 60 min with repeated vortexing, followed by centrifugation at 9,300 x *g* for 10 min at 4°C. The supernatant, containing the nuclear fraction was mixed with 75 µl buffer (10 mM Hepes pH7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mmol/L DTT, 0.5 mmol/L PMSF), and stored at -20°C for future protein estimation.

2.16.4 Isolation of total cell lysates from 3T3-L1s

3T3-L1s were cultured with and without fatty acids in the presence or absence of recombinant murine TNFα as described in Section 2.9.7. After this incubation period, cell culture supernatants were removed and cell monolayers were washed twice with sterile ice-cold PBS to stop the reaction. 1.5 ml of lysis buffer (20 mM Tris (pH7.5), 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 50 units/ml aprotinin, 1 mM Na_3VO_4, 1 mM PMSF and 50 mM NaF) was added directly to each well and cells were lysed by repetitive scraping and pipetting. Lysates were transferred into microfuge tubes, incubated on ice for 30 min with regular vortexing, followed by centrifugation at 9300 x *g* for 10 min at 4°C to remove insoluble material (Yoshizaki *et al*, 2004). Supernatants were removed, transferred to fresh microfuge tubes, vortexed and stored at -20°C for future protein estimation.

2.16.5 Determination of protein concentrations using the Modified Lowry Assay

The protein concentration in total protein extracts, plasma membrane extracts and total cell lysates was determined according to the Modified Lowry technique. This assay is based on the principle that when a protein is reacted with alkaline cupric sulphate in the presence of tartrate,

a tetradentate copper complex forms, yielding a pale blue colour. Following incubation with Folin phenol reagent it is believed that electrons transfer from the tetradentate copper complex to the Folin phenol reagent, resulting in a deeper blue colour formation, which intensifies during the incubation period (Lowry *et al*, 1951; Legler *et al*, 1985).

Total protein samples were diluted 1:20 in distilled water. Plasma membrane fractions and total cell lysates were diluted 1:15 in distilled water. A standard curve was prepared from a stock solution of protein standard [1mg BSA (Sigma, Poole, Dorset, UK) per ml distilled water] ranging from 0 µg/ml to 250 µg/ml. 300 µl of each standard and sample was incubated with 900 µl of buffer [Buffer = 100 volume Reagent A (2% w/v Na_2CO_3, 0.4% w/v NaOH, 0.6% w/v K/Na Tartrate, 1% w/v SDS) + 1 volume Reagent B (4% w/v $CuSO_4.5H_2O$)]. Samples were mixed thoroughly and incubated at room temperature for 10 min. 90 µl Folin phenol Reagent was added (1:3 dilution in distilled water) and colour was allowed to develop for 45 min. The absorbance of the protein-solutions was measured at 660 nm. Duplicate measurements were recorded and readings averaged. A standard curve was constructed using the BSA samples, from which the concentration of protein in the samples was determined.

2.16.6 Determination of protein concentrations using the Bradford Assay

Determination of the protein concentration of the membrane and cytosolic fraction and the nuclear fraction was performed according to the method originally described by Bradford (1976) using the Bio-Rad Protein Assay dye reagent concentrate (Bio-Rad Laboratories Inc., CA, USA). In the acidic environment of the reagent, protein binds to the Coomassie dye, resulting in a spectral shift from the reddish/brown colour of the dye to the blue form of the dye. The difference between the two forms of the dye is greatest at 595 nm, and can be measured spectrophotometrically.

For nuclear extracts, samples were diluted 1:80 in Buffer (10 mM Hepes pH7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF). For cytosolic and membrane fractions, samples were diluted 1:160 in PBS. Standards were prepared from a stock solution of protein standard (1 mg BSA/ml; Sigma-Aldrich) and ranged from 0 µg/ml to 15 µg/ml in buffer for nuclear samples and in PBS for cytosolic and membrane samples. 800 µl of each standard or sample was incubated with 200 µl of Bio-Rad dye. Colour was allowed to develop for 10 min and absorbance was measured at 590 nm. Duplicate measurements were recorded and readings averaged. The concentration of protein in the samples was then calculated from the standard curve generated with the BSA samples.

2.16.7 Preparation of samples for gel electrophoresis and Western blotting

Depending on the protein fraction and the antigen under investigation, between 10-250 µg of protein was acetone precipitated in 5 volumes of ice-cold acetone overnight at -20°C. Samples

were then centrifuged at 13,400 x *g* for 5 min, the acetone aspirated and the pellet allowed air-dry. The pellet was resuspended in 20 µl of sample buffer (62.5 mM Tris-HCL pH 6.8, 10% v/v glycerol, 2% w/v SDS, 5% v/v β-mercaptoethanol, 0.05% w/v bromophenol blue) and boiled at 100°C for 5 min, thereby reducing the disulphide bonds and denaturing the protein. The samples were centrifuged briefly and frozen at -20°C until SDS-PAGE electrophoresis was performed.

2.17 PRINCIPLE OF IMMUNOBLOTTING

Western Blotting, or immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. The proteins are first separated by electrophoresis through SDS-polyacrylamide gels, and then transferred electophoretically from the gel to a membrane (usually nitrocellulose or polyvinylidene fluoride, PVDF). The specificity of the subsequent antibody-antigen interaction enables a single protein to be identified in the midst of a complex mixture of proteins. A suitable secondary antibody, which is conjugated to an enzyme e.g. HRP, binds to the primary antibody. An appropriate substrate is then added to the enzyme, and together they produce a detectable product, the intensity of which correlates with the abundance of the antigen on the blotting membrane. Molecular weight markers are included on each gel to aid in the identification of the detected proteins and to confirm the sensitivity of the antibody.

2.17.1 SDS polyacrylamide gel electrophoresis (SDS PAGE)

Proteins were separated using a 6% or a 10% acrylamide resolving gel. The 6% resolving gel was used for the future determination of IRS-1(ser307) due to the high molecular weight of this protein (detected at 170kDa). Other proteins under investigation were separated using a 10% resolving gel. The resolving gel was cast between two glass plates and allowed to set, prior to casting the stacking gel, as described below:

- *Resolving gel*:

  6%: 2.0 ml 30% acrylamide, bisacrylamide mix (BDH, Sigma-Aldrich, Poole, Dorset, UK), 2.5 ml 1.5 M Tris (pH 8.8), 5.3 ml distilled water, 100 µl 10% ammonium persulphate, 100 µl 10% SDS and 30 µl TEMED

  10%: 4.0 ml 30% acrylamide, bisacrylamide mix (BDH, Poole, UK), 2.5 ml 1.5 M Tris (pH 8.8), 3.3 ml distilled water, 100 µl 10% ammonium persulphate, 100 µl 10% SDS and 30 µl TEMED
- *Stacking gel*: 0.325 ml 30% acryamide, bisacrylamide mix, 0.625 ml 0.5 M Tris (pH 6.8), 1.525 ml distilled water, 25 µl 10% ammonium persulphate, 25 µl 10% SDS and 15 µl TEMED

Electrode running buffer (25 mM Tris Base, 200 mM glycine, 0.5% w/v SDS) was added to the upper and lower reservoirs. Following standard (Precision Plus Protein™ Standards Dual Colour, Bio-Rad Laboratories Inc., CA, USA) and sample loading, electrophoresis was carried out at 30 mA per gel for ~30mins until the migration dye front was approximately 5 cm from the gel base. The electrophoresed proteins were then ready for transfer.

2.17.2 Western blotting

The semi-dry method for transfer of electrophoresed proteins to immobilizing membranes as described by Towbin *et al* (1979), was preformed using a semi-dry blot system (Sigma-Aldrich, Poole, Dorset, UK). Biotrace™ polyvinylidene fluoride (PVDF) transfer membrane (0.45 μm, Pall Life Sciences/Pall Corporation, FL, USA) was soaked in 100% methanol for 1 min and then in transfer buffer (25 mM Tris Base, 192 mM glycine, 20% v/v methanol, 0.05% w/v SDS) on a rotating platform for at least 30 min prior to use. The blot sandwich was prepared as follows: cathode, 2 sheets of Whatman 3mm filter paper (Whatman International Ltd., Maidstone, UK) pre-soaked in transfer buffer, PVDF membrane, gel, another 2 sheets of pre-soaked filter paper and finally the anode. Care was taken to ensure the absence of air bubbles and to note the orientation of the gel. Electrophoresis transfer was performed at 225 mA for 1 hr 15 min.

2.17.3 Detection of antigens

After transfer, the blot sandwich was carefully opened, and the membrane removed with a forceps. The choice of block buffer (used to suppress non-specific absorption of antibodies), wash buffer and secondary antibody depends on the antigen under investigation and the choice of antibody. *Table 2.4* and *Table 2.5* outline the conditions used to detect the antigens investigated in experiments outlined in Chapter 4 and Chapter 5. All incubations and wash steps were performed on a roller to ensure thorough mixing.

Antigens were detected by incubating the membrane in a 1:1 solution of luminol/enhancer solution: stable peroxide solution (Supersignal® West Pico Chemiluminescent Substrate, Pierce, IL, USA) for 5 min at room temperature. Chemiluminescence involves oxidation of luminol in the presence of HRP and hydrogen peroxidase to form an excited state product (3-aminophthalate), resulting in light emission at 425 nm as it decays to the ground state. The enhancer increases the intensity of the light emission and reduces background light emission due to oxidation of luminol by peroxide or other oxidants. This system allows multiple exposures to be performed to obtain the best image. Following the 5 min incubation, excess reagent was drained from the membrane, which was then placed between two sheets of transparent acetate in an autoradiography cassette (Sigma, Poole, Dorset, UK) before being exposed to Fugi RX film (Amersham

Bioscience, Buckinghamshire, England) for 2 min – overnight, depending on the exposure required. The films were developed using an AGFA CURIX 60, Type 9462/100/140 (AGFA-Gevaert, AG Munich, Germany).

Table 2.4a: Detection of the insulin receptor and Glut 4 by Western blotting

Antibody / **STEP**	**Insulin receptor** (IRS-1 antibody, Cell Signalling Technology, Beverly, MA, USA)[1]	**Glut 4** (Chemicon International, Inc, Temecula, CA, USA)
Block - solution	5% w/v Marvel non-fat dried milk in TBS/Tween 20 (0.1%)	10% w/v Marvel non-fat dried milk in TBS/Tween 20 (0.1%)
- incubation	1 hr at RT	1 hr at RT
Wash Step	6 x 5 min with TBS/Tween 20 (0.1%)	
Primary antibody - dilution	1:1000 dilution in 5% w/v BSA-TBS/Tween 20 (0.1%)	1:5000 dilution for adipose tissue plasma membrane, and 1:1000 dilution for total cell lysates in 5% w/v Marvel-TBS/Tween 20 (0.1%)
- incubation	1 hr at RT, overnight at 4°C, 2 hr at RT the following day	Overnight at 4°C, 30 min at RT the following day
Wash Step	6 x 5 min with TBS/Tween 20 (0.1%)	
Secondary Antibody[2] - dilution	1:2000 dilution in 10% w/v Marvel-TBS/Tween 20 (0.1%)	1:2000 dilution in 10% w/v Marvel-TBS/Tween 20 (0.1%)
- incubation	1 hr at RT	1 hr at RT
Wash Step	10 x 5 min with TBS/Tween 20 (0.1%)	

RT, room temperature; TBS, Tris buffered saline.

[1] Band between 78 and 120kDa represents the insulin receptor as described by Rui *et al*, 2001

[2] polyclonal goat anti-rabbit immunoglobulin G-peroxidase-conjugated immunoglobulins (Sigma-Aldrich, Poole, Dorset, UK)

Table 2.4b: Detection of IRS-1(ser307), IκBα and IKKα by Western blotting

Antibody / STEP	IRS-1(ser307) Upstate, Lake Placid, NY, USA	IκBα (C-21), *sc*-371 (Santa Cruz Biotechnology, Inc, CA, USA)	p65-NFκB (C20) *sc*-372 (Santa Cruz Biotechnology, Inc, CA, USA)
Block - solution - incubation	5% Marvel in TBS/Tween (0.05%) containing 1% BSA 45 min at RT	10% w/v Marvel non-fat dried milk in PBS/Tween 20 (0.05%) 1 hr at RT	10% w/v Marvel non-fat dried milk in PBS/Tween 20 (0.05%) 1 hr at RT
Wash step	1 x 1min with distilled water	6 x 5 min with PBS/Tween 20 (0.05%)	
Primary antibody - dilution - incubation	1:250 dilution in 5% Marvel in TBS/Tween (0.05%) containing 1% BSA 1 hr at RT, overnight at 4°C, 2 hr at RT the following day	1:1000 dilution in 5% w/v BSA-PBS/Tween 20 (0.05%) overnight at 4°C	1:1000 dilution in 5% w/v Marvel-PBS/Tween 20 (0.05%) overnight at 4°C
Wash step	2 x 5 min with distilled water	6 x 5 min with PBS/Tween 20 (0.05%)	
Secondary Antibody [1] - dilution - incubation	1:1000 dilution in 5% Marvel in TBS/Tween (0.05%) containing 1% BSA 1 hr at RT	1:1000 dilution in 10% w/v Marvel-PBS/Tween 20 (0.05%) 1 hr at RT	1:1000 dilution in 5% w/v Marvel-PBS/Tween 20 (0.05%) 1 hr at RT
Wash step	2 x 5 min with distilled water, 5 x 5 min with TBS/Tween (0.01%), 5 x 5 min with distilled water	10 x 5 min with PBS/Tween 20 (0.05%)	

PBS, phosphate buffered saline; RT, room temperature; TBS, Tris buffered saline.

[1] polyclonal goat anti-rabbit immunoglobulin G-peroxidase-conjugated immunoglobulins (Sigma-Aldrich, Poole, Dorset, UK)

2.17.4 Reuse of Western blots

Before probing for a different protein, the blot was initially washed in TBS/Tween 20 (0.1%) or PBS/Tween 20 (0.05%) for 3 x 10 min, followed by incubation at 50°C for 30 min in stripping buffer (31.25 mM Tris (pH 6.8), 100 mM β-mercaptoethanol, 2% w/v SDS). After re-washing the blot for a further 6 x 5min in TBS/Tween 20 (0.1%), it was ready to use again commencing with the blocking stage in the Western blot protocol.

2.17.5 Quantification of protein bands

Quantification of protein bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation and Analysis System, Syngene). Molecular weight estimation was confirmed by the inclusion of a suitable standard (Precision Plus Protein™ Standards Dual Colour, Bio-Rad Laboratories Inc.). In some blots a positive control was also included. Results were expressed as arbitrary units, normalised to controls and expressed as fold change relative to control.

2.18 ELECTROPHORETIC MOBILITY SHIFT ASSAY

The electrophoretic mobility shift assay (EMSA) detects the interaction of DNA binding proteins e.g. NF-κB with their cognate DNA recognition sequences (e.g. κB enhancer elements) in a qualitative and quantative manner. In summary, the purified nuclear proteins are incubated with a ^{32}P-radiolabelled DNA probe, in this case specific for NF-κB, followed by separation of the complexes from the free probe through a non-denaturing PAGE. The DNA-protein bound complexes migrate at a slower rate than the free DNA molecules and can be easily detected (Revzin, 1989). The gels are transferred to sturdy blotting paper by drying. Exposure of the dried gel to X-Ray film at -70°C, allows for detection of the DNA-protein complexes by autoradiography.

2.18.1 Detection of antigens

Nuclear extracts from adipose tissue were prepared as outlined in Section 2.15.3 and the protein concentration in each sample determined using the Bradford assay as described in Section 2.15.6. 4 μg of nuclear protein was incubated for 30 min at room temperature with 10,000 cpm [^{32}P]-labelled oligonucleotide probe (Perkin-Elmer Life Sciences, Inc., MA, USA), 2 μg Poly dI-dC (Amersham-Pharmacia, UK), 100 mM Tris (pH 7.5), 500 mM NaCl, 40% glycerol (w/v), 5 mM EDTA, 5 mM DTT, 1 mg/ml nuclease-free BSA and sterile water to make up a final volume of 20 μl. The binding reaction was arrested by the addition of one tenth of a volume of gel loading dye (0.25% Bromophenol blue, 30% glycerol in sterile water) prior to loading the samples onto the gel for electrophoresis. The probes used in the EMSA

contained the consensus binding sequence for NF-κB 5'-AGT TGA GGG GAC TTT CCC AGG C-3', which had been previously labelled with [^{32}P] ATP by T4 polynucleoide kinase (Promega, Madison, WI, USA). DNA-protein complexes were resolved on a non-denaturing 4% (v/v) polyacrylamide gels comprising 3.125 ml Acrylamide mix (40%, Accugel 29:1 National Diagnostics, Atlanta, Georgia, US), 2.5 ml 10X Tris borate buffer (TBE buffer: 0.6 M Tris Base, 0.87 M Boric acid, 20 mM EDTA), 0.05 g Ammonium persulphate (nuclease free), 15 µl TEMED, 0.5 mM DTT and 19.4 ml distilled water, and allowed to run for approximately 1¾ hr at 140 V in 0.5X Tris borate buffer. The gels were transferred with appropriate precautions to a piece of firm blotting paper and wrapped in cling film, dried in an automatic drier for approximately 30 min at 80°C and exposed to Fuji RX film (Amersham Bioscience, Buckinghamshire, England) in autoradiography cassettes (Kodak, UK) with intensifying screens for 24-48 hr at -70°C. Films were developed as for Western blots (Section 2.16.5) but with appropriate care.

2.19 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to allow the identification of specific tissue/cell components by means of specific antigen-antibody reaction tagged to a visible label. By using IHC, it is possible to visualise the distribution and localisation of specific cellular components (Marrack, 1934). The steps involved are similar to immunoblotting protocol, encompassing blocking non-specific sites, incubating with primary antibody, followed by incubating with secondary antibody. Additional steps which are specific for IHC are detailed below.

2.19.1 Detection of antigens

Tissues sections were prepared in the Histopathology Department of St James's Hospital by fixing the tissue in neutral buffered formalin, embedding them in paraffin, and sectioning at 5 µm onto poly-L-lysine-coated slides. Section were stained with hematoxylin and eosin for histology. For immunohistochemistry, sections were deparaffinised and hydrated through xylene and alcohol as follows: 3 xylene washes for 5 min each, 100% methanol for 3 min, 90% methanol for 3 min, 70% methanol for 3 min, 50% methanol for 3 min, 30% methanol for 3 min. Antigen unmasking was performed by heating the sections at 95°C for 20 min in 10 mM sodium citrate buffer (pH 6.0), and allowing to cool for 30 min. Sections were washed twice with distilled water before incubating with normal blocking serum (10% block in PBS, 0.05% Tween 20, for 1 hr at room temperature) from which the secondary antibody was raised, to reduce non-specific background staining. Sections were then incubated overnight at 4°C in a humid chamber with a rat primary antibody to MAC-3 (BD Pharmingen, CA, USA) diluted 1:10 in block serum. Sections were washed three times in PBS containing 0.05% Tween 20

for 2 min each, prior to incubation with 20 μg/ml fluorescein isothiocyanate-conjugated antibody against rat IgG (Molecular Probes, Leiden, Netherlands) for 30 min at room temperature. Slides were washed a further three times with PBS containing Tween 20 for 2 min each. Nuclear stain (Hoechst stain, Molecular Probes Europe, Leiden, Netherlands) diluted 1:10,000 in PBS was added for 3 min, slides washed once and subsequently mounted with DakoCytomation fluorescent mounting medium (DakoCytomation, CA, USA) and a coverslip applied.

2.20 GLUCOSE UPTAKE ASSAY

Measuring glucose uptake provides the most relevant end point for assessing insulin sensitivity. 2-deoxyglucose (2-DOG) is an analogue of glucose and is commonly used in uptake studies as it is rapidly phosphorylated following uptake thereby preventing saturation of the transporter, Glut4. However, unlike glucose it is not further metabolised (Shepherd *et al*, 1995).

Fibroblasts were grown to confluency as described in Section 2.9.2. Cells were removed from the 150 mm plates using 0.25X trypsin (without EDTA) resuspended in media and 100 μl added to each well of a 96-well Cytostar-T™ scintillating microplate (Amersham Biosciences, Buckinghamshire, UK). Cells were allowed to settle for 24 hr, checked for confluency, and then differentiated into adipocytes as described in Section 2.9.4. Fatty acid and TNFα treatments were performed as outlined in Section 2.9.7. Wells containing undifferentiated fibroblasts were included as controls. Cells were grown overnight in serum-free DMEM containing 0.5% (w/v) BSA. This was removed and cells were washed with 200 μl buffer (50 mM HEPES (pH 7.4), 120 mM NaCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 4.8 mM KCL). Cells were cultured for 5 hr in buffer to ensure glucose depletion. Insulin (0.1 nM, 1 nM, 10 nM; Sigma-Aldrich, Poole, Dorset, UK) was added to the appropriate wells and the cells cultured for a further 20 min. This was followed by the addition of 10 μl (0.1 μCi) 2-DOG (Amersham Biosciences, Buckinghamshire, UK) for 10 min. The reaction was stopped by disposing of the buffer, and replacing it with PBS. Glucose uptake was monitored as an increase in scintillation counts, using a Wallac MicroBeta™ plate counter (Perkin Elmer, MA, USA).

Chapter Three

Conjugated linoleic acid supplementation, insulin sensitivity and lipoprotein metabolism in subjects with Type 2 Diabetes Mellitus

Part of this work has been published in the *American Journal of Clinical Nutrition:* Moloney *et al,* (2004), **80**, 887-895.

3.1 ABSTRACT

Background: Some animal studies have suggested that conjugated linoleic acid (CLA) supplementation may have therapeutic potential with respect to insulin sensitivity and lipid metabolism which are important cardiovascular disease (CVD) risk factors associated with type 2 diabetes mellitus (T2DM).

Objective: To investigate the effect of CLA supplementation on markers of glucose and insulin metabolism, lipoprotein metabolism, and inflammatory markers of CVD in subjects with T2DM.

Design: The study was a randomised, double-blinded, placebo-controlled trial. 32 subjects with stable, diet-controlled T2DM received 3.0g/day CLA (50:50 blend of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) or control for 8 wk. A 3 hr 75g oral glucose tolerance test was performed, and fasting plasma lipid concentrations and inflammatory markers were measured before and after the intervention.

Results: CLA supplementation significantly increased fasting glucose concentrations (6.3%; $P<0.05$) and reduced insulin sensitivity as measured by homeostasis model assessment, oral glucose insulin sensitivity, and insulin sensitivity index (composite) ($P=0.05$). Total HDL-cholesterol concentrations increased by 8% ($P<0.05$), which was due to a significant increase in HDL_2-cholesterol concentrations ($P<0.05$). The ratio of LDL to HDL cholesterol was significantly reduced ($P<0.01$). CLA supplementation reduced fibrinogen concentrations ($P<0.01$), but had no effect on the inflammatory markers of CVD (C-reactive protein and interleukin 6).

Conclusions: CLA supplementation did not improve insulin and glucose metabolism. Whereas CLA had positive effects on HDL metabolism and fibrinogen, a therapeutic nutrient should not be associated with potentially adverse effects on other clinical markers of T2DM.

3.2 INTRODUCTION

In parallel with the global epidemic of type 2 diabetes mellitus (T2DM) are significant health and socioeconomic burdens (Hu *et al*, 2001). Thus, there is a need to identify effective dietary strategies to attenuate the impact of T2DM, which is a heterogeneous disease characterised by target-tissue insulin resistance that cannot be overcome by ß-cell hypersecretion (Steppan *et al*, 2001). The World Health Organization (WHO) defined the clustering of metabolic abnormalities associated with T2DM as the metabolic syndrome. Features include impaired insulin sensitivity, glucose intolerance, or diabetes mellitus coupled with 2 metabolic derangements including obesity, insulin resistance, hypertension, dyslipidaemia [increased plasma triacylglycerol (TAG) and low high density lipoprotein (HDL)-cholesterol concentrations] and microalbuminuria (Alberti & Zimmet, 1998). Low-grade inflammation, also a feature of T2DM (Das, 2002) has been implicated in the development of atherosclerosis (Leinonen *et al*, 2003). Cardiovascular disease (CVD) is the leading cause of morbidity and mortality among subjects with T2DM, as a result of the presence of several primary risk factors for CVD in this patient population (Erkelens, 2001).

Although little can be done to avert a genetic predisposition to T2DM, attenuation of the effect of modifiable risk factors through dietary and lifestyle factors is important. Conjugated linoleic acid (CLA) has received attention as a potential therapeutic nutrient with respect to insulin resistance and hyperlipidaemia (Roche *et al*, 2002), which are key characteristics of T2DM. The term CLA refers to the positional and geometric isomers of linoleic acid with a conjugated double-bond system (Gavino *et al*, 2000). Animal feeding studies have shown that CLA reduces body fat, increases lean body mass (Park *et al*, 1997, West *et al*, 1998, DeLany *et al*, 1999), improves plasma lipid metabolism, and inhibits the progression and promotes the regression of atherosclerosis (Wilson et al, 2000, Kritchevsky *et al*, 2000, Toomey *et al*, 2003). There are relatively few human intervention studies, and the results of those few are mixed. Most of the evidence regarding body composition suggests that CLA supplementation does not reduce body weight or body fat or increase lean body mass in humans (Larsen *et al*, 2003). Small, non-significant reductions in low density lipoprotein (LDL)-cholesterol (Blankson *et al*, 2000, Noone *et al*, 2002) and conflicting effects regarding HDL cholesterol (Blankson *et al*, 2000, Benito *et al*, 2001, Mougios *et al*, 2001) have been observed following CLA supplementation in humans. A recent study indicated that *trans*-10, *cis*-12 CLA (t10, c12-CLA) supplementation had negative effects on insulin resistance and biomarkers of oxidative stress and inflammation in obese men who had signs of the metabolic syndrome (Riserus *et al*, 2002a, Riserus *et al*, 2002b). In contrast, Noone *et al* (2002) reported that a supplement combining *cis*-9, *trans*-11 (c9, t11-CLA) and t10, c12-CLA improved plasma TAG concentrations and very low density lipoprotein (VLDL) metabolism, without exerting adverse effects on insulin and glucose metabolism in healthy subjects. These

conflicting results may reflect differences in study duration, cohort composition, study settings, and most important, supplement composition. CLA is a heterogeneous compound, and distinct isomer-specific effects have been identified (Roche *et al*, 2002, Noone *et al*, 2001). c9, t11-CLA and t10, c12-CLA are thought to have most biological activity, and most research has investigated these two isoforms. Research has shown that c9, t11-CLA and t10, c12-CLA, have contrasting metabolic and molecular effects. Feeding a c9, t11-CLA enriched diet improved lipid and glucose metabolism, whereas feeding a t10, c12-CLA enriched diet promoted insulin resistance and dyslipidaemia in ob/ob mice (Roche *et al*, 2002, Noone *et al*, 2001).

The objective of the present study was to determine the effect of CLA supplementation, providing equal proportions of c9, t11-CLA and t10, c12-CLA, in subjects with stable, diet-controlled T2DM. To date no study has investigated the metabolic effects of CLA supplementation in these patients. Several key aspects of the metabolic syndrome, including insulin and glucose metabolism, lipoprotein metabolism, and markers of coagulation and inflammation, were measured to ascertain the effect of CLA in persons with diet-controlled T2DM.

3.3 SUBJECTS AND METHODS

This study was approved by the Joint Ethics Committee of St James's Hospital and the Federated Dublin Voluntary Hospitals, Ireland. The purpose, nature, and potential risks of the study were explained before written informed consent was obtained from each volunteer. Sample size was estimated by the ability to detect a 20-30% change in TAG concentrations, assuming a type I error of 0.05 and a power of 0.9. 32 subjects with type 2 diabetes who attended the Diabetic Day Care Centre at St James's Hospital in Dublin and whose diabetes was controlled by diet therapy alone completed the trial. All subjects had stable metabolic control with a glycated haemoglobin (HbA_{1c}) concentration of 6.83 ± 0.18 mmol/L and a mean fasting blood glucose concentration of 7.33 ± 0.24 mmol/L. None of the subjects were receiving pharmacological treatment for glycaemic control or lipid-lowering purposes. Hypertension was present in approximately one-half of the subjects. However, only subjects whose blood pressure was under stable control participated in the study. There was no difference in the prevalence of hypertension between the two groups. In addition, there was no documentation of macrovascular disease in the medical notes of any participant. Blood screening samples indicated that the liver and kidney profiles and haemoglobin concentrations were within the routine acceptable ranges for St. James's Hospital. All subjects had stable body weight. All were following healthy eating guidelines as recommended by the American Diabetes Association (American Diabetes Association, 2002). The study subjects did not consume fatty acid supplements or other dietary products known to affect metabolic markers of T2DM.

3.3.1 Study design

This randomised, double-blinded, placebo-controlled study was conducted on a free-living, outpatient basis. Subjects received 3.0g CLA/day (6 x 0.5 g capsules comprising a 50:50 isomer blend of c9, t11-CLA and t10, c12-CLA) or placebo (6 x 0.5 g capsules comprising a blend of palm oil and soya bean oil) for 8 wk. The placebo was designed to contain a blend of fatty acids that was representative of a habitual western diet (Gregory *et al*, 1990, Wallace *et al*, 2002). The fatty acid compositions of the CLA supplement and the placebo are shown in *Table 3.1*. All supplements were supplied by Loders Croklann (Wormeveer, Netherlands). Each volunteer received his or her capsules in 2 batches, at baseline and after wk 4. A capsule count was completed midway and at the end of the supplementary period. All participants were asked to maintain their usual dietary and lifestyle habits. The effect of changes in diet, weight, and physical activity was explained to the subjects. There was no change in prescribed medication i.e. anti-hypertensives throughout the trial.

Table 3.1: Fatty acid composition of the CLA supplement and the control supplement.

	Supplement	
Ingredient	Control	CLA
	% by wt of total fatty acids	
Fatty acid composition (%)		
16:0	38.52	4.82
18:0	4.70	2.28
18:1	34.84	10.17
18:2n-6	17.89	1.73
Saturated Fatty Acids	44.97	8.16
CLA isomers (%)		
Total CLA	0.06	73.83
cis-9, *trans*-11 CLA	0.06	35.62
trans-10, *cis*-12 CLA	undetectable	38.21

ND, not detectable.

Supplements were given as free fatty acid. Supplement details provided by Consultus Laboratories, Cork, Ireland.

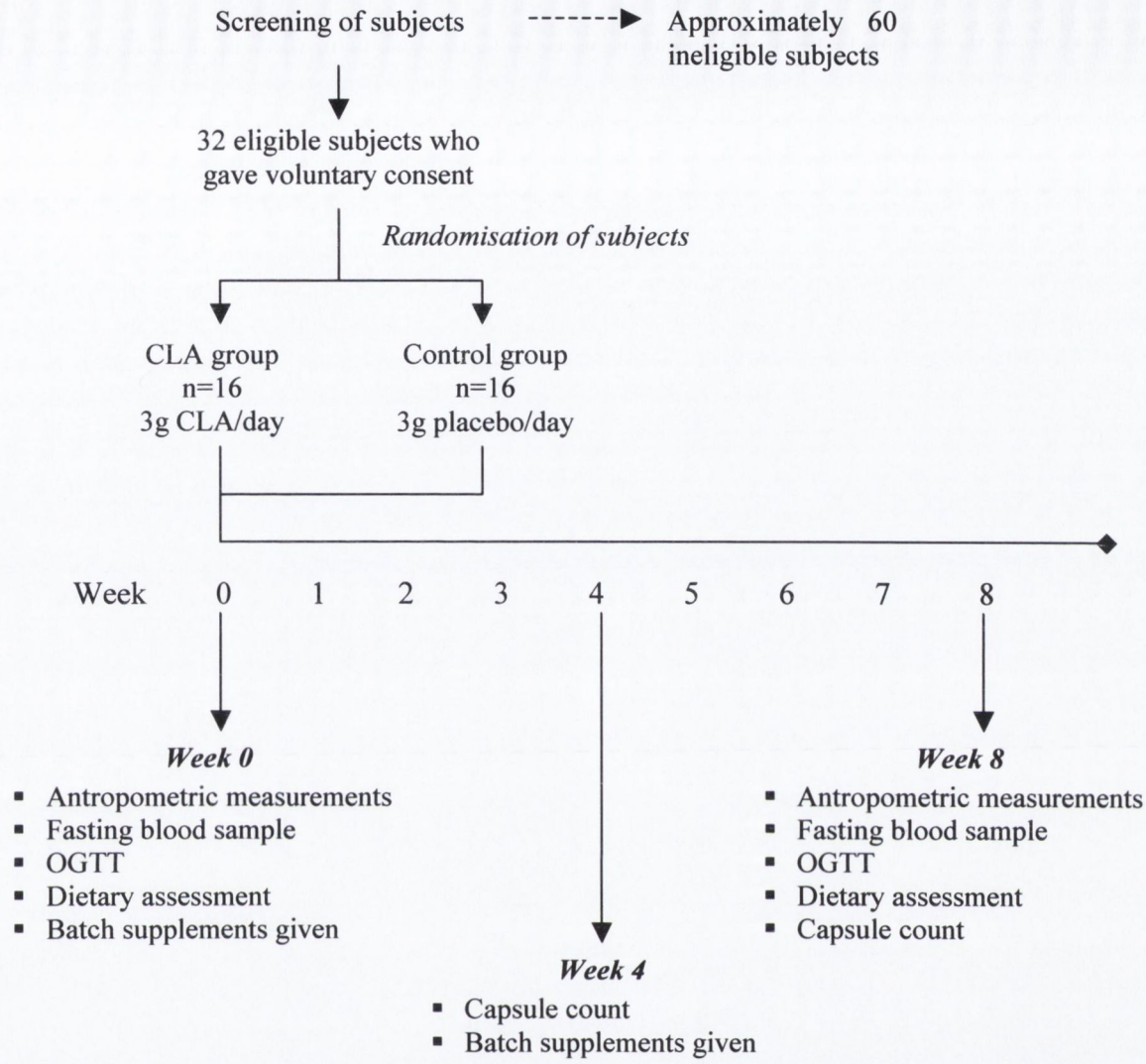


Fig. 3.1 Study Design

OGTT, oral glucose tolerance test.

3.3.2 Dietary assessment

Mean daily dietary intake was assessed by using two 4-day food records, one completed immediately before the study and the other completed at the end of the supplementation period (Korhonen *et al*, 2003). Dietary information was recorded in the food diary used in the North-South Food Consumption Survey, 2001 (Harrington *et al*, 2001). A detailed description of the ingredients or food brand, food preparation and cooking method was gained. Household measures, standard food portions, and a photographic food atlas were used to quantify food and fluid portion sizes (Harrington *et al*, 2001). This dietary information was analysed for macronutrient composition with the use of NETWISP software (version 2.0; Tinuviel Software, Warrington, UK), which was modified to include the composition of frequently

consumed foods that were not part of the database. This additional information was obtained from the nutrient information panel supplied by the manufacturers.

3.3.3 Clinical investigations

Subjects attended the Metabolic Unit, Department of Endocrinology, St James's Hospital, for blood sampling after a 12 hr overnight fast before and after intervention (wk 0 and wk 8). Subjects abstained from strenuous exercise and alcohol intake for 24 hr before the examination and refrained from smoking on the morning of the examination. Medications e.g. anti-hypertensives were not taken until the clinical investigations were complete. On the morning of each investigation, a fasting blood sample was drawn and a standard 3 hr, 75g oral glucose tolerance test (OGTT) was performed. The 75g anhydrous glucose equivalent (Polycal; Nutricia Clinical, Trowbridge, UK) was consumed in 300 ml water within 5 min. Blood samples were collected at 0, 30, 60, 90, 120, and 180 min for the measurement of plasma glucose and serum insulin and C-peptide concentrations.

3.3.4 Collection of blood samples

Blood for the future measurement of cholesterol, TAG, low density lipoprotein (LDL), interleukin (IL)-6, insulin, and C-peptide concentrations was collected in serum tubes (Becton Dickinson, Oxford, UK). Blood for HDL and VLDL analysis was collected in EDTA-coated evacuated tubes (Becton Dickinson, Oxford, UK). Samples for glucose analysis were collected in tubes containing sodium fluoride, and blood for fibrinogen, C-reactive protein (CRP), and fatty acid analyses was collected in tubes containing citrate (Becton Dickinson, Oxford, UK). Serum samples were allowed to clot for 1 hr. Samples for lipid, glucose, insulin, C-peptide, and IL-6 analyses were centrifuged immediately at 1400 x *g* for 10 min at room temperature. Citrated samples for CRP and fibrinogen measurements were collected and then centrifuged at 2000 x *g* for 20 min at room temperature. Blood samples collected for fatty acid analysis were centrifuged at 200 x *g* for 20 min at room temperature to harvest the platelet rich plasma. All of the samples listed above were frozen immediately and stored at -70°C until subsequent analysis.

3.3.5 Biochemical analysis

Plasma samples were analysed for cholesterol and TAG concentrations using enzymatic colorimetric assays on an ILAB 600 clinical chemistry analyser (Instrumentation Laboratory, Warrington, UK) as described in Section 2.2.2 and Section 2.2.3, respectively. Total HDL-, HDL_3-, and HDL_2-cholesterol concentrations were determined after precipitation with Quantolip total HDL Precipitation Reagent and Quantolip HDL_3 Precipitation Reagent (both: Immuno Ag, Vienna, Austria) as described in Section 2.3.2. LDL-cholesterol concentrations

were measured after precipitation of LDL using Randox Precipitation Reagent (Randox, Antrim, Ireland) as outlined in Section 2.3.1. VLDL was isolated using ultracentrifugation techniques as described in Section 2.3.3. TAG-poor lipoprotein (TPL) was also harvested. VLDL, TPL, LDL, and HDL lipid compositions were determined using colorimetric assays as described in Section 2.3.2 and Section 2.3.3. VLDL apolipoprotein B (apoB) was measured using a turbidimetric assay (Instrumentation Laboratory, Warrington, UK) on the ILAB 600 clinical chemistry analyser, as detailed in Section 2.4. Plasma glucose concentrations were measured using enzymatic determination based on the glucose hexokinase principle (BioMerieux, Marcy-l'Etoile, France) (Section 2.2.4). Serum insulin and C-peptide concentrations were measured by solid-phase, two-site fluoroimmunometric assays (AutoDELFIA Insulin kit and AutoDELFIA C-Peptide kit, respectively; Wallac Oy, Turku, Finland) on a 1235 AutoDELFIA automatic immunoassay system (Wallac Oy, Turku, Finland) (Section 2.6). HbA_{1c} was assessed using a fully automated HPLC analyser (Menarini-Arkray HA 8140; Arkray KDK, Kyoto, Japan) in the Haematology laboratory in St. James's Hospital. IL-6 and CRP assays were performed using human high-sensitivity immunoassay test kits (Biosource, Camarillo, CA, USA and BioCheck Inc, Burlingame, CA, USA respectively) according to each manufacturer's instructions (Section 2.7). Fibrinogen clotting activity was measured by using an automated clotting assay as described in Section 2.5.

For each marker, both pre-intervention and post-intervention samples for each subject were analysed within a single batch. The inter-assay coefficient of variation (CV) for total cholesterol and triacylglycerol was 1.25% and 3.15%, respectively. The inter-assay CV for fibrinogen was 3.25%. Insulin, C-peptide, HbA_{1c}, glucose, and microalbumin concentrations were analysed in the laboratory of St James's Hospital and were acceptable according to the routine internal and external standards applied.

3.3.6 Fatty acid compositional analysis

Protocol compliance was verified by conducting a capsule count and by measurement of plasma fatty acid composition with the use of gas liquid chromatography. Total plasma lipids were isolated using the method of Folch *et al* (1957), as described in Section 2.8.1. Methyl esters of total plasma lipid were prepared by adding 0.5 ml of 0.01 M NaOH in dry methanol and then adding 0.5 ml boron trifluoride as outlined in Section 2.8.2. Total plasma lipid fatty acid composition was determined by using a Shimadzu GC-14A Gas Liquid Chromatograph (Mason Technologies, Dublin, Ireland) fitted with a Shimadzu C-16A integrator (Mason Technologies) and a CP Sil 88 fused silica column (50 m x 0.22 mm, 0.2 μm file thickness; Chrompack Ltd, Middelburg, Netherlands). Plasma fatty acids were identified according to their retention times in comparison with a fatty acid methyl ester standard (Sigma-Aldrich, Poole, Dorset, UK) spiked with known concentrations of transmethylated c9, t11-CLA and

t10, c12-CLA isomers (Cayman Chemical, MI, USA). Plasma fatty acid composition was calculated as a percentage of total fatty acids (Section 2.8.3).

3.3.7 Anthropometric measurements

Body weight was measured on an electronic balance, measuring to the nearest 0.1kg, with subjects wearing light clothing but no shoes. Height was assessed using a stadiometer that measured to the nearest 0.1 cm. Waist girth was measured at the minimum circumference between the iliac crest and the rib cage. Hip girth was measured at the maximum width over the greater trochanters. Waist-to-hip ratio (WHR) was calculated from these measurements. Bioelectric impedance analysis was measured in subjects in the erect position using a body fat analyser (TBF-300; Tanita Corporation, Arlington Heights, IL, USA). Percentage body fat was calculated using the manufacturer's programmed equations. Appendix I provides a more detailed description of the methodology employed to record the anthropometric measures.

3.3.8 Data preparation and statistical analysis

All statistical analysis was conducted by using Data Desk software (version 6.0, Data Description Inc, Ithaca, NY, USA). Results are presented as mean ± SEM. When necessary, values were transformed to give a normal Gaussian distribution. The postprandial data were expressed in summary form i.e. area under the postprandial curve (AUC), incremental area under the postprandial curve (IAUC), maximum postprandial concentration (C_{max}), and time to maximal postprandial concentration (T_{max}). These data were used to investigate postprandial variations between the supplement groups. AUC was calculated using the trapezium rule (Matthews *et al*, 1990), and IAUC was calculated as the total incremental AUC according to Le Floch (Allison *et al*, 1995). Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). Quantitative insulin sensitivity check index (QUICKI) was calculated as 1/(log fasting insulin concentration + log fasting glucose concentration) (Katz *et al*, 2000). The insulin sensitivity index (ISI) composite was calculated as proposed by Matsuda & De Fronzo (1999). Oral glucose insulin sensitivity (OGIS) was calculated using the published formula (Mari *et al*, 2001).

Analysis of covariance (ANCOVA), after control for the baseline value of the outcome variable, was used to identify significant changes in biochemical values after the supplementation period (ANCOVA 1). Baseline microalbumin and cholesterol concentrations were also controlled for (in addition to the baseline value of the control variable) because wk 0 (baseline) values differed between the groups (ANCOVA 2). ANCOVA 1 and ANCOVA 2 did not detect different statistical effects. ANCOVA 2 is reported in the results section. 3-way

analysis of variance (ANOVA) with subject, treatment, and time as independent variables and a treatment-by-time interaction was used to identify significant changes in plasma fatty acid composition. Repeated-measures ANOVA with a treatment-by-time interaction was used to investigate significant differences in postprandial responses between the study groups. Post hoc statistical analysis was conducted by using Scheffe's test. A P value < 0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 Subject characteristics and anthropometric measurement details

Baseline characteristics of the 2 study groups are summarized in *Table 3.2a*. There was no difference between the groups at baseline regarding mean age, weight, body mass index (BMI; kg/m^2), waist circumference, hip circumference, WHR, percentage body fat or duration of diabetes. There was no difference in any of the anthropometric measurements after either the CLA or control supplements *(Table 3.2b)*.

Table 3.2a: Baseline characteristics of the study population by treatment group. Values represent mean ± SEM. There were no significant differences between the groups (1-way ANOVA).

	Control group (n=16)	CLA group (n=16)
Age (y)	58.1 ± 2.7	63.8 ± 2.2
Weight (kg)	88.2 ± 3.8	84.4 ± 3.1
BMI (kg/m^2)	30.7 ± 1.2	29.1 ± 1.0
Waist circumference (cm)	101.1 ± 2.6	102.7 ± 2.5
Hip circumference (cm)	110.0 ± 2.8	108.2 ± 1.9
WHR	0.92 ± 0.02	0.95 ± 0.02
Percentage Body Fat	33.2 ± 2.3	32.9 ± 2.5
Duration of Diabetes (months)	32.9 ± 7.4	34.5 ± 5.7

Table 3.2b: Mean anthropometric measurements before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. There were no significant differences between the groups at wk 0 or following 8 wk supplementation (ANCOVA or 3-way ANOVA).

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
Weight (kg)	88.2 ± 3.8	87.8 ± 3.7	84.4 ± 3.1	84.1 ± 3.2
BMI (kg/m^2)	30.7 ± 1.2	30.6 ± 1.2	29.1 ± 1.0	29.0 ± 1.0
Waist circumference (cm)	101.1 ± 2.6	100.9 ± 2.4	102.7 ± 2.5	102.0 ± 2.4
Hip circumference (cm)	110.0 ± 2.8	110.4 ± 2.8	108.2 ± 1.9	108.1 ± 2.1
WHR	0.92 ± 0.02	0.92 ± 0.02	0.95 ± 0.02	0.94 ± 0.01
Percentage Body Fat	33.2 ± 2.3	34.4 ± 2.3	32.9 ± 2.5	30.7 ± 2.8

3.4.2 Study compliance

Study compliance was assessed by conducting a capsule count, completing dietary assessments and by determining total plasma fatty acid composition. The subjects consumed 96.16% of the prescribed supplements. Compliance did not differ significantly between the study groups: 96.33% and 95.83% of supplements were used by the control and CLA groups, respectively. Dietary analysis, based on two 4-day food records, one completed before and the other completed after the intervention, showed that mean daily energy, macronutrient, dietary fibre, cholesterol and alcohol intake did not differ significantly after CLA or placebo supplementation. In addition, the percentage contribution of macronutrients to total energy and food energy did not differ after either supplement. Mean daily nutrient intakes did not differ significantly between groups before or after intervention. Appendix II, at the end of this chapter, tabulates these results. The fatty acid compositions of total plasma lipids are shown in *Table 3.3*. Total plasma c9, t11-CLA concentrations increased by 89% after CLA supplementation ($P<0.001$). The t10, c12-CLA isomer was undetectable in most samples and was not affected by CLA supplementation. Plasma linoleic acid (18:2n–6) concentrations increased significantly within the CLA group ($P<0.01$) and α-linoleic acid (18:3n-3) concentrations increased significantly within the control group ($P<0.01$), but statistical differences were not observed between the groups.

Table 3.3: Fatty acid composition of total plasma lipids before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. Significant difference within the group after 8 wk supplementation (3-way ANOVA) † P<0.01. Significant difference between groups after 8 wk supplementation (treatment x time interaction) (3-way ANOVA) ‡ P<0.001.

Fatty Acid	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
	% by wt of total fatty acids			
16:0	24.80 ± 0.55	25.52 ± 0.58	25.75 ± 0.57	26.3 ± 0.50
16:1	2.16 ± 0.22	1.79 ± 0.13	2.174 ± 0.22	2.24 ± 0.19
18:0	9.96 ± 0.40	10.97 ± 0.55	10.3 ± 0.40	10.78 ± 0.40
18:1	18.58 ± 0.85	17.66 ± 0.80	18.46 ± 0.88	19.66 ± 0.96
18:2n-6	20.73 ± 0.72	21.26 ± 0.91	19.21 ± 0.74	21.90 ± 1.18 †
18:3n-6	0.26 ± 0.02	0.23 ± 0.01	0.20 ± 0.02	0.24 ± 0.01
18:3n-3	0.49 ± 0.08	0.57 ± 0.1 †	0.44 ± 0.06	0.32 ± 0.05
CLA	0.28 ± 0.04	0.32 ± 0.05	0.37 ± 0.06	0.7 ± 0.08 ‡
20:2	0.27 ± 0.02	0.29 ± 0.02	0.30 ± 0.01	0.29 ± 0.02
20:3n-6	2.52 ± 0.15	2.59 ± 0.24	2.31 ± 0.25	2.30 ± 0.14
20:4n-6	8.58 ± 0.37	9.06 ± 0.53	9.36 ± 0.56	8.66 ± 0.46
20:5n-3	1.30 ± 0.16	1.24 ± 0.24	1.67 ± 0.32	1.18 ± 0.14
22:4n-6	0.41 ± 0.03	0.43 ± 0.06	0.70 ± 0.18	0.40 ± 0.03
22:6n-3	2.94 ± 0.25	2.47 ± 0.23	3.15 ± 0.29	3.16 ± 0.26

3.4.3 Insulin and glucose metabolism

The effect of CLA supplementation on a range of measures of insulin sensitivity and glycaemic control is shown in *Table 3.4a.* Fasting glucose concentrations were significantly increased after CLA supplementation (P<0.05). HOMA-IR was also significantly higher after CLA supplementation than after control supplementation (P=0.05). Both OGIS and the ISI (composite) were significantly lower after CLA supplementation than after control supplementation (P=0.05 and P<0.05, respectively). HOMA-2 IR or HOMA-2 % insulin sensitivity, the QUICKI measure of insulin sensitivity, HbA_{1c} and microalbumin concentrations were not significantly altered by CLA supplementation.

Table 3.4a: Measurements of insulin sensitivity and glycaemic control before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. Significant difference between groups after 8 wk of supplementation (ANCOVA) ** P<0.05, * P=0.05. [1] Significant difference between the groups at baseline (Wk 0).

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
Fasting Glucose (mmol/L)	7.33 ± 0.40	7.09 ± 0.35	7.34 ± 0.27	7.8 ± 0.28 **
Fasting Insulin (pmol/L)	54.94 ± 5.10	50.68 ± 7.60	52.13 ± 7.90	57.87 ± 8.86
HOMA-IR	3.00 ± 0.35	2.59 ± 0.42	2.81 ± 0.42	3.35 ± 0.54 *
HOMA 2[1]				
IR	1.29 ± 0.12	1.33 ± 0.15	1.22 ± 0.17	0.82 ± 0.21
% insulin sensitivity	88.5 ± 8.21	92.25 ± 12.33	106.6 ± 13.55	100.59 ± 15.02
QUICKI	0.33 ± 0.005	0.36 ± 0.025	0.34 ± 0.007	0.33 ± 0.007
ISI (composite)	3.98 ± 0.40	6.26 ± 1.89	4.58 ± 0.64	4.11 ± 0.54 **
OGIS	353.63 ± 19.0	362.40 ± 16.41	341.08 ± 15.89	329.84 ± 16.38 *
HbA_1c	6.73 ± 0.12	6.59 ± 0.18	6.93 ± 0.33	6.63 ±0.16
Microalbumin	3.22 ± 0.77	3.32 ± 0.72	1.61 ± 0.31[1]	1.49 ± 0.21

[1] derived from the updated version of the model. This is available as a computer model (see Wallace *et al*, 2004).

The postprandial glucose, insulin, and C-peptide concentrations in response to the OGTT are shown in *Fig. 3.2.* Repeated-measures ANOVA showed that plasma glucose, serum insulin and C-peptide concentrations increased significantly (P<0.001) during the OGTT. Both before and after the intervention, postprandial glucose concentrations were significantly higher in the CLA group (P<0.01), whereas postprandial C-peptide concentrations were significantly higher in the control group (P<0.01). Fasting glucose concentrations were significantly higher after CLA supplementation than after control supplementation (P<0.05). Postprandial glucose concentrations were not significantly changed by either supplement. In addition, neither supplement had a significant effect on fasting or postprandial insulin or C-peptide concentrations. Summary variables, AUC, IAUC, T_{max}, C_{max}, of the postprandial glucose, insulin and C-peptide responses after the OGTT were not significantly altered by CLA or control supplementation *(Table 3.4b).*

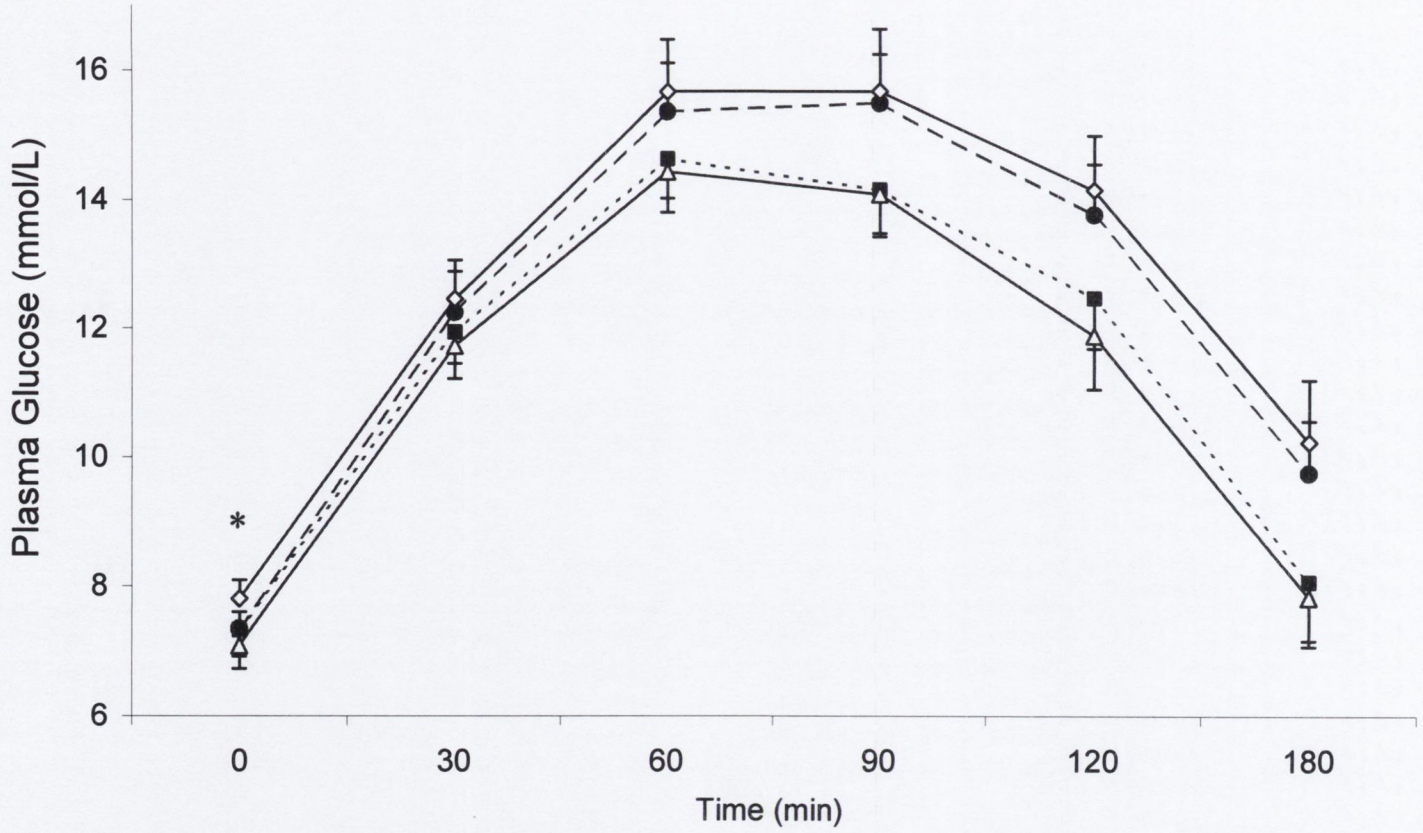


Fig. 3.1a: Mean (± SEM) plasma glucose concentrations in response to the administration of 75 g anhydrous glucose before and after CLA supplementation (3 g/d) or placebo for 8 wk: control group (week 0, ■; week 8, △), CLA group (week 0, ●; week 8, ◇). By repeated-measures ANOVA, there was a significant time effect for glucose ($P<0.001$) and a treatment-by-time effect for plasma glucose ($P<0.01$). Fasting plasma glucose concentrations were significantly higher after CLA supplementation, $P<0.05$ (ANCOVA).

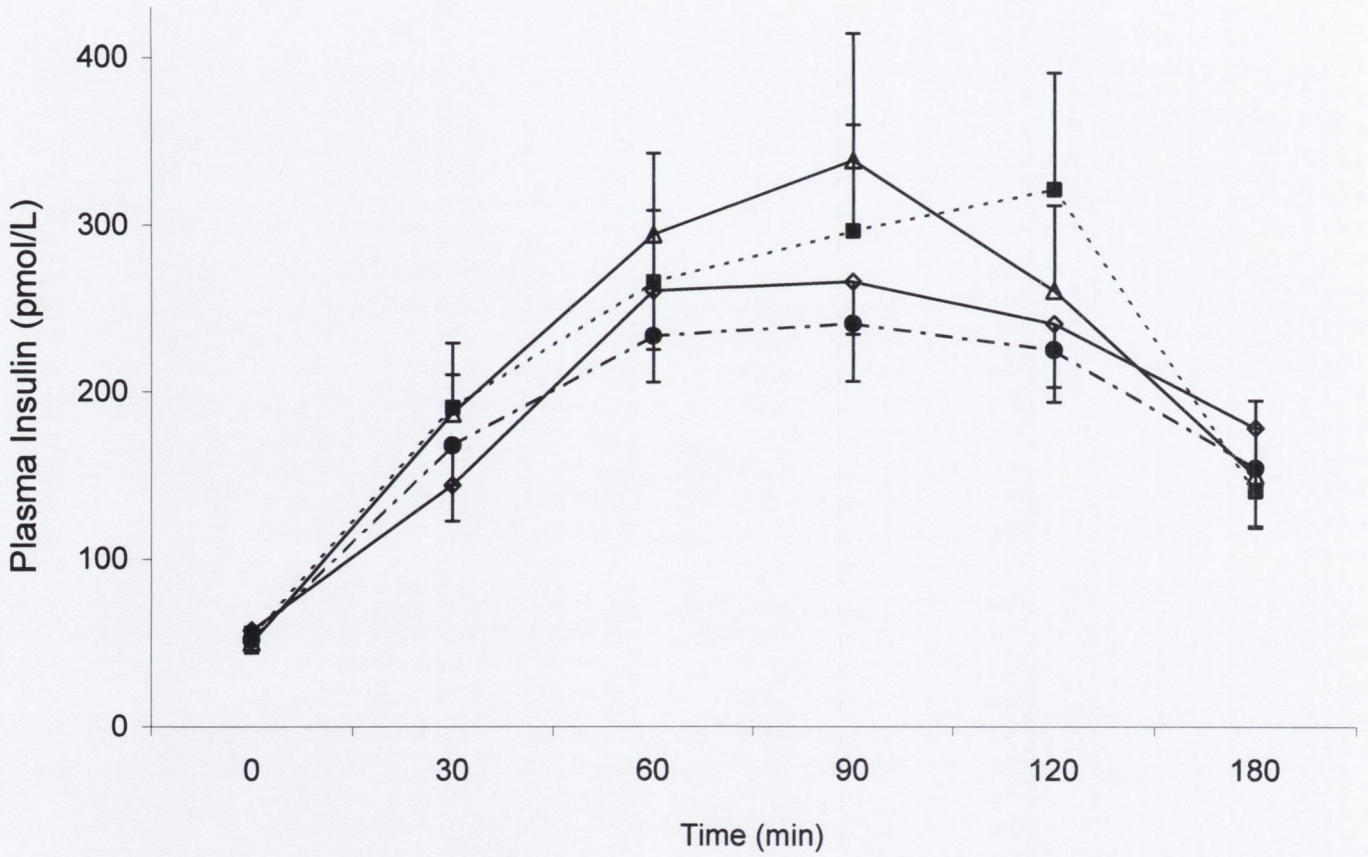


Fig. 3.2b: Mean (± SEM) serum insulin concentrations in response to the administration of 75 g anhydrous glucose before and after CLA supplementation (3 g/d) or placebo for 8 wk: control group (week 0, ■; week 8, △), CLA group (week 0, ●; week 8, ◇). By repeated-measures ANOVA, there was a significant time effect for insulin ($P<0.001$).

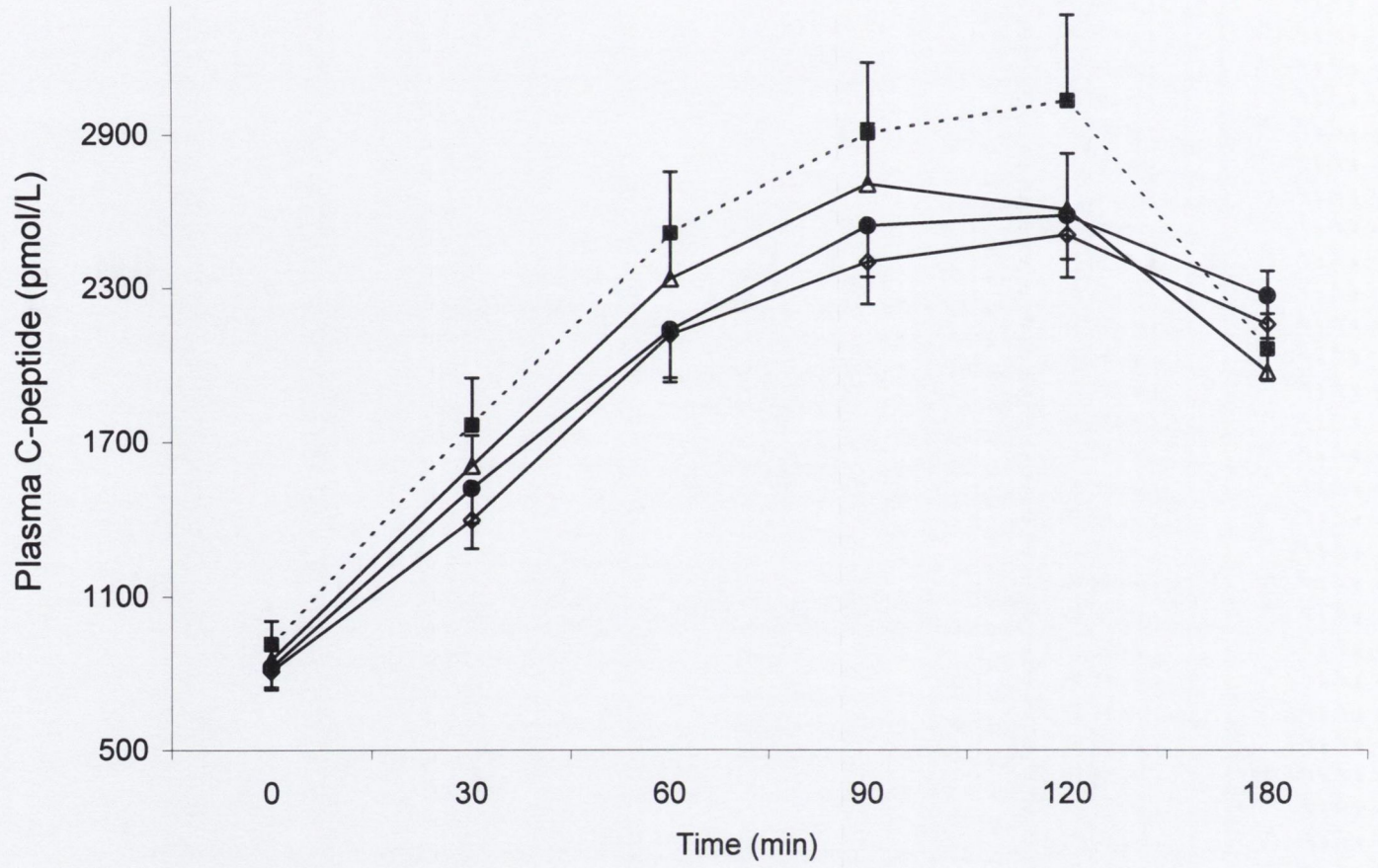


Fig. 3.1c: Mean (± SEM) serum C-peptide concentrations in response to the administration of 75 g anhydrous glucose before and after CLA supplementation (3 g/d) or placebo for 8 wk: control group (week 0, ■; week 8, △), CLA group (week 0, ●; week 8, ◇). By repeated-measures ANOVA, there was a significant time effect for C-peptide ($P < 0.001$) and a treatment-by-time effect for serum C-peptide ($P < 0.01$).

Table 3.4b: Summary variables (AUC, iAUC, C_{max}, T_{max}) of the postprandial glucose, insulin and C-peptide responses before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. There were no significant differences between the groups at wk 0 or following 8 wk supplementation (ANCOVA or 3-way ANOVA).

Glucose

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
AUC (mmol/L·min)	35.56 ± 1.94	34.70 ± 1.82	38.56 ± 1.86	39.55 ± 2.18
IAUC mmol/L·min)	13.58 ± 0.94	13.43 ± 1.05	16.55 ± 1.30	16.15 ± 1.47
C_{max} (mmol/L)	14.79 ± 0.62	14.84 ± 0.70	16.09 ± 0.76	16.25 ± 0.80
T_{max} (min)	71.25 ± 3.75	75.00 ± 4.74	76.88 ± 4.72	78.75 ± 4.64

Insulin

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
AUC (pmol/L·min)	700.70 ± 133.06	692.75 ± 125.35	579.26 ± 72.51	619.15 ± 89.46
IAUC (pmol/L·min)	535.90 ± 122.35	540.70 ± 108.28	422.88 ± 53.47	445.55 ± 66.96
C_{max} (pmol/L)	366.68 ± 67.70	372.23 ± 75.01	288.31 ± 35.96	337.05 ± 55.67
T_{max} (min)	90.0 ± 7.25	80.63 ± 6.55	89.28 ± 10.55	97.5 ± 10.06

C-peptide

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
AUC (pmol/L·min)	7118.05 ± 708.37	6473.64 ± 522.67	6373.33 ± 438.55	6113.16 ± 413.07
IAUC (pmol/L·min)	4374.89 ± 567.77	3916.66 ± 338.66	3903.25 ± 281.87	3686.51 ± 285.88
C_{max} (pmol/L)	3119.68 ± 322.81	2753.51 ± 288.45	2748.40 ± 191.25	2734.89 ± 225.75
T_{max} (min)	110.63 ± 6.55	99.38 ± 3.59	120.0 ± 8.22	114.38 ± 7.85

3.4.4 Lipoprotein metabolism

Fasting serum and lipoprotein lipid concentrations before and after CLA or placebo supplementation are shown in *Table 3.5a*. There was no significant effect of either CLA or control supplementation on total or LDL-cholesterol concentrations. Total HDL-cholesterol concentrations were significantly increased after CLA supplementation ($P<0.05$). The 7.9% increase in total HDL-cholesterol concentration was due to a significant increase in HDL_2-cholesterol concentrations after CLA supplementation ($P<0.05$). LDL:HDL cholesterol was significantly reduced after CLA supplementation ($P<0.01$). Serum TAG, VLDL-TAG and VLDL-cholesterol concentrations were not significantly altered by either supplement. VLDL apoB concentrations were significantly higher after CLA supplementation than after control supplementation ($P<0.05$).

Table 3.5a: Mean fasting plasma and lipoprotein cholesterol and TAG concentrations before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. Significant difference within the group after 8 wk supplementation (3-way ANOVA) * $P<0.05$, ** $P<0.01$. Significant difference between groups after 8 wk supplementation (ANCOVA) ‡ $P<0.05$. [1] Significant difference between the groups at baseline (Wk 0).

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
Plasma cholesterol (mmol/L)	4.73 ± 0.26	4.78 ± 0.23	5.16 ± 0.17[1]	5.06 ± 0.14
Cholesterol (mmol/L)				
LDL	3.10 ± 0.22	3.18 ± 0.20	3.63 ± 0.18	3.31 ± 0.19
Total HDL	1.50 ± 0.10	1.54 ± 0.08	1.44 ± 0.07	1.55 ± 0.08 *
HDL_2	0.23 ± 0.03	0.25 ± 0.03	0.23 ± 0.04	0.34 ± 0.05 *
HDL_3	1.26 ± 0.08	1.29 ± 0.07	1.21 ± 0.05	1.21 ± 0.05
HDL_2:HDL_3	0.20 ± 0.03	0.21 ± 0.03	0.19 ± 0.03	0.28 ± 0.05
LDL:HDL	2.15 ± 0.17	2.13 ± 0.16	2.63 ± 0.21	2.26 ± 0.21 **
Plasma TAG (mmol/L)	1.60 ± 0.16	1.51 ± 0.18	1.62 ± 0.12	1.64 ± 0.13
VLDL TAG (mmol/L)	0.66 ± 0.08	0.56 ± 0.07	0.76 ± 0.09	0.78 ± 0.7
VLDL cholesterol (mmol/L)	0.28 ± 0.05	0.24 ± 0.03	0.30 ± 0.04	0.33 ± 0.04
VLDL apoB (μg/ml)	43.91 ± 6.36	39.93 ± 5.0	42.03 ± 3.57	49.09 ± 4.64 ‡

The ratio of VLDL TAG:VLDL apoB reflects the the proportion of TAG being carried by apoB. Results in *Table 3.5b* indicate that neither supplement altered this ratio. In addition there was no effect on VLDL Chol:VLDL TAG indicating that the relative proportion of each component was unchanged.

In the fasting state the majority of TAG are of endogenous, or hepatic origin and are transported as VLDL. In the postprandial state most TAG are of dietary origin, and are transported as chylomicrons. In T2DM, chylomicrons may be present in plasma following an overnight fast due to dyslipidaemia, and therefore VLDL is sometimes referred to as TAG-rich lipoprotein (TRL). The lipoprotein fractions which contain only a small amount of TAG i.e. HDL and LDL are referred to as TAG-poor lipoprotein (TPL). Excessive enrichment of HDL and LDL with TAG and subsequent hydrolysis by hepatic lipase leads to the formation of small, dense LDL and HDL particles, which are associated with increased risk of CVD (Betteridge, 2000). Neither TPL-TAG nor TPL-cholesterol concentrations were significantly altered by the CLA or placebo supplements *(Table 3.5b)*.

In addition plasma TAG:HDL-cholesterol and total cholesterol:HDl-cholesterol have been proposed as indicators of CVD risk. The percentage of individuals having small, dense LDL is increased by at least 2 fold in T2DM, even in the absence of dyslipidaemia (Siegel *et al*, 1996). Consequently, Boizel *et al* (2000) proposed the ratio of plasma TAG:HDL-cholesterol as an indicator of LDL particle size in subjects with T2DM and normal HDL cholesterol level. They suggested a ratio > 1.33 to distinguish the small, dense LDL particle. Data from the Framingham study indicates that as the ratio of total cholesterol:Total HDL-cholesterol increases, so does the risk of CHD, conjestive heart failure and intermittent claudication (Kannel, 1987; Kannel, 1994). Total cholesterol:HDL-cholesterol determines the initial risk of CHD events at any serum total or HDL cholesterol, thereby reflecting the importance of the balance of the lipoprotein fractions in serum. In populations with low CHD incidences, average values of total cholesterol/HDL-cholesterol are below 4.0 (Kannel, 1987). An optimum ratio of approximately 3.5 has been proposed (Kannel, 1997). As illustrated in *Table 3.5b*, the values obtained for both these ratio's is below these proposals, indicating that this population is a quite healthy despite a diabetic profile. Neither ratio was significantly altered by either the CLA or placebo supplement.

Table 3.5b: TAG-poor lipoprotein cholesterol and triacylglycerol concentrations and lipoprotein ratios before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. There were no significant differences between the groups at wk 0 or following 8 wk supplementation (ANCOVA or 3-way ANOVA).

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
VLDL TAG: VLDL apo B	0.016 ± 0.001	0.014 ± 0.001	0.018 ± 0.001	0.016 ± 0.001
VLDL Chol:VLDL TAG	0.40 ± 0.02	0.41± 0.19	0.40 ± 0.02	0.43 ± 0.02
TPL triglyceride (mmol)	0.59 ± 0.07	0.55 ± 0.05	0.50 ± 0.03	0.49 ± 0.02
TPL cholesterol (mmol)	4.09± 0.18	4.19 ± 0.23	4.34 ± 0.18	4.50 ± 0.19
Plasma TAG:Total HDL	1.19 ± 0.17	1.11 ± 0.21	1.19 ± 0.13	1.14 ± 0.14
Plasma cholesterol:Total HDL	3.12 ± 0.14	3.12 ± 0.17	3.60 ± 0.20	3.40 ± 0.22

3.3.5 Markers of inflammation and coagulation

The effect of the CLA and control supplements on inflammation and coagulation was also determined, and results are shown in *Table 3.6*. Plasma fibrinogen concentrations were significantly lower after CLA supplementation than after control supplementation ($P<0.01$). Serum IL-6 and plasma CRP concentrations were not altered by either CLA or control supplements.

Table 3.6: Measurements of coagulation and inflammation before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. Significant difference between groups after 8 wk supplementation (ANCOVA) † $P<0.01$.

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
Fibrinogen (mg/dl)	315.11 ± 7.34	317.36 ± 5.56	310.15 ± 5.84	294.30 ± 5.87 †
IL-6 (pg/mL)	3.05 ± 1.64	2.94 ± 1.49	5.18 ± 2.43	6.20 ± 3.86
CRP (mg/L)	2.91 ± 0.74	2.97 ± 0.78	2.94 ± 0.68	2.50 ± 0.72

3.5 DISCUSSION

This study determined the effect of CLA supplementation on several key CVD risk factors in a group of healthy subjects with well-controlled T2DM that was treated by diet therapy alone. In view of the increased risk of CVD associated with diabetes, it is important to investigate the ability of nutritional therapies to attenuate the effect of modifiable risk factors associated with CVD in this high-risk patient group. Glucose tolerance and insulin sensitivity are central to T2DM, and therefore an OGTT was performed, and a number of fasting and postprandial indices were investigated. CLA supplementation did not improve fasting glucose concentrations. HOMA-IR is a common measure of insulin resistance, derived from fasting insulin and glucose concentrations. In our study, CLA supplementation had an adverse effect on insulin resistance, increasing HOMA-IR by 19%. However neither HOMA-2 IR or HOMA-2 % insulin sensitivity were significantly altered following CLA supplementation. HOMA-2 is an updated version of the original HOMA model, as it has been modified following a greater understanding of physiology e.g. insulin secretion, glucose metabolism, renal glucose losses in hyperglycaemic states (Rudenski *et al*, 1988; Rudenski *et al*, 1991; Levy *et al*, 1998). Therefore it should represent a better measure of insulin sensitivity. Postprandial plasma glucose concentration has been cited as an independent risk factor for CVD in persons with T2DM (Bonora & Muggeo, 2001). Hepatic and peripheral insulin sensitivities differ considerably within subjects, and a combination of fasting and postprandial measures provides a more comprehensive measure of insulin sensitivity than does either measure alone (Matsuda & De Fronzo, 1999). Postprandial glucose and insulin concentrations did not increase significantly after CLA supplementation, but the ISI (composite), a measure of insulin sensitivity which is derived from both fasting and postprandial measures, decreased significantly after CLA supplementation. In addition, OGIS, which is also based on both fasting and postprandial data, was significantly reduced by CLA supplementation. It has been proposed that OGIS represents a better index of insulin sensitivity because it has a more physiologic basis than the empirical formulas (Mari *et al*, 2001). Moreover, OGIS strongly correlates with the hyperinsulinemic-euglycaemic glucose clamp measures of insulin sensitivity in diabetic subjects, whereas HOMA-IR and ISI (composite) may not (Mari *et al*, 2001).

This study investigated the effect of a CLA supplement that provides equal proportions of the c9, t11 and t10, c12-CLA isomers, the composition of which reflects most commercially available CLA supplements. It is interesting that this supplement did not adversely affect insulin and glucose metabolism in healthy subjects (Noone *et al*, 2002). Other groups have shown that feeding a blend of CLA isomers induced marked lipodystrophic insulin resistance and glucose intolerance in the C57BL/6J mouse model of diabetes (Tsuboyama-Kasaoka *et al*, 2000). In contrast, another group showed that feeding a 50:50 blend of c9, t11-CLA and

t10, c12-CLA reduced glucose and insulin concentrations in male ZDF rats (Houseknecht *et al*, 1998, Ryder *et al*, 2001). Recent studies reflect the diverse isomer-specific metabolic and molecular effects of CLA. Roche *et al* (2002) reported that feeding a t10, c12-CLA rich diet induced a profound prodiabetic effect, whereas feeding the c9, t11-CLA isomer improved lipid and glucose metabolism in ob/ob mice (Roche *et al*, 2002, Noone *et al*, 2001). Riserus *et al* (2002a) showed that t10, c12-CLA supplementation had a negative effect on insulin resistance in obese men with signs of the metabolic syndrome. The discrepancy between studies clearly reflects that the effect of CLA supplementation is isomer-specific and dependent on the diabetic risk of the experimental model. In the case of subjects with T2DM, a CLA blend containing equal quantities of c9, t11-CLA and t10, c12 CLA does not improve insulin and glucose metabolism.

However it should be noted that the HbA_{1C} concentration did not increase following CLA supplementation. Rather there was a small decrease (4%) in the value. Glycated haemoglobin (GHb) is a measure of integrated glycaemic control in the preceding 2-3 months, with extra weighting for the preceding month i.e. the blood glucose levels in the 1 month before sampling contribute about 50% of the GHb value, whereas blood glucose concentrations 3-4 months previously contributes only about 10% (Pickup, 2003). Since there are wide day-to-day intra-individual variation in fasting blood glucose, HbA_{1C} may represent a better measure of glycaemic control following a treatment intervention. Serum proteins, mostly albumin, are also glycated in diabetes. Ideally therefore fructosamine could be measured as this assesses glycated serum protein, thereby providing an index of glycaemic control over the preceding 2-3 weeks (the lifetime of albumin). The fructosamine test is the generic name for the measurement of all serum glycated proteins, although the bulk is albumin. Since the average half-life of serum proteins is 17 days, fructosamine measures glycaemic control over a shorter period than GHb i.e. approximately 2-3 weeks compared to 2-3 months. Like GHb, it is a weighted measure of preceding control. Although this assay is not typically implemented used in clinical practice, it is a useful when information about control is required over a shorter period (Pickup, 2003) and could help to confirm or refute whether there is true insulin resistance following CLA supplementation. However future studies should preferentially use the euglycaemic clamp if insulin sensitivity is being assessed.

CLA supplementation had a positive effect on HDL-cholesterol concentrations in this study. It has been reported that for each increase of 0.03 mmol/L in HDL-cholesterol concentration, there is a 2-3% reduction in coronary heart disease (CHD) risk (NIH Consensus Statement, 1993). Thus, the increase in HDL-cholesterol after CLA supplementation could represent a 7.3–11% reduction in CHD risk. The increased HDL_2-cholesterol concentrations could promote reverse cholesterol transport (RCT) (Tall *et al*, 2000). Since RCT is impaired in persons with T2DM (Autran *et al*, 2000), the potential ability of CLA to restore RCT would

be favourable. CLA supplementation reduced LDL-cholesterol concentrations by 8.8%, albeit not significantly, but that reduction contributed to a greater reduction (14.5%) in LDL:HDL cholesterol. The Helsinki Heart Study showed that LDL:HDL cholesterol was the single best predictor of cardiac events (Rader *et al*, 2003). Therefore, the effect of CLA supplementation on LDL:HDL cholesterol in T2DM may be of clinical benefit.

The lack of effect on CLA on TAG metabolism contrasts with Noone *et al* (2002). It is possible that this patient cohort may be resistant to CLA induced improvements in TAG metabolism, an effect that may be linked to the adverse effects of CLA on glucose and insulin metabolism. VLDL apoB concentrations increased after CLA supplementation. Smedman & Vessby (2001) also showed an increase in plasma apoB concentrations after CLA supplementation. Increased intrahepatic lipid substrate delivery (TAG and cholesterol) is a critical factor regulating hepatic VLDL apoB secretion. However, neither TAG nor cholesterol concentrations were significantly altered after CLA supplementation. Increased VLDL apoB concentrations are associated with insulin resistance (Ginsberg *et al*, 2005; Cummings *et al*, 1995). Therefore, the increase in VLDL apoB concentrations in this study may be related to the increase in insulin resistance.

Elevated plasma concentrations of fibrinogen, CRP, and IL-6 are associated with increased risk of CHD and the severity of atherosclerosis (Yudkin *et al*, 1999). Both low-grade inflammation (Das, 2002) and a pro-thrombotic risk profile have been reported in persons with T2DM (Frohlich & Steiner, 2000). CLA supplementation reduced plasma fibrinogen concentrations, which has a key role in coagulation (Norris, 2003). CRP concentrations showed a non-significant reduction (15%) after CLA supplementation. A previous study showed that CLA supplementation had no effect on markers of blood coagulation and platelet function in a small group of healthy women who were given a CLA supplement containing a mixture of 10 CLA isomers for 63 days (Benito *et al*, 2001). This isomeric blend is in contrast to our supplement. CRP concentrations were significantly increased in obese men with signs of the metabolic syndrome (Riserus, *et al*, 2002b). The latter result may reflect the isomer-specific effect of t10, c12-CLA. CRP stimulates tissue factor production which is the main stimulus for initiating coagulation (Yudkin *et al*, 1999). Therefore the reduction in plasma fibrinogen and CRP concentrations observed in this study may indicate that CLA could attenuate thrombosis. This warrants further investigation.

Baseline plasma concentrations of c9, t11-CLA and the percentage increase after CLA supplementation are comparable to those in previous supplementation studies (Noone *et al*, 2002, Jiang *et al*, 1999, Britton *et al*, 1992). In contrast, the t10, c12-CLA isomer was undetectable in most samples and was not affected by CLA supplementation, despite the fact that the supplement provided equal quantities of c9, t11-CLA and t10, c12-CLA. The difficulty in detecting the t10, c12-CLA isomer has been documented previously (Noone *et al*,

2002). Two hypotheses for this observation have been suggested. First, the t10, c12-CLA may be more easily oxidized because of its structure, and this would allow it to bypass a number of rate-limiting steps in the peroxisomal ß-oxidation pathway (Martin *et al*, 2000). Alternatively, Sebedio *et al* (1997) showed that the t10, c12-CLA isomer is metabolised into 20:4 Δ5,8,12,14 and 20:3 Δ8,12,14 via desaturation and elongation pathways. Consequently, t10, c12-CLA may not be efficiently incorporated into plasma lipids.

In conclusion, CLA supplementation did not improve plasma insulin and glucose concentrations in persons with T2DM. Whereas we did show an increase in HDL-cholesterol concentrations and a decrease in fibrinogen concentrations following CLA supplementation, the relative importance of one CVD risk factor over another is unknown. Compliance was ensured, given the significant increase in c9, t11-CLA in total plasma lipid fraction, which was comparable with previously reported data (Noone *et al*, 2002, Jiang *et al*, 1999). Any truly effective dietary strategy would improve CVD risk factors without any negative effect on other components of the metabolic syndrome.

ACKNOWLEDGEMENTS

- Insulin, C-peptide, HbA_1c and microalbumin analysis were completed in the Laboratory of St. James's Hospital. Data preparation and statistical analysis was completed by the author.
- Dr. Toh-Peng Yeow, Metabolic Unit, Department of Endocrinology, St James's Hospital assisted with the clinical investigations.
- Anne Mullen, Nutrigenomics Research Group, Institute of Molecular, Trinity College Dublin conducted the IL-6 ELISA.

3.6 APPENDICES

3.6.1 Appendix I: Dietary assessment information

Table 3.7: Mean daily nutrient intakes estimated, using a 4-day food diary, before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. There were no significant differences between the groups at baseline or following 8 wk supplementation (ANCOVA or 3-way ANOVA).

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
Energy (MJ)	8.11 ± 0.53	8.02 ± 0.60	8.38 ± 0.55	8.25 ± 0.62
Protein (g)	98.3 ± 6.6	92.1 ± 7.2	102.4 ± 4.8	99.9 ± 5.8
% total energy	20.7 ± 0.8	19.8 ± 1.0	21.3 ± 1.0	21.3 ± 1.2
% food energy	21.91 ± 0.79	21.27 ± 1.21	22.95 ± 1.07	23.46 ± 1.22
Carbohydrate (g)	225.7 ± 10.2	219.0 ± 11.6	223.7 ± 16.2	226.4 ± 14.9
% total energy	48.3 ± 1.9	47.8 ± 2.1	45.2 ± 1.7	47.3 ± 1.7
% food energy	50.98 ± 1.51	50.86 ± 1.78	48.7 ± 1.43	51.81 ± 1.25
Total Fat (g)	62.9 ± 5.6	63.83 ± 9.0	65.0 ± 5.5	57.2 ± 6.0
% total energy	28.9 ± 1.4	29.7 ± 2.4	29.5 ± 1.6	25.8 ± 1.3
% food energy	30.68 ± 1.47	31.34 ± 2.32	31.68 ± 1.45	28.31 ± 1.21
Alcohol (g)	17.9 ± 7.4	20.6 ± 9.3	22.8 ± 7.4	28.6 ± 8.3
% total energy	5.5 ± 1.9	6.0 ± 2.4	7.1 ± 2.0	8.8 ± 2.2
Saturated Fat (g)	20.3 ± 2.1	20.6 ± 3.2	22.2 ± 2.4	17.5 ± 1.8
% total energy	9.31 ± 0.66	9.54 ± 0.95	10.01 ± 0.85	7.89 ± 0.42
% food energy	9.89 ± 0.69	10.16 ± 0.97	10.74 ± 0.85	8.70 ± 0.47
Monounsaturated Fat (g)	18.5 ± 2.2	19.0 ± 3.9	19.1 ± 2.1	15.8 ± 1.8
% total energy	8.32 ± 0.58	8.60 ± 1.14	8.63 ± 0.78	7.14 ± 0.47
% food energy	8.95 ± 0.75	9.12 ± 1.16	9.23 ± 0.74	7.87 ± 0.50
Polyunsaturated Fat (g)	10.3 ± 1.0	10.1 ± 1.3	11.3 ± 1.2	11.4 ± 2.0
% total energy	4.76 ± 0.35	4.76 ± 0.43	5.18 ± 0.42	5.02 ± 0.64
% food energy	5.03 ± 0.34	4.99 ± 0.40	5.55 ± 0.43	5.50 ± 0.67
Sugars (g)	74.6 ± 5.5	71.3 ± 5.8	80.2 ± 10.2	81.2 ± 8.3
Starch (g)	150.0 ± 9.0	146.8 ± 8.5	142.4 ± 9.7	143.8 ± 9.9
Dietary Fibre (g)	21.9 ± 1.4	20.7 ± 1.4	23.7 ± 2.3	23.5 ± 1.5
Cholesterol (mg)	265.4 ± 36.5	246.3 ± 27.7	287.3 ± 30.7	259.7 ± 19.7

3.6.2 Appendix II: Detailed description of the methodology employed to record the anthropometric measurements

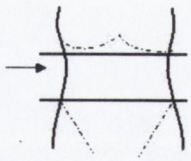
All anthropometric measures were taken with subjects wearing light clothing. Shoes and belts were removed, and pockets emptied prior to any measurements being performed. In addition, all subjects were asked to clear their bladder. To minimise variability, all measures were taken by the same investigator throughout the study.

I. **Body weight** was measured on an electronic balance recording to the nearest 0.1 kg, after a subject held his breath shortly after normal expiration. Two measurements were performed, with the mean value recorded as body weight.

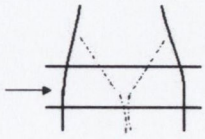
II. **Height** was assessed using a wall-mounted stadiometer measuring to the nearest 0.1 cm. Subjects were asked to stand upright with their heels, buttocks, and occiput touching the backboard of the stadiometer. The measurement was taken with the subjects's head in the Frankfurt plane which is the line passing through the tragion and the lowest point of the eye socket. Subjects were asked to relax their shoulders and inhale. Two measurements were recorded, the mean of which was taken as height.

Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2).

III. **Waist circumference** was measured at the minimum circumference between the iliac crest and the rib cage, as illustrated. Measurements were taken using a non-stretch tape measure, at the end of normal expiration, ensuring the tape was taut, without compressing the skin. All measures were made directly on the skin. Duplicate measures were performed recording the nearest 0.1 cm, and the mean value calculated.



IV. **Hip circumference** was measured at the maximum width over the greater trochanters, without compressing the skin. The measure was recorded to the nearest 0.1 cm.



Waist-to-hip-ratio (WHR) was calculated as waist (cm)/hip (cm) (Taylor *et al*, 1998).

V. **Bioelectric impedance analysis** was measured in the erect position using a body fat analyser TBF-300 (Tanita Corporation, Illinois, USA). Bioelectric impedance measures the opposition of body cells and tissues to the flow of electric current (Ellis *et al*, 1999). Impedance is measured as a voltage drop between electrodes and occurs in fat tissue and bone. In contrast, lean tissues and fluids containing electrolytes conduct the current. An

additional opposition or reactance occurs due to the capacitance of cell membranes, tissue interfaces and non-ionic substances, which is accounted for in the prediction equations (Zarowitz & Pilla, 1989; Ellis *et al*, 1999). Height, weight, age and gender are included in a number of multiple regression relationships to predict body composition compartments such as fat-free mass and lean body mass. A measure of body fat is calculated as the difference between body weight and the derived estimate for fat-free mass (Ellis *et al*, 1999).

Factors known to affect the measurement of bioelectric impedance including hydration status, consumption of food and beverages, ambient air and skin temperature, recent physical activity, and conductance of the examining table (Ellis *et al*, 1999) were minimised where possible. Duplicate measures were taken, and the mean value recorded. Percent body fat was calculated using the manufacturers pre-programmed equations.

3.6.3 Appendix III: Assessment of pancreatic β-cell function

Glucose homeostasis is determined primarily by a fine balance between β-cell secretory function and insulin action in target tissues. The observed reduction in insulin sensitivity in subjects with T2DM following supplementation with CLA may have been a consequence of attenuated insulin secretion from the β-cells of the pancreas, resulting in insufficient insulin to mount the expected physiological response. The IVGTT and the hyperglycaemic clamp represent the gold standard for the measurement of β-cell function, as both first-phase and second-phase insulin secretion may be determined. However, a number of surrogate measures of β-cell function have been proposed derived from data collected during the OGTT. The HOMA index is probably the best known and validated measure of β-cell function, and has been shown to discriminate subjects across the range of glucose tolerances, from normoglycaemia to impaired glucose tolerance to T2DM (Hermans *et al*, 1999). Additionally, the insulinogenic index correlates well with the measure of first-phase insulin secretion obtained after IV glucose infusion, in subjects with NGT or IGT (Vague & Nguten, 2001). Both of the HOMA index of β-cell function and the insulinogenic index were employed to determine whether supplementation with a 50:50 blend of c9, t11-CLA and t10, c12-CLA had any influence on β-cell function.

Table 3.8: Measurements of β-cell function before and after CLA supplementation or placebo for 8 wk. Values represent mean ± SEM. There were no significant differences between the groups at baseline or following 8 wk supplementation (ANCOVA or 3-way ANOVA).

	Control group (n=16)		CLA group (n=16)	
	Wk 0	Wk 8	Wk 0	Wk 8
β -cell function HOMA [1]	58.60 ± 10.36	58.60 ± 11.38	48.62 ± 8.22	47.52 ± 7.80
β -cell function HOMA-2 [2]	57.14 ± 7.81	64.09 ± 8.26	49.99 ± 6.18	47.81 ± 5.96
Insulinogenic index [3]	5.2 ± 1.0	5.22 ± 0.80	4.62 ± 0.87	4.04 ± 0.83

[1] calculated as [20 x (fasting insulin/fasting glucose - 3.5)] (Albareda *et al*, 2000).

[2] calculated from the updated computer model, available as a computer model (see Wallace *et al*, 2004).

[3] calculated as [(insulin 30 min - fasting insulin)/(glucose 30 min - fasting glucose)] (Albareda *et al*, 2000).

There were no significant differences between the groups at baseline or following 8 wk supplementation. This suggests that the insulin resistance was a consequence of an impaired response of target tissues to insulin rather that a reduction in insulin secretion from the pancreas. Ideally this result should be confirmed using one of the gold standard methods for the assessment of β-cell secretion.

Chapter Four

Anti-diabetic effects of *cis*-9, *trans*-11 conjugated linoleic acid may be mediated via anti-inflammatory effects in adipose tissue

4.1 ABSTRACT

Background: Some animal studies have suggested that conjugated linoleic acid (CLA) supplementation may have therapeutic potential with respect to insulin sensitivity and lipid metabolism. Evidence has emerged that the *trans*-10, *cis*-12 CLA (t10, c12-CLA) isomer is pro-diabetic. However there is little information regarding the metabolic or molecular effects of the *cis*-9, *trans*-11 CLA (c9, t11-CLA) isomer.

Objective: To determine the *in vivo* isomer specific effects of c9, t11-CLA on metabolic and molecular markers of glucose homeostasis, focusing in particular on altered adipose tissue biology, by investigating the effect of the isomer on the insulin signalling pathway, adipokine expression and transcription factors known to regulate them.

Design: Sixteen 6-wk old male ob/ob mice were randomly allocated to receive a high-fat diet supplying 30% energy supplemented with c9, t11-CLA, t10, c12-CLA or linoleic acid for 6 wk. All investigations were completed in the fasting state. Plasma was collected for biochemical analysis. Tissue samples were collected for gene expression and protein analysis.

Results: Feeding a diet enriched with c9, t11-CLA significantly reduced fasting glucose ($P<0.05$), insulin ($P<0.05$) and triacylglycerol concentrations ($P<0.01$). Insulin receptor substrate-1, glucose transporter (Glut)-1 and Glut4 mRNA, key targets involved in glucose uptake in adipose tissue, were increased by 1.4-, 1.4- and 1.6-fold respectively, although statistical significance was not attained. Protein expression of the insulin receptor increased by 23% ($P<0.05$), while the expression of Glut4 at the plasma membrane increased by 17% compared to the control diet ($P<0.05$). The mRNA expression of several inflammatory mediators was decreased, in particular tumour necrosis factor (TNF)-α ($P<0.05$). This was associated with a down-regulation in nuclear factor-κB (NF- κB) p65 expression in adipose tissue ($P<0.01$) and NF-κB DNA binding ($P<0.01$).

Conclusions: This study demonstrated a favourable effect of c9, t11-CLA on insulin sensitivity and dyslipidaemia in ob/ob mice. These metabolic effects may be ascribed to a reduction in inflammatory mediators, in particular TNFα. Increased expression of peroxisome proliferator activated receptor γ and liver X receptor and a reduction in the NF-κB complex

may offer insight into a potential mechanism for the insulin sensitising effect. Nevertheless, the isomer specific effect of CLA supplementation on insulin sensitivity and lipoprotein metabolism in man requires investigation.

4.2 INTRODUCTION

Insulin resistance is a fundamental defect in type 2 diabetes mellitus (T2DM) and the metabolic syndrome, and aberrations in the insulin signalling pathways underpin this resistance. Circulating insulin rapidly reaches its target where it interacts with its receptor. Under normal conditions, insulin binds to the extracellular α subunits of the insulin receptor which leads to intracellular autophosphorylation of the β subunits of the receptor (Patti & Kahn, 1998). Subsequent tyrosine phosphorylation of the insulin receptor substrate (IRS) family of proteins leads to the phosphorylation and activation of the phosphoinositide-dependent protein kinase, which phosphorylates and activates downstream serine/threonine kinases, protein kinase C (PKC) and protein kinase B (Saltiel & Kahn, 2001; Lizcano & Alessi, 2002; Brown & McIntosh, 2003). This cascade results in the translocation of the insulin-stimulated glucose transporter (Glut)-4, to the plasma membrane where it facilitates intracellular flux of glucose, thereby reducing circulating glucose concentrations (Brown & McIntosh, 2003)

Since skeletal muscle accounts for up to 85% of glucose uptake and adipose tissue accounts for less than 10%, it was originally thought that adipose tissue did not play a physiologically significant role in maintaining glucose homeostasis (DeFronzo *et al,* 1981). However, tissue specific gene targeting of Glut4 provided an insight into the importance of adipose tissue in maintaining whole-body insulin sensitivity. Interestingly adipose tissue specific ablation of Glut4 caused a similar degree of insulin resistance as muscle specific Glut4 ablation. Glucose uptake was impaired in skeletal muscle despite preserved Glut4 expression. In addition, insulin-stimulated suppression of hepatic glucose production was also impaired (Abel, 2001; Minokoshi *et al*, 2003). It was postulated that an adipokine, or lack thereof, was a causative factor for the insulin resistance in muscle and liver as basal and insulin-stimulated glucose uptake in muscle were normal from these mice *ex vivo* (Minokoshi *et al*, 2003).

It is now well established that that adipose tissue is far more than a lipid-storing organ (Trayhurn & Wood, 2004; Hauner, 2005). White adipose tissue is a secretory organ with potent autocrine, paracrine and endocrine functions, allowing it to maintain a complex communication with many other organs, and modulate a variety of biological and physiological processes, including insulin sensitivity (Berg *et al*, 2002; Hauner, 2005). As part of the myriad of secretory products, a significant body of research suggests that adipose tissue synthesises and secretes a number of insulin de-sensitising inflammatory molecules that predispose to insulin resistance (Weisberg *et al,* 2003; Xu *et al*, 2003). Increased expression of tumour necrosis factor (TNF)-α, interleukin (IL)-6 and resistin are associated with obesity and insulin resistance states (Hotamisligil *et al*, 1993; Hauner, 2005; Ridker *et al*, 2000; Steppan *et al*, 2001). In contrast, adiponectin which is known to have insulin sensitising

effects is reduced in T2DM and with increasing adiposity, especially in the visceral depot (Cancello *et al*, 2004; Hauner, 2005). Thus, altered expression and activity of adipokines may predispose to insulin resistance and consequently other components of the metabolic syndrome (Doria, 2003).

Regulation of metabolic pathways involves the rapid modulation of the activity of specific proteins including enzymes and transporters but also, on a longer-term basis, changes in the quantity of the protein. This can be achieved by modulating their transcription rate through the action of specific transcription factors (Ferre, 2004). A number of transcription factors have been identified which modulate insulin sensitivity by controlling the expression of genes which directly or indirectly influence insulin sensitivity. The key transcription factors related to insulin sensitivity are peroxisome proliferator activated receptor (PPAR)-γ, PPARα, liver X receptor (LXR) and nuclear factor-κB (NF-κB).

The thiazolidinediones (TZDs) are high affinity pharmacological ligands for PPARγ (Sewter & Vidal-Puig, 2002). They markedly improve whole body insulin sensitivity by increasing the expression of Glut4 and adiponectin (Young *et al*, 1995;Combs *et al*, 2002). PPARγ activation via TZD's also sequesters non-esterified fatty acids (NEFAs) from the circulation, thereby reducing the deleterious effects on muscle, hepatic and pancreatic insulin sensitivity (Sewter & Vidal-Puig, 2002; Jiang *et al*, 1998a). TZDs may also reduce the risk of atherosclerosis by increasing HDL-cholesterol, decreasing the intima and media thickness in carotid arteries, and inhibiting the production of several pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 (Minamikawa *et al*, 1998). PPARα ligands induce the expression of the fatty acid transporters and enzymes, which contribute to fatty acid catabolism, thereby lowering triacylglycerol (TAG) concentrations (Moller & Berger, 2003; Choi *et al*, 2004). PPARα ligands may indirectly exert insulin-sensitising effects by reducing hepatic or muscle lipid content (Moller & Berger, 2003). LXR was originally reported to modulate cholesterol and fatty acid metabolism (DeBose-Boyd *et al*, 2001; Joseph *et al*, 2002; Schultz *et al*, 2000). However recent reports indicate that activation of LXR in the liver leads to the suppression of key gluconeogenic enzymes and the induction of glucokinase which promotes hepatic glucose utilisation (Laffitte *et al*, 2003). In adipose tissue, activation of LXR inhibits glycolysis, and LXRα activation in particular, enhances transcriptional induction of Glut4 (Dalen *et al*, 2003; Laffitte *et al*, 2003; Steffensen & Gustafsson, 2004). LXR also induces the expression of sterol regulatory element binding protein (SREPB)-1c, a membrane-bound transcription factors which activate genes involved in lipogenesis (Clarke, 2001). In contrast activation of NK-κB induces insulin resistance by up-regulating the production of a host of inflammatory markers and mediators, including C-reactive protein, plasminogen activated inhibitor (PAI)-1, IL-6, TNFα and IL-1β. These proteins exert paracrine and systemic effects on key organs which regulate glucose homeostasis, and consequently reduce insulin sensitivity (Cai *et al*, 2005).

Systemic inhibition of inhibitor of κB kinase (IKK)-β attenuates NK-κB activity, and has been shown to improve insulin sensitivity in rodents and to improve glycaemia, insulin sensitivity and hyperlipidaemia in subjects with T2DM (Yuan *et al*, 2001; Hundal *et al*, 2002).

Evidence to date has indicated divergent effects of conjugated linoleic acid (CLA) on glucose and insulin metabolism. However, it is becoming increasingly obvious that the physiological effects of CLA on risk factors relevant to the metabolic syndrome are isomer specific. Initial reports demonstrated enhanced insulin sensitivity and glucose tolerance in Zucker Diabetic fa/fa rats following a CLA enriched diet providing equal quantities of *cis*-9, *trans*-11 CLA (c9, t11-CLA) and *trans*-10, *cis*-12 CLA (t10, c12-CLA) (designated a 50:50 blend) (Houseknecht *et al*, 1998; Ryder *et al*, 2001). In contrast, a 50:50 blend induced insulin resistance and hyperlipidaemia in C57BL/6J mice (Tsuboyama-Kasaoka *et al*, 2000). More recently both animal and human studies have indicated that the pro-diabetic effects of CLA may be attributable to the t10, c12-CLA isomer (Roche *et al*, 2002; Riserus *et al*, 2002a). However there is a paucity of information regarding the effects of c9, t11-CLA on metabolic and molecular markers of insulin sensitivity. The objective of the present study was to focus on the potential anti-diabetic actions of the c9, t11-CLA isomer *in vivo*. A previous study in our group showed that feeding a c9, t11-CLA enriched diet to ob/ob mice had no effect on glucose or insulin metabolism, despite improvements in both TAG and NEFA concentrations (Roche *et al*, 2002). This current study differs as investigations were completed in the fasted state as opposed to the fed state as was the case in the previous study. In addition a further objective of the present study was to investigate whether there were alterations in molecular markers of insulin sensitivity and inflammation in adipose tissue of ob/ob mice, the model chosen for the study.

Ob/ob mice are characterised by marked obesity, hyperphagia, transient hyperglycaemia and markedly elevated plasma insulin concentrations associated with pancreatic β-cell hypertrophy and hyperplasia. Hepatic glucose production is excessive (Bray & York, 1979; Herberg & Coleman, 1977). In addition hepatic lipogenesis is increased, which contributes to hepatic steatosis (Yahagi *et al*, 2004). This phenotype is a consequence of an inherited deficiency of the appetite-suppressing hormone, leptin. The ob/ob model therefore represents a suitable model for obesity, insulin resistance and related syndromes. The *in vivo* model is preferential to a cell culture system as whole body glucose tolerance and insulin sensitivity may be investigated. Since a number of organs, primarily adipose tissue, skeletal muscle, liver and pancreatic β-cells, are involved in maintaining glucose homeostatis, the *in vivo* model is more physiologically relevant.

A limited number of studies have examined the *in vivo* effect of CLA on inflammatory mediators in murine models of insulin resistance or T2DM. In these studies the serum concentrations and mRNA expression of TNFα was investigated, as it is well established that

TNFα secretion from adipose tissue plays a crucial role in insulin resistance in T2DM (Hotamisligil *et al*, 1994). Both Tsuboyama-Kasaoka *et al* (2000) and Ohashi *et al* (2004) reported that the TNFα messenger ribonucleic acid (mRNA) was increased in murine white adipose tissue following a CLA-enriched diet providing a 50:50 blend of c9, t11-CLA and t10, c12-CLA. In both studies insulin sensitivity was attenuated, and there was an excessive accumulation of TAG in the liver. Choi *et al* (2004) reported raised serum TNFα concentrations in Sprague-Dawley rats following a t10, c12-CLA enriched diet, however it was not associated with increased insulin resistance or raised serum glucose levels. Wargent *et al* (2005) reported that feeding a CLA enriched diet (50:50 blend) for 10 wk had no effect on plasma TNFα concentration in female ob/ob mice. In contrast Roche *et al* (2002) reported that TNFα mRNA levels were decreased in white adipose tissue following feeding a c9, t11-CLA enriched diet to male ob/ob mice; no alteration was observed in insulin sensitivity although TAG and NEFA concentrations were reduced. The ability of CLA consumption to modulate the expression of other cytokines which have been shown to influence insulin sensitivity e.g. IL-6, has not been investigated within the context of an insulin resistant milieu. In addition, there is little information regarding the effect of CLA on the insulin signalling pathway, adipokines, or transcription factors known to regulate them. An investigation of these parameters is a key focus of this current study.

4.3 RESEARCH DESIGN AND METHODS

4.3.1 Animal experiment and intervention details

This study was conducted at the BioResources Unit at Trinity College, Dublin, Ireland according to good animal welfare protocols that comply with European legislation governing the use of animals in research. The mice were exposed to a 12 hr light/12 hr dark cycle and maintained at a constant temperature of 22°C. Sixteen 6-wk old male ob/ob mice, purchased from Harlan UK were randomly assigned to receive a high-fat diet enriched with c9, t11-CLA, t10, c12-CLA or linoleic acid for 6 wk. Feed consumption and mouse weight were measured weekly. The diets were prepared by Unilever Ltd (Vlaardingen, Netherlands) as previously described (Roche *et al*, 2002). Each diet contained 131g fat/kg (30% energy) and provided equal amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The PUFA fraction of the control diet contained linoleic acid as free fatty acids and the CLA-enriched diets contained an equivalent amount of the CLA isomers as free fatty acids *(Table 4.1)*. The CLA was supplied by Looders Croklaan (Wormerveer, Netherlands). In this study the investigations were completed in the fasted state. Food was removed at 6pm and the animals were sacrificed the following morning between 8-10 am. The mice were euthanised and blood collected by cardiac puncture. White adipose tissue and liver were also harvested.

Table 4.1: The fatty acid composition (w/w%) of the control (linoleic acid), *cis*-9 *trans*-11 CLA (c9, t11-CLA) and *trans*-10 *cis*-12 CLA (t10, c12-CLA) enriched diets.

	Control (linoleic acid)	c9, t11-CLA	t10, c12-CLA
SFA	40.4	39.0	40.4
MUFA	32.6	34.1	32.6
PUFA	26.7	26.6	26.7
Free fatty acid composition of the PUFA fraction			
Linoleic acid (18:2n-6)	6.65	0.27	0.32
Cis-9, *trans*-11 CLA	-	4.43	1.03
Trans-10, *cis*-12 CLA	-	0.84	4.59
Other CLA isomers	-	0.11	0.30

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

4.3.2 Sample preparation and handling

Blood was centrifuged, serum was harvested and stored at -20°C. Serum glucose (Glucose PAP, BioMerieux SA, Lyon, France), TAG (TAG PAP, BioMerieux SA, Lyon, France), cholesterol (Cholesterol PAP, BioMerieux SA, Lyon, France) and NEFA (NEFA, Randox Laboratories, Antrim, Ireland) concentrations were determined using enzymatic colorimetric assays on a Technicon RA-XT analyser (Bayer, Dublin, Ireland) as previously described Noone *et al* (2002). Serum insulin concentrations were measured using a Rat Insulin ELISA kit (Crystal Chem Inc., Chicago, IL, USA) (Roche *et al*, 2002).

Tissue samples for future gene expression analysis were immediately immersed in RNALater (Ambion, Cambridgeshire, UK) and stored at -70°C. Tissue samples for protein analysis were snap frozen in liquid N_2, and stored at -70°C.

4.3.3 mRNA expression analysis

Total RNA was extracted from adipose tissue and liver as described in Section 2.13.1, and quantified spectrophotometrically (Section 2.13.2). The integrity of the RNA was assessed by RNA gel electrophoresis (Section 2.13.3). RNA was deoxyribonuclease (DNase) treated and subsequently first strand complementary deoxyribonucleic acid (cDNA) was synthesised by reverse transcription (Sections 2.14.1 and 2.14.2). Gene expression was quantified by real-time RT-PCR on an ABI 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Warrington, UK) (Section 2.14.3). Custom made oligonucleotide primers and TaqMan probes were designed using Primer express (version 1.0 Perkin-Elmer Applied Biosystems, Warrington, UK). The sequences of these primers and probes are presented in *Table 4.2*. The remaining target genes were investigated using pre-developed primer and probe kits (Perkin-Elmer Applied Biosystems, Warrington, UK). 2X TaqMan® Universal PCR Mastermix (Applied Biosystems, Warrington, UK) was used in the polymerase chain reaction (PCR). After PCR, standard curves were constructed and the fractional cycle number of each of the unknown samples was used to calculate the amount of target gene relative to the standard (Section 2.14). Target gene mRNA expression was normalised to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed relative to the control group i.e. animals fed the diet enriched with linoleic acid.

Table 4.2: Sequences of primers and probes for Taqman® PCR

Target Gene	Oligonucleotides (5' → 3')	Final Conc (nM)
SREBP1a	F: CGGTTTTGAACGACATCGAA	F 500
	R: TCAAATAGGCCAGGGAAGTCA	R 500
	P: ACATGCTTCAGCTCATCAACAACCAAGACA	P 200
SREBP1c	F: GCCATGGATTGCACATTTGA	F 500
	R: TCAAATAGGCCAGGGAAGTCA	R 500
	P: ACATGCTTCAGCTCATCAACAACCAAGACA	P 400
aP2	F: CACCGCAGACGACAGGAAG	F 150
	R: GCACCTGCACCAGGG	R 150
	P: TGAAGAGCATCAAACCCTAGATGGCGG	P 175
PPARγ1	F: AACCACTGATATTCAGGACATTTTTAAA	F 500
	R: AGAGGTCCACAGAGCTGATTCC	R 250
	P: AGAGATGCCATTCTGGCCCACCAACTT	P 200
PPARγ2	F: GCCTATGAGCACTTCACAAG	F 500
	R: ATGTCAAAGGAATGCGAGTG	R 500
	P: CCATCACGGAGAGGTCCACAGAGC	P 250
IRS-2	F: GCGAGTACATCAACATTGACTT	F 250
	R: GACTACTAGCTGAGAGCAGTGA	R 50
	P: ACCCGTCTGTCTCCGCCTGCC	P 175
LPL	F: CCG GGA GAT GGA GAG CAA A	F 500
	R: CGG TCA AAC TCT GGA GCC AA	R 250
	P: CCT GCT CCT GGT GGT CCT GGG A	P 125
FAS	F: TGC GCC CCA CGC A	F 500
	R: GAA TGA TGG TGC AGC TGG ACT	R 500
	P: AGG CTG TCC TGA AGC GAG GCG TG	P 150
UCP-3	F: CCC TGC ACT ACC CAA CCT TG	F 500
	R: GCT TGC CTG GCA ATC TTT TG	R 250
	P: CGC ACA GCT TCC TCC CTG AAC TGA A	P 250
DGAT	F: CGT GGG CGA CGG CTA CT	F 500
	R: GAT AAT TGC TGA AAC CAC TGT CTG A	R 500
	P: CTG AGG TGC CAT CGT CTG CAA GAT TCT	P 175
ABCA1	F: CTA ATG AGG CTT CCT TCT GTC TGT C	F 500
	R: TGA GCC TGA CTT CTG TCG GA	R 500
	P: TGT GTC AAC CTG AAC AAG CTG GAA CCC	P 400
MTP	F: GAT CAT CAT TGG AGC CCT AGT CA	F 500
	R: CAA GTC CTC CCA GGA TCA GC	R 500
	P: CTT CCA CCA CTG CCT TGA GCT TGC AG	P 175

F, R and P indicate forward primer, reverse primer and probe, respectively
Final concentration of forward primer (F), reverse primer (R) and probe (P).

4.3.4 Protein analysis

Different protein fractions were isolated from white adipose tissue using specific lysis buffers as described in Sections 2.15.1 to 2.15.4. All work was done on ice using ice-cold buffers. The plasma membrane fraction was prepared from the pooled fat of two animals according to dietary intervention to gather a sufficient amount of extract. Protein concentrations were determined using the Modified Lowry or BCA assays (Section 2.15.6 and Section 2.15.7), using BSA as a standard. Equal quantities of protein from each sample were acetone precipitated overnight (Section 2.15.8). Samples were used for Western blot analysis and nuclear extracts were also used in the electrophoretic mobility shift assay.

4.3.5 Western blotting

Proteins were separated by electrophoresis through SDS-polyacrylamide gels (Section 2.16.1). The quantity of protein loaded (between 10-250 μg) was determined by protein fraction and the antigen under investigation. Western blotting was performed as outlined in Section 2.16.2. Blots were probed for Glut4 (Chemicon International, Temecula, CA, USA), IRS-1 (Cell Signalling Technology, Beverly, MA, USA), Phospho-IRS-1 (Ser307), (Upstate, Lake Placid, NY, USA), IκBα (C-21, *sc*-371, Santa Cruz Biotechnology, CA, USA) and NF-κB p65 (C20, *sc*-372, Santa Cruz Biotechnology, CA, USA). A detailed protocol outlining the specific steps for each antibody is provided in *Table 2.4a* and *Table 2.4b* in Section 2.16.3. Samples treated with thiazolidinedione (TZD) were included as a positive control in some experiments. An appropriate molecular weight marker was run concurrently to aid protein identification. Immunoreactive bands were visualized using an enhanced chemiluminescence reaction (Supersignal® West Pico Chemiluminescent Substrate, Pierce, IL, USA) (Section 2.16.3).

4.3.6 Electrophoretic mobility shift assay (EMSA)

4 μg white adipose tissue nuclear extracts were incubated with ^{32}P-labelled oligonucleotide probe containing the consensus sequence for NF-κB (Perkin-Elmer Life Sciences, Inc., MA, USA) (Section 2.17.1). DNA-protein complexes were resolved on a nondenaturing 4% (v/v) polyacrylamide gels, gels were dried, exposed to film overnight and developed using an AGFA X-ray processor.

4.3.7 Quantification of protein bands

Quantification of Western blotting and EMSA autoradiographic bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation and Analysis System, Syngene) (Section 2.16.5). Results are expressed as arbitrary units.

4.3.8 Data preparation and statistical analysis

Results are expressed as mean ± SEM for the number of observations indicated. All statistical analysis was performed using Data Desk (version 6.0, Data Description Inc, NY, USA). The distribution of the data for each variable was assessed. When necessary, values were transformed to give a normal Gaussian distribution. 1-way analysis of variance (ANOVA) was employed to investigate differences between treatments. If ANOVA highlighted inter-group differences, post-hoc statistical analysis was performed using the Scheffe test to identify individual differences. A statistical probability of $P<0.05$ was considered statistically significant.

4.4 RESULTS

4.4.1 Biochemical analysis

Table 4.3 presents the effect of the dietary interventions on markers of the metabolic syndrome. Results indicate that feeding a diet enriched with the c9, t11-CLA isomer improved several markers of the metabolic syndrome compared to the linoleic acid enriched diet, whereas the t10, c12-CLA enriched diet had no effect. The c9, t11-CLA diet significantly reduced fasting serum glucose ($P<0.05$), insulin ($P<0.05$) and TAG ($P<0.01$) concentrations, compared to the control diet. In contrast, mice fed a diet enriched with t10, c12-CLA had significantly higher glucose concentrations ($P=0.05$), were more hyperinsulinaemic ($P<0.01$) and hypertriacylglycerolaemic ($P<0.01$) compared to those fed the c9, t11-CLA diet. Neither of the CLA rich diets had a significant effect on serum NEFA or cholesterol concentrations. The mice who received the t10, c12-CLA enriched diet had significantly lower body weight compared to the mice fed the control and c9, t11-CLA diets ($P<0.001$). The change in body weight was largely accounted for by loss of adipose tissue. The epididymal fat pad weight was significantly lower after the t10, c12-CLA enriched diet compared to the control and c9, t11-CLA enriched diets ($P<0.001$). There was no significant difference between the groups in the amount of feed consumed throughout the dietary intervention period (data not shown).

Table 4.3: Effect of the control (linoleic acid), c9, t11-CLA and t10, c12-CLA enriched diets on fasting serum glucose, insulin and lipid concentrations, and body weight. Values represent group means ± SEM. Significantly different to the control diet * $P<0.05$; ** $P<0.01$. Significantly different to c9, t11-CLA diet † $P<0.05$; ‡ $P<0.01$. Significantly different to control and c9, t11-CLA diets § $P<0.001$.

	Control (n = 6)	**c9, t11-CLA** (n = 5)	**t10, c12-CLA** (n = 5)
Glucose (mmol/l)	12.06 ± 0.61	10.76 ± 0.71 *	12.24 ± 0.89 †
Insulin (ng/ml)	1.16 ± 0.32	0.65 ± 0.21 *	1.82 ± 0.42 ‡
TAG (mmol/l)	1.11 ± 0.07	0.95 ± 0.04 **	1.26 ± 0.06 ‡
NEFA (mmol/l)	0.74 ± 0.04	0.65 ± 0.12	0.71 ± 0.10
Cholesterol (mmol/l)	6.46 ± 0.32	6.98 ± 0.17	7.17 ± 0.18
Body weight (g)	47.59±2.08	46.72±1.34	40.48±1.98 §
Epididymal fat pad weight (g)	2.29 ± 0.19	2.27 ± 0.21	1.79 ± 0.19 §

Since the t10, c12-CLA dietary treatment had a profound effect on adipose tissue mass by inducing lipoatrophy, the effect of this fatty acid isomer was not investigated further as any change could be due to gross tissue changes rather than a specific effect of the fatty acid intervention. Furthermore, a number of studies have indicated that t10, c12-CLA exerts a pro-diabetic effect (Roche *et al*, 2002; Riserus *et al*, 2002a)

4.4.2 Gene expression analysis

(i) Molecular markers of insulin sensitivity in white adipose tissue

Adipose tissue is a key organ involved in systemic insulin sensitivity, and therefore the isomer specific effects of c9, t11-CLA on the expression of several genes involved in regulating insulin sensitivity and glucose homeostasis were investigated *(Fig. 4.1a* and *Fig. 4.1b)*. IRS-1 expression was increased (1.4 fold) following the c9, t11-CLA enriched diet, although this change did not reach statistical significance ($P=0.08$). In contrast, the c9, t11-CLA diet marginally lowered the expression of IRS-2 (17% reduction). Glut1 and Glut4 expression were also increased (1.4 fold and 1.6 fold, respectively) but neither reached statistical significance. The most remarkable effect of c9, t11-CLA on adipokine mRNA expression was evident for TNFα which was significantly reduced by almost 50% ($P<0.05$). Other inflammatory markers, IL-6 and monocyte chemoattractant protein (MCP)-1 were also down-regulated by c9, t11-CLA (52% and 31% respectively) but did not reach statistical significance. IL-10, a cytokine with anti-inflammatory properties was up-regulated (1.2 fold) but did not reach statistical significance. The c9, t11-CLA diet had no effect on the expression of macrophage inflammatory protein (MIP)-1α. Adiponectin/Acrp30 mRNA expression was significantly reduced by c9, t11-CLA as compared to the control diet (42% reduction, $P<0.05$). Feeding a c9, t11-CLA enriched diet had no statistically significant effect on resistin mRNA expression despite a 1.42 fold increase in expression.

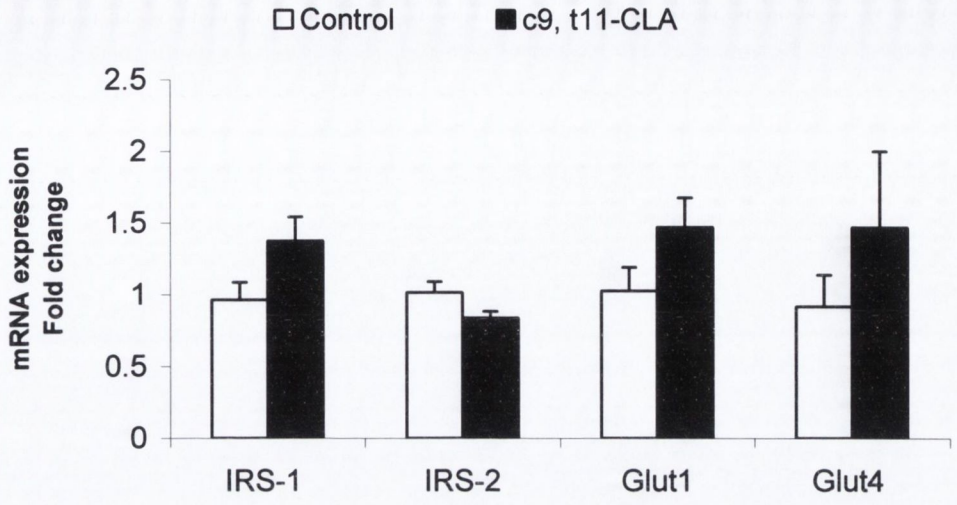


Fig. 4.1a: mRNA expression of key proteins involved in the insulin signalling cascade in white adipose tissue following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. There were no statistical differences detected.

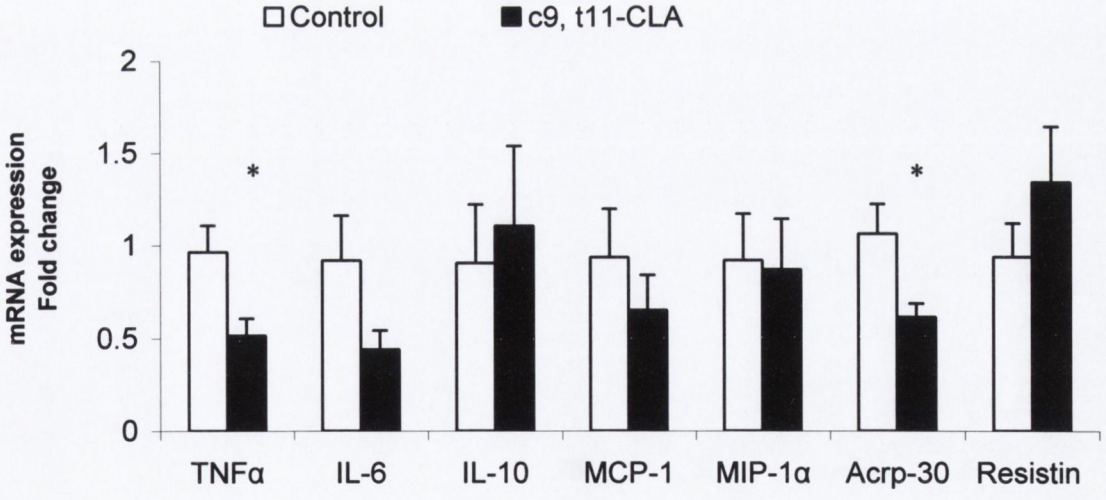


Fig. 4.1b: mRNA expression of adipokines in white adipose tissue following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. Significantly different from the control diet (linoleic acid) * $P<0.05$.

Examination of transcription factors *(Fig. 4.2)* revealed that LXR was significantly increased following the c9, t11-CLA enriched diet (1.85 fold increase, $P<0.05$). SREBP-1c mRNA expression was increased 1.4 fold following c9, t11-CLA ($P=0.055$). White adipose tissue PPARγ2 mRNA expression was increased 1.5 fold, and aP2 mRNA, a PPARγ responsive gene, was increased 1.2 fold following the c9, t11-CLA enriched diet, but neither reached statistical significance. No effect was observed with respect to either PPARγ1 or SREBP1c mRNA.

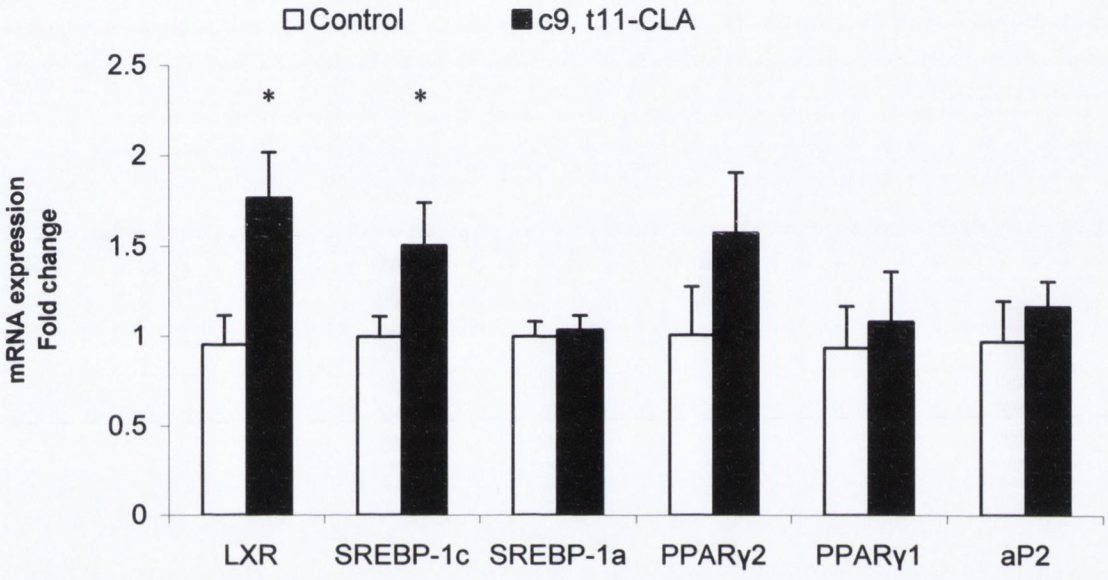


Fig. 4.2: mRNA expression of transcription factors in white adipose tissue which regulate insulin sensitivity and lipid metabolism following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. Significantly different from the control diet (linoleic acid) * $P<0.05$.

(ii) Molecular markers of glucose homeostatis in liver

The liver is a key organ in regulating whole body glucose metabolism, as it is involved in both *de novo* gluconeogenesis and glycogen synthesis. *Fig. 4.3* displays the effect of feeding a c9, t11-CLA enriched diet on the expression of genes involved in regulating glucose homeostatis in liver. IRS-1 mRNA was significantly increased (1.4 fold, $P<0.05$), while the expression of the IRS-2 isoform was increased 1.2 fold, but this did not reach statistical significance. Results show a significant decrease in the expression of glucose-6-phosphatase (G6Pase) following the c9, t11-CLA enriched diet (43% reduction, $P<0.05$). TNFα mRNA expression was significantly increased (1.8 fold, $P<0.05$).

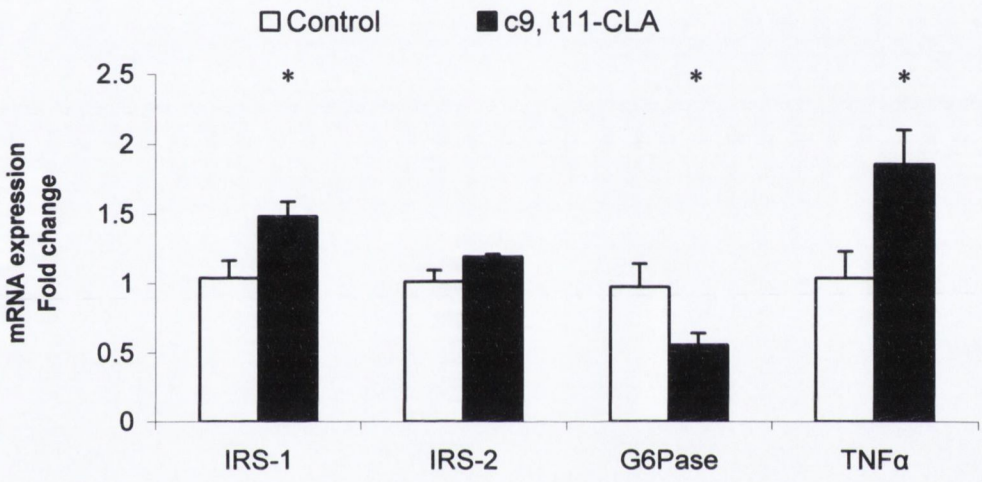


Fig. 4.3: mRNA expression of molecular markers of glucose homeostasis in the liver following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. Significantly different from the control diet (linoleic acid) * $P<0.05$.

Feeding a c9, t11-CLA enriched diet had no effect on the level of expression of the transcription factors LXR, SREBP-1c or PPARα in the liver *(Fig. 4.4)*.

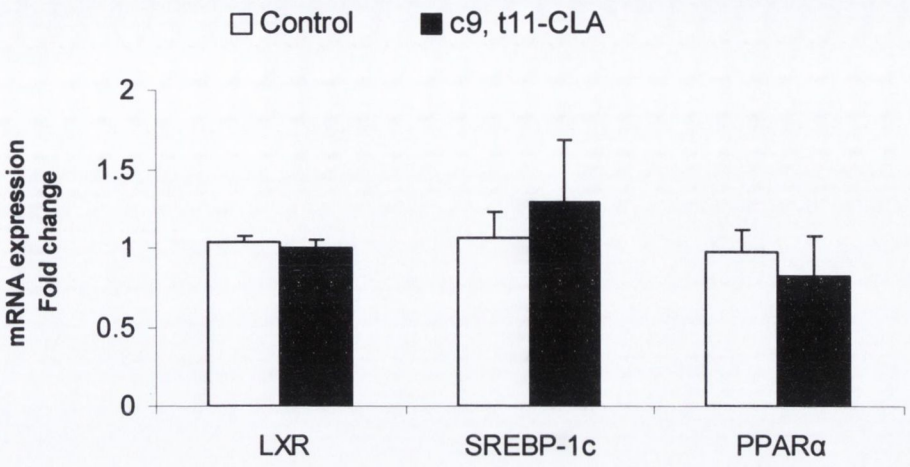


Fig. 4.4: mRNA expression of transcription factors in liver which regulate insulin sensitivity and lipid metabolism following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. There were no statistical differences detected.

(iii) Molecular markers of lipid metabolism in white adipose tissue

A number of genes involved in the regulation of fatty acid metabolism were also investigated *(Fig. 4.5)*. Feeding a c9, t11-CLA enriched diet resulted in a significant reduction in the expression of lipoprotein lipase (LPL) (27% reduction, $P<0.05$). Both fatty acid synthase (FAS) and diacylglycerol acyltransferase (DGAT) were increased (1.5 fold and 1.32 fold, respectively), but neither reached statistical significance. In contrast uncoupling protein (UCP)-3 expression was significantly increased (3.6 fold, $P<0.01$).

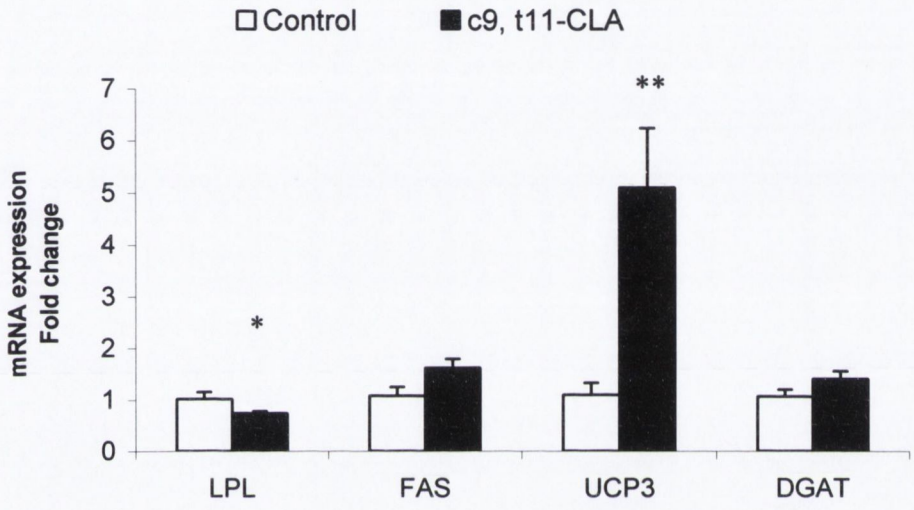


Fig. 4.5: mRNA expression of molecular markers of fatty acid metabolism in white adipose tissue following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. Significantly different from the control diet (linoleic acid) * $P<0.05$, ** $P<0.01$.

(iv) Molecular markers of lipid metabolism in liver

Feeding a c9, t11-CLA enriched diet had a significant impact on several key genes which regulate hepatic lipid metabolism *(Fig. 4.6)*. The expression of adenosine triphosphate-binging cassette (ABC)-A1, was significantly lower following the c9, t11-CLA diet ($P<0.05$ for ABCA1 (60% reduction). In addition, the expression of DGAT, FAS and microsomal triglyceride transfer protein (MTP), key proteins which regulate TAG synthesis, were significantly lower following the c9, t11-CLA diet [$P<0.05$ for DGAT (46% reduction); $P<0.05$ for FAS (21% reduction); $P<0.01$ for MTP (72% reduction)]. LPL mRNA was significantly increased (2.5 fold, $P < 0.01$).

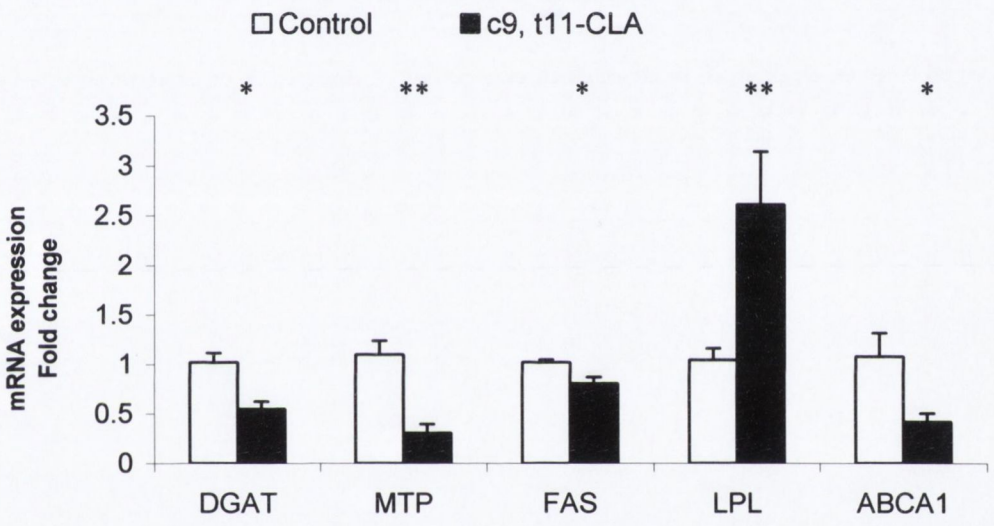


Fig. 4.6: mRNA expression on molecular markers of fatty acid metabolism in liver following a control (linoleic acid) or c9, t11-CLA enriched diet. Target gene mRNA levels were normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=5 in the control group and n=6 in the CLA group. Significantly different from the control diet (linoleic acid) * $P<0.05$, ** $P<0.01$.

4.4.3 Expression of proteins involved in the insulin signalling cascade in white adipose tissue

Key targets involved in glucose uptake into adipose tissue - the insulin receptor, IRS-1 and Glut4, were investigated to determine whether feeding a c9, t11-CLA enriched diet altered the expression of these proteins. *Fig 4.7* shows that feeding a diet enriched with c9, t11-CLA increased the expression of the insulin receptor in the membrane and cytosolic fraction by 23% compared to the control diet ($P<0.05$). In samples treated with TZD which served as a positive control, expression of the insulin receptor increased by 29.5%.

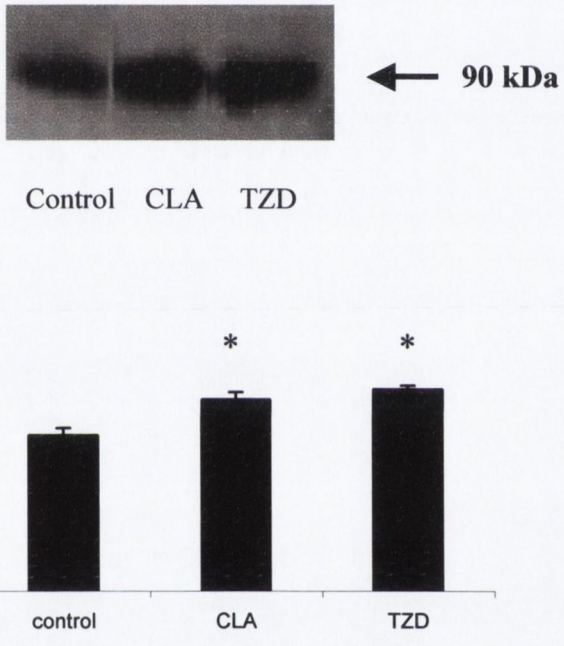


Fig. 4.7: Insulin Receptor expression in membrane and cytosolic extracts from control (linoleic acid), c9, t11-CLA (CLA) dietary treatments and thiazolidinedione (TZD) treated samples (n=5 in the control and CLA groups and n=2 in the TZD group). Representative blots are displayed. Histogram from densitometric analysis expressed as arbitrary units are means ± SEM. Significantly different from control (linoleic acid) * $P<0.05$.

Glut4 expression was measured in the plasma membrane fraction. Results indicate a 17% increase in expression following the c9, t11-CLA enriched diet compared to the control diet ($P<0.05$). In the samples treated with TZD, Glut4 expression increased by 15% *(Fig. 4.8)*.

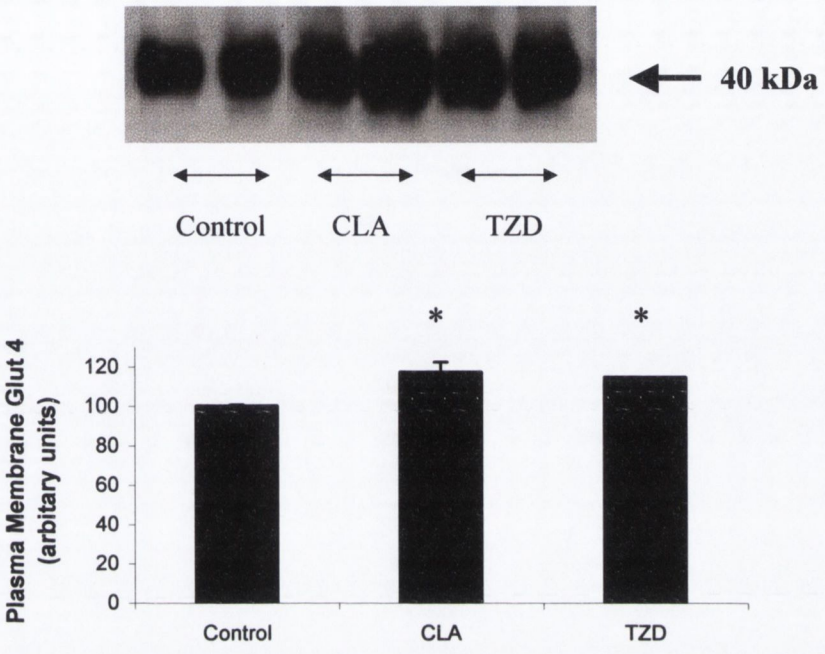


Fig. 4.8: Glut4 expression in plasma membrane extracts (pooled samples) from control (linoleic acid), c9, t11-CLA (CLA) dietary treatments and thiazolidinedione (TZD) treated samples (n=3 for the control and CLA groups and n=2 for the TZD group). Representative blots are displayed. Histogram from densitometric analysis expressed as arbitrary units are means ± SEM. Significantly different from control (linoleic acid) * $P<0.05$.

IRS-1 Serine (Ser)307 is recognised as a molecular marker of insulin resistance. Feeding a c9, t11-CLA enriched diet had no effect on the expression of phosphoIRS-1 (ser307) in the total protein fraction *(Fig. 4.9)*.

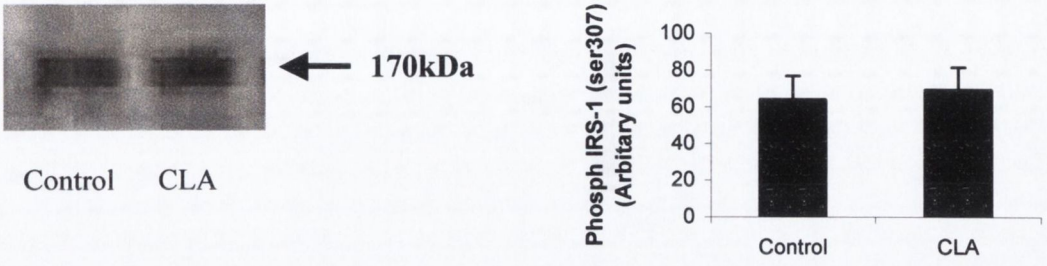


Fig. 4.9: IRS-1 Ser (307) expression in total protein fraction from control (linoleic acid), and c9, t11-CLA (CLA) dietary treatments (n=4 per group). Representative blots are displayed. Histogram from densitometric analysis expressed as arbitrary units are means ± SEM. There were no statistical differences detected.

4.4.4 NF-κB p65 and IκBα expression, and NF-κB:DNA-binding in white adipose tissue

Given the marked reduction in TNFα mRNA expression following the c9, t11-CLA treatment, the effect of the c9, t11-CLA diet on several components of the NF-κB expression complex was investigated *(Fig. 4.10a-d)*. The c9, t11-CLA enriched diet down-regulated nuclear NF-κB p65 expression in adipose tissue (15% reduction, $P<0.01$). Cytosolic NF-κB p65 expression was also decreased (24%) and this tended towards statistical significance ($P=0.0682$). IκBα expression was decreased in both nuclear and cytosolic fractions from white adipose tissue (10% and 9% respectively, $P<0.001$ for both). Furthermore, EMSA showed that the c9, t11-CLA diet reduced white adipose tissue NF-κB DNA binding (23% reduction, $P<0.01$) *(Fig. 4.11)*.

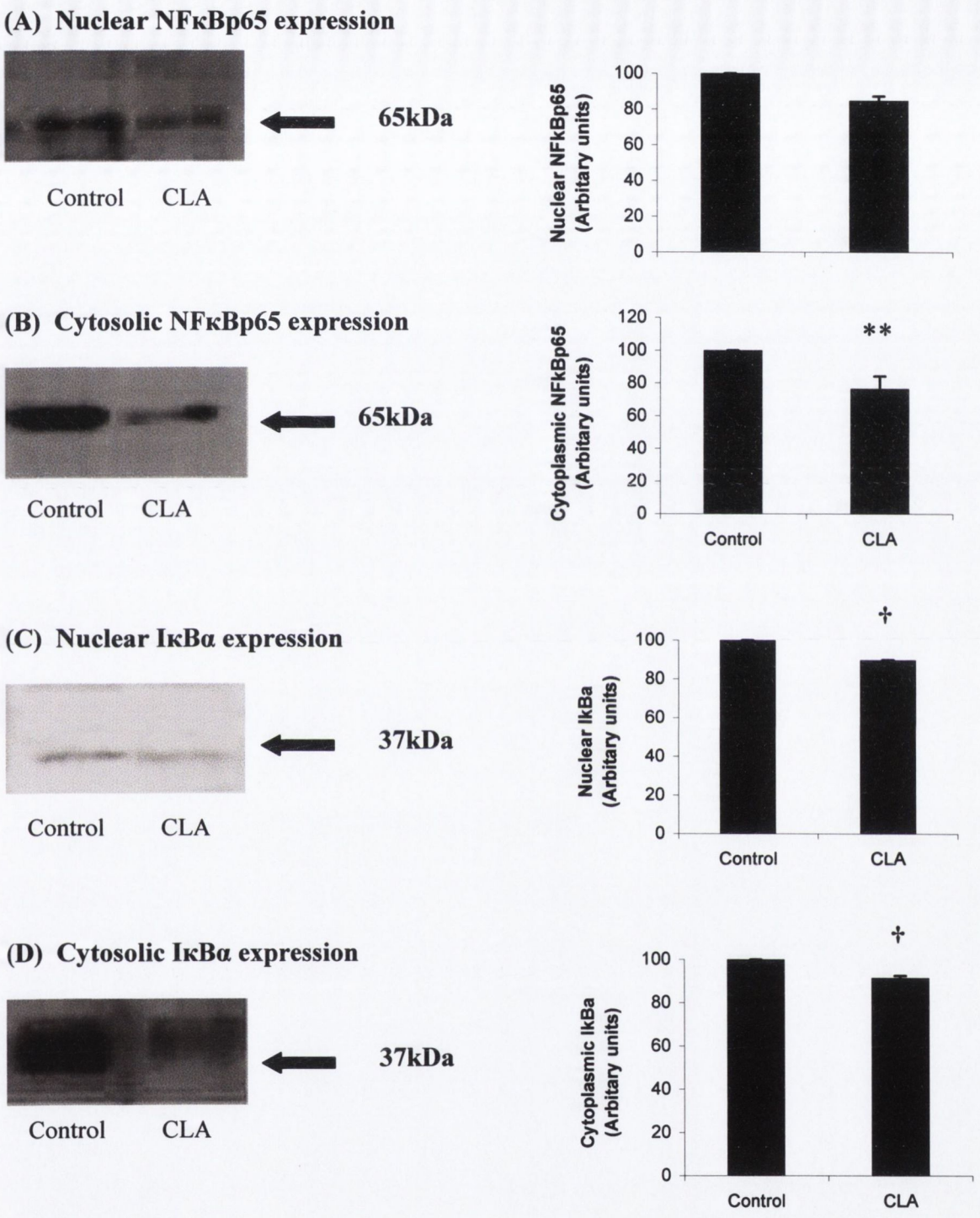
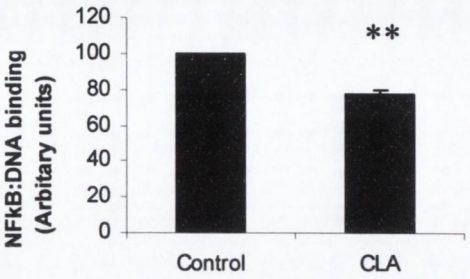


Fig. 4.10: NF-κB p65 and IκBα expression in nuclear and cytosolic extracts from white adipose tissue following a linoleic acid (control) or c9, t11-CLA enriched diet (n=3 per group). (A) Nuclear NF-κB p65 expression, (B) Cytosolic NF-κB p65 expression, (C) Nuclear IκBα expression, (D) Cytosolic IκBα expression. Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. Significantly different from control (linoleic acid) † $P<0.001$, ** $P<0.01$.

NF-κB



Control CLA

Fig. 4.11: Nuclear NF-κB:DNA binding in white adipose tissue following a linoleic acid (control) or c9, t11-CLA enriched diet (n=3 per group). Representative blot is displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. Significantly different from control (linoleic acid) ** $P<0.01$.

4.5 DISCUSSION

This study demonstrated that feeding a c9, t11-CLA enriched diet significantly reduced plasma insulin, glucose and TAG concentrations in the well characterised ob/ob mouse model that displays an obese and diabetic phenotype. Plasma insulin and glucose concentrations were reduced by almost 44% and 11%, respectively. IRS-1, Glut1 and Glut4 mRNA, key targets involved in glucose uptake in adipose tissue were increased by 1.4 fold, 1.4 fold and 1.6 fold, respectively. In addition, key proteins involved in insulin signalling were significantly increased: the expression of the insulin receptor in the membrane and cytosolic fraction was increased by 23%, and the expression of Glut4 at the plasma membrane was increased by 17%, when compared to the control diet. This was comparable with the positive controls, animals treated with the pharmacological agent TZD, whereby the expression of the insulin receptor increased by 29.5% and Glut4 expression at the plasma membrane increased by 15%. This increased expression of the insulin receptor, IRS-1, Glut1 and Glut 4 is likely to promote glucose uptake and storage in cells, thereby contributing to the lower circulating glucose and insulin concentrations observed in the biochemical assays. The improvement in insulin sensitivity following the c9, t11-CLA enriched diet cannot be ascribed to reduced adiposity, as neither body weight or epidymal fat pad mass were different compared to the control group.

One of the primary objectives of the present study was to investigate if altered expression of adipokines might explain the molecular basis of the favourable effect of c9, t11-CLA on insulin sensitivity. This study showed that c9, t11-CLA did not mediate its effect via altered adiponectin or resistin expression. Resistin has been shown to induce insulin resistance. Both gene expression and circulating levels of resistin are markedly increased in obese, insulin resistant mice (Way *et al*, 2001; Le Lay *et al*, 2001). Administration of recombinant protein to wild-type mice impaired glucose tolerance and insulin action, while treatment with antibodies for resistin improved glycaemia and reversed insulin resistance (Steppan *et al*, 2001). In contrast, adiponectin is a potent insulin enhancer and has been found to ameliorate insulin resistance in murine models of obesity and diabetes. Adiponectin promotes insulin sensitivity by decreasing gluconeogenesis in the liver and decreasing lipid accumulation in non-adipose tissues (Guerre-Millo, 2004). The insulin sensitising effect of c9, t11-CLA in this study cannot be ascribed to altered adiponectin or resistin expression, since the attenuation of adiponection (42% reduction, $P<0.05$) and the rise in resistin expression (1.42 fold) would be associated with an increase in insulin resistance.

Some recent studies have investigated the impact of CLA on adipokines. In line with the results of this study, Ohashi *et al* (2004) and Wargent *et al* (2005) reported that feeding a CLA enriched diet (50:50 blend) decreased plasma adiponectin concentrations and expression levels in white adipose tissue, in both normoglycaemic mice or those with a metabolic abnormality (ob/ob, mildly obese and diabetic KK and morbidly obese and diabetic KKAy).

Warren *et al* (2003) identified an isomer specific effect and reported that t10, c12-CLA but not c9, t11-CLA reduced the level of adiponectin mRNA in white adipose tissue of lean C57/BL6N mice. In contrast Nagao *et al* (2003) fed 1% CLA (50:50 blend) to Zucker diabetic (fa/fa) rats and reported elevated plasma adiponectin concentrations and increased expression in white adipose tissue. This was associated with improvements in plasma insulin and glucose concentrations in agreement with other CLA feeding studies in Zucker rats (Houseknecht *et al*, 1998; Ryder *et al*, 2001; Henriksen *et al,* 2003). This may indicate a species difference. Ohashi *et al* (2004) reported that CLA feeding significantly decreased plasma resisitin concentrations in both KK and KKAy mice. However, plasma leptin and adiponectin concentrations were also reduced and an induction in insulin resistance was observed.

It may be ruled out that the improvement in insulin sensitivity was mediated by altered leptin concentrations, since this study was carried out in mice which carry an inherited deficiency of the ob gene. Other studies have reported a reduction in both plasma leptin concentrations and expression levels in white adipose tissue following a CLA enriched diet, which was associated with an attenuation in insulin sensitivity in most cases (Ohashi *et al*, 2004; Clement *et al*, 2002; Rahman *et al*, 2001; DeLany *et al*, 1999; Tsuboyama-Kasaoka *et al*, 2000), although not all (Ryder *et al*, 2001; Faulconnier *et al*, 2004). The reduction in leptin concentration is thought to be associated with the t10, c12-CLA induced reduction in adiposity (Clement *et al*, 2002: Ryder *et al*, 2001). Both Medina *et al* (2000) and Riserus *et al* (2002a) reported decreased leptin concentrations in humans following CLA supplementation, in the latter case this was associated with a reduction in sagittal abdominal diameter and percentage body fat.

Adipokine expression of inflammatory mediators may provide an insight into a potential mechanism of the insulin sensitising effect of c9, t11-CLA. Gene expression analysis in white adipose tissue demonstrated an attenuation in the expression of pro-inflammatory mediators, with approximately 50% reduction in both TNFα and IL-6 mRNA and a 1.2-fold increase in mRNA of the anti-inflammatory cytokine IL-10. In addition, a 30% reduction in MCP-1 expression was observed after the c9, t11-CLA enriched diet. MCP-1 is a key modulator of atherogenesis by inducing chemotaxis of monocytes to the atherosclerotic intima, whereby they differentiate into macrophages and exacerbate the progression of atherosclerosis (Boisvert, 2004; Linton & Fazio, 2003). Adipose tissue comprises not only mature adipocytes but also endothelial cells, sympathetic nerve fibres and poorly-identified cells present in the stromal-vascular fraction, all of which may contribute to adipose tissue function (Hauner, 2005). Several studies have suggested that adipose tissue is the source of pro-inflammatory molecules that mediate obesity-induced insulin resistance. Nevertheless, adipocytes produce relatively lower cytokine levels compared to the classical inflammatory cells. Recent reports demonstrating that obesity is associated with progressive infiltration of monocytes and

macrophages into adipose tissue and that adipose tissue macrophages are the source of inflammatory pathways that are activated within adipose tissue of obese individuals, clearly suggests that the non-adipocyte fraction may mediate a significant component of the inflammatory state within adipose tissue (Trayhurn & Wood, 2004; Weisberg *et al*, 2003; Xu *et al*, 2003). Expression analysis of macrophage and non-macrophage cell populations isolated from adipose tissue indicates that adipose tissue macrophages are responsible for almost all adipose tissue TNFα expression and significant amounts of inducible nitric oxide synthase (iNOS) and IL-6 expression. *Table 4.4* summarises some recent data regarding the cellular origin of factors released from adipose tissue

Table 4.4: Cellular origin of adipokines secreted from human adipose tissue (Hauner, 2005)

	Adipocytes	Stromal Cells	Macrophages
IL-6	+	+	++
TNFα	+	+	++
PAI-1	+	+	?
MCP-1	(+)	+	++
MIP-1α	(+)	+	++
MIF	+	+	++

(+), Data not yet completely clear; +, clear evidence, ++, very strong evidence.
MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α; MIF, macrophage inhibitory factor; PAI-1, plasminogne activator inhibitor-1.

A recent paper by Charriere *et al* (2003) demonstrates the close relationship between the adipocyte and macrophage lineages. Injection of the well-established 3T3-L1 preadipocyte cell line into the peritoneum of nude mice resulted in the induction of a number of highly specific macrophage-surface markers, indicating that these cells effectively trans-differentiate into macrophages *in vivo*. A key question is what actually triggers the inflammatory responses in adipose tissue. Wellen & Hotamisligil (2003) suggest that obesity-induced inflammatory changes in adipose tissue is initiated by adipocytes that secrete low levels of TNF-α, which in turn stimulates pre-adipocyte and endothelial cells to secrete MCP-1, increase adipokine expression and NEFA release to further advance a pro-inflammatory and pro-oxidative state, ultimately leading to impaired adipocyte function and insulin resistance. This is illustrated in *Fig. 4.12*.

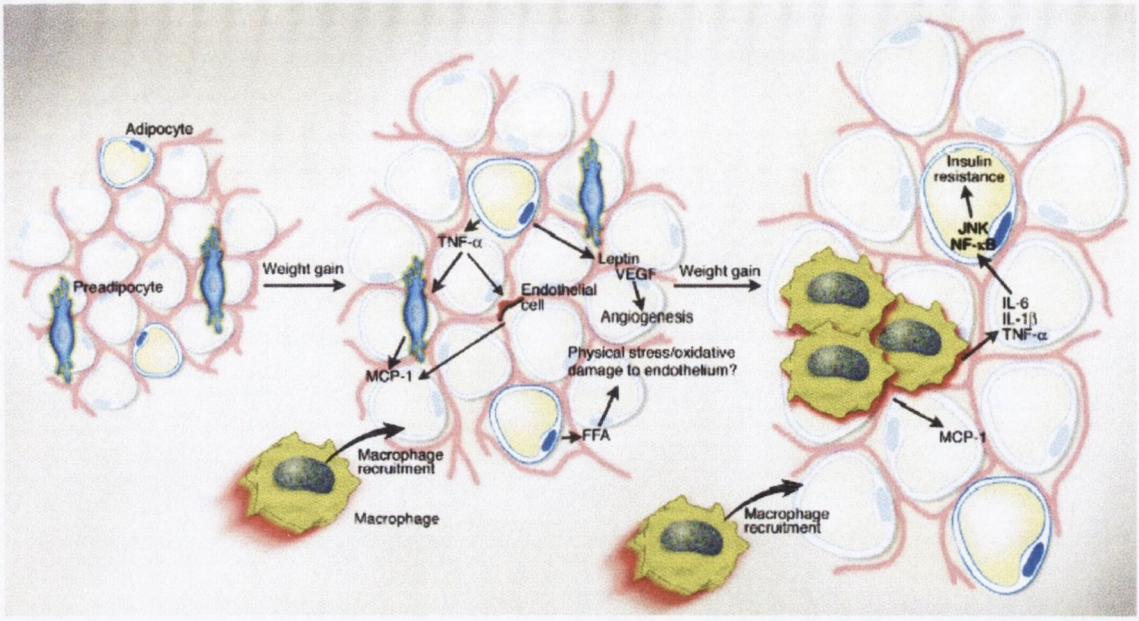


Fig. 4.12: Consequences of macrophage infiltration into adipose tissue (Wellen & Hotamisligil, 2003).

FFA, free fatty acid; IL, interleukin, JNK, c-Jun N-terminal j-kinase; MCP-1, monocyte chemoattractant protein 1; NF-κB, Nuclear factor-κB; TNFα, tumour necrosis facror-α; VEGF, vascular endothelial growth factor.

Adipose tissue TNFα mRNA expression was halved following the c9, t11-CLA diet. The insulin de-sensitising effects of TNFα are well characterised. TNFα increases insulin resistance by inhibiting tyrosine autophosphorylation of insulin receptor, decreasing Glut4 mRNA, altering the expression of other adipokines e.g. increasing IL-6 and PAI-1, decreasing adiponectin, all of which impede insulin signalling (Hotamisligil *et al*, 1994; Stephens & Pekala, 1991; Sonnenberg *et al*, 2004; Guerre-Millo, 2004). TNFα is also known to induce serine phosphorylation of IRS-1 at residue 307, which disrupts the interaction between the insulin receptor and the phosphotyrosine binding domain of IRS-1, thereby inhibiting propagation of the insulin signal (Aguirre *et al*, 2000). Ruan *et al* (2002a) showed that NF-κB is an obligatory mediator of the TNFα induced changes in gene expression that could lead to insulin resistance in 3T3-L1 adipocytes. Blocking NF-κB activation by expressing a dominant inhibitor of NF-κB (IκBα-DN) abolished the suppression of most of the genes normally suppressed by TNFα, and blocked induction of 60-70% of the genes normally induced by TNFα. Under basal conditions, NF-κB is inhibited by inhibitor of κB (IκB) and remains within the cytoplasm. Pro-inflammaory stimuli results in activation of IκB kinase (IKK) which subsequently catalyses the phosphorylation of IκB, leading to its degradation. This liberates NF-κB to translocate into the cell nucleus and stimulate the transcription of

inflammatory mediators including IL-2, IL-6, IL-8, intercellular adhesion molecule, vascular cell adhesion molecule and E-selectin (Baldwin, 1996; Shoelson *et al*, 2003).

Reports from the turn of the last century describe the experimental use of high doses (5-7.5g/day) of salicylates to treat diabetes mellitus (Shoelson *et al*, 2003). However it is only recently that Yuan *et al* (2001) established that high-dose aspirin exerts its insulin sensitising effects through inhibition of the pro-inflammatory pathway IKK/NF-κB. Heterozygous gene deletion ($IKKB^{+/-}$) or salicylates treatment protected against the insulin-resistant effects of lipid infusion in rats (Kim *et al*, 2001). In addition, a 50% decrease in the IKKβ gene in normal and in ob/ob mice led to reductions in blood glucose levels and improved insulin sensitivity (Yuan *et al*, 2001). More specifically salicyclates attenuate phosphorylation of IRS-1 at Ser(307) through reduced IKKβ expression/activation which promotes a positive effect on glucose tolerance (Yuan *et al*, 2001). Other groups have proposed that IKK-β is central to the interplay between dietary fatty acids and insulin resistance (Zick, 2003). Elevated lipids lead to an increase in metabolites such as diacylglycerol and ceramide, which are both potent activators of PKCθ and PKCζ, both known to activate IKKβ.

Hence, it may be possible to reduce the impact of metabolic stressors either by altering dietary fatty acid composition and/or using nutrient based anti-inflammatory strategies to improve insulin sensitivity and impede progression towards the metabolic syndrome and T2DM. The results of this study demonstrate that adipose tissue TNFα mRNA expression was halved following the c9, t11-CLA diet, and this was coupled with important immuno-regulatory changes on several components of the NF-κB expression complex, namely marked down-regulated of NF-κB p65 (in the nuclear fraction) and a non-significant reduction of cytosolic NF-κB p65. Additionally white adipose tissue NF-κB:DNA binding was significantly reduced. However, despite the attenuation in inflammatory stressors, feeding a c9, t11-CLA enriched diet did not reduce the phosphorylation at IRS-1 at Ser(307) contrary to the hypothesis. Elevations in TNFα are known to increase phosphorylation at this site, so a reduction in Ser phosphorylation would be expected with a reduction in TNFα. Nevertheless, it would be worthwhile to investigate the impact of c9, t11-CLA on IRS-1 Ser(307) phosphorylation in the fed state, when insulin stimulates glucose uptake, rather than in the background quiescent state which is currently being investigated.

Other key transcription factors regulating insulin sensitivity were also investigated. Results indicated that the expression of LXR and SREBP-1c in white adipose tissue were significantly increased (1.85 fold and 1.4 fold, respectively) while white adipose tissue PPARγ2 was non-significantly increased (1.5 fold). No effect was observed with respect to either PPARγ1 (15% increase) or SREBP1c mRNA (3.7% increase). The increase in expression of LXR may have contributed to the induction of Glut4 observed. LXR may also

inhibit glycolysis in white adipose tissue although this was not investigated in this study. This is the first study to examine the effect of CLA on the two different isoforms of PPARγ, PPARγ1 and PPARγ2. Although neither reached statistical significance, it is noteworthy that c9, t11-CLA increased the expression of PPARγ2 (1.5 fold) while PPARγ1 was increased by only 15%. PPARγ2 has been shown to be the more potent adipogenic isoform and TZDs can increase adipocyte differentiation through enhanced expression of PPARγ2 (Chawla *et al*, 1994; Sewter & Vidal-Puig, 2002). Both PPARγ2 and SREBP-1c are influenced/regulated more by nutritional state than the alternative isoform (Medina-Gomez *et al*, 2005).

Reports have suggested that CLA represents a natural PPARγ ligand (Moya-Camerena & Belury, 1999; Belury, 2002; Yu *et al*, 2002c). However, conflicting information has emerged regarding the effect of CLA and individual isomers on PPARγ activation and expression levels (Houseknecht *et al*, 1998; Clement *et al*, 2002; Choi *et al*, 2004; Brown & McIntosh, 2003; Takahashi *et al*, 2002; Ryder *et al*, 2001). Additionally although activation of PPARγ is associated with enhanced insulin sensitivity, this was not observed in all studies (Clement *et al*, 2002; Choi *et al*, 2004). Differences cannot be attributed to isomer specific effects. Nevertheless, it is possible that the effect of CLA on PPARγ activity and expression may vary across species (Wargent e*t al*, 2005).

The effect of CLA on hepatic LXR activation has been assessed, whereby t10, c12-CLA and c9, t11-CLA are equally efficient in inhibiting LXR activation following *in vitro* transcription assays in COS-1 cells (Clement *et al*, 2002). However information is lacking regarding the effect of CLA on LXR activation in white adipose tissue and subsequently on influencing insulin sensitivity.

It is important to note that the transcription factors do not work in isolation, but rather can influence the activity of each other. PPARγ agonist, 15-deoxy-$\Delta^{12, 14}$ prostaglandin J_2 has been shown to antagonise the activity of NF-κB, by inhibiting IKK complex activity, thereby preventing IκBκ degradation. It has also been shown to reduce NF-κB binding by alkylating p50/p65 dimers (Delerive *et al*, 2001). Joseph *et al* (2003) suggested that inhibition of the inflammatory mediators iNOS and cyclooxygenase-2 genes by LXR agonists is accomplished through antagonism of NF-κB, downstream of NF-κB:DNA binding. This trans-repression of NF-κB is thought to underlie the anti-inflammatory actions of these nuclear receptors. Interestingly, the expression of both PPARγ and LXR was increased in this study, while nuclear NF-κB p65 and NF-κB DNA binding were markedly down-regulated. However, in order to elucidate whether c9, t11-CLA activates LXR or PPARγ and thereby down-regulates the NF-κB system, cells would need to be transfected with a LXR- or PPARγ-expression vector, luciferase activity quantified and a NF-κB:DNA binding assay performed. The activity of NF-κB target genes could then be investigated to assess for functionality.

Hepatic insulin resistance is an important pathophysiological feature of T2DM and the metabolic syndrome. Insulin signalling in the liver functions to activate glycogen synthesis for energy storage and to suppress hepatic glucose output by inhibiting phosphoenol pyruvate carboxykinase (PEPCK) and G6Pase (Ide *et al*, 2004). Fasting hyperglycaemia in T2DM is believed mainly to be a consequence of an increase in gluconeogenesis (Valverde *et al*, 2003). Therefore an improvement in glucose and insulin homeostasis may also be a consequence of a reduction in hepatic gluconeogenesis. In insulin-resistant and hyperinsulinaemic states, there are usually simultaneous reductions in both IRS-1 and IRS-2 in the liver (Anai *et al*, 1998). Both IRS-1 and IRS-2 expression were increased following the c9, t11-CLA enriched diet. IRS-1 and IRS-2 have complementary roles in the control of hepatic metabolism and can compensate for each other (Taniguchi *et al*, 2005). However recent work has suggested that IRS-1 is more closely linked to glucose homeostasis by regulating the expression of gluconeogenic enzymes, while IRS-2 may be more closely linked to hepatic lipid metabolism, by limiting the upregulation of lipogenic enzymes and preventing hepatic steatosis. In association with the significant increase in hepatic IRS-1 expression a significant decrease was observed in hepatic G6Pase mRNA expression following the CLA-enriched diet. G6Pase catalyses the hydrolysis of the terminal step in the gluconeogenic and glycogenolytic pathways allowing the release of glucose into the bloodstream. TNFα has been shown to suppress G6Pase expression *in vitro* and *in vivo* (Metzger *et al*, 1997). This tissue specific increase in TNFα mRNA following the c9, t11-CLA enriched diet may have contributed to the decrease in G6Pase expression. Further investigation is required to confirm or refute this hypothesis. Additionally other enzymes involved in gluconeogenesis including PEPCK and fructose-1, 6-bisphosphatase, and transcription factors which regulate *de novo* glucose synthesis e.g. Foxo1 and peroxisome proliferator-activated receptor-γ coactivator-1 (Barthel & Schmoll, 2003) could also be investigated.

A dyslipidaemic profile is associated with insulin resistance. Therefore, systemic lipid parameters and key genes which regulate lipid metabolism were investigated. This study showed that feeding the c9, t11-CLA significantly reduced fasting serum TAG concentrations. This isomer specific effect agrees with a previous study wherein c9, t11-CLA significantly reduced postprandial serum TAG concentrations (Roche *et al*, 2002). The reduction in serum TAG concentrations was associated with significant down-regulation of hepatic DGAT, MTP and FAS mRNA expressions, key determinants of hepatic lipogenesis. FAS catalyses the conversion of acetyl-CoA and malonyl CoA to form palmitate (Sul *et al*, 2000). DGAT catalyses the final reaction in the glycerol phosphate pathway, which is considered the main pathway of TAG synthesis in mammals, converting diacylglycerol into TAG (Chen & Farese, 2000). MTP catalyses the assembly of VLDL by promoting the fusion of apolipoprotein B with TAG (Gibbons *et al*, 2004). Therefore, reduced expression of these genes would

contribute to lower fasting TAG concentrations following the c9, t11-CLA diet. PPARγ agonist drugs are associated with fatty liver which is possibly due to an increase in TAG synthesis and/or increase in fatty acid uptake mediated by an increase in hepatic LPL. LPL expression was also significantly increased following the c9, t11-CLA enriched diet. In white adipose tissue LPL hydrolyses TAG-rich lipoproteins resulting in the formation of chylomicron remnants, VLDL remnants, intermediate density lipoproteins and LDL. Fatty acids are used as an energy source in muscle or are stored in adipose tissue as TAG. The reason for the induction in hepatic LPL following the c9, t11-CLA enriched diet is unknown. It is unlikely that hepatic steatosis was induced as plasma TAG concentrations were reduced, important lipogeneic enzymes in the liver were down-regulated and additionally neither body weight nor epidymal fat pad mass changed. ABCA1 mRNA was significantly decreased following the c9, t11-CLA enriched diet. ABCA1 promotes the efflux of cellular cholesterol to lipid-poor ApoA1 and therefore plays a key role in HDL synthesis (Brewer *et al*, 2004). Macrophage and other peripheral tissues also express ABCA1 (Joyce *et al*, 2003). Since plasma HDL concentrations were not investigated in this study it is impossible to determine whether the down-regulation in hepatic ABCA1 exerted a negative consequence on HDL synthesis or whether other tissues exerted a compensatory effect. However, the previous study in subjects with T2DM (Chapter 3) indicated that total HDL- and HDL_2-cholesterol concentrations increased following CLA supplementation (Moloney *et al*, 2004).

The transcription factors PPARα and SREBP-1c regulate hepatic lipoprotein metabolism. Hepatic PPARα and SREBP-1c expressions are nutritionally regulated, such that PPARα level is induced by fasting and suppressed by refeeding, whereas the reverse is true for SREBP-1c. In the fasted state hepatic PPARα induces fatty acids oxidation to form acetyl-CoA and subsequently ketone bodies. In the re-fed state, lipogenesis is induced through an increased in expression of SREPB-1. It has been suggested that PUFA could contribute to this reciprocal energy regulation by PPARα and SREBP-1c through activation of PPARα ligands and direct inhibition of SREBP-1c (Yoshikawa *et al*, 2003). In contrast to a previous study (Roche *et al*, 2002) hepatic SREBP-1c mRNA expression was not reduced by c9, t11-CLA. This apparent discrepancy probably reflects that different nutritional states of the animals in the studies i.e. fasting in the current study, while non-fasting in the former study. LXR a key regulator of SREBP-1c is not subject to nutritional regulation (Yoshikawa *et al*, 2001). Hepatic LXR mRNA was not altered by the c9, t11-CLA enriched diet. Surprisingly PPARα expression was not affected by c9, t11-CLA consumption. It has been suggested that CLA is a more potent ligand PPARα than PPARγ (Clement et al, 2002) with the c9, t11-CLA isomer more efficient at activating PPARα than t10, c12-CLA (Moya-Camarena & Belury, 1999; Clement *et al*, 2002).

Feeding c9, t11-CLA slightly increased the expression of FAS and DGAT in white adipose tissue, although neither reached statistical significance. Ideally other insulin markers of lipid metabolism, including acetyl CoA carboxylase and hormone sensitive lipase could be measured in future experiments. SREPB-1 induces the expression of lipogenic genes and was significantly increased, which probably explains the increase in FAS and DGAT mRNAs. In contrast, LPL mRNA was significantly reduced. Potentially this could lead to a reduction in HDL concentrations due to reduced availability of substrates including phospholipids and apolipoproteins. However, as mentioned previously HDL concentrations were not determined in this study. Studies in rodents have demonstrated that CLA inhibits the progression and promotes the regression of atherosclerosis (Kritchevsky *et al*, 2000, Toomey *et al*, 2003). Nevertheless the literature indicates that CLA exerts differential effects on lipid profiles in animal models, depending on the feeding regimen used and/or the animal model employed (Wilson *et al*, 2000; Khosla & Fungwe, 2001). c9, t11-CLA has been shown to increase HDL-cholesterol, decrease TAG, and increase HDL-cholesterol:LDL-cholesterol, indicating a potential for an attenuation in atherogenesis (Gavino *et al*, 2000; Valeille *et al*, 2004; Akahoshi *et al*, 2003; Roche *et al*, 2002).

It should be noted that in view of the large number of genes measured, further statistical analysis could be performed prior to publication of the data e.g. by completing a Bonferroni adjustment, which allocates the error rate among several hypothesis tests or confidence intervals so that the total error rate is only as large as the specified alpha level. This minimises the possibility of making a Type 1 error i.e. rejecting the null hypothesis when the null hypothesis is true.

This is not the first study to report lipoatrophy following a t10, c12-CLA enriched diet. Atrophic adipose tissue observed after starvation, in cachexia or in lipodystrophy, contain small and poorly developed adipocytes that exert profound alterations in adipose gene expression and adipokine release into serum (Nawrocki & Scherer, 2004). In addition, metabolic abnormalities may develop as a consequence of fatty acid storage in peripheral tissues, which is associated with whole-body insulin resistance (Rajala & Schere, 2003). Tsuboyama-Kasaoka *et al* (2000) reported lipodystrophy, comprising ablation of brown adipose tissue, a marked reduction in white adipose tissue, marked hepatomegaly, and marked insulin resistance, in female C57BL/6J mice fed 1% CLA (50:50 product) as part of a low-fat diet (11% kcal from fat) for 17 wk. Clement *et al* (2002) reported that the t10, c12-CLA isomer was responsible for this phenotype. In addition, several studies have indicated hepatomegaly as a result of fatty liver (DeLany *et al*, 1999; Clement *et al*, 2002; Tsuboyama-Kasaoka *et al*, 2000; Ohashi *et al*, 2004). The anti-obesity effects of CLA are attributed to the t10, c12-CLA isomer and may be ascribed to increased energy expenditure, increased fat oxidation, decreased lipogenesis and a reduction in adipocyte size (Wang & Jones, 2004;

Roche *et al*, 2002). t10, c12-CLA has also been shown to induce apotosis (Evans *et al*, 2000). Consequently, the effect of the t10, c12-CLA isomer was not explored in-depth since any change observed could be due to gross tissue changes rather than a specific effect of the fatty acid intervention. Additionally in view of the negative effects associated with the isomer, it is unlikely to be employed as a therapeutic nutrient.

In conclusion, this study demonstrated a favourable effect of c9, t11-CLA on insulin sensitivity in ob/ob mice. An improvement was also observed in the lipid profile. These metabolic effects may be ascribed to a reduction in inflammatory mediators, in particular TNFα. Increased expression of PPARγ and LXR and a reduction in the NF-κB complex may offer an insight into a potential mechanism for the insulin sensitising effect. Nevertheless, the isomer specific effect of CLA supplementation on insulin sensitivity and lipoprotein metabolism in man requires in-depth investigation.

ACKNOWLEDGEMENTS

- Dr. Enda Noone, Nutrition Laboratory, Department of Clinical Medicine, Trinity College Dublin completed the animal feeding study, in addition to performing the biochemical assays and gene expression analysis regarding lipid metabolism in liver and white adipose tissue.
- Dr. Christine Loscher, Nutrigenomics Research Group, Institute of Molecular Medicine, Trinity College Dublin completed the NF-κB p65 and IκBα Western blot analysis and assisted with the EMSA.
- The primer and probe sequences for RT-PCR were designed either by Dr. Enda Noone (Nutrition Laboratory, Department of Clinical Medicine, Trinity College Dublin), Dr. John Browne (Nutrigenomics Research Group, Institute of Molecular Medicine, Trinity College Dublin) or Dr. Helen Roche (Nutrigenomics Research Group, Institute of Molecular Medicine, Trinity College Dublin).

Chapter Five

The effects of *cis*-9, *trans*-11 conjugated linoleic acid and *trans*-10, *cis*-12 conjugated linoleic acid on insulin sensitivity in normal and insulin resistant 3T3-L1 adipocytes

5.1 ABSTRACT

Background: The different isomers of conjugated linoleic acid (CLA) exert distinct physiological effects. Evidence has emerged that *trans*-10, *cis*-12 CLA (t10, c12-CLA) is pro-diabetic. However, the previous study in ob/ob mice (chapter 4), indicated that feeding a *cis*-9, *trans*-11 CLA (c9, t11-CLA) enriched diet resulted in improvements in plasma insulin, glucose and TAG concentrations. Key targets of the insulin signalling cascade, namely the insulin receptor, insulin receptor substrate (IRS)-1 and glucose transporter (Glut)-4 were also increased.

Objective: To investigate whether the insulin sensitising effect of c9, t11-CLA observed *in vivo* (chapter 4) may be replicated in an *in vitro* model. To discern whether the improvement in insulin sensitivity was a direct effect of the particular fatty acid isomer on adipose tissue biology, rather than being secondary to a fatty acid/dietary intervention.

Design: 3T3-L1s were incubated with 50 μM of linoleic acid, c9, t11-CLA, t10, c12-CLA, or an equivalent volume of dimethyl sulfoxide (DMSO) which served as the vehicle control. Fatty acids were added in parallel with the differentiation media to test whether chronic treatment with the fatty acids is effective. In addition fatty acids were added following differentiation to an adipocyte phenotype to test whether acute treatment is effective. Investigations were performed in normal adipocytes and adipocytes which were made insulin resistant following treatment with the pro-inflammatory cytokine, tumour necrosis factor (TNF)-α. Gene expression analysis, protein analysis and a glucose uptake assay were performed.

Results: Chronic treatment with c9, t11-CLA in 3T3-L1 adipocytes resulted in a significant increase in IRS-1 and Glut4 mRNA in both normal adipocytes (IRS-1: $P<0.001$ compared to cells treated with t10, c12-CLA, Glut4: $P<0.001$ compared to cells treated with DMSO, linoleic acid or t10, c12-CLA) and cells which had been made insulin resistant following treatment with TNFα (IRS-1: $P<0.05$ compared to cells treated with linoleic acid, Glut4: $P<0.05$ compared to cells treated with DMSO). In contrast, acute treatment with c9, t11-CLA did not modulate the expression levels of these markers. However, Glut4 protein expression, basal

glucose uptake and insulin-stimulated glucose uptake following chronic treatment with c9, t11-CLA was comparable with cells treated with the vehicle control.

Conclusions: The lack of effect of c9, t11-CLA on glucose uptake despite an increase in expression in both IRS-1 and Glut4 may indicate a failure to translate the increase in gene expression into functional proteins, or attenuated protein activity by post-translational modification. Differences exist between *in vivo* and *in vitro* models. Investigation of the impact of c9, t11-CLA on insulin sensitivity and indeed on whole body metabolism in physiological and pathological situations remains to be of critical importance.

5.2 INTRODUCTION

Insulin resistance occurs when target tissues fail to respond appropriately to physiological concentrations of insulin (Werner *et al*, 2004). A major metabolic consequence of insulin resistance is hyperglycaemia, which leads to type 2 diabetes mellitus (T2DM) when the pancreatic β-cells are no longer able secrete an adequate supply of insulin to maintain normoglycaemia (Ruan & Lodish, 2003). Genetics plays a role in the pathogenesis of T2DM, but cannot account for the worldwide epidemic of T2DM which we are currently experiencing. Obesity, poor diet and a sedentary lifestyle directly promote insulin resistance, while exercise and weight loss reverse it (Mokdad *et al,* 2003). Recent findings have suggested potential roles for pro-inflammatory cytokines, adipokines and non-esterified fatty acids (NEFAs) as possible mediators of insulin resistance (Doria, 2003; Kahn & Flier, 2000; Hauner, 2005).

The insulin de-sensitising effects of the pleiotrophic cytokine tumour necrosis factor (TNF)-α are well characterised. Although other factors may contribute to the development of insulin resistance, TNFα initiated autocrine and paracrine pathways in adipose tissue mediate a reduction in insulin sensitivity (Ruan & Lodish, 2003). Both TNFα mRNA and protein are elevated in adipose tissue of obese rodents (Hotamisligil *et al*, 1994; Hotamisligil *et al*, 1996; Hofmann *et al*, 1994; Hamann *et al*, 1995; Ruan & Lodish, 2003). TNFα knockout animals have lower insulin and NEFA concentrations, and increased insulin sensitivity compared to their wild type littermates when placed on an equivalent diet (Uysal *et al*, 1997; Ventre *et al*, 1997). Additionally, rodents with TNFα receptor knocked out also show better insulin sensitivity even when obese (Coppack, 2001). TNFα inhibits insulin-induced glucose uptake by targeting several components of the insulin signalling cascade. It has been shown to inhibit tyrosine phosphorylation of the insulin receptor in a variety of cell types, including murine and human adipocytes, fibroblasts and hepatoma cells, and also in muscle and adipose tissue (Hotamisligil *et al*, 1994; Hotamisligil *et al*, 1996; Feinstein *et al*, 1993; Kroder *et al*, 1996). Increased serine phosphorylation of insulin receptor substrate (IRS)-1 by TNFα inhibits tyrosine phosphorylation of the protein due to a disruption of the interaction between the insulin receptor and the phosphotyrosine binding domain of IRS-1 (Aguirre *et al*, 2000; Hotamisligil *et al*, 1996). Additionally serine phosphorylation of IRS-1 promotes proteasome-mediated degradation (Pederson *et al*, 2001). TNFα also reduces transcription of the insulin receptor, IRS-1and glucose transporter (Glut)-4 (Stephens & Pekala, 1991; Stephens & Pekala, 1992). By altering the expression of other adipocyte secretory products, namely increasing the expression of NEFAs, interleukin-6, plasminogen activated inhibitor-1, and decreasing the expression of adiponectin, TNFα further impedes insulin signalling through systemic mechanisms (Sonnenberg *et al*, 2004 Guerre-Millo, 2004). Furthermore, TNFα has been shown to increase plasma triacylglycerol (TAG) and very low density lipoprotein concentrations, as well as stimulate lipolysis (Peraldi & Spiegelman, 1998; Lang *et al*, 1992).

There is substantial evidence that altering dietary fatty acid composition may play a role in the development and progression of insulin resistance. Several lines of evidence suggest that diets rich in saturated fatty acids de-sensitises peripheral tissue responsiveness to insulin, whereas some polyunsaturated fatty acids may counteract this effect (Roche, 2005; Feskens *et al*, 1994; Vessby *et al*, 2001). The previous *in vivo* study in ob/ob mice (chapter 4) demonstrated an improvement in plasma insulin, glucose and TAG concentrations following a cis-9, trans-11 conjugated linoleic acid (c9, t11-CLA) enriched diet. Key targets of the insulin signalling cascade (insulin receptor, IRS-1 and Glut4) were also increased. This metabolic effect may be ascribed, at least partially, to a reduction in inflammatory mediators, in particular TNFα, the expression of which was reduced by 50% in white adipose tissue. This was coupled with an increased expression of peroxisome proliferator activated receptor (PPAR)-γ2, liver X receptor (LXR) and a reduction in the nuclear factor-κB (NF-κB) complex. This data supports a role for c9, t11-CLA in improving insulin resistance through anti-inflammatory pathways, in a murine model of obesity and insulin resistance. The current set of experiments will investigate whether the insulin sensitising effect of c9, t11-CLA observed in ob/ob mice may be replicated in an *in vitro* model. Investigations will be performed in normal adipocytes and adipocytes which have been made insulin resistant following treatment with TNFα. Another aim of this work is to discern whether the improvement in insulin sensitivity was a direct effect of the particular fatty acid isomer on adipose tissue biology, rather than being secondary to a fatty acid/dietary intervention.

The well-characterised murine 3T3-L1 adipocyte cell line was the model of choice for these experiments. Human preadipocytes can be induced to differentiate into adipocytes in primary culture, enabling adipocyte biology in man to be investigated (Hauner *et al*, 2001). More recently a human fat cell line has become available (Rodriguez *et al*, 2004). However, since humans and rodents may potentially respond differently to stimuli as a consequence of different physiology, this follow-up work was also performed in a murine model. The 3T3-L1 cell line offers a number of advantages as an adipocyte cell line. 3T3-L1 cells are morphologically similar to fibroblastic preadipose cells found in the stroma of adipose tissue, and once differentiated, they exhibit virtually all of the characteristics associated with adipocytes present within adipose tissue (Cowherd *et al*, 1999; Novikoff *et al*, 1980). The formation and appearance of developing fat droplets also minic live tissue (Green & Kehinde, 1975). 3T3-L1 also express many of the same genes as adipocytes *in vivo*, such as TNFα, angiotensinogen and adipsin (Cowherd *et al*, 1999). When injected into mice, 3T3-L1 preadipocytes differentiate and form fat pads which are indistinguishable from normal adipose tissue (Green & Kehinde, 1979). Therefore, they represent an invaluable model to aid our understanding of adipocyte cell biology.

5.3 MATERIALS AND METHODS

5.3.1 Cell culture and experimental design

3T3-L1s were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-25mM glucose containing 10% foetal bovine serum (FBS) and penicillin/streptomycin as described in Section 2.9.2. When fibroblasts were approximately 70% confluent, differentiation was initiated by culturing the cells in the presence of 0.6 μM insulin, 2.5 μM dexamethasone and 517 μM isobutyl-1-methylxanthine (IBMX) for 4 days. This media was then replaced with DMEM-25mM glucose containing 10% FBS for a further 3 days (Section 2.9.4). 3T3-L1s were incubated with 50 μM of linoleic acid, c9, t11-CLA, t10, c12-CLA or an equivalent volume of dimethyl sulfoxide (DMSO) which served as the vehicle control. A dose of 50 μM fatty acids was chosen as previous *in vitro* work in 3T3-L1s have used this concentration of CLA isomers (Evans et al, 2002b) without reporting toxicological effects. For all treatments, the final concentration of DMSO in the culture medium was ≤0.1%. Both DMSO and linoleic acid were purchased from Sigma-Aldrich (Poole, Dorset, UK) and the CLA isomers were purchased from Cayman Chemical (MI, USA). Fatty acids were added in parallel with the differentiation media to test whether chronic treatment with the fatty acids is effective. In addition fatty acids were added following differentiation to test whether acute treatment is effective. Fresh fatty acids were added with each media change. For all treatments, the final concentration of DMSO in the culture medium was ≤0.1%. Insulin resistance was induced in adipocytes by incubating the differentiated adipocytes for 3 days in the presence of 3 ng/ml TNFα (R&D Systems, Minneapolis, MN), with fresh media changes each day. This TNFα treatment has previously been shown to significantly reduce the rate of insulin-stimulated glucose uptake, by approximately 60%, without inducing morphological changes in mature adipocytes (Sartipy & Loskutoff, 2003a). Adipocytes treated in parallel but without TNFα were considered normal adipocytes. *Figure 5.1* illustrates an outline of the cell culture experimental conditions.

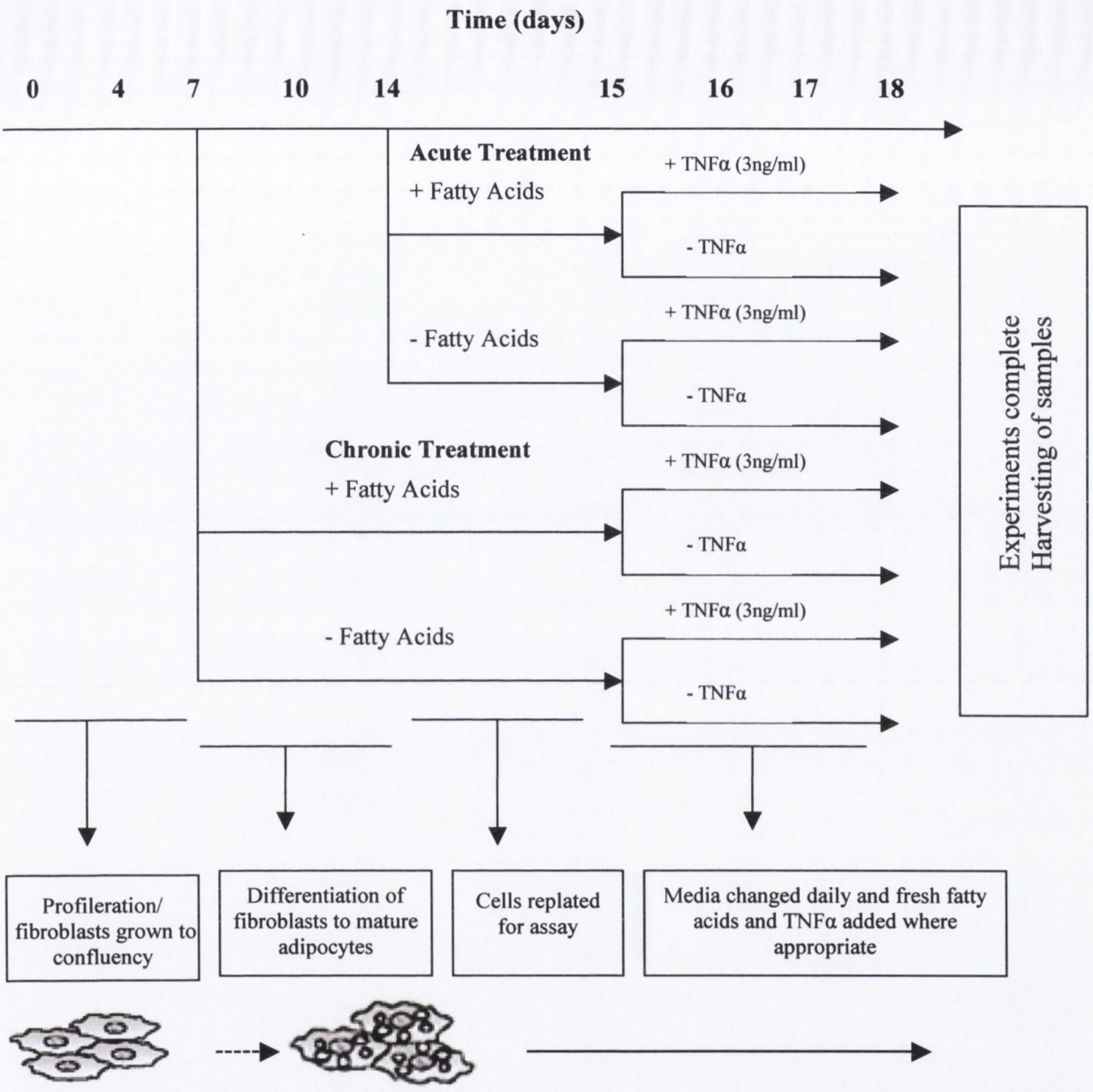


Fig. 5.1: Outline of cell culture experiments

5.3.2 Oil Red O staining

Fibroblasts were grown to approximately 70% confluency, replated in 96-well microtitre plates, and differentiated to adipocyte as outlined in Section 2.9.4. Cells were treated with fatty acid and TNFα as required. Oil Red O (ORO) staining was performed on day 15 to test that the untreated and fatty acid-treated fibroblasts differentiated into mature adipocytes *(Fig. 5.1)*. The effect of TNFα treatment was assessed on day 18 *(Fig. 5.1)*. Cells were fixed with formalin and stained with ORO stain as outlined in Section 2.12. Micrographs were taken. Cells were dissolved in isopropanol prior to quanification at 490 nm. Control cells, labelled nil, were left untreated. 4 independent experiments were conducted.

5.3.3 MTS assay

Fibroblasts were grown to approximately 70% confluency, replated in 96-well microtitre plates, and differentiated as outlined in Section 2.9.4. An MTS assay was employed at the end of the experimental protocol i.e. day 18, in order to determine if the concentrations of fatty acids and TNFα used in the experiments affect cellular viability (Section 2.11). 6 independent experiments were conducted. Results are expressed as a % viability of control cells.

5.3.4 mRNA expression analysis

1 x 10^6 mature adipocytes were plated and cultured with fatty acid as detailed in *Fig. 5.1.* To allow examination of fatty acids on gene expression in normal and insulin resistant adipocytes, cells were cultured in the presence and absence of TNFα as required. Total RNA was extracted from cells as outlined in Section 2.13.1 and quantified spectrophotometrically (Section 2.13.2). The integrity of the ribonucleic acid (RNA) was assessed by RNA gel electrophoresis (Section 2.13.3). RNA was deoxyribonuclease (DNase) treated and subsequently reverse transcribed to synthesise complementary deoxyribonucleic acid (cDNA) (Sections 2.14.1 and 2.14.2). Gene expression was quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) on an ABI 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Warrington, UK) (Section 2.14.3). IRS-1 and Glut4 were investigated using pre-developed primer and probe kits (Perkin-Elmer Applied Biosystems, Warrington, UK). Primers and probes for PPARγ1, PPARγ2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were custom made. The sequences of custom made primers and probes are presented in *Table 5.1.* JumpStart™ Taq ReadyMix (Sigma-Aldrich, Poole, Dorset, UK) was used in the polymerase chain reaction (PCR). After PCR, standard curves were constructed and the fractional cycle number of each of the unknown samples was used to calculate the amount of target gene relative to the standard (Section 2.14). Target gene mRNA expression was normalised to the internal control GAPDH and expressed relative to the vehicle control group i.e. cells which received an acute or chronic treatment of DMSO, in the presence or absence of TNFα as appropriate. (DMSO). 6 independent experiments were conducted.

Table 5.1: Sequences of primers and probes for Taqman® PCR

Target Gene	Oligonucleotides (5' → 3')	Final Conc (nM)
GAPDH	F: ATGTGTCCGTCGTGGATC	F 250
	R: TCAGATGCCTGCTTCACC	R 50
	P: ATGTCATCATACTTGGCAGGTT	P 175
PPARγ1	F: AACCACTGATATTCAGGACATTTTTAAA	F 500
	R: AGAGGTCCACAGAGCTGATTCC	R 250
	P: AGAGATGCCATTCTGGCCCACCAACTT	P 200
PPARγ2	F: GCCTATGAGCACTTCACAAG	F 500
	R: ATGTCAAAGGAATGCGAGTG	R 500
	P: CCATCACGGAGAGGTCCACAGAGC	P 250

F, R and P indicate forward primer, reverse primer and probe, respectively
Final concentration of forward primer (F), reverse primer (R) and probe (P).

5.3.5 Protein analysis and Western blotting

1 x 10^6 mature adipocytes were plated and cultured with fatty acid as detailed in *Fig. 5.1.* To allow examination of fatty acids on gene expression in normal and insulin resistant adipocytes, cells were cultured in the presence and absence of TNFα as required. Cells were washed, and total cell lysates was prepared as outlined in Section 2.15.5. Protein concentrations were assessed using the Modified Lowry assay (Section 2.15.6), and equal concentrations of samples were acetone precipitated overnight (Section 2.15.8). Proteins were separated by electrophoresis through SDS-polyacrylamide gels (Section 2.16.1) and Western blotting was performed as outlined in Section 2.16.2. 250μg of protein was loaded and blots were probed for Glut4 (Chemicon International, CA, USA) and IκBα (C-21), *sc*-371, Santa Cruz Biotechnology, CA, USA). Immunoreactive bands were visualized using an enhanced chemiluminescence reaction (Supersignal® West Pico Chemiluminescent Substrate, Pierce, IL, USA) (2.16.3). Quantification of Western blotting autoradiographic bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation and Analysis System, Syngene). The position of the bands was confirmed by the inclusion of suitable molecular weight markers during the experiment. Results are expressed as arbitrary units. Four independent experiments were conducted.

5.3.6 Glucose uptake assay

Fibroblasts were grown to confluency as described in Section 2.9.2 and replated in Cytostar-T™ scintillating microplates (Amersham Biosciences, Buckinghamshire, UK). Cells were differentiated into adipocytes as described in Section 2.9.4, and fatty acid and TNFα treatments were performed as outlined in Section 2.9.7. Cells were grown overnight in serum-

free DMEM containing 0.5% (w/v) bovine serum albumin (BSA). This was removed and cells were washed with 200 µl buffer and cultured for 5 hr to ensure glucose depletion as described in Section 2.18. Insulin (0.1 nM, 1 nM, 10 nM) was added to the appropriate wells and the cells cultured for a further 20 min. This was followed by the addition of 10 µl (0.1 µCi) 2-DOG (Amersham Biosciences, Buckinghamshire, UK) for 10 min. Glucose uptake was monitored as an increase in scintillation counts, using a Wallac MicroBeta^{TM} 1450 liquid scintillation counter (Perkin Elmer, MA, USA). 4 independent experiments were conducted. Undifferentiated fibroblasts were included as a negative control.

5.3.7 Investigation of the effect of TNFα *in vivo*

In a previous study performed in the Nutrition Group four male C57/BL6 mice received an intraperitoneal injection (75 αg/kg) of murine recombinant TNFα. Two control mice received an intraperitoneal injection of an equal volume of saline. Adipose tissue was available to perform RNA extractions, and subsequent gene expression analysis as outlined in Section 4.3.3.

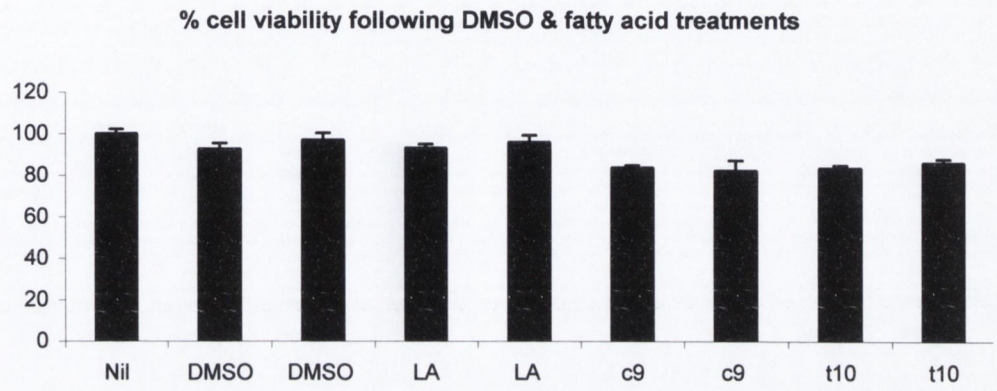
5.3.8 Data preparation and statistical analysis

Statistical analysis was performed using Data Desk 6.0 (Data Description Inc., NY, USA). The distribution of the data was assessed and variables were transformed as necessary to give the data a normal Gaussian distribution. Multiple comparisons were performed by 1-way analysis of variance (ANOVA). If ANOVA highlighted inter-group differences, post-hoc statistical analysis was performed using the Scheffe test to identify individual differences. A statistical probability of $P<0.05$ was considered statistically significant.

5.4 RESULTS

5.4.1 MTS assay

Fig 5.2 shows the effect of the fatty acids and TNFα treatment on cellular viability. Treatment with 50 μM fatty acid or an equivalent volume of DMSO had no significant effect on cellular viability.



TNFα (3 ng/ml for 3 days)	-	-	-	-	-	-	-	-	-
Fatty acid added to differentiation media	-	-	+	-	+	-	+	-	+
Fatty acids added after differentiation	-	+	+	+	+	+	+	+	+

Fig. 5.2: MTS Assay in normal adipocytes. 3T3-L1s were treated with vehicle control (DMSO) or 50 μM fatty acids as described. Results are expressed as a percentage of untreated control cells (nil=100%). Values represent mean ± SEM of 6 independent experiments. There were no statistical differences detected. DMSO, dimethyl sulfoxide; LA, linoleic acid; c9, c9, t11-CLA; t10, t10, c12-CLA.

The addition of TNFα significantly lowered the viability of all cells treated with fatty acid or DMSO compared to the untreated control cells *(Fig 5.3)*: P<0.001 for linoleic acid and DMSO added to the differentiation media, both c9, t11-CLA treatments, both t10, c12-CLA treatments; P<0.01 for linoleic acid added after differentiation; P<0.05 for DMSO added after differentiation. There was no statistical difference in the viability of control cells treated in the presence or absence of TNFα, although percentage viability was reduced by 13%. Comparing treatments that were cultured in the presence of TNFα, indicated that cells cultured with t10, c12-CLA (added to the differentiation media), had significantly lower viability than control cells (P<0.001), cells treated with DMSO (added after differentiation) and cells treated with linoleic acid (added after differentiation) (P<0.01 and P<0.05, respectively).

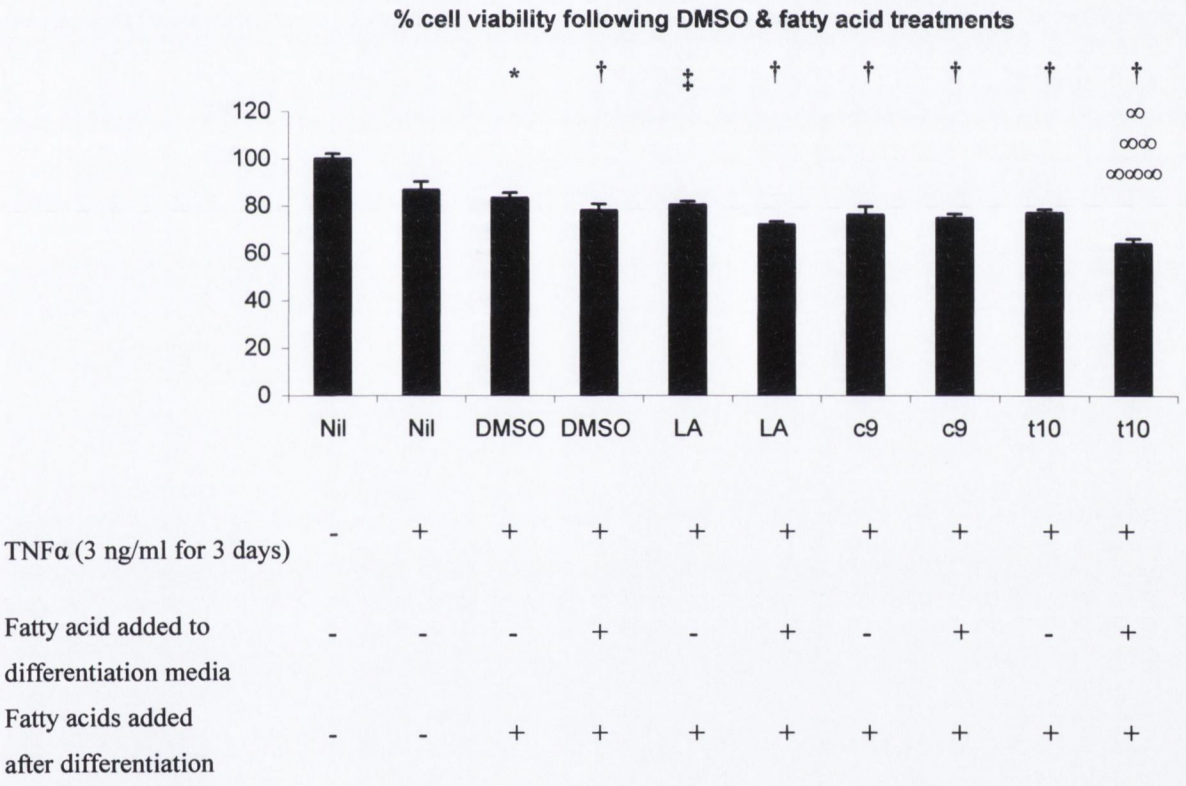


Fig. 5.3: MTS assay in insulin resistant adipocytes. 3T3-L1s were treated with vehicle control (DMSO), 50 μM fatty acids and TNFα (3 ng/ml for 3 days) as described. Results are expressed as a percentage of untreated control cells (nil=100%). Values represent mean ± SEM of 6 independent experiments. Significantly different from untreated control cell † P<0.001, ‡ P<0.01, * P<0.05. Significantly different from treated control cells ∞∞∞∞ P<0.001, cells treated with DMSO added after differentiation ∞∞∞ P<0.01, cells treated with linoleic acid after differentiation ∞ P<0.05. DMSO, dimethyl sulfoxide; LA, linoleic acid; c9, c9, t11-CLA; t10, t10, c12-CLA.

5.4.2 Oil Red O staining

Fig. 5.4 depicts absorbance values of the cells following ORO staining. Results show that the quantity of lipid in fibroblasts is significantly lower than in differentiated adipocytes, and differentiated adipocyte treated with 50 μM fatty acid or an equivalent volume of DMSO ($P<0.001$ for untreated adipocytes, DMSO, linoleic acid and c9, t11-CLA, $P<0.01$ for t10, c12-CLA). Neither fatty acid nor vehicle control treatments had any effect on the differentiation of the fibroblast to mature adipocyte. Micrographs of the cells are displayed in *Fig 5.5*.

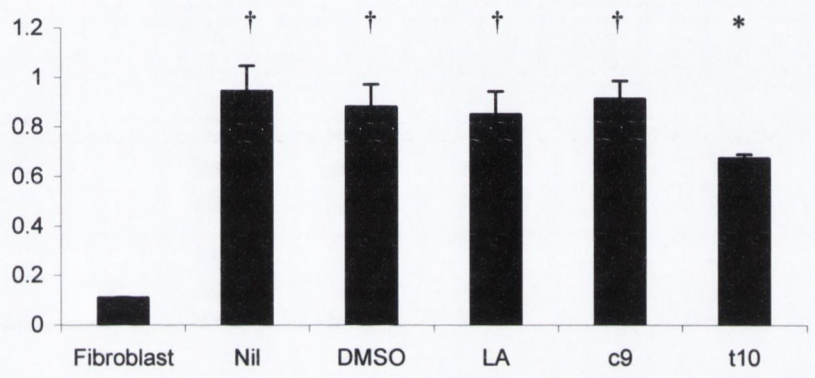


Fig. 5.4: ORO staining at the end of the differentiation period (day 15). 3T3-L1s were treated with vehicle control (DMSO) or 50 μM fatty acids and differentiated as described. Results represent mean ± SEM of 4 independent experiments. Significantly different from fibroblast † $P<0.001$, * $P<0.01$. DMSO, dimethyl sulfoxide; LA, linoleic acid; c9, c9, t11-CLA; t10, t10, c12-CLA.

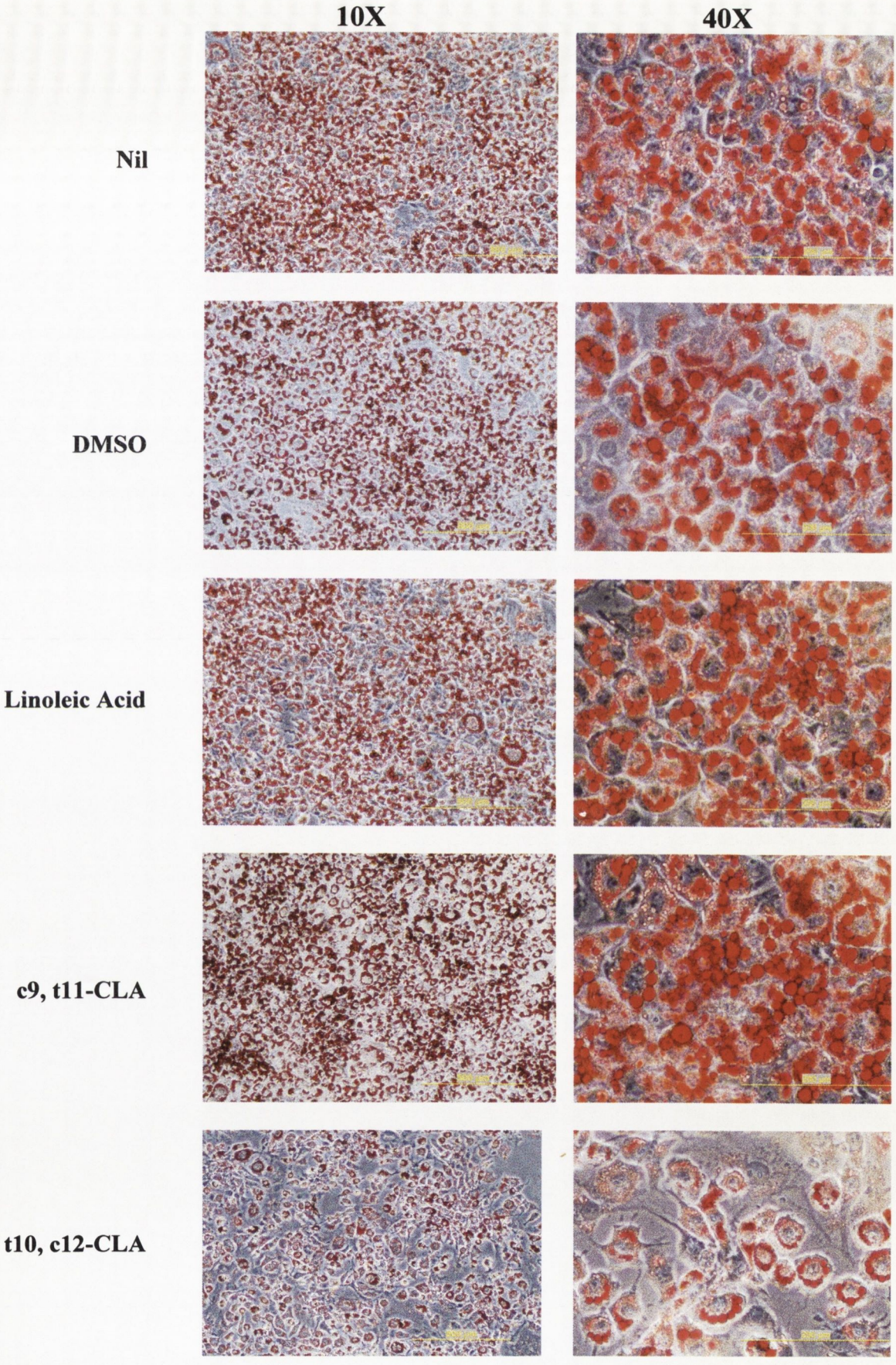


Fig. 5.5: Oil Red O staining at the end of the differentiation period (day 15). DMSO and fatty acids added in parallel with the differentiation media. Magnification 10X and 40X.

As mentioned previously, TNFα can induce the de-differentiation of adipocytes. Therefore ORO staining was repeated at the end of the experimental period to check the differentiation status of the cells. *Fig. 5.6* depicts absorbance values of the cells following Oil red O staining at day 18. The absorbance values for the fibroblast group were significantly lower than all other treatments ($P<0.001$). In addition, cells which were treated with t10, c12-CLA, displayed significant differences to the other treatments. Cells cultured with t10, c12-CLA (added after differentiation) either in the presence or the absence of TNFα had significantly lower absorbance values than untreated control cells ($P<0.01$ and $P<0.05$, respectively). When t10, c12-CLA was added to the differentiation media (in the absence of TNFα), absorbance values were significantly lower compared to the untreated control cells i.e. cells labelled nil in the absence of TNFα ($P<0.001$), DMSO and linoleic acid treatments (both added after differentiation, in the absence of TNFα) ($P<0.01$) and control cells in the presence of TNFα ($P<0.05$). When t10, c12-CLA was added to the differentiation media, and cells were subsequently treated with TNFα, absorbance values were significantly lower compared to all other treatments: $P<0.001$ compared to both nil treatments, all c9, t11-CLA treatments, linoleic acid (added after differentiation, in the absence of TNFα) and DMSO (added after differentiation, both in the presence and the absence of TNFα) and DMSO added during differentiation (in the absence of TNFα); $P<0.05$ compared to DMSO (added during differentiation, in the presence of TNFα), linoleic acid (added after differentiation, in the presence of TNFα; added during differentiation, both in the presence and absence of TNFα. Micrographs of the cells are displayed in *Fig. 5.7a* and *Fig. 5.7b*.

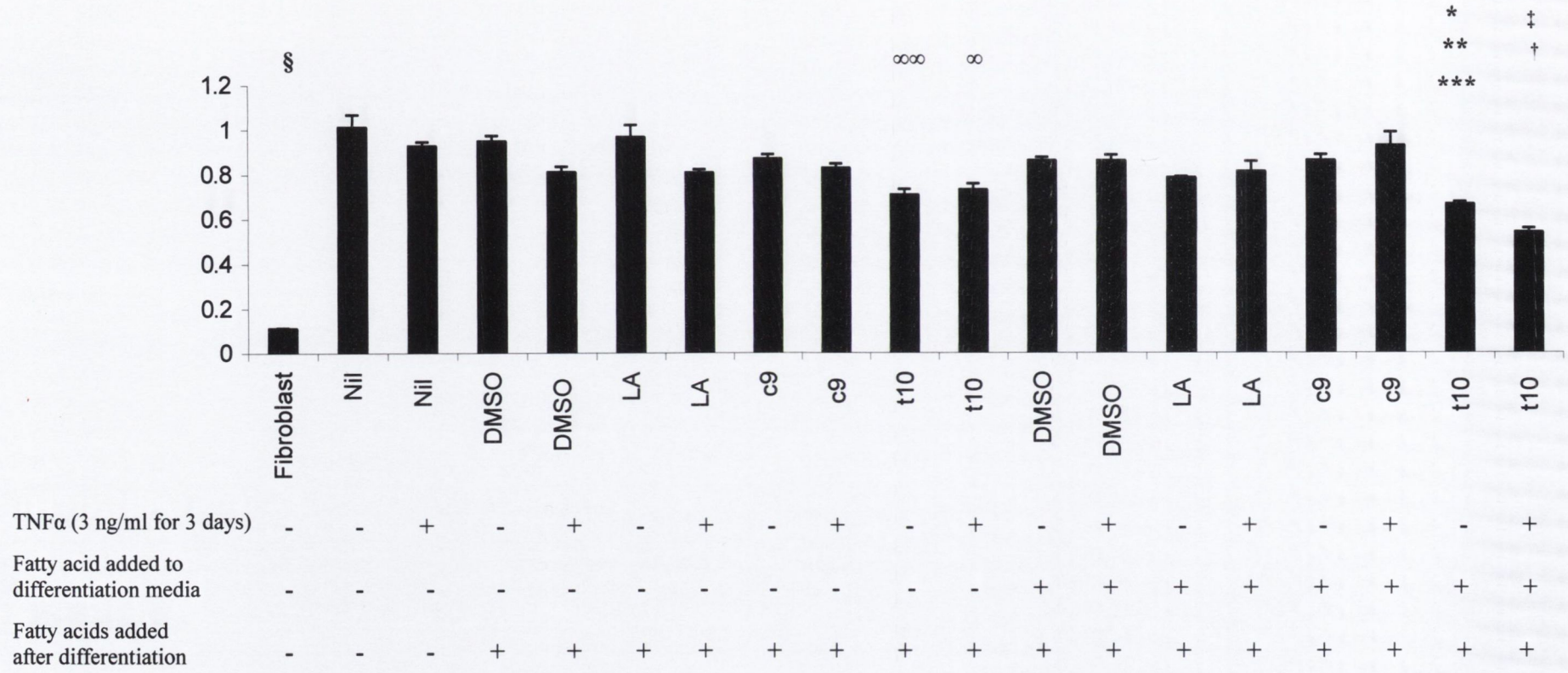


Fig. 5.5: ORO staining at the end of the experiment (day 18). 3T3-L1s were treated with vehicle control (DMSO), fatty acids, and TNFα as described. Results represent mean and SEM of 4 independent experiments. Fibroblast significantly different to all treatments § $P<0.001$. Significantly different to nil (in the absence of TNFα) treatment ∞∞ $P<0.01$, ∞ $P<0.05$. Significantly different to all c9, t11-CLA treatments, both nil treatments, linoleic acid (added after differentiation, in the absence of TNFα) and DMSO (added after differentiation, both in the presence and the absence of TNFα) and DMSO added during differentiation (in the absence of TNFα) ‡ $P<0.001$. Significantly different to DMSO (added during differentiation, with TNFα), linoleic acid (added after differentiation, with TNFα; added after differentiation, both with and without TNFα) † $P<0.05$. Significantly different to nil (without TNFα) *** $P<0.01$. Significantly different to DMSO and linoleic acid (both added after differentiation, without TNFα) ** $P<0.01$. Significantly different to nil (with TNFα) * $P<0.05$. DMSO, dimethyl sulfoxide; LA, linoleic acid; c9, c9, t11-CLA; t10, t10, c12-CLA.

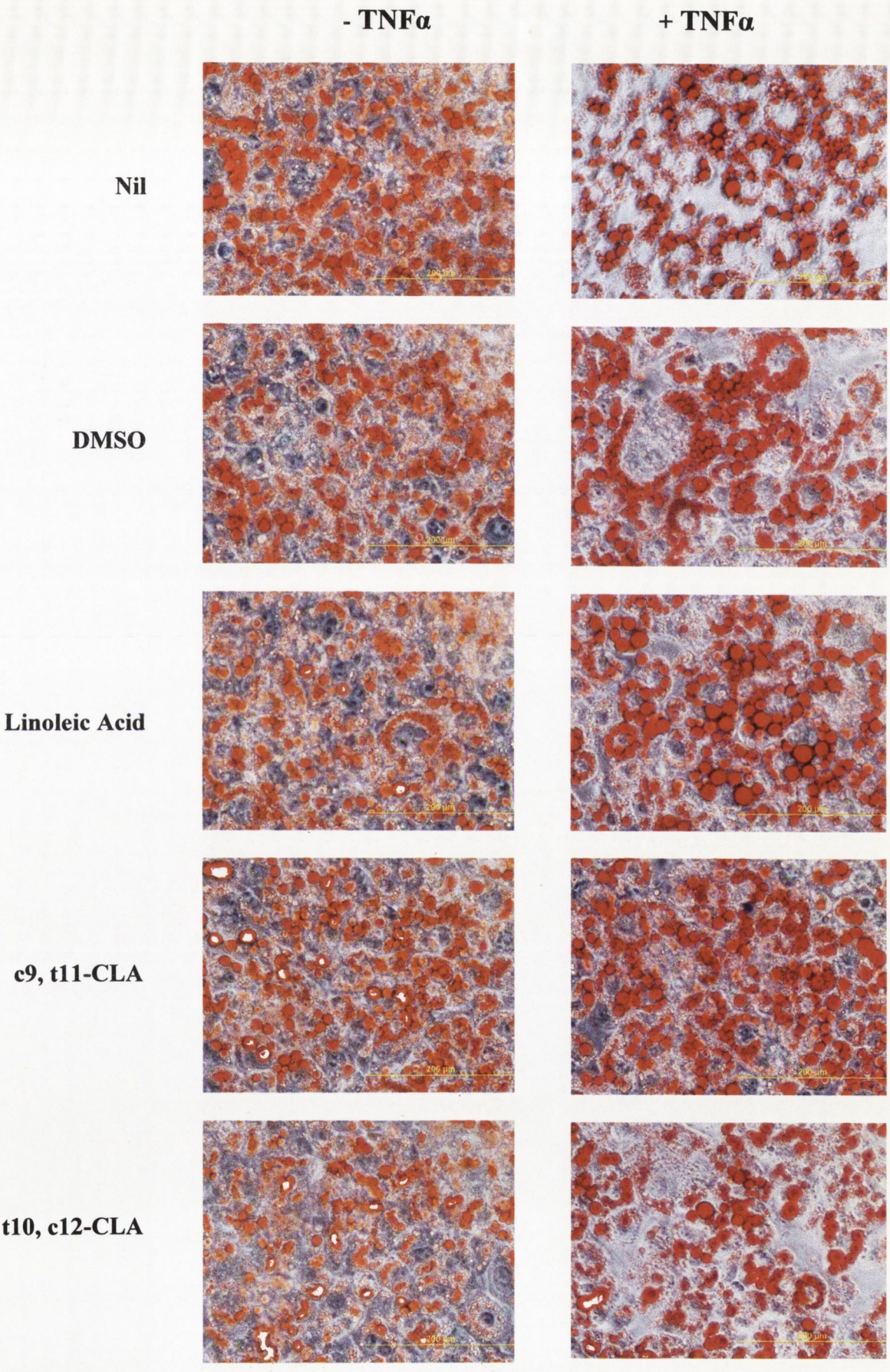


Fig. 5.7a: Oil Red O staining at the end of the experiment (day 18). DMSO and fatty acids added in parallel with the differentiation media and cultured in the presence or the absence of TNFα. Magnification 40X.

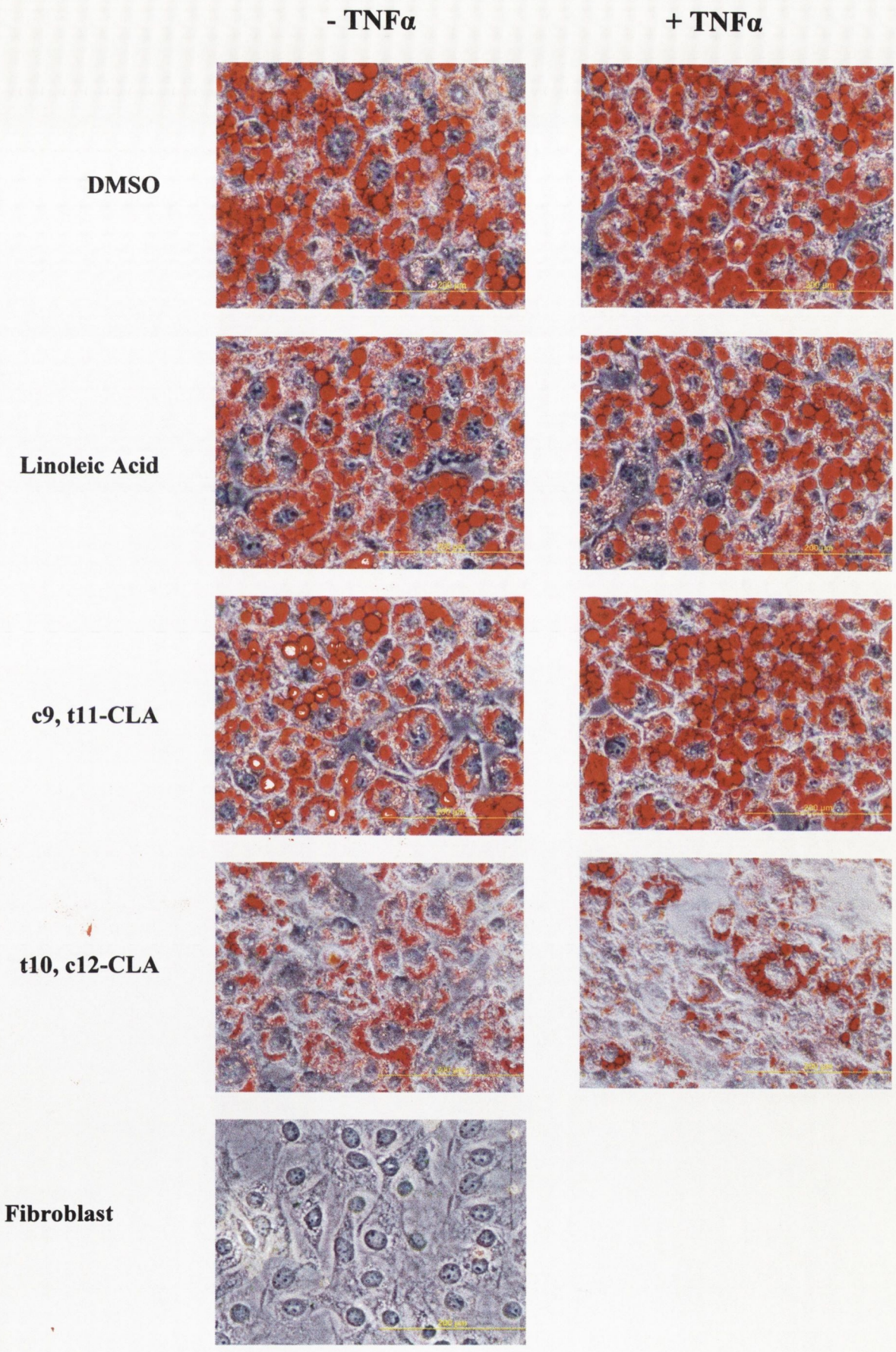


Fig. 5.7b: Oil Red O staining at the end of the experiment (day 18). DMSO and fatty acids added after differentiation to adipocyte and cultured in the presence or the absence of TNFα. Magnification 40X.

5.4.3 Gene expression analysis

Prior to commencing the gene expression work, it was necessary to choose a suitable endogenous control to normalise the target genes to. Both GAPDH and 36B4 were tested. Since GAPDH was custom made, a magnesium curve was performed to optimise the performance of the primer/probe set. Since 36B4 was purchased pre-designed (Taqman® Gene Expression Assays, Applied Biosystems, Warrington, UK) it was already optimised and so it was not necessary to complete this step. Results in *Fig. 5.8* show that the variability in the data was low for both targets. In addition, the presence of TNFα at a concentration of 3 ng/ml for 3 days had no effect on the expression of either gene. A decision was made to use GAPDH as the housekeeping or normalising gene in future experiments due its higher abundance in the extracts.

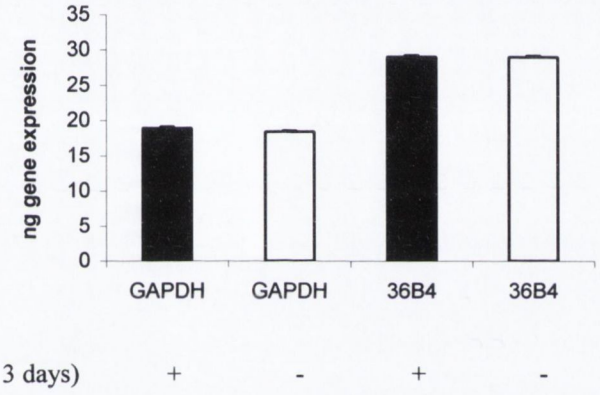


Fig. 5.8: Expression of normalising genes in extracts from 3T3-L1s. Values represent mean ± SEM of 6 independent experiments. There were no statistical differences detected.

The effect of fatty acid treatment on mRNA expression in 3T3-L1s

Gene expression was investigated in 3T3-L1 adipocytes to test whether 50 μM c9, t11-CLA had any impact on the expression of key proteins involved in the insulin signalling cascade. Fatty acids were added in parallel with the differentiation media to test whether chronic treatment with the fatty acids is effective. In addition fatty acids were added after differentiation to an adipocyte phenotype to test whether acute treatment is effective.

(i) Glut4 and IRS-1 mRNA following chronic treatment with fatty acids

Fig. 5.9 shows the effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 expression in normal adipocytes. Treatment of adipocytes with c9, t11-CLA resulted in significantly higher expression of Glut4 compared to DMSO, linoleic acid and t10, c12-CLA (1.5 fold increase compared to DMSO, $P<0.001$ for all). 50 μM t10, c12-CLA resulted in significantly lower expression of Glut4 compared to all other treatments ($P<0.001$ for all). In addition, 50 μM t10, c12-CLA resulted in significantly lower expression of IRS-1 compared to all other treatments ($P<0.001$ compared to c9, t11-CLA; $P<0.01$ compared to DMSO and linoleic acid). c9, t11-CLA resulted in a 1.26 fold increase in IRS-1 expression, which showed a trend towards significance compared to the vehicle control, DMSO ($P=0.06$).

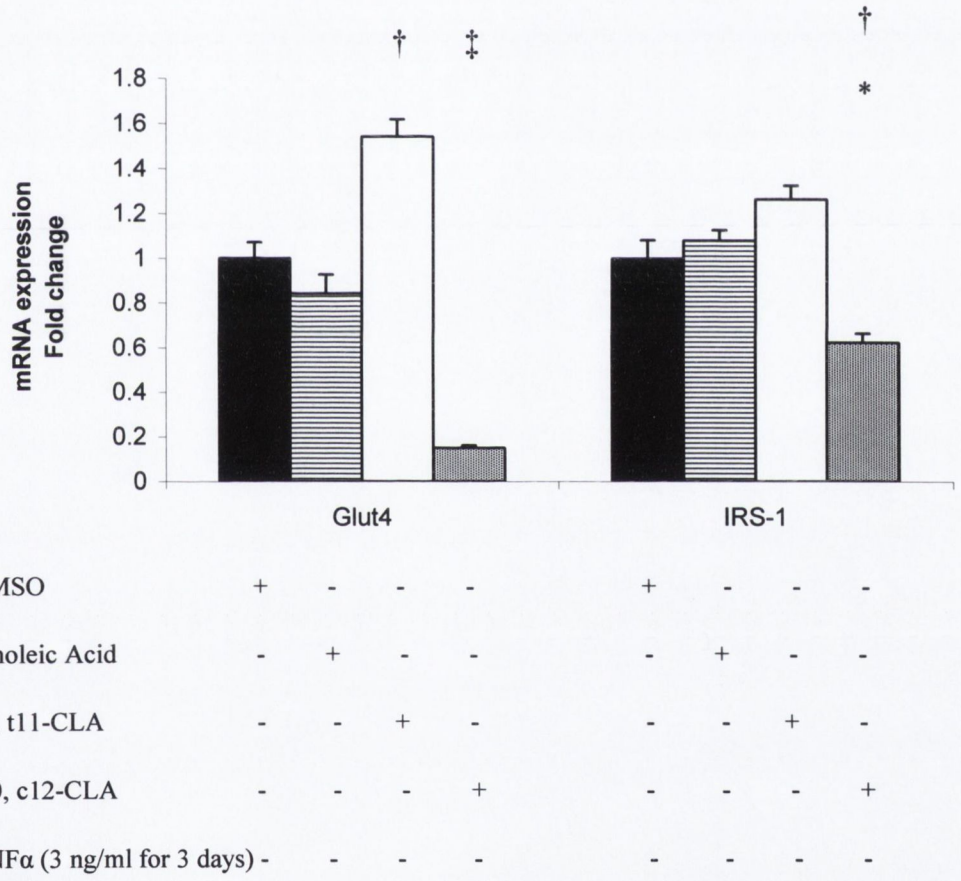


Fig. 5.9: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 mRNA expression in normal 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. For Glut4, † $P<0.001$ compared to DMSO, linoleic acid and t10, c12-CLA, ‡ $P<0.001$ compared to DMSO, linoleic acid and c9, t11-CLA. For IRS-1, † $P<0.001$ compared to c9, t11-CLA, * $P<0.01$ compared to DMSO and linoleic acid.

Fig. 5.10 shows the effect of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 mRNA expression in adipocytes made insulin resistant by treating with 3 ng/ml TNFα for 3 days. Treatment of adipocytes with 50 μM c9, t11-CLA resulted in significantly higher expression of Glut4 compared to the vehicle control (DMSO) (1.46 fold increase, $P<0.05$). There was a trend towards a statistically significant difference in Glut4 expression between c9, t11-CLA and t10, c12-CLA treatments ($P=0.077$). Examining IRS-1 mRNA indicated that treatment with 50 μM c9, t11-CLA resulted in a 1.3 fold increase in expression compared to DMSO controls, which was statistically significant compared to cells treated with linoleic acid ($P<0.05$).

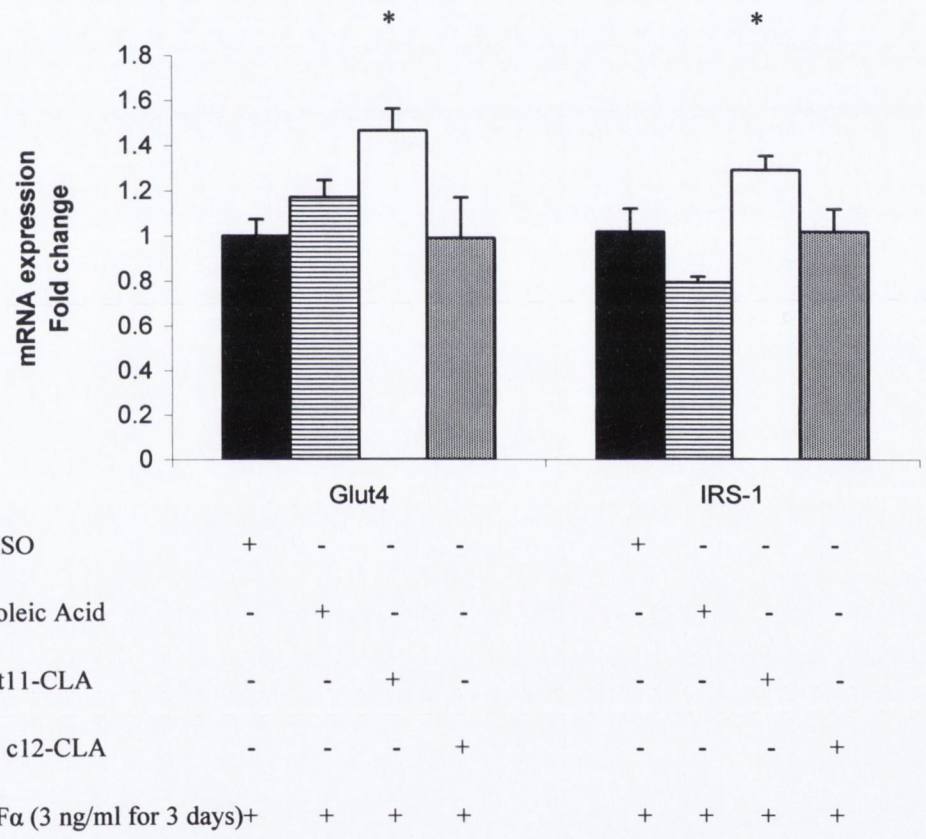
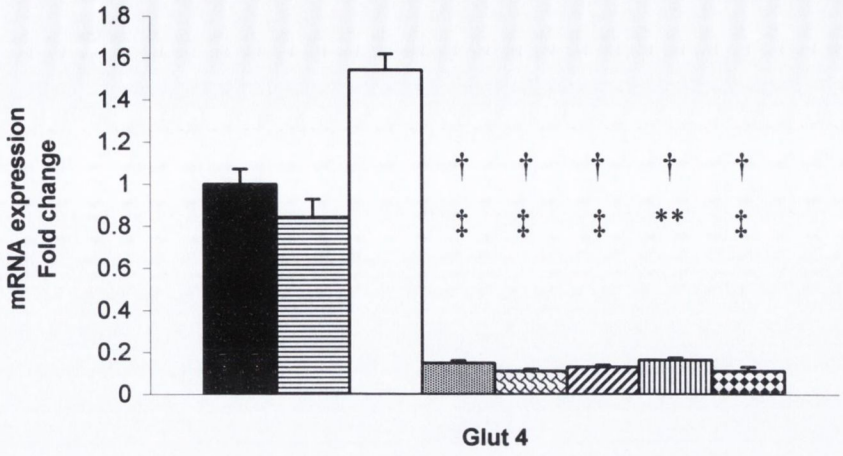


Fig. 5.10: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 mRNA expression in insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. For Glut4, * $P<0.05$ compared to DMSO. For IRS-1, * $P<0.05$ compared to linoleic acid.

Data was expressed to examine the effect of TNFα treatment on adipocyte Glut4 expression, and to determine whether fatty acids could modulate the insulin de-sensitising effect of TNFα *(Fig. 5.11)*. The presence of TNFα resulted in 89% reduction in Glut4 mRNA ($P<0.001$ comparing untreated and treated DMSO controls). All treatments cultured with 3 ng/ml TNFα for 3 days had significantly lower Glut4 expression than untreated DMSO control, untreated linoleic acid and untreated c9, t11-CLA samples ($P<0.001$ for treated DMSO, linoleic acid and t10, c12-CLA compared to cells treated in the absence of TNFα; $P<0.001$ for treated c9, t11-CLA compared to untreated DMSO and c9, t11-CLA, $P<0.01$ compared to untreated linoleic acid). In addition, the expression of Glut4 was significantly lower in cells treated with t10, c12-CLA (in the absence of TNFα) compared to untreated DMSO controls, untreated linoleic and untreated c9, t11-CLA samples ($P<0.001$ for all treatments). Expression of Glut4 following treatment with t10, c12-CLA was similar to samples treated with TNFα *(Fig. 5.11)*, whereas untreated DMSO controls, untreated linoleic acid and untreated c9, t11-CLA samples exhibited approximately 10 fold higher Glut4 expression than samples treated with TNFα. However the down-regulation of Glut4 expression following treatment with TNFα was not as great in samples cultured in the presence of t10, c12-CLA and TNFα as that observed in other treatment (26% reduction compared to 89-96% reduction observed with other treatments).

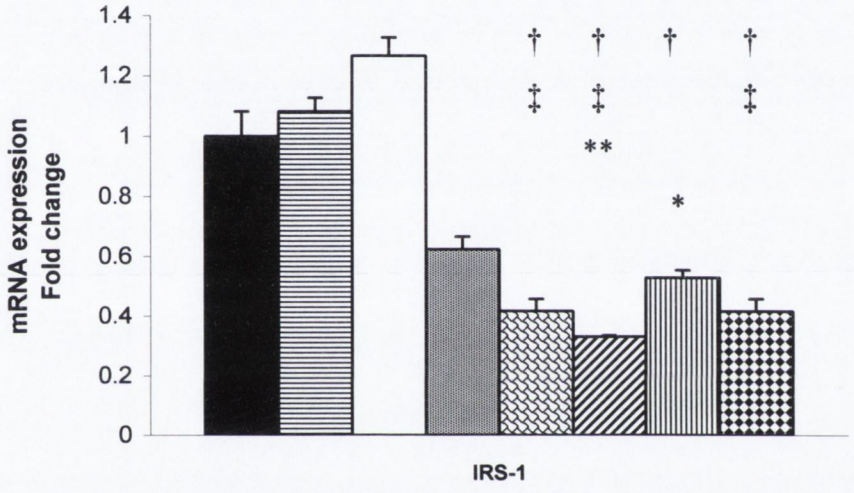


DMSO	+	-	-	-	+	-	-	-
Linoleic Acid	-	+	-	-	-	+	-	-
c9, t11-CLA	-	-	+	-	-	-	+	-
t10, c12-CLA	-	-	-	+	-	-	-	+
TNFα (3 ng/ml for 3 days)	-	-	-	-	+	+	+	+

Fig. 5.11: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 mRNA expression in normal and insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. † $P<0.001$ compared to DMSO and c9, t11-CLA (both in the absence of TNFα), ‡ $P<0.001$ compared to linoleic acid (in the absence of TNFα), ** $P<0.01$ compared to linoleic acid (in the absence of TNFα).

In addition data was expressed to examine the effect of TNFα treatment on adipocyte IRS-1 expression, and to determine whether fatty acids could modulate the insulin de-sensitising effect of TNFα *(Fig. 5.12)*. The presence of TNFα resulted in 58% reduction in IRS-1 mRNA ($P<0.001$ comparing untreated and treated DMSO controls). All treatments cultured with 3 ng/ml TNFα for 3 days had significantly lower IRS-1 expression than untreated c9, t11-CLA cells ($P<0.001$). In addition, samples treated with DMSO, linoleic acid or t10, c12-CLA, in the presence of TNFα, had significantly lower IRS-1 mRNA compared to untreated DMSO samples and untreated linoleic samples ($P<0.001$). However, in contrast samples treated with c9, t11-CLA and TNFα did not reach statistical significance compared to untreated DMSO and linoleic acid samples ($P=0.077$, and $P=0.068$ respectively). Samples cultured with linoleic acid and in the presence of TNFα also had significantly lower expression of IRS-1 compared to

untreated t10, c12-CLA samples (P<0.01), and samples cultured with c9, t11-CLA and TNFα (P<0.05). Of note is that the expression of IRS-1 following treatment with t10, c12-CLA (in the absence of TNFα is similar to the samples treated with TNFα (1.4 fold higher expression compared to untreated samples versus approximately 2 fold higher expression in untreated DMSO controls, untreated linoleic acid and untreated c9, t11-CLA, *Fig 5.12*). However similar to Glut4 expression, the down-regulation of IRS-1 expression following treatment with TNFα was not as great in samples cultured with in the presence of t10, c12-CLA and TNFα as that observed in other treatment (33% reduction compared to 58-69% reduction observed with other treatments).



DMSO	+	-	-	-	+	-	-	-
Linoleic Acid	-	+	-	-	-	+	-	-
c9, t11-CLA	-	-	+	-	-	-	+	-
t10, c12-CLA	-	-	-	+	-	-	-	+
TNFα (3 ng/ml for 3 days)	-	-	-	-	+	+	+	+

Fig. 5.12: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on IRS-1 mRNA expression in normal and insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. † P<0.001 compared to c9, t11-CLA (in the absence of TNFα), ‡ P<0.001 compared to DMSO and linoleic acid (both in the absence of TNFα), ** P<0.01 compared to t10, c12-CLA (in the absence of TNFα), * P<0.05 compared to linoleic acid (in the presence of TNFα).

(ii) Glut4 and IRS-1 mRNA following acute treatment with fatty acids

Fig. 5.13 shows the effect of acute treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 mRNA expression in normal adipocytes. Treatment of adipocytes with 50 μM t10, c12-CLA resulted in significantly lower expression of both Glut4 and IRS-1 compared to all other treatments ($P<0.001$ for all). Acute treatment of c9, t11-CLA had no effect on either Glut4 or IRS-1 expression.

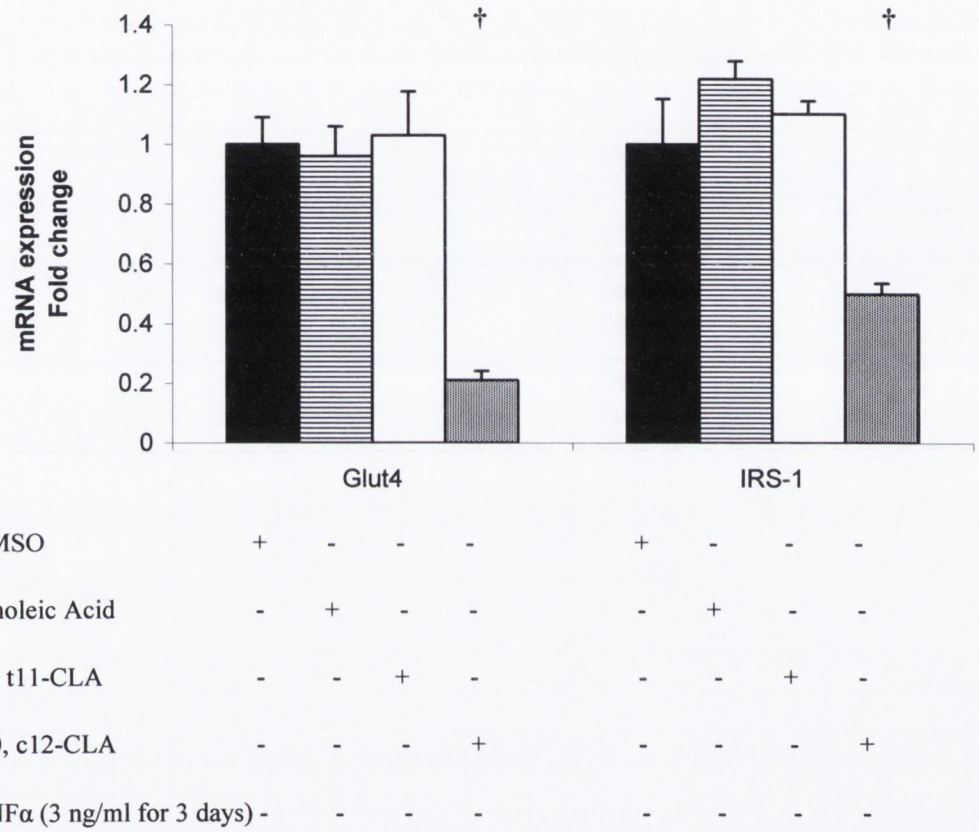


Fig. 5.13: Effect of acute treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 mRNA expression in normal 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. For Glut4, † $P<0.001$ compared to DMSO, linoleic acid and c9, t11-CLA. For IRS-1, * $P<0.001$ compared to DMSO, linoleic acid and c9, t11-CLA.

Fig. 5.14 shows the effect of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 and IRS-1 mRNA expression in adipocytes made insulin resistant by treating with 3ng/ml TNFα for 3 days. Treatment of adipocytes with 50μM t10, c12-CLA resulted in significantly lower expression of Glut4 compared to all other treatments ($P<0.01$ compared to DMSO and c9, t11-CLA; $P<0.05$ compared to linoleic acid). In addition, 50 μM t10, c12-CLA resulted in significantly lower expression of IRS-1 compared to all other treatments ($P<0.001$ for all). c9, t11-CLA had no effect on either Glut4 or IRS-1 expression in insulin resistant adipocytes.

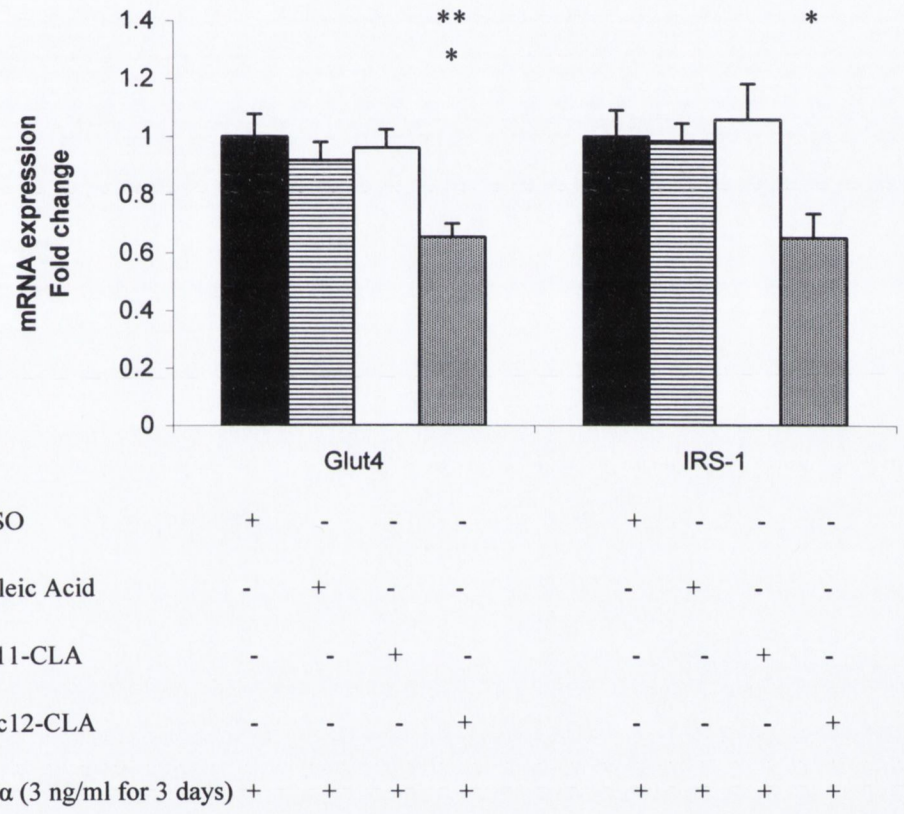


Fig. 5.14: Effect of acute treatment of linoleic acid, c9,t11-CLA and t10,c12-CLA on Glut4 and IRS-1 mRNA expression in insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means and SEM of 6 independent experiments. For Glut4, ** $P<0.01$ compared to DMSO and c9, t11-CLA, * $P<0.05$ for linoleic acid. For IRS-1, * $P<0.05$ compared to DMSO, linoleic and c9, t11-CLA.

Data was expressed to examine the effect of TNFα treatment on adipocyte Glut4 expression, and to determine whether fatty acids could modulate the insulin de-sensitising effect of TNFα *(Fig. 5.15)*. The presence of TNFα resulted in 91% reduction in Glut4 mRNA ($P<0.001$ comparing untreated and treated DMSO controls). All treatments cultured with TNFα had significantly lower Glut4 expression than untreated DMSO control, untreated linoleic acid and untreated c9, t11-CLA samples ($P<0.001$ for all). In addition, the expression of Glut4 was significantly lower in cells treated with t10, c12-CLA (in the absence of TNFα) compared to untreated DMSO controls, untreated linoleic acid and untreated c9, t11-CLA ($P<0.001$ for all). Glut4 expression following treatment with t10, c12-CLA, was only approximately 2 fold higher than samples treated with TNFα, whereas untreated DMSO controls, untreated linoleic acid and untreated c9, t11-CLA samples exhibited approximately 10 fold higher Glut4 expression than samples treated with TNFα. However the down-regulation in Glut4 expression following treatment with TNFα was not as great in samples cultured in the presence of t10, c12-CLA and TNFα as that observed in other treatments (74% reduction compared to 92% reduction observed with other treatments).

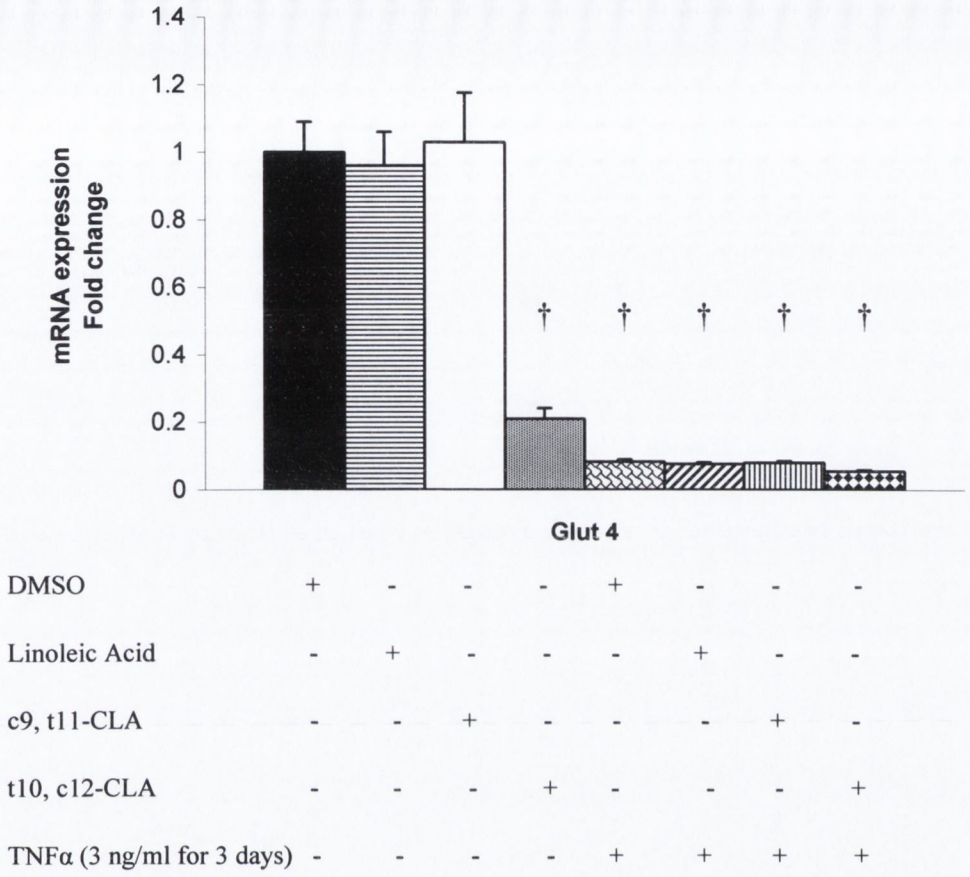


Fig. 5.15: Effect of acute treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on Glut4 mRNA expression in normal and insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. † $P<0.001$ compared to DMSO, linoleic acid and c9, t11-CLA (all in the absence of TNFα).

In addition data was expressed to examine the effect of TNFα treatment on adipocyte IRS-1 expression, and to determine whether fatty acids could modulate the insulin de-sensitising effect of TNFα *(Fig. 5.16)*. The presence of TNFα resulted in 61% reduction in IRS-1 mRNA ($P<0.01$ comparing untreated and treated DMSO controls). All treatments cultured with TNFα had significantly lower IRS-1 expression than untreated DMSO control, untreated linoleic and untreated c9, t11-CLA cells ($P<0.001$ for DMSO, linoleic acid and c9, t11-CLA compared to untreated linoleic acid and untreated c9, t11-CLA; $P <0.01$ compared to untreated DMSO; $P <0.001$ for t10, c12-CLA compared to untreated DMSO, untreated linoleic acid, untreated c9, t11-CLA and untreated t10, c12-CLA). Cells treated with t10, c12-CLA (in the presence of

TNFα) had significantly lower expression of IRS-1 compared to c9, t11-CLA treated samples (also in the presence of TNFα) ($P<0.05$). In addition, a trend was observed towards a statistically lower expression of IRS-1 in samples treated with t10, c12-CLA (in the presence of TNFα) compared to linoleic acid ($P=0.057$) and DMSO ($P=0.076$) (both also in the presence of TNFα). IRS-1 expression following treatment with t10, c12-CLA (in the absence of TNFα) is very similar to samples treated with TNFα (1.2 fold increase compared to 2.75 fold increase with DMSO, linoleic acid and c9, t11-CLA samples treated with TNFα). However the negative impact of TNFα on IRS-1 expression was not as great in samples cultured in the presence of t10, c12-CLA and TNFα as that observed in other treatments (49% reduction compared to 60-68% reduction observed with other treatments).

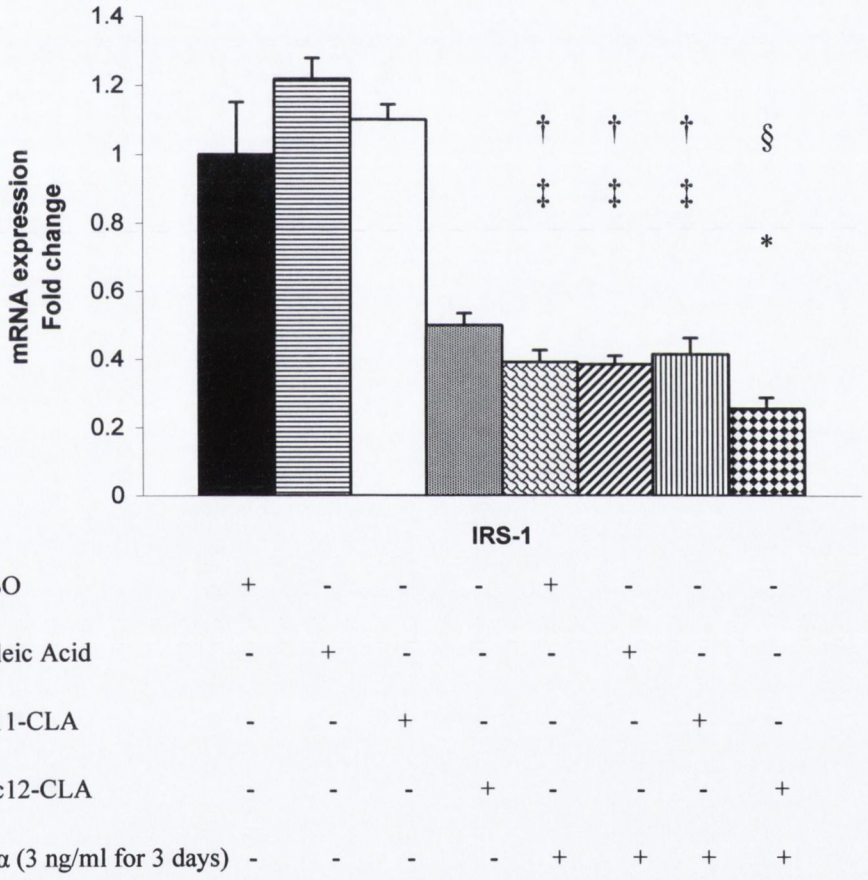


Fig. 5.16: Effect of acute treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on IRS-1 mRNA expression in normal and insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. † $P<0.001$ compared to linoleic acid and c9, t11-CLA (both in the absence of TNFα), ‡ $P<0.01$ compared to DMSO (in the absence of TNFα), § $P<0.01$ compared to DMSO, linoleic acid, c9, t11-CLA and t10, c12-CLA (all in the absence of TNFα), * $P<0.05$ compared to c9, t11-CLA (in the presence of TNFα).

(iii) PPARγ1 and PPARγ2 mRNA following chronic treatment with fatty acids

Fig. 5.17 shows the effect of linoleic acid, c9, t11-CLA and t10, c12-CLA on PPARγ1 and PPARγ2 mRNA expression in normal adipocytes. Treatment of adipocytes with 50 μM t10, c12-CLA resulted in significantly lower expression of PPARγ1 compared to all other treatments ($P<0.001$ for all). In addition 50 μM t10, c12-CLA resulted in significantly lower expression of PPARγ2 compared to all other treatments ($P<0.01$ compared to DMSO and c9, t11-CLA; $P<0.05$ compared to linoleic acid). c9, t11-CLA resulted in a 1.15 fold and 1.18 fold increase in PPARγ1 and PPARγ2 expression respectively, but neither reached statistical significance. Interestingly although PPARγ1 mRNA was slightly increased following linoleic acid treatment (1.18 fold increase), PPARγ2 mRNA expression was decreased by 8% compared to DMSO control group. Neither reached statistical significance.

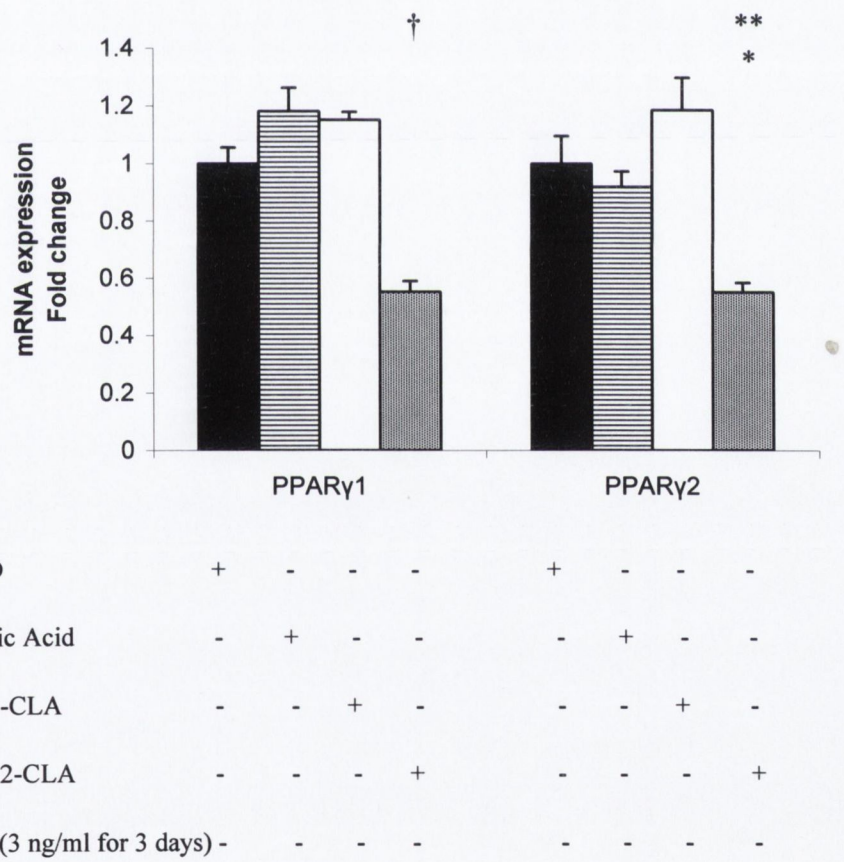


Fig. 5.17: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10,c12-CLA on PPARγ1 and PPARγ2 mRNA expression in normal 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. For PPARγ1, † $P<0.001$ compared to DMSO, linoleic acid and c9, t11-CLA. For PPARγ2, ** $P<0.01$ compared to DMSO and c9, t11-CLA, * $P<0.05$ compared to linoleic acid.

Fig. 5.18 shows the effect of linoleic acid, c9, t11-CLA and t10, c12-CLA on PPARγ1 and PPARγ2 mRNA expression in adipocytes made insulin resistant following treatment with 3 ng/ml TNFα for 3 days. Treatment of adipocytes with 50 μM c9, t11-CLA resulted in a 1.46 fold increase in PPARγ1 expression, which was statistically significant compared to DMSO ($P<0.01$). With respect to PPARγ2 expression, 50μM t10, c12-CLA resulted in a 2 fold increase in expression (compared to DMSO). This was statistically significant compared to all treatments ($P<0.001$ compared to DMSO and linoleic acid treatments; $P<0.01$ compared to c9, t11-CLA). c9, t11-CLA resulted in a 1.3 fold increase in PPARγ2 expression, which tended towards statistical significance compared to vehicle control (DMSO, $P=0.092$) and linoleic acid ($P = 0.07$).

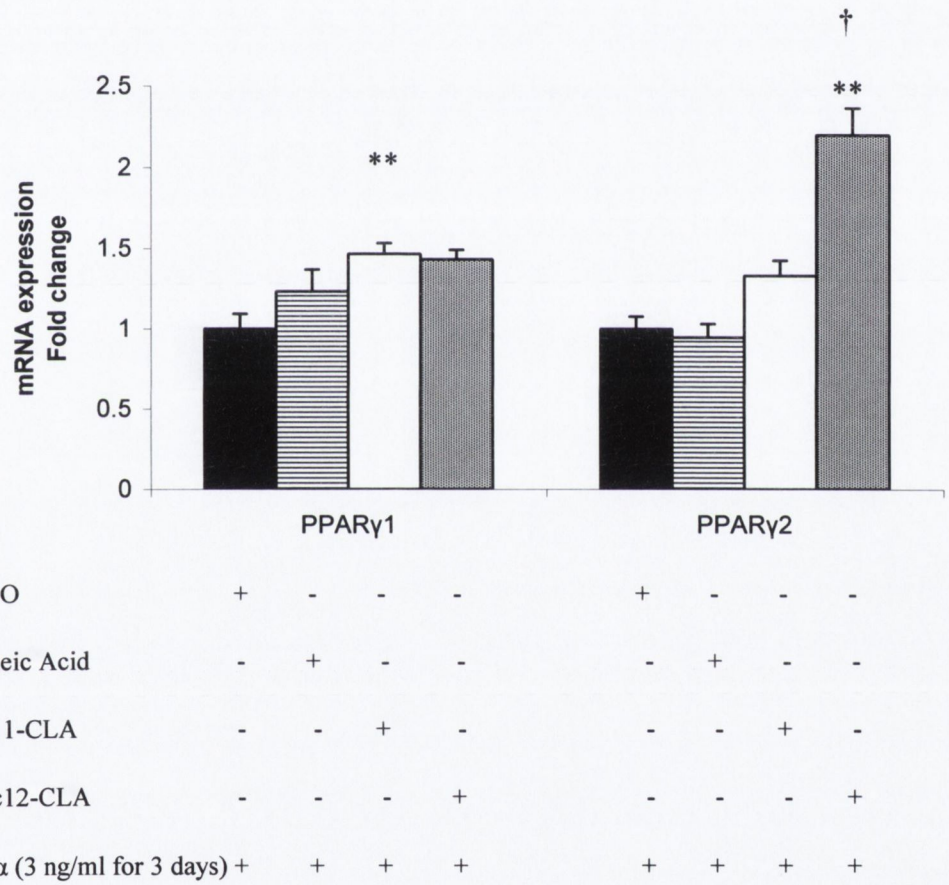
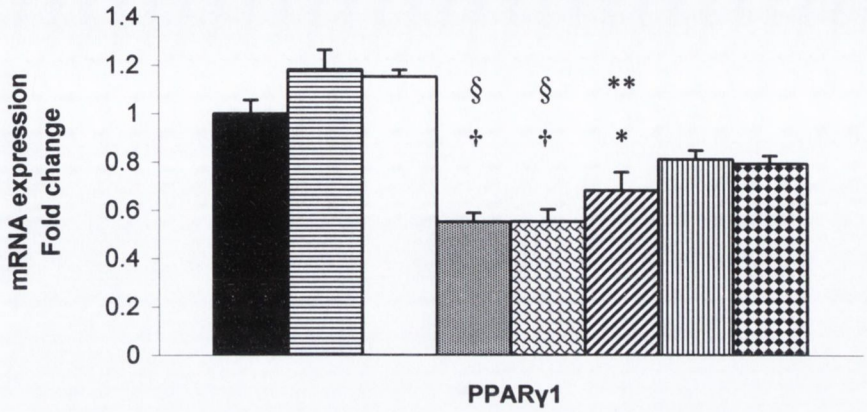


Fig. 5.18: Effect of chronic treatment of linoleic acid, c9,t11-CLA and t10,c12-CLA on PPARγ1 and PPARγ2 mRNA expression in insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. For PPARγ1, ** $P<0.01$ compared to DMSO. For PPARγ2, † $P<0.001$ compared to DMSO and linoleic, ** $P<0.01$ compared to c9, t11-CLA.

Data was expressed to examine the effect of TNFα treatment on adipocyte PPARγ1 expression, and to determine whether fatty acids could modulate the insulin de-sensitising effect of TNFα *(Fig. 5.19)*. The presence of TNFα resulted in 45% reduction in PPARγ1 mRNA ($P<0.001$ comparing untreated and treated DMSO controls). Cells cultured with t10, c12-CLA (in the absence of TNFα) and cells cultured with DMSO (in the presence of TNFα) had significantly lower expression of PPARγ1 compared to cells treated with DMSO, linoleic acid and c9, t11-CLA (cultured in the absence of TNFα) ($P<0.001$ for all) and cells cultured with c9, t11-CLA (in the presence of TNFα) ($P<0.01$). Additionally, cells cultured with linoleic acid (in the presence of TNFα) had significantly lower expression than linoleic acid and c9, t11-CLA (both in the absence of TNFα, $P<0.01$) and cells treated with DMSO (in the absence of TNFα, $P<0.05$). Of note is that the expression of PPARγ1 following treatment with t10, c12-CLA is comparable to cells treated with DMSO (in the presence of TNFα). Interestingly, the expression of PPARγ1 increased in samples cultured with t10, c12-CLA and TNFα compared to untreated t10, c12-CLA samples (1.43 fold increase). All other treatments demonstrated a down-regulation of PPARγ1 following TNFα treatment of similar magnitude (29-44% decrease).

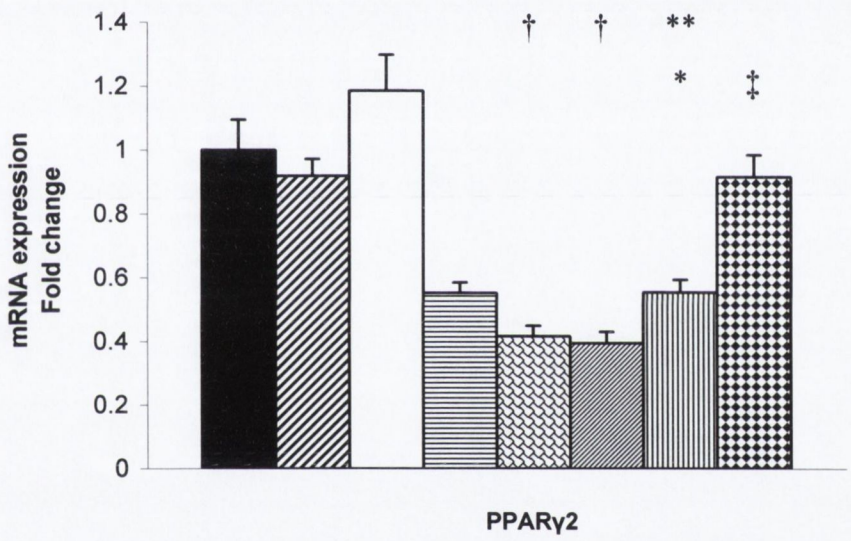


DMSO	+	-	-	-	+	-	-	-
Linoleic Acid	-	+	-	-	-	+	-	-
c9, t11-CLA	-	-	+	-	-	-	+	-
t10, c12-CLA	-	-	-	+	-	-	-	+
TNFα (3 ng/ml for 3 days)	-	-	-	-	+	+	+	+

Fig. 5.19: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on PPARγ1 mRNA expression in normal and insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. § $P<0.001$ compared to DMSO, linoleic acid and c9, t11-CLA (all in the absence of TNFα), † $P<0.05$ compared to c9, t11-CLA (in the presence of TNFα), ** $P<0.01$ compared to linoleic acid and c9, t11-CLA (both in the absence of TNFα), * $P<0.05$ compared to DMSO (in the absence of TNFα).

In addition data was expressed to examine the effect of TNFα treatment on adipocyte PPARγ2 expression, and to determine whether fatty acids could modulate the insulin de-sensitising effect of TNFα *(Fig. 5.20)*. The presence of TNFα resulted in 58% reduction in PPARγ2 mRNA ($P<0.001$ comparing untreated and treated DMSO controls). Cells cultured with DMSO, linoleic acid or c9, t11-CLA (in the presence of TNFα) had significantly lower expression of PPARγ2 compared to untreated cells: $P<0.001$ for DMSO and linoleic acid (cultured in the presence of TNFα) compared to DMSO, linoleic acid and c9, t11-CLA (cultured in the absence of TNFα); $P<0.01$ for c9, t11-CLA (cultured in the presence of TNFα) compared to c9, t11-CLA cultured in the absence of TNFα, and $P < 0.05$ compared to DMSO

(cultured in the absence of TNFα). There was a trend towards higher expression of PPARγ2 in samples treated with c9, t11-CLA compared to samples treated with t10, c12-CLA (both in the absence of TNFα) (P=0.062). Of note is that the expression of PPARγ2 following treatment with t10, c12-CLA is quiet similar to the samples treated with TNFα *(Fig 5.20)*. Interestingly, the expression of PPARγ2 increased in samples cultured with t10, c12-CLA and TNFα compared to untreated t10, c12-CLA samples (1.65 fold). All other treatments demonstrated a down-regulation of PPARγ2 following TNFα treatment of similar magnitude (53-58% decrease). Samples treated with t10, c12-CLA and TNFα had significantly higher expression of PPARγ2 compared to samples treated with DMSO or linoleic acid (both in the presence of TNFα) (P<0.01).



DMSO	+	-	-	-	+	-	-	-
Linoleic Acid	-	+	-	-	-	+	-	-
c9, t11-CLA	-	-	+	-	-	-	+	-
t10, c12-CLA	-	-	-	+	-	-	-	+
TNFα (3 ng/ml for 3 days)	-	-	-	-	+	+	+	+

Fig. 5.20: Effect of chronic treatment of linoleic acid, c9, t11-CLA and t10, c12-CLA on PPARγ2 mRNA expression in normal and insulin resistant 3T3-L1 adipocytes. Target gene mRNA levels were normalised to GAPDH and expressed relative to DMSO. Values represent group means ± SEM of 6 independent experiments. † P<0.001 compared to DMSO, linoleic and c9, t11-CLA (all in the absence of TNFα), ** P<0.01 compared to c9, t11-CLA (in the absence of TNFα), * P<0.05 compared to DMSO (in the absence of TNFα), ‡ P<0.01 compared to DMSO and linoleic acid (both in the presence of TNFα).

(iv) *In vivo* effect of TNFα administration on the expression of genes regulating insulin sensitivity and glucose homeostasis in white adipose tissue

Intraperitoneal injection of mice with recombinant TNFα (75 μg/kg) had a significant impact on gene expression in white adipose tissue *(Fig. 5.21)*. As expected, there was massive induction of TNFα in the mice that received the TNFα injection (15 fold) compared to controls. Although statistical analysis was not performed due to the small number of samples (n=2 in the control group, n=4 in the group who received the TNFα injection) results show a 60% decrease in IRS-1 mRNA, and a 30% decrease in IRS-2 mRNA following the TNFα load. Glut1 and Glut4 expression were decreased by 30% and 60% respectively. In addition, PPARγ1 and PPARγ2 mRNA were reduced by 70% and 90%, respectively.

Fig. 5.21: *In vivo* effect of TNFα administration on the expression of genes regulating insulin sensitivity and glucose homeostasis in white adipose tissue. mRNA levels are normalised to GAPDH and expressed relative to the control group. Values represent group means ± SEM with n=2 in the control group and n=4 in the TNFα group. Statistical analysis was not performed due to the small sample size.

5.4.4 Western Blotting

(i) Glut4 protein expression following chronic treatment of fatty acids

Glut4 expression was investigated in total cell lysates of 3T3-L1's to determine whether fatty acids could alter the expression of the protein. Results indicated no difference in the level of expression of Glut4 following DMSO, linoleic acid, c9, t10-CLA or t10, c12-CLA treatment in normal adipocytes *(Fig. 5.22)*.

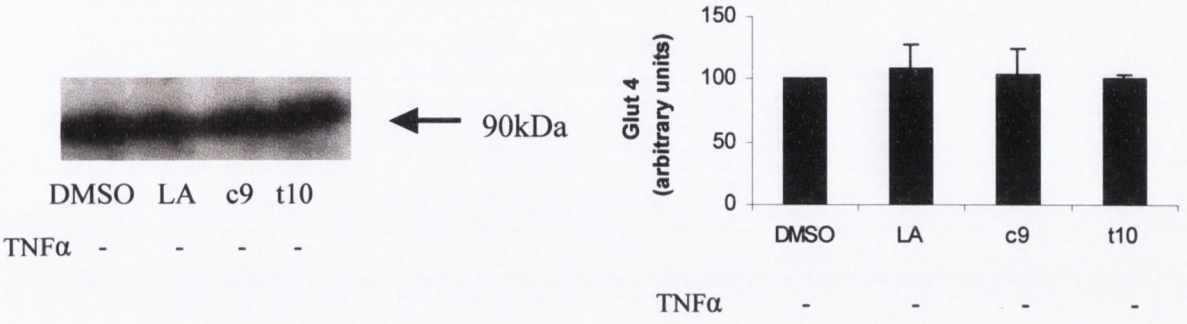


Fig. 5.22: Glut4 protein expression in normal adipocytes. Total cell lysates from control (DMSO), linoleic acid (LA), c9, t11-CLA (c9) and t10, c12-CLA (t10) treated samples (n=4 per treatment). Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. No significant differences were observed.

Additionally, it was investigated whether fatty acids can modulate the inflammatory, insulin de-sensitising effect of the pro-inflammatory cytokine, TNFα. No fatty acid treatment effect was observed in insulin resistant adipocytes *(Fig. 5.23)*.

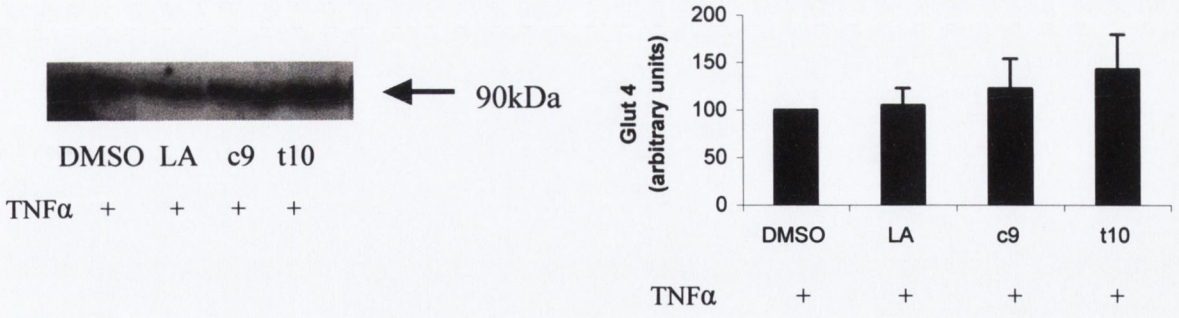


Fig. 5.23: Glut4 protein expression in insulin resistant adipocytes. Total cell lysates from control (DMSO), linoleic acid (LA), c9, t11-CLA (c9) and t10, c12-CLA (t10) treated samples (n=4 per treatment). Insulin resistance was induced in adipocytes by incubating the differentiated adipocytes for 3 days in the presence of 3 ng/ml TNFα. Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. No significant differences were observed.

All data was combined to examine the effect of TNFα treatment on adipocyte Glut4 expression i.e. combining all samples treated in the presence or the absence of TNFα. Treatment of adipocytes with 3 ng/ml TNFα for 3 days resulted in a 48% reduction in the expression of the protein ($P<0.05$ comparing treated and untreated DMSO samples). No fatty acid treatment effect was observed *(Fig. 5.24)*.

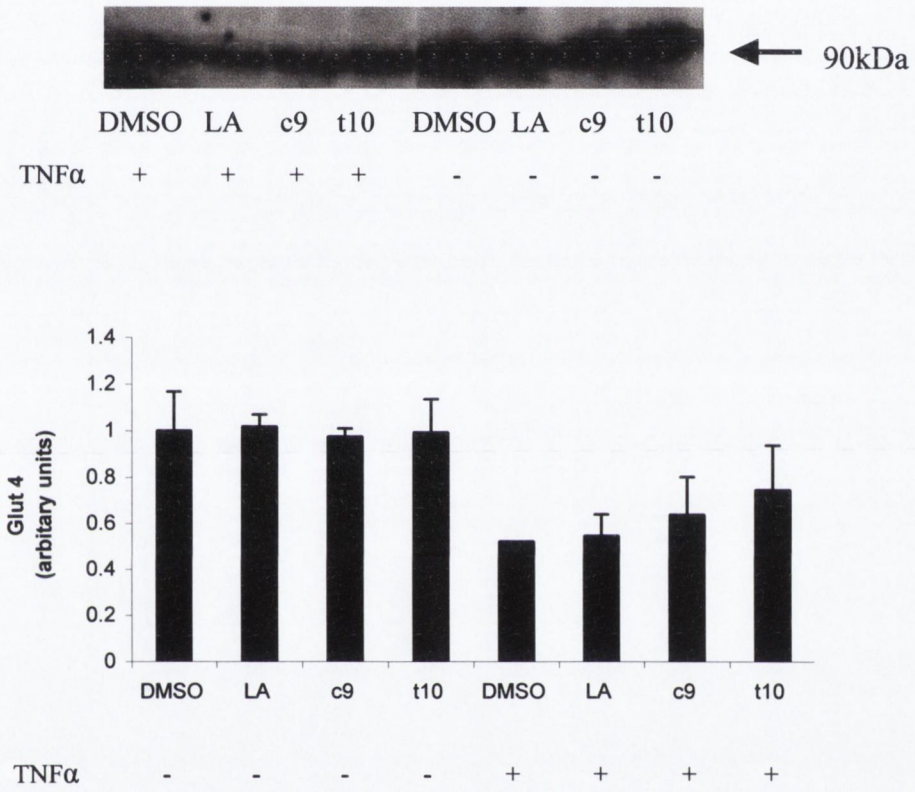


Fig. 5.24: Glut4 protein expression in normal and insulin resistant adipocytes. Total cell lysates from control (DMSO), linoleic acid (LA), c9, t11-CLA (c9) and t10, c12-CLA (t10) treated samples, in the presence or absence of 3 ng/ml TNFα for 3 days (n=4 per treatment). Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. No significant differences were observed.

(ii) IκBα expression following chronic treatment of fatty acids

IκBα expression was also examined in total cell lysates following 50μM fatty acid treatment. In normal adipocytes, there was no difference in the expression of IκBα following the different treatments *(Fig. 5.25)*.

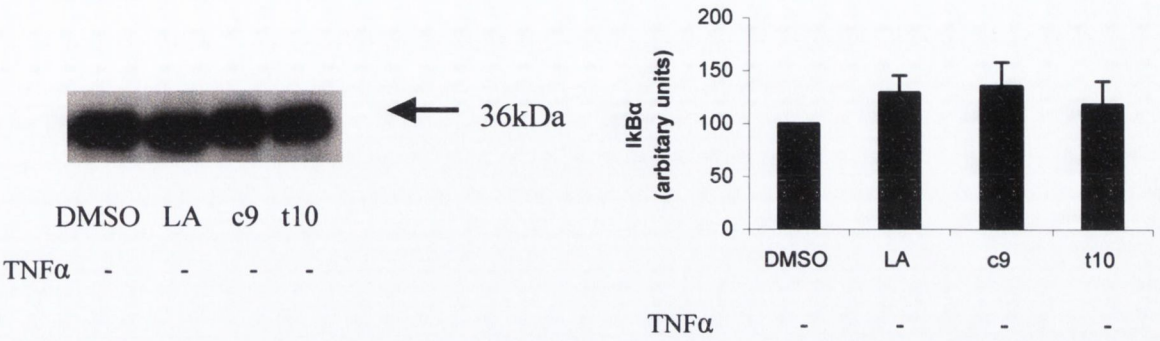


Fig. 5.25: IκBα expression in normal adipocytes. Total cell lysates from control (DMSO), linoleic acid (LA), c9, t11-CLA (c9) and t10, c12-CLA (t10) treated samples (n=4 per treatment). Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. No significant differences were observed.

In insulin adipocytes, a trend was observed towards higher expression of IκBα following c9, t11-CLA treatment (1.8 fold increase in expression, P=0.085) *(Fig. 5.26)*.

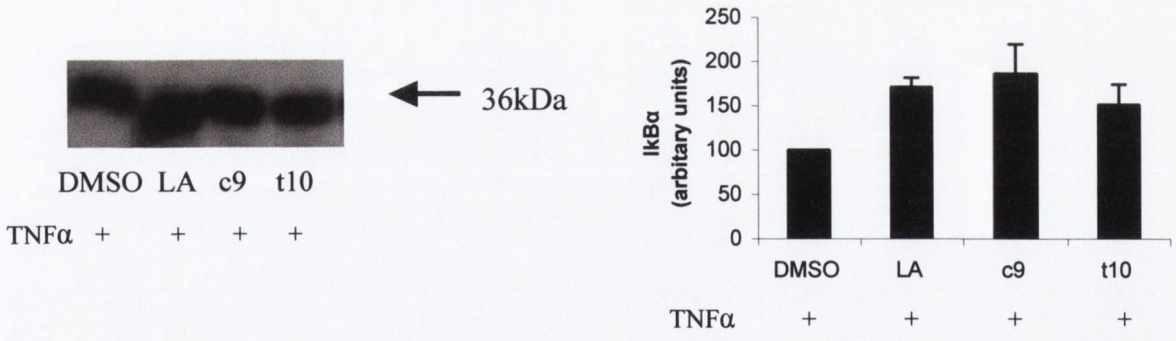


Fig. 5.26: IκBα expression in insulin resistant adipocytes. Total cell lysates from control (DMSO), linoleic acid (LA), c9, t11-CLA (c9) and t10, c12-CLA (t10) treated samples (n=4 per treatment). Insulin resistance was induced in adipocytes by incubating the differentiated adipocytes for 3 days in the presence of 3 ng/ml TNFα. Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. A trend was observed towards higher expression of IκBα following c9, t11-CLA treatment (P=0.085).

All data was combined to examine the effect of TNFα treatment on adipocyte IκBα expression i.e. combining all samples treated in the presence or the absence of TNFα. Treatment of

adipocytes with 3ng/ml TNFα for 3 days resulted in a 55% reduction in the expression of the protein ($P<0.05$, comparing treated and untreated DMSO samples). All samples cultured in the absence of TNFα had significantly higher expression of IκBα compared to adipocytes cultured in the presence of DMSO and TNFα ($P<0.001$ for linoleic acid, c9, t11-CLA and t10, c12-CLA; $P<0.01$ for DMSO). In addition, adipocytes cultured with c9, t11-CLA or linoleic acid (in the presence of TNFα) had significantly higher IκBα expression than adipocytes cultured in the presence of DMSO and TNFα ($P<0.01$ for both) *(Fig. 5.27)*.

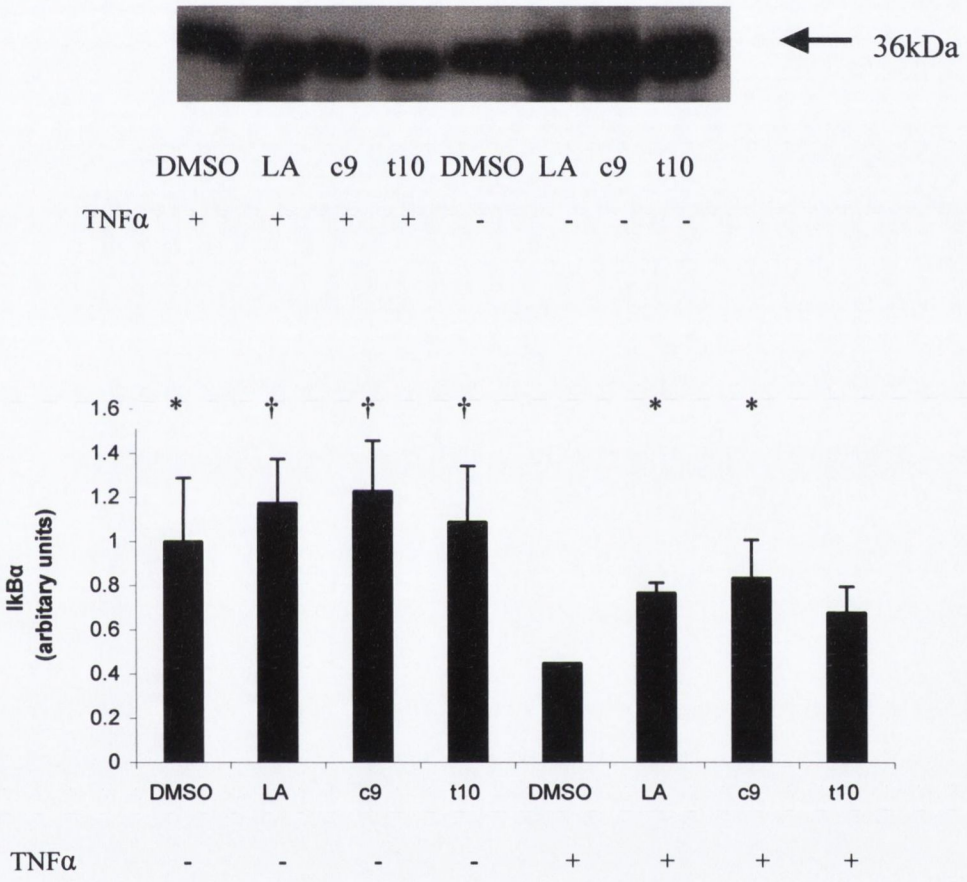
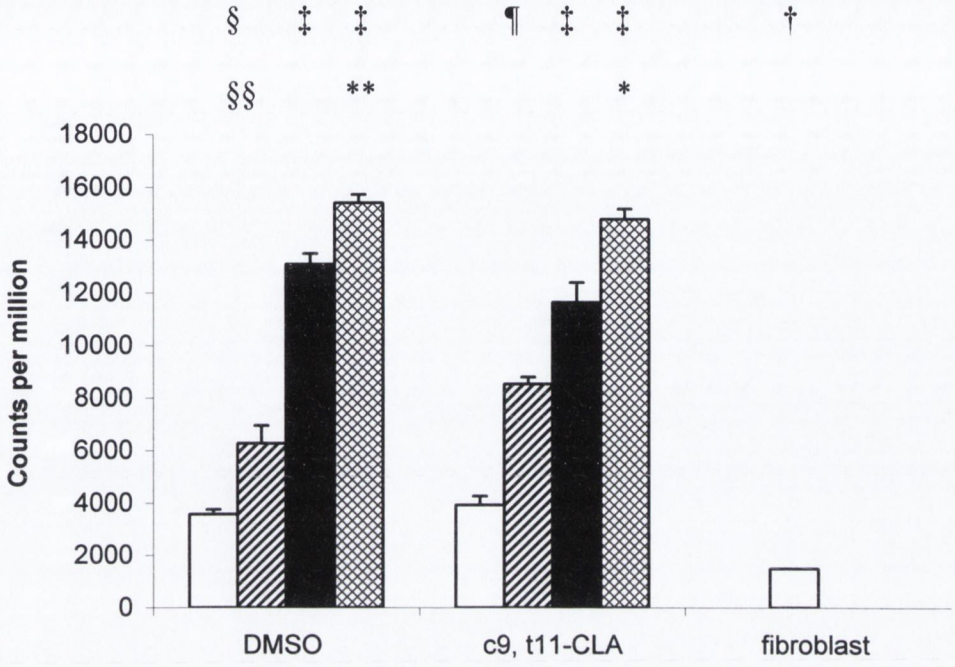


Fig. 5.27: IκBα expression in normal and insulin resistant adipocytes. Total cell lysates from control (DMSO), linoleic acid (LA), c9, t11-CLA (c9) and t10, c12-CLA (t10) treated samples, in the presence or absence of 3 ng/ml TNFα for 3 days (n=4 per treatment). Representative blots are displayed. Histograms from densitometric analysis expressed as arbitrary units are means ± SEM. Significantly different from DMSO control (in the presence of TNFα) † $P<0.001$, * $P<0.01$.

5.4.5 Glucose uptake assay

A glucose uptake assay was performed in normal adipocytes and adipocytes made insulin resistant following TNFα treatment (3 ng/ml for 3 days). These results are displayed in *Fig. 5.28* and *Fig. 5.29* respectively. Basal glucose uptake refers to glucose uptake in the absence of an insulin stimulus. Initially data was assessed to determine whether statistical differences existed between groups treated with the same dose of insulin. There was a trend towards higher glucose uptake in cells cultured with c9, t11-CLA and 0.1nM insulin compared to cells treated with DMSO and an equivalent concentration of insulin ($P=0.0847$). No other group differences were detected. Glucose uptake was significantly higher in all cells cultured with either DMSO or c9, t11-CLA compared to fibroblasts ($P<0.001$).

Data was combined to determine whether there were differences depending on the dose of insulin administered. Insulin treatment resulted in a dose-responsive increase in glucose uptake in all cells compared to glucose uptake under basal conditions. The specific differences detected are outlined. Glucose uptake was significantly higher in both groups of adipocytes treated with 0.1 nM insulin compared to basal glucose uptake ($P<0.001$ for cells cultured with c9, t11-CLA plus 0.1nM insulin compared to basal glucose uptake in cells cultured with DMSO or c9, t11-CLA; $P<0.01$ for cells cultured with DMSO and 0.1nM insulin compared to basal glucose uptake in cells treated with DMSO and $P<0.05$ compared to basal glucose uptake in cells treated with c9, t11-CLA.). Treatment of cells (either DMSO or c9, t11-CLA) with either 1 nM or 10 nM insulin resulted in significantly higher glucose uptake compared to basal glucose uptake in cells treated with c9, t11-CLA or DMSO ($P<0.001$ for all) and glucose uptake in cells treated with DMSO and 0.1 nM insulin ($P<0.001$ for all). Treatment of cells with 10 nM insulin and either DMSO or c9, t11-CLA resulted in significantly higher glucose uptake compared to cells treated with c9, t11-CLA plus 0.1nM insulin ($P<0.01$ for cells cultured with DMSO and 10 nM insulin, $P<0.05$ for cells cultured with c9, t11-CLA and 10 nM insulin). Glucose uptake following treatment with 1.0 nM insulin and either DMSO or c9, t11-CLA was not significantly higher than glucose uptake in cells cultured with c9, t11-CLA and 0.1 nM insulin.

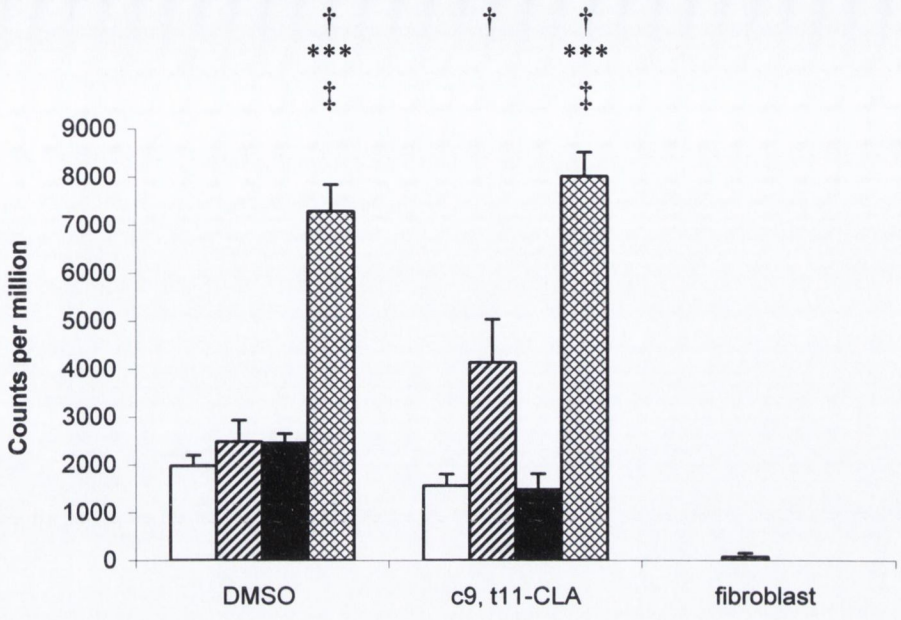


Insulin (nM)									
0	+	-	-	-	+	-	-	-	-
0.1	-	+	-	-	-	+	-	-	-
1	-	-	+	-	-	-	+	-	-
10	-	-	-	+	-	-	-	+	
TNFα (3 ng/ml for 3 days)	-	-	-	-	-	-	-	-	-

Fig. 5.28: Glucose uptake in normal adipocytes cultured with c9, t11-CLA or DMSO (chronic treatment). Values represent group means ± SEM of 4 independent experiments. † $P<0.001$ compared to all other treatments. ‡ $P<0.001$ compared to DMSO and c9, t11-CLA (no insulin), DMSO (0.1nM insulin). ** $P<0.01$ compared c9, t11-CLA (0.1nM insulin). * $P<0.05$ compared c9, t11-CLA (0.1nM insulin). ¶ $P<0.001$ compared to DMSO and c9, t11-CLA (no insulin). §§ $P<0.01$ compared to DMSO (no insulin). § $P<0.05$ compared to c9, t11-CLA (no insulin).

Results of the glucose uptake assay in insulin resistant adipocytes is displayed in *Fig. 5.29*. In insulin resistant cells there was a trend towards higher basal glucose uptake and glucose uptake after 1.0 nM insulin in cells treated with DMSO compared to cells cultured with c9, t11-CLA ($P=0.0535$ and $P=0.0893$, respectively). No group differences were observed following 0.1nM or 10nM insulin. Glucose uptake was significantly higher in cells treated with DMSO or c9, t11-CLA and 10 nM insulin, and cells treated with c9, t11-CLA and 0.1nM insulin compared to glucose uptake in fibroblasts.

Data was combined to determine whether there were differences depending on the dose of insulin administered. Glucose uptake was significantly higher in cells cultured with DMSO or c9, t11-CLA and 10 nM insulin compared to basal glucose uptake in cells treated with DMSO or c9, t11-CLA ($P<0.001$ for both), glucose uptake in cells treated with DMSO and 0.1 nM insulin ($P<0.01$) or 1.0 nM insulin ($P<0.01$), and glucose uptake in cells treated with c9, t11-CLA and 1.0nM insulin ($P<0.001$). Glucose uptake did not differ significantly between cells treated with c9, t11-CLA and 0.1 nM insulin and cells treated with 10 nM insulin.



Insulin (nM)									
0	+	-	-	-	+	-	-	-	-
0.1	-	+	-	-	-	+	-	-	-
1	-	-	+	-	-	-	+	-	-
10	-	-	-	+	-	-	-	+	
TNFα (3ng/ml for 3 days)	-	-	-	-	-	-	-	-	-

Fig. 5.29: Glucose uptake in insulin resistant adipocytes cultured with c9, t11-CLA or DMSO (chronic treatment). Values represent group means ± SEM of 4 independent experiments. † $P<0.001$ compared to fibroblast. *** $P<0.001$ compared to DMSO (no insulin), c9, t11-CLA (no insulin) and c9, t11-CLA (1.0nM insulin). ‡ $P<0.01$ compared to DMSO (0.1nM insulin) and DMSO (1nM insulin).

5.5 DISCUSSION

In this study two experimental conditions were examined. Firstly fatty acids were added in parallel with the differentiation media to test whether chronic treatment with the fatty acids is effective in altering the metabolic and molecular markers. In addition fatty acids were added following full differentiation of the cells to an adipocyte phenotype, to test whether acute treatment of fatty acids is effective. Experiments were performed in normal adipocytes, and adipocytes made insulin resistant cells following treatment with recombinant murine TNFα. Several groups have shown that TNFα treatment causes insulin resistance in 3T3-L1 adipocytes and specifically down-regulates many adipocyte-abundant genes that are critical for insulin responsiveness (Ruan *et al*, 2002b; Hotamisligil, 2003; Ruan *et al*, 2003). The results of the present study show a significant down-regulation of both IRS-1 and Glut4 mRNA following TNFα administration. In both experimental conditions TNFα treatment in vehicle control cells lowered expression levels to a similar extent: IRS-1 and Glut4 were lowered by 58% and 89% respectively following chronic fatty acid treatment, and by 61% and 91% respectively following acute fatty acid treatment. This was comparable with the 60% reduction in both IRS-1 expression and Glut4 expression observed *in vivo* following administration of recombinant TNFα to mice. Although other studies have investigated the impact of CLA on TNFα gene expression and plasma concentrations in animals models of insulin resistance (Tsuboyama-Kasaoka *et al,* 2000; Ohashi *et al,* 2004; Choi *et al,* 2004; Wargent *et al,* 2005; Roche *et al;* 2002), this is the first study to examine the direct effects of CLA on markers of insulin sensitivity in an *in vitro* TNFα induced insulin resistant state.

The timing of fatty acid treatment had an important effect on the mRNA expression of both IRS-1 and Glut4. Examination of the c9, t11-CLA isomer was of particular interest as feeding this isomer to ob/ob mice resulted in a significant reduction in plasma glucose, insulin and TAG concentrations (chapter 4). Results from gene expression analysis indicated a potential positive effect of c9, t11-CLA in murine adipocytes. Chronic treatment with c9, t11-CLA in both normal adipocytes and in insulin resistant adipocytes resulted in increased expression of both IRS-1 and Glut4 mRNA. In normal adipocytes Glut4 expression was significantly increased compared to both linoleic acid, t10, c12-CLA and DMSO treated samples. IRS-1 mRNA expression was significantly higher compared to samples treated with t10, c12-CLA and there was a trend towards higher mRNA expression compared to samples treated with DMSO. In insulin resistant adipocytes, Glut4 expression was significantly increased compared to DMSO and IRS-1 expression was significantly higher compared to linoleic acid. Thus the mRNA expression of two key components of the insulin signalling cascade are up-regulated in both normal and insulin resistant adipocytes. Data from normal and insulin resistant adipocytes was merged to examine the effect of the fatty acids following

TNFα treatment. Although c9, t11-CLA increased the expression of IRS-1 and Glut4 in the insulin resistant state, the positive effect of c9, t11-CLA was not sufficient to exert a significant rescuing effect from the down-regulation in expression caused by TNFα treatment. Acute treatment of 3T3-L1s with c9, t11-CLA did not significantly alter the expression of IRS-1 or Glut4 mRNA in either normal adipocytes or those which are insulin resistant, indicating that long-term treatment with c9, t11-CLA is necessary to induce a positive effect. Alternatively a priming effect may be required prior to treatment with TNFα. We may assume that the altered expression levels are independent of an effect on differentiation as studies have indicated that c9, t11-CLA does not alter differentiation of adipocytes (Brown *et al*, 2001a; Brown *et al*, 2001b; Brown *et al*, 2004). In support of this, c9, t11-CLA had no effect on lipid accumulation (as observed after ORO staining) either at the end of the differentiation period or at the end of the experimental period (in the presence or absence of TNFα treatment). Additionally cytotoxicity was judged to be negligible at a concentration of 50 uM (evidence from MTS data).

Cell treated with linoleic acid served as fatty acid controls to determine if the effects of CLA are unique to its geometric structure or a generic effect of unsaturated fatty acids (Evans *et al*, 2000). Of interest is that the expression of IRS-1 or Glut4 was not significantly higher than the vehicle control or the 9, t11-CLA isomer following chronic treatment in normal or insulin resistant adipocytes *(Fig. 5.9* and *Fig 5.10)*, which may suggest a specific effect of c9, t11-CLA on these markers rather than a generic fatty acid effect.

Evidence has emerged that the t10, c12-CLA isomer is pro-diabetic (Roche *et al*, 2002; Riserus *et al*, 2002a), and the isomer specific effects of t10, c12-CLA were also apparent in this study. Following chronic treatment of t10, c12-CLA in normal adipocytes, IRS-1 expression was significantly lower than all other treatments, and Glut4 expression was significantly lower compared to cells treated with c9, t11-CLA. Both normal adipocytes and insulin resistant adipocytes, which received an acute treatment of t10, c12-CLA exhibited lower expression levels of IRS-1 and Glut4. When data from normal and insulin resistant adipocytes was merged, the negative effect of acute or chronic treatment of t10, c12-CLA in normal adipocytes was more pronounced as the expression levels of IRS-1 and Glut4 were similar to what was observed following TNFα treatment in other cells. Interestingly however, chronic treatment of t10, c12-CLA in insulin resistant adipocytes resulted in expression levels of IRS-1 and Glut4 which were comparable to the vehicle control.

Both t10, c12-CLA and TNFα exerted a negative effect on both IRS-1 and Glut4 expression. Striking similarities have been reported between the effects of TNFα and t10, c12-CLA in murine and human adipocytes. *In vitro* TNFα has been shown to induce apotosis both in mature adipocytes and in preadipocytes (Prins *et al*, 1997), with omental

preadipocytes more sensitive to this effect than subcutaneous preadipocytes (Niesler *et al*, 1998). TNFα also blocks the differentiation of new adipocytes and induces the de-differentiation of both preadipocytes and mature adipocytes (Pekala et al, 1983; Torti et al, 1989; Petruschke & Hauner, 1993). This may be mediated by repression of PPARγ gene expression (Xing *et al*, 1997). TNFα also increases lipolysis (Sumida *et al*, 1997) and inhibits lipogenesis (Feingold & Grunfeld, 1992), thereby increasing plasma NEFA concentrations. Consequently it has been hypothesised that TNFα functions to limit white adipose tissue expansion, and that insulin resistance and the concomitant disturbances in glucose and lipid metabolism is a consequence of that. ORO staining was performed at the end of the experimental period to check whether TNFα induced de-differentiation of the adipocytes. Our results indicate that although lipid accumulation was reduced following TNFα treatment, this was only significantly different to the control group when cells were also cultured in the presence of t10, c12-CLA. Cell viability was not reduced in control cells cultured with TNFα, in the absence of either vehicle control or fatty acid. However, treatment with TNFα and DMSO or any fatty acid (either for acute or chronic treatment) significantly lowered cell viability compared to untreated control cells.

Both animal feeding studies and *in vitro* work in 3T3-L1 preadipocytes and in human primary cultures of differentiating preadipocytes, have provided evidence that t10, c12-CLA is responsible for the observed reductions in body fat and attenuation in the accumulation of TAG associated with CLA intervention (Keim, 2003; Evans *et al*, 2002a; Evans *et al*, 2000; Brown *et al*, 2001a; Brown *et al*, 2001b). Results from ORO staining were in agreement with this observation. At the end of the differentiation period absorbance values were lower following treatment with t10, c12-CLA, indicating reduced lipid accumulation, although this was not statistically significant. However at the end of the 18-day experimental period, adipocytes which were treated with t10, c12-CLA had significantly lower lipid accumulation compared to the other treatments. The addition of TNFα reduced lipid accumulation further. Several potential mechanisms of action have been proposed to explain how t10, c12-CLA decreases TAG content in adipocytes, many of which overlap with those previously mentioned for TNFα (Pariza *et al*, 2000) including reduced lipoprotein lipase activity (Park *et al*, 1999; Lin *et al*, 2001), reduced glucose and fatty acid uptake and *de novo* lipogenesis (Brown *et al*, 2003), reduced stearoyl Co-A desaturase (SCD)-1 expression and activity (Choi *et al*, 2000), increased lipolysis (Park *et al*, 1999), increased fatty acid oxidation (Evans *et al*, 2002b), interference with the preadipocyte differentiation programme (Brown *et al*, 2001b; Evans *et al*, 2000; Kang *et al*, 2003) and induced apotosis (Evans *et al*, 2000). These are isomer specific effects associated with the t10, c12-CLA isomer but not with c9, t11-CLA. As indicated previously lipid accumulation was not reduced after either acute or chronic treatment with

c9, t11-CLA in normal adipocytes or in insulin resistant adipocytes. Inhibition of glucose and lipid uptake into the adipocyte by t10, c12-CLA and subsequent elevated plasma NEFA concentrations and lipid accumulation in non-adipose tissue has been proposed as a potential mechanism to explain how t10, c12-CLA mediates a reduction in whole-body insulin sensitivity (Brown & McIntosh, 2003). Treatment with t10, c12-CLA had no effect on cell viability in normal adipocytes. However chronic treatment with t10, c12-CLA in the presence of TNFα significantly lowed cell viability compared to some treatments.

Since the positive effect of c9, t11-CLA was only observed following chronic treatment of fatty acids, further investigations were only performed in this sub-section of experiments. PPARγ was of interest since it is a key transcription factor which influences insulin sensitivity. The expression of both PPARγ1 and PPARγ2 was examined as they encode for two different protein, with PPARγ2 producing a protein with an additional 30 amino acids at the NH_2-terminus compared to PPARγ1 (Kintscher & Law, 2005). PPARγ2 is the more potent adipogenic isoform (Chawla *et al*, 1994), and the ligand-dependent activation domain of PPARγ2 has been reported to be 5-10 fold more efficient than PPARγ1 (Wang & Tafuri, 2003). Previous reports have indicated that treatment with TNFα attenuates the expression of PPARγ (Xing *et al*, 1997; Ruan *et al*, 2002a; Ruan *et al*, 2002b), with most reports focusing on the examination of the PPARγ2 isoform (Kudo *et al*, 2004; Rosenbaum & Greenberg, 1998; Zhang *et al*, 1996; Ajuwon & Spurlock, 2005). *In vivo* we showed that TNFα reduced PPARγ1 expression by 70%, and PPARγ2 expression by 90%. *In vitro*, TNFα treatment down-regulated the expression of both PPARγ1 and PPARγ2 by 45% and 58%, respectively. c9, t11-CLA had minimal effect on the expression of both isoforms in normal adipocytes, with an 18% increase in PPARγ1 mRNA and a 15% increase in PPARγ2 mRNA levels compared to vehicle control cells. In insulin resistant adipocytes the expression of PPARγ1 mRNA was 46% higher and PPARγ2 mRNA was 30% higher compared to DMSO vehicle control cells. However statistical significance was only reached in insulin resistant adipocytes. This increased expression of PPARγ, albeit small, may have contributed to the up-regulation of IRS-1 and Glut4 mRNA.

It has been suggested that down-regulation of PPARγ expression and/or activity by t10, c12-CLA decreases both lipogenesis and adipogenesis (Brown & McIntosh, 2003; Granlund *et al*, 2003; Kang *et al*, 2003) by down-regulating PPARγ responsive/target genes including fatty acid binding protein (aP2), acyl-CoA binding protein, LPL and Glut4 (Brown & McIntosh, 2003; Brown *et al*, 2003; Granlund *et al*, 2003). In normal adipocytes both PPARγ1 and PPARγ2 expression was significantly lower following t10, c12-CLA treatment compared to all other treatments, and this was coupled with reduced expression of IRS-1 and Glut4. Of interest is the altered expression profile in insulin resistant adipocytes, in which the expression of both PPARγ isoforms was significantly higher, with that of PPARγ2 being

especially pronounced. In addition the negative effects of TNFα on PPARγ2 expression were reversed by t10, c12-CLA. The reason for this is unclear, but it may explain at least in part, the increase in expression of IRS-1 and Glut4 observed in these cells. Other experiments have shown a decrease in PPARγ expression following t10, c12-CLA, but the effect of this isomer has not been investigated in an insulin resistant state *in vitro*. The increase in IRS-1 and Glut4 mRNA may represent gene specific effects as lipid accumulation was significantly lower in cells cultured with both t10, c12-CLA and TNFα, perhaps indicating that other lipogenic and adipogenic genes were not up-regulated simultaneously or translated into a functional protein.

There is conflicting evidence regarding the influence of CLA on PPARγ2 expression based on the specific type and amount of isomer used and the tissue site of action. Future studies measuring the impact of specific CLA isomers on PPARγ1 and PPARγ2 activity and down-stream effectors in preadipocytes and mature adipocytes will provide greater insight into CLA's potential mechanism of action (Brown *et al*, 2001a; Brown *et al*, 2003).

On the basis of the improvement in inflammatory markers in the previous set of experiments outlined in chapter 4, the protein expression of the inhibitory subunit of nuclear factor-κB (NF-κB) termed inhibitor of κB (IκBα) was investigated. In unstimulated cells, NK-κB is maintained in an inactive form in the cytoplasm through interaction with IκB (Schmitz *et al*, 2001). TNFα activates the IκB kinase (IKK) complex leading to phosphorylation and subsequent ubiquitination and proteolytic degradation of IκBα (Schmitz *et al*, 2001). This releases the NF-κB dimer which then translocates to the nucleus, binds to its cognate DNA element and activates the transcription of numerous target genes encoding proteins involved with immune or inflammatatory responses (Schmitz *et al*, 2001; Baldwin, 1996). Thus, IκBα is critical to attenuate NK-κB signalling. A fatty acid modulating effect was observed, in particular in insulin resistant adipocytes. Additionally, protein expression of IκBα was slightly higher following treatment with c9, t11-CLA compared to linoleic acid in both normal and insulin resistant adipocytes.

Glut4 protein expression was examined in total cell lysates. TNFα treatment lowered Glut4 protein expression by half. However no fatty acid treatment effect was observed either in normal or in insulin resistant adipocytes. Analysis of Glut4 protein expression was examined in this fraction due to the large number of treatments being performed. Translocation of the Glut4 vesicle to the plasma membrane is necessary for glucose uptake. However isolation of the plasma membrane fraction requires subcellular fractionation and ultracentrifugation steps to be performed (Tamori *et al*, 1998). Alternatively translocation of Glut4 to the plasma membrane may be assessed using the plasma membrane lawn assay (Tamori *et al*, 1998). As a consequence our result informs us that the balance between the production and degradation of Glut4 is the same i.e. there is no net difference between the fatty

acid treatments. However we are limited as we know nothing regarding the distribution of the Glut4 vesicle which may be different between the treatment groups. It is noteworthy that both mRNA and protein expression were measured in the total cellular fraction. While an increase in Glut4 mRNA was observed relative to the other treatments in both normal and insulin resistant adipocytes, there was no difference between any of the treatments with respect to protein expression. The abundance of a gene is determined by the transcriptional rate and mRNA stability or degradation. However there are a number of post-transcriptional mechanisms including mRNA processing and transport which determine whether mRNA is actively translated to produce a protein. Studies examining Glut1 have shown that regulatory elements in the 5' untranslated region (UTR) of the mRNA interacts with certain proteins to control translational efficiency, while those in the 3' UTR bind specific cytosolic proteins and mediate post-transcriptional regulation of Glut1 gene expression. It has been suggested that such mechanisms may occur throughout the Glut family (Hesketh *et al,* 1998). The reason why Glut4 protein expression did not increase despite an increase in Glut4 mRNA following c9, t11-CLA is unknown at present, but presumably is due to one or more post-transcriptional modifications.

Measuring glucose uptake in cells provides the most relevant end point for assessing insulin sensitivity. Consequently a glucose uptake assay was performed to determine whether c9, t11-CLA improves glucose uptake relative to the vehicle control. Glucose uptake increased with increasing concentrations of insulin, with a 5 fold increase observed comparing basal glucose uptake and glucose uptake following 10 nM insulin treatment, indicating that the cells were responsive to insulin. Cells treated with TNFα were less insulin responsive, with glucose uptake following 0.1 nM insulin or 1 nm insulin comparable to basal glucose uptake, thus highlighting the insulin de-sensitising effect of this pro-inflammatory cytokine. Glucose uptake was not significantly higher following treatment with c9, t11-CLA compared to the vehicle control. There are several potential reasons for this. Nutrients and pharmacological treatments may increase the expression of various genes. However if this gene is not translated into a protein which has a functional effect, there is no net outcome effect/measure. By this rational, although treatment with c9, t11-CLA increased both IRS-1 and Glut4 expression in normal and insulin resistant adipocytes, it is possible that this increase in mRNA did produce a functional protein. As discussed above there was no difference in Glut4 protein expression between the treatments despite the fact that mRNA was higher in both normal and insulin resistant adipocytes following c9, t11-CLA treatment. In addition down-regulation of the insulin-signalling cascade via post-translational mechanisms is evident from several studies. Protein tyrosine phosphatases inhibits insulin receptor phosphorylation (Saltiel & Kahn, 2001), Ser/Thr phosphorylation of IRS-1 disrupts the interaction of IRS-1 with the insulin receptor (Aguirre *et al*, 2000; Hotamisligil *et al*, 1996) and furthermore may promote proteasome-

mediated degradation of IRS-1 (Pederson *et al*, 2001). The suppressors of cytokine signalling compete for crucial receptor binding sites (Emanuelli *et al*, 2000) and additionally have been implicated in promoting the ubiquitination and subsequent degradation of IRS-1 by the proteosome (Rui *et al*, 2002). Lipid phosphatases may dephosphorylate phosphatidlyinositol (3,4,5)-triphosphate thereby inhibiting the insulin signal and attenuating insulin stimulated Glut4 translocation to the plasma membrane. A combination of these mechanisms may have prevented increased glucose uptake following treatment with c9, t11-CLA. Alternatively although feeding a c9, t11-CLA enriched diet to ob/ob mice significantly lowered both plasma insulin and glucose concentrations (chapter 4), the lack of stimulatory effect of c9, t11-CLA on glucose uptake in 3T3-L1 adipocytes may simply reflect the differences observed between *in vivo* and *in vitro* situations. Cells may exert different responses to stimuli when they are cultured in isolation to than when they are present in more physiological conditions (Morik, 1991). Alternatively since white adipose tissue depots comprises not only unilocular adipocytes, but also stromal-vascular cells, blood vessels, lymph nodes and nerves (Cinti, 2001; Hausman *et al*, 2001), the impact of c9, t11-CLA on these cell types may have been responsible for the improvement in insulin sensitivity observed *in vivo*. A limiting factor on commencement of the present study was access to a radiation suite to perform the glucose uptake assay. Future *in vitro* work should firstly involve performing a dose response curve to determine if c9, t11-CLA can increase glucose uptake across different fatty acid concentrations in both normal and insulin resistant adipocytes. Additionally time course data should be performed as a lag could exist before c9, t11-CLA improves glucose uptake i.e. glucose uptake was measured in this study following 20 min culture with various doses of insulin, however this length of time could be extended or shortened.

Previous work has focused on investigating mechanisms to understand how t10, c12-CLA attenuates lipid accumulation in adipocytes as outlined above. Consequently there is little information regarding the effect of c9, t11-CLA on insulin sensitivity *in vivo* or *in vitro*. Results from Brown *et al* (2004) failed to see an increase in basal glucose uptake in human stromal vascular cells following 3 days treatment with c9, t11-CLA. Indeed following 100 nM insulin, glucose uptake was significantly lower than vehicle controls treated with the same dose of insulin. Nevertheless gene expression analysis revealed increased mRNA expression levels of PPARγ1, PPARγ2, Glut4 and adiponectin in stromal vascular cells following 9 days treatment with c9, t11-CLA, although statistical significance is not reported. At 3 days when the glucose uptake was performed the expression of these genes was comparable with the vehicle control. Unfortunately glucose uptake was not performed at day 9. Glucose uptake was also measured in human adipocytes following fatty acid treatment for

12 days during differentiation. Results indicated that glucose uptake was comparable following treatment with c9, t11-CLA or linoleic acid (Brown et al, 2003).

In vitro studies use a number of agents as a vehicle to deliver fatty acids including DMSO, ethanol and BSA. *In vivo* fatty acids are transported bound to albumin (Evans *et al*, 2000). Consequently delivery of fatty acids using BSA has been claimed to be more physiological than use of an organic solvent (Evans *et al*, 2000). The method of choice in our laboratory is DMSO, which has also been used as the vehicle to deliver fatty acids in other experiments in 3T3-L1 adipocytes (Brodie *et al,* 1999; Kang *et al,* 2003). Some groups supplement culture media with antioxidants e.g. α-tocopherol when performing *in vitro* work to prevent fatty acid peroxidation. An advantage of DMSO is that it functions as an antioxidant itself (Xing & Remick, 2005).

It is important to note that fatty acids may exert their effects through direct mechanisms or indirectly through the action of a fatty acid metabolite e.g. an eicosanoid created through the arachidonic acid cascade. Future work therefore should also examine the fatty acid composition of cells and cell culture media to determine the incorporation of c9, t11-CLA into the cell membranes and lipid fractions.

In conclusion, chronic treatment with c9, t11-CLA in 3T3-L1 adipocytes resulted in a significant increase in IRS-1 and Glut4 mRNA in both normal adipocytes and those which had been made insulin resistant following treatment with the pro-inflammatory cytokine TNFα. In contrast acute treatment with c9, t11-CLA did not modulate the expression levels of these markers. Basal and insulin-stimulated glucose uptake following chronic treatment with c9, t11-CLA was comparable with the vehicle control. This may indicate a failure to translate the increase in gene expression into functional proteins, or attenuation in protein activity by post-translational modification. Nevertheless feeding a c9, t11-CLA enriched diet to ob/ob mice improved plasma glucose and insulin concentrations (chapter 4). Differences in response to stimuli between *in vivo* and *in vitro* models are evident. Investigation of the impact of c9, t11-CLA on insulin sensitivity and indeed on whole body metabolism in humans in both physiological and pathological situations remains to be of critical importance.

ACKNOWLEDGEMENTS

- Dr. Helen Roche, Nutrigenomics Research Group, Institute Molecular Medicine, Trinity College completed the *in vivo* TNFα experiment.

Chapter Six
General Discussion

Dietary fatty acids play an inherent role in the development of obesity, the metabolic syndrome and type 2 diabetes mellitus (T2DM). Since dietary fats are an enriched source of energy, excess can induce obesity and subsequent insulin resistance. In addition, nutrient derived metabolic stressors including a diet high in saturated fatty acids (SFA) induces insulin resistance by inhibiting insulin signalling (Le Marchand-Brustel *et al*, 2003). Insulin sensitivity may be modulated by altering the composition of the diet. Isoenergetic substitution of monounsaturated fatty acids (MUFA) for SFA has been shown to improve insulin sensitivity in man (Vessby *et al*, 2001; Perez-Jimenez *et al*, 2001). However in view of the limited success of individuals reaching nutrition targets for dietary fat composition, the use of natural food constituents with bioactive properties with a view to forming functional foods represents an attractive strategy to attenuate the clinical consequences of insulin resistance. Conjugated linoleic acid (CLA) offers potential as such an option. A human intervention study, examination of samples following a murine feeding study and *in vitro* investigations were employed to examine the relationship between CLA and insulin sensitivity and the possible contribution as a therapeutic mediator in reducing the risk of other cardiovascular disease (CVD) risk factors. Particular attention was focused on white adipose tissue as tissue specific gene targeting of the glucose transporter (Glut)-4 has revealed the importance of this tissue in whole-body insulin sensitivity. In addition several adipokines secreted from adipose tissue have been shown to exert paracrine and systemic affects and thereby modulate insulin sensitivity.

There is substantial evidence that the isomers of CLA exert distinct physiological effects. The *trans*-10, *cis*-12 CLA (t10, c12-CLA) isomer is responsible for the reduction in body fat in animal feeding studies. However evidence has emerged that this isomer also exerts a pro-diabetic effect in both humans and mice (Roche *et al*, 2002; Riserus *et al*, 2002a). Consequently chapter 5 was designed to examine the effect of the individual isomers with particular interest in determining whether *cis*-9, *trans*-11 CLA (c9, t11-CLA) had any beneficial effect on key markers in the insulin signalling pathway. To achieve this an *in vitro* model was employed whereby the effects of culturing murine 3T3-L1 adipocytes in the presence of exogenous fatty acid was investigated. 3T3-L1 cells are morphologically similar to fibroblastic preadipose cells found in the stroma of adipose tissue, and once differentiated they exhibit virtually all of the characteristics associated with adipocytes present within adipose tissue (Cowherd *et al*, 1999; Novikoff *et al*, 1980). However, a limitation of all *in vitro* investigations is that conditions, although tightly controlled, are often unphysiological in

nature. Therefore *in vitro* observations may not necessarily represent the *in vivo* situation (Morik, 1991). Nevertheless the use of a cell line permits a focused examination of the effects of a treatment in a homogenous population of cells. Insulin resistant adipocytes were formed following treatment with tumour necrosis factor-α (TNFα) and were examined in parallel with normal adipocytes to determine whether the effect of the fatty acids differed according to the metabolic environment. Two culturing conditions were designed: fatty acids were added when differentiation of the fibroblasts to adipocyte was induced to determine whether chronic treatment was effective; in addition, fatty acids were added after differentiation to an adipocyte phenotype to test whether acute treatment was effective. Differentiation of fibroblast to adipocyte involves the uptake of glucose and fatty acids and the accumulation of triacylglycerol (TAG). Consequently differentiation of fibroblasts to adipocytes was checked by Oil Red O staining to determine if the observed effects may be a consequence of altered or impaired differentiation. Cells were cultured with 50 μM fatty acids prepared in dimethyl sulfoxide and fresh fatty acids were added each time the media was changed. Petridou *et al* (2003) reported that the CLA content of the total serum lipid fraction was approximately 50.4 μmol/L, with almost equal concentrations of c9, t11-CLA and *trans*-9, *trans*-11 CLA, and a lesser quantity of t10, c12-CLA. Thus although the individual isomer doses used in the *in vitro* experiments is supraphysiological, they are comparable with the total CLA content in the lipid fraction of fasting plasma. This same concentration has been used in other *in vitro* studies both in the same cell model and in other cell cultures.

The results in this chapter demonstrated that c9, t11-CLA increased the expression of Glut4 and IRS-1 both in normal and insulin resistant adipocytes. Analysis of cells treated with t10, c12-CLA concurred with the previous reports of a negative effect of the isomer, with a reduction in mRNA expression of both Glut4 and insulin receptor substrate (IRS)-1 observed following both acute and chronic treatments. In contrast linoleic acid which is the non-conjugated form of CLA had no effect on the mRNA expression of either protein when compared to the vehicle control. Despite these findings there was no difference between the treatments regarding the protein expression of Glut4. This is of interest as despite alterations in mRNA abundance, the expression of protein was comparable. Characterisation of the post-transcriptional regulation of Glut4 by the individual fatty acids, and in particular the isomers of CLA, warrants investigation as they would appear to have differential effects. Future work should also examine translocation of the Glut4 vesicle to the plasma membrane, as this step is critical to facilitate glucose uptake. This study investigated the effect of the fatty acids in the basal state. In addition future experiments should be performed following insulin stimulation when the expression of Glut4 at the plasma membrane dramatically alters due to a small decrease in internalisation of the transporter and a massive induction in exocytosis from intracellular depots. This net effect facilitates increased glucose uptake. The effect of fatty

acid treatment may differ in the basal state and following insulin stimulation. A glucose uptake assay was performed as part of this set of experiments. c9, t11-CLA had no additive effect compared to the vehicle control at the fatty acid concentration and time-point chosen. However these conditions may need to be further optimised and a time course performed to determine whether a lag-effect exists. Ideally a dose-response study should be performed to establish the optimum concentration of fatty acids in this system. A glucose uptake assay should be employed for determining this as measuring glucose uptake in cells is the most relevant end point for assessing insulin sensitivity *in vitro*. Future work should also examine the fatty acid composition of the cells and cell culture media to determine the incorporation of the fatty acids and their metabolites into the cell membranes and lipid fractions.

In contrast, a beneficial effect of c9, t11-CLA was observed *in vivo* as outlined in chapter four. Plasma glucose, insulin and TAG concentrations were significantly reduced following feeding a c9, t11-CLA enriched diet to ob/ob mice. IRS-1, Glut1 and Glut4 mRNA, were increased, although statistical significance was not attained. Nevertheless the protein expression of the insulin receptor and Glut4 were significantly increased compared to the control diet. The difference in study outcomes between the chapter four and chapter five may simply highlight diversity between *in vivo* and *in vitro* situations. However in comparison to the homeogenous population of cells *in vitro*, adipose tissue comprises not only mature adipocytes but also endothelial cells, sympathetic nerve fibres and poorly-identified cells present in the stromal-vascular fraction, all of which may contribute to adipose tissue function (Hauner, 2005). Reports have demonstrated that obesity is associated with progressive infiltration of monocytes and macrophages into adipose tissue. In addition adipose tissue macrophages are the source of inflammatory pathways that are activated within adipose tissue of obese individuals. This clearly suggests that the non-adipocyte fraction may mediate a significant component of the inflammatory state within adipose tissue (Trayhurn & Wood, 2004; Weisberg *et al*, 2003; Xu *et al*, 2003). Indeed the mRNA expression of several inflammatory mediators was decreased following the c9, t11-CLA enriched diet including the mRNA for interleukin-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, with statistical significance achieved for the 50% reduction in TNF-α mRNA. This attenuation in the mRNA expression levels of inflammatory mediators was associated with a down-regulation in nuclear factor-κB (NF-κB) p65 expression in adipose tissue and NF-κB:DNA binding. Additionally in chapter five, treatment of normal and insulin resistant cells with c9, t11-CLA and to a lesser extent linoleic acid increased the expression of the inhibitor of NF-κB, IκBα. These results indicate that it may be possible to reduce the impact of metabolic stressors by nutrient based anti-inflammatory strategies and thereby ameliorate insulin sensitivity.

Preliminary work has commenced examining the presence of macrophage in sections of white adipose tissue from the ob/ob feeding study. The details of the assay are outlined in Section 2.18. It will be intriguing to investigate whether the attenuation of inflammatory mediator expression and the associated improvement in insulin sensitivity could be due to reduced infiltration of macrophage into adipose tissue in mice fed the c9, t11-CLA enriched diet.

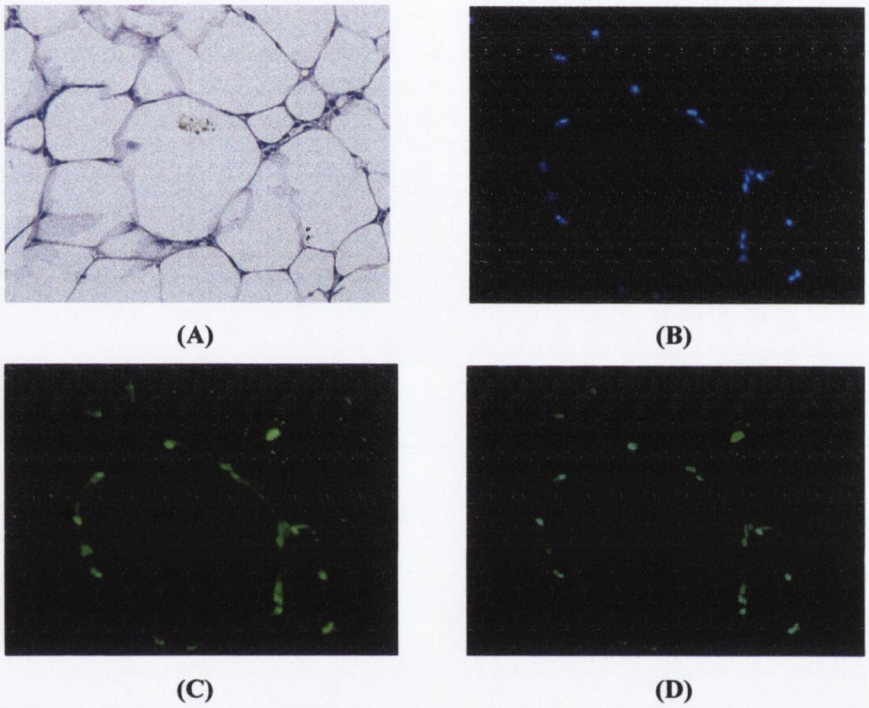


Fig. 5.1: Preliminary work investigating the presence of macrophage in adipose tissue. (A) hematoxylin and eosin staining, (B) nuclear staining using Hoechst stain, (C) detection of activated macropahge using MAC-3 antibody, (D) co-localisation of Hoechst staining and MAC-3 antibody.

Future work could utilise laser-capture microdissection to isolate the macrophages from adipose tissue thereby allowing the examination of cell specific effects.

The original hypothesis was that serine phosphorylation at site 307 of IRS-1 would be reduced following the c9, t11-CLA enriched diet due to the down-regulation in TNFα mRNA, and the observed improvement in insulin sensitivity. Although no effect was observed, other factors uncouple IRS-mediated downstream signals and/or promote IRS degradation including the suppressors of cytokine signalling and 14-3-3, a phosphoserine binding protein. In addition phosphorylation of a number of other serine residues on IRS-1 e.g. 612 and 632, impedes the transduction of the insulin signal. Potentially the favourable effect of c9, t11-CLA on insulin sensitivity involved modulation of one or more of these processes and this deserves

investigation. Alternatively some of the effects may be mediated by up-regulation of the phosphoinositide 3-kinase independent pathway. This was beyond the scope of the current set of experiments.

This *in vivo* investigation was completed in the fasting state. Pertubations in the postprandial state also exist in T2DM. A previous study indicated that c9, t11-CLA had no effect on plasma glucose or insulin concentrations when investigations were completed in the fed state (Roche *et al*, 2002). Future work should examine insulin sensitivity by assessing plasma glucose excursions following a more controlled stimulus by means of both glucose tolerance tests and insulin tolerance tests.

In chapter three the effect of CLA supplementation on CVD risk factors was investigated in subjects with diet-controlled T2DM. Volunteers consumed 3g CLA per day during the dietary intervention study representing approximately 1.5% total energy. This compared well with the animal feeding study in which CLA contributed approximately 1.6% of total energy of the diet. However the isomer blends in the studies differed. Ideally an enriched source of the c9, t11-CLA isomer would have been utilised in the human intervention study as was the case in the animal feeding study. Unfortunately this was not available. Instead a blend comprising equal quantities of c9, t11-CLA and t10, c12-CLA was given. Nevertheless the work is important and yielded interesting results. Other human intervention studies utilising a similar blend of isomers reported that plasma glucose and insulin concentrations and insulin sensitivity were unaltered by the supplement. In some cases positive effects were reported with respect to lipid metabolism and body composition. This study represents the first report of the effects of CLA in subjects with T2DM and it is also the first to demonstrate a negative effect of a 50:50 blend of isomers on insulin sensitivity. While positive effects were observed with respect to both high density lipoprotein concentrations and fibrinogen concentrations, a therapeutic nutrient should not be associated with potentially adverse effects on other clinical markers of T2DM.

These results have important implications. At present there is a plethorea of CLA supplements available for purchase in health food shops and on the internet, comprising a similar blend of isomers to the supplement used in the intervention trial. The accompanying promotional literature claims a variety of health benefits. Cardiovascular disease is characterised by many risk factors. Since the relative merit of one risk factor over another is unknown, any negative effect of a potential therapeutic nutrient must be investigated. The difference observed in the human intervention study compared to other studies may reflect differences in the metabolic status of the volunteers i.e. T2DM compared to normal glucose tolerance or the metabolic syndrome. It is tempting to assume that this negative effect was a consequence of the t10, c12-CLA isomer. However this is impossible to confirm without performing studies using purified isomers. In view of the recent human intervention study

indicating that c9, t11-CLA reduced insulin sensitivity in Caucasian men with abdominal obesity (Riserus *et al*, 2004), the isomer specific effect of CLA supplementation on insulin sensitivity and other CVD risk factors in man requires in-depth investigation. Currently there are attempts to enrich the amount of c9, t11-CLA in foods by altering cattle feeding and also through the potential development of functional foods using probiotic bacteria. Animal studies provide an indication of a potential effect and allow for more in-depth analysis due to the availability of samples. However, differences between species exist so one may not assume that the results obtained from murine and rodent studies are always identical to the effects observed in man.

In conclusion, this series of experiments has yielded many results. Although positive effects on insulin sensitivity were observed in the murine ob/ob model and on lipid metabolism in subjects with T2DM, negative effects were also seen. CLA may not represent the ideal in terms of a therapeutic nutrient, but results support the hypothesis that the composition of the diet has a dramatic impact on metabolic processes. These experiments demonstrate the ability of different fatty acids to exert distinct effects. Linoleic acid, c9, t11-CLA and t10, c12-CLA are all 18-carbon fatty acids and differ only by the position and geometry of their double bonds. Yet in spite of this, distinct metabolic and molecular effects were observed. Fatty acids may exert their effects through multiple mechanisms either by exerting a direct effect or through the production of fatty acid derived metabolites. In addition to modulating gene expression and post-transcriptional processes, alterations in the composition of membrane phospholipids and lipid rafts can alter downstream signalling events. Of particular interest is the ability of fatty acids to modulate inflammatory processes as an increased number of diseases are shown to have an underlying inflammatory component associated with them. However the challenge exists to ensure that any therapy, either nutrient or pharmacological, does not increase the risk of other diseases while attenuating risk factors associated with another. Down-regulation of inflammatory pathways for example should not compromise immune function and defence mechanisms. Therefore it is critical to consider the safety and efficacy of potential therapeutic nutrients on whole body metabolism in both physiological and pathological situations and to examine the underlying mechanisms involved to ensure that consumers are protected.

Chapter Seven

Bibliography

A

Abbasi F & Reaven GM (2002) Evaluation of the quantitative insulin sensitivity check index as an estimate of insulin sensitivity in humans. *Metabolism* **51**, 235-237.

Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, Minnemann T, Shulman GI & Kahn BB (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* **409**, 729-733.

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI & Westphal H (1996) Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nature Genetics* **12**, 106-109.

Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK & O'Rahilly S (1997) Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *Journal of Clinical Investigation* **100**, 3149-3153.

Adlof RO, Duval S & Emken EA (2000) Biosynthesis of conjugated linoleic acid in humans. *Lipids* **35**, 131-135.

Aguilera AA, Diaz GH, Barcelata ML, Guerrero OA & Ros RM (2004) Effects of fish oil on hypertension, plasma lipids, and tumor necrosis factor-alpha in rats with sucrose-induced metabolic syndrome. *Journal of Nutritional Biochemistry* **15**, 350-357.

Aguirre V, Uchida T, Yenush L, Davis R & White MF (2000) The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *Journal of Biological Chemistry* **275**, 9047-9054.

Ajuwon KM & Spurlock ME (2005) Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. *American Journal of Physiology. Regulatory,Integrative and Comparative Physiology* **288**, R1220-R1225.

Akahoshi A, Koba K, Ohkura-Kaku O, Kaneda N, Goto C, Sano H, Iwata T, Yamauchi Y, Tsutsumi K & Sugano M (2003) Metabolic effects of dietary conjugated linoleic acid isomers in rats. *Nutrition Research* **23**, 1691-1701.

Akinmokun A, Selby PL, Ramaiya K & Alberti KG (1992) The short insulin tolerance test for determination of insulin sensitivity: a comparison with the euglycaemic clamp. *Diabetic Medicine* **9**, 432-437.

Alasnier C, Berdeaux O, Chardigny JM & Sebedio JL (2002) Fatty acid composition and conjugated linoleic acid content of different tissues in rats fed individual conjugated

linoleic acid isomers given as triacylglycerols. *Journal of Nutritional Biochemistry* **13**, 337-345.

Albareda M, Rodriguez-Espinosa J, Murugo M, de Leiva A & Corcoy R (2000) Assessment of insulin sensitivity and beta-cell function form measurements in the fasting state and during an oral glucose tolerance test. *Diabetologia* **43**, 1507-1511.

Alberti KGMM & Zimmet P (1998) Definition, diagnosis and classification of diabetes mellitus and its complications: Part 1: Diagnosis and classification of diabetes mellitus: Provisional report of a WHO consultation. *Diabetic Medicine* **15**, 539-553.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB & Cohen P (1997) Characterisation of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current Biology* **7**, 261-269.

Allison DB, Paultre F, Maggio C, Mezzitis N & Pi-Sunyer FX (1995) The use of area under curves in diabetes research. *Diabetes Care* **18**, 245-250.

American Diabetes Association (1998) Consensus Development Conference on Insulin Resistance. 5-6 November 1997. *Diabetes Care* **21**, 310-314.

American Diabetes Association (2002) Evidence-based nutrition principles and recommendation for the treatment and prevention of diabetes and related complications. *Diabetes Care* **25**, 202-212.

Amos AF, McCArty DJ & Zimmet P (1997) The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabetic Medicine* **14** (suppl 5), S1-S5.

Anai M, Funaki M, Ogihara T, Terasaki J, Inukai K, Katagiri H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y & Asano T (1998) Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* **47**, 13-23.

Anderson RL, Hamman RF, Savage PJ, Saad MF, Laws A, Kades WW, Sands RE & Cefalu W (1995) Exploration of simple insulin sensitivity measures derived from frequently sampled intravenous glucose tolerance (FSIGT) tests. The Insulin Resistance Atherosclerosis Study. *American Journal of Epidemiology* **142**, 724-732.

Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS & Kahn CR (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* **372**, 186-190.

Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T & Matsuzawa Y (2002) Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* **105**, 2893-2898.

Asano T, Kanda A, Katagiri H, Nawano M, Ogihara T, Inukai K, Anai M, Fukushima Y, Yazaki Y, Kikuchi M, Hooshmand-Rad R, Heldin CH, Oka Y & Funaki M (2000) p110beta is up-regulated during differentiation of 3T3-L1 cells and contributes to the highly insulin-responsive glucose transport activity. *Journal of Biological Chemistry* **275**, 17671-17676.

Autran D, Attia N, Debecjus M, Durlach V & Girard-Globa A (2000) Postprandial reverse cholesterol transport in type 2 diabetic patients: effect of lipid lowering treatment. *Atherosclerosis* **153**, 453-460.

Azain MJ, Hausman DB, Sisk MB, Flatt WP & Jewell DE (2000) Dietary conjugated linoleic acid reduces rat adipose tissue cell size rather than cell number. *Journal of Nutrition* **130**, 1548-1554.

B

Bailey CJ (1999) Insulin resistance and antidiabetic drugs. *Biochemical Pharmacology* **58**, 1511-1520.

Baldwin AS, Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annual Review of Immunology* **14**, 649-683.

Balkau B, Bertrais S, Ducimetiere P & Eschwege E (1999) Is there a glycemic threshold for mortality risk? Diabetes Care 22, 696-699.

Bandyopadhyay G, Kanoh Y, Sajan MP, Standaert ML & Farese RV (2000) Effects of adenoviral gene transfer of wild-type, constitutively active, and kinase-defective protein kinase C-lambda on insulin-stimulated glucose transport in L6 myotubes. *Endocrinology* **141**, 4120-4127.

Banerjee RR & Lazar MA (2003) Resistin: molecular history and prognosis. *Journal of Molecular Medicine* **81**, 218-226.

Banerjee RR, Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, Wang J, Rajala MW, Pocai A, Scherer PE, Steppan CM, Ahima RS, Obici S, Rossetti L & Lazar MA (2004) Regulation of fasted blood glucose by resistin. *Science* **303**, 1195-1198.

Banni S (2002) Conjugated linoleic acid metabolism. *Current Opinion in Lipidology* **13**, 261-266.

Banni S, Angioni E, Carta G, Casu V, Deiana M, Dessì MA, Lucchi L, Melis MP, Rosa A, Vargiolu & Corongiu FP (1999) Influence of dietary conjugated linoleic acid on lipid metabolism in relation to its anticarcinogenic activity. In *Advances in Conjugated Linoleic Acid Research, Volume 1*, eds. Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW & Nelson GJ, pp 307-318. AOCS Press, Champaign, Illinois.

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A & Evans RM (1999) PPAR gamma is required for placental, cardiac, and adipose tissue development. *Molecular Cell* **4**, 585-595.

Barrett-Connor E & Ferrara A (1998) Isolated postchallenge hyperglycemia and the risk of fatal cardiovascular disease in older women and men. The Rancho Bernardo Study. *Diabetes Care* **21**, 1236-1239.

Barthel A & Schmoll D (2003) Novel concepts in insulin regulation of hepatic gluconeogenesis. *American Journal of Physiology, Endocrinolgy and Metabolism* **285**, (suppl) E685-E692.

Bastard JP, Robert JJ, Jardel C, Bruckert E, Grimaldi A & Hainque B (2001) Is quantitative insulin sensitivity check index, a fair insulin sensitivity index in humans? *Diabetes and Metabolism* **27**, 69-70.

Basu S, Riserus U, Turpeinen A & Vessby B (2000) Conjugated linoleic acid induces lipid peroxidation in men with abdominal obesity. *Clinical Science* **99**, 511-516.

Basu S, Smedman A & Vessby B (2000) Conjugated linoleic acid induces lipid peroxidation in humans. *FEBS Letters* **468**, 33-36.

Baumann CA, Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, Bickel PE, Pessin JE & Saltiel AR (2000) CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* **407**, 202-207.

Bee GJ (2000) Dietary conjugated linoleic acids consumption during pregnancy and lactation influences growth and tissue composition in weaned pigs. *Journal of Nutrition* **130**, 2981-2899.

Beard J, Bergman R, Ward W & Porte DJ (1986) The insulin sensitivity index in nondiabetic man. Correlation between clamp-derived and IVGTT-derived values. *Diabetes*, **35**, 362-369.

Beck-Nielson H & European Group for the study of Insulin Resistance (1999) Generla characteristics of the insulin resistance syndrome: prevalence and heritabilty. *Drugs* **58**, 7-10.

Belury MA (2002) Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. *Annual Review of Nutrition* **22**, 505-531.

Belury MA, Nickel KP, Bird CE & Wu Y (1996) Dietary conjugated linoleic acid modulation of phorbol ester skin tumor promotion. *Nutrition and Cancer* **26**, 149-157.

Benito P, Nelson GJ, Kelley DS, Bartolini G, Schmidt PC & Simon V (2001) The effect of conjugated linoleic acid on platelet function, platelet fatty acid composition, and blood coagulation in humans. *Lipids* **36**, 221-227.

Berg AH, Combs TP & Scherer PE (2002) ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends in Endocrinology and Metabolism* **13**, 84-89.

Bergman R, Ider Y, Bowden C & Cobelli C. Quantitative estimation of insulin sensitivity. *American Journal of Physiology* **236**: E667-677.

Bergman RN & Ader M (2000) Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends in Endocrinology and Metabolism* **11**, 351-356.

Bergman RN (1989) Lilly lecture. Toward physiological understanding of glucose tolerance. Minimal-model approach. *Diabetes* **38**:1512-1527.

Bergman RN, Finegood DT & Ader M (1985) Assessment of insulin sensitivity in vivo. *Endocrine Reviews* **6**, 45-86.

Betteridge DJ (2000) Diabetic dyslipidaemia. *Diabetes, Obesity & Metabolism* **2** (suppl 1), S31-S36.

Bi L, Okade I, Bernard Dj & Nussbaum RL (2002) Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. *Mammalian Genome* **13**, 169-172.

Bi L, Okade I, Bernard DJ, Wynshaw-Bories A & Nussbaum RL (1999) Proliferative defect and embryonic lethality in mice homozygous for a deletion in the p110alpha subunit of phosphoinositide 3-kinase. *Journal of Biological Chemistry* **16**, 10963-10968.

Bjornholm M, Al-Khalili L, Dicker A, Naslund E, Rossner S, Zierath JR & Arner P (2002) Insulin signal transduction and glucose transport in human adipocytes: effects of obesity and low calorie diet. *Diabetologia* **45**, 1128-1135.

Blagoev B, Kratchmarova I, Nielsen MM, Fernandez MM, Voldby J, Andersen JS, Kristiansen K, Pandey A & Mann M (2002) . Inhibition of adipocyte differentiation by resistin-like molecule alpha. *Journal of Biological Chemistry* **277**, 42011-42016.

Blankson H, Stakkestad JA, Fagertun H, Thom E, Wadstein J & Gudmundsen O (2000) Conjugated linoleic acid reduces body fat mass in overweight and obese humans. *Journal of Nutrition* **130**, 2943-2948.

Boden G (2002) Interaction between free fatty acids and glucose metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care* **5**, 545-549.

Boisvert WA (2004) Modulation of atherogenesis by chemokines. *Trends in Cardiovascular Medicine* **14**, 161-165.

Boizel R, Benhamou PY, Lardy B, Laporte F, Foulon T & Halimi S (2000) Ratio of triglycerides to HDL cholesterol is an indicator of LDL particle size in patients with type 2 diabetes and normal HDL cholesterol levels. *Diabetes Care* **23**, 1679-1685.

Bonora E & Muggeo M (2001) Postprandial blood glucose as a risk factor for cardiovascular disease in type II diabetes: the epidemiological evidence. *Diabetologia* **4**, 2107-2114.

Bonora E, Formentini G, Calcaterra F, Lombardi S, Marini F, Zenari L, Saggiani F, Poli M, Perbellini S, Raffaelli A, Cacciatori V, Santi L, Targher G, Bonadonna R & Muggeo M

(2002) HOMA-estimated insulin resistance is an independent predictor of cardiovascular disease in type 2 diabetic subjects: prospective data from the Verona Diabetes Complications Study. *Diabetes Care* **25**, 1135-1141.

Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Targher G, Alberiche M, Bonadonna RC & Muggeo M (1998) Prevalence of insulin resistance in metabolic disorders: the Bruneck Study. *Diabetes* **47**, 1643-1649.

Bonora E, Moghetti P, Zancanaro C, Cigolini M, Querena M, Cacciatori V, Corgnati A & Muggeo M (1986) Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. *Journal of Clinical Endocrinology and Metabolism* **68**, 374-378.

Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T & Muggeo M (2000) Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* **23**, 57-63.

Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ & Campbell LV (1993) The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *New England Journal of Medicine* **328**, 238-244.

Boston RC, Stefanovski D, Moate PJ, Sumner AE, Watanabe RM & Bergman RN (2003) MINMOD Millennium: a computer program to calculate glucose effectiveness and insulin sensitivity from the frequently sampled intravenous glucose tolerance test. *Diabetes Technology and Therapeutics* **5**, 1003-1015.

Bouche C, Serdy S, Kahn CR & Goldfine AB (2004) The cellular fate of glucose and its relevance in type 2 diabetes. *Endocrine Reviews* **25**, 807-830.

Boyko EJ, Fujimoto WY, Leonetti DL & Newell-Morris L (2000) Visceral adiposity and risk of type 2 diabetes: a prospective study among Japanese Americans. *Diabetes Care* **23**, 465-471.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

Brady LM, Lovegrove SS, Lesauvage SV, Gower BA, Minihane AM, Williams CM & Lovegrove JA (2004) Increased n-6 polyunsaturated fatty acids do not attenuate the effects of long-chain n-3 polyunsaturated fatty acids on insulin sensitivity or triacylglycerol reduction in Indian Asians. *American Journal of Clinical Nutrition* **79**, 983-991.

Bray GA & York DA (1979) Hypothalamic and genetic obesity in experimental animals: an autonomic and endocrine hypothesis. *Physiological Reviews* **59**, 719-809.

Breda E, Cavaghan MK, Toffolo G, Polonsky KS & Cobelli (2001) Oral glucose tolerance test minimal model indexes of beta-cell function and insulin sensitivity. *Diabetes* **50**, 150-158.

Brewer HB, Jr., Remaley AT, Neufeld EB, Basso F & Joyce C (2004) Regulation of plasma high-density lipoprotein levels by the ABCA1 transporter and the emerging role of high-density lipoprotein in the treatment of cardiovascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* **24**, 1755-1760.

Britton M, Fong C, Wickens D & Yudkin J (1992) Diet as a source of phospholipid esterified 9,11-octadecadienoic acid in humans. *Clinical Science* **83**, 97-101.

Brodie AE, Manning VA, Ferguson KR, Jewell DE & Hu CY (1999) Conjugated linoleic acid inhibits differentiation of pre- and post- confluent 3T3-L1 preadipocytes but inhibits cell proliferation only in preconfluent cells. *Journal of Nutrition* **129**, 602-606.

Brown JM & McIntosh MK (2003) Conjugated linoleic acid in humans: regulation of adiposity and insulin sensitivity. *Journal of Nutrition* **133**, 3041-3046.

Brown JM, Boysen MS, Chung S, Fabiyi O, Morrison RF, Mandrup S & McIntosh MK (2004) Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. *Journal of Biological Chemistry* **279**, 26735-26747.

Brown JM, Boysen MS, Jensen SS, Morrison RF, Storkson J, Lea-Currie R, Pariza M, Mandrup S & McIntosh MK (2003) Isomer-specific regulation of metabolism and PPARgamma signaling by CLA in human preadipocytes. *Journal of Lipid Research* **44**, 1287-1300.

Brown JM, Halvorsen YD, Lea-Currie YR, Geigerman C & McIntosh M (2001a) Trans-10, cis-12, but not cis-9, trans-11, conjugated linoleic acid attenuates lipogenesis in primary cultures of stromal vascular cells from human adipose tissue. *Journal of Nutrition* **131**, 2316-2321.

Brown M, Evans M & McIntosh M (2001b) Linoleic acid partially restores the triglyceride content of conjugated linoleic acid-treated cultures of 3T3-L1 preadipocytes. *Journal of Nutritional Biochemistry* **12**, 381-387.

Brown MA & Goldstein JL (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331-340

Brun JF, Raynaud E & Mercier J (2000) Homeostasis model assessment and related simplified evaluations of insulin sensitivity from fasting insulin and glucose. *Diabetes Care* **23**, 1037-1038.

Bruning JC, Winnay J, Bonner-Weir S, Taylor SI, Accili D & Kahn CR (1997) Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. *Cell* **88**, 561-572.

Brustolin D, Maierna M, Aguzzi F, Zoppi F, Tarenghi G & Berti G (1991) Immunoturbidimetric method for routine determinations of apolipoproteins A1 and B. *Clinical Chemistry* **37**, 742-747.

Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A & Richelsen B (2003) Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *American Journal of Physiology, Endocrinology and Metabolism* **285**, E527-E533.

Bullo M, Garcia-Lorda P, Megias I & Salas-Salvado J (2003) Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. *Obesity Research* **11**, 525-531.

Burdge GC, Lupoli B, Russell JJ, Tricon S, Kew S, Banerjee T, Shingfield KJ, Beever DE, Grimble RF, Williams CM, Yaqoob P & Calder PC (2004) Incorporation of cis-9, trans-11 or trans-10, cis-12 conjugated linoleic acid into plasma and cellular lipids in healthy men. *Journal of Lipid Research* **45**, 736-741.

C

Cagnacci A, Arangino S, Renzi A, Cagnacci P & Volpe A (2001) Insulin sensitivity in women: a comparison among values derived from intravenous glucose tolerance tests with different sampling frequency, oral glucose tolerance test or fasting. *European Journal of Endocrinology* **145**, 281-287.

Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J & Shoelson SE (2005) Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nature Medicine* **11**, 183-190.

Camejo G, Hurt-Camejo E, Wiklund O & Bondjers G (1998) Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. *Atherosclerosis* **139**, 205-222.

Cancello R, Tounian A, Poitou C & Clement K (2004) Adiposity signals, genetic and body weight regulation in humans. *Diabetes & Metabolism* **30**, 215-227.

Cao G, Liang Y, Broderick CL, Oldham BA, Beyer TP, Schmidt RJ, Zhang Y, Stayrook KR, Suen C, Otto KA, Miller AR, Dai J, Foxworthy P, Gao H, Ryan TP, Jiang XC, Burris TP, Eacho PI & Etgen GJ (2003) Antidiabetic action of a liver X receptor agonist mediated by inhibition of hepatic gluconeogenesis. *Journal of Biological Chemistry* **278**, 1131-1136.

Carantoni M, Abbasi F, Warmerdam F, Klebanov M, Wang PW, Chen YD, Azhar S & Reaven GM (1998) Relationship between insulin resistance and partially oxidised LDL particles in healthy, nondiabetic volunteers. *Arteriosclerosis, Thrombosis, and Vascular Biology* **18**, 762-767.

Caumo A & Cobelli C (1993) Hepatic glucose production during the labeled IVGTT: estimation by deconvolution with a new minimal model. *American Journal of Physiology* **264,** E829-E841.

Charriere G, Cousin B, Arnaud E, Andre M, Bacou F, Penicaud L & Casteilla L (2003) Preadipocyte conversion to macrophage. Evidence of plasticity. *Journal of Biological Chemistry* **278**, 9850-9855.

Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM & Tontonoz P (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Molecular Cell* **7**, 161-171.

Chawla A, Schwarz EJ, Dimaculangan DD & Lazar MA (1994) Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology* **135**, 798-800.

Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J & Kahn CR (1994) Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Molecular and Cellular Biology* **14**, 4902-4911.

Cheatham B, Volchuk A, Kahn CR, Wang L, Rhodes CJ & Klip A (1996) Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins. *Proceedings of the National Academy of Sciences, USA* **93**, 15169-15173.

Chen HC & Farese RV, Jr. (2000) DGAT and triglyceride synthesis: a new target for obesity treatment? *Trends in Cardiovascular Medicine* **10**, 188-192.

Chevenne D, Trivin F & Porquet D (1999) Insulin assays and reference values. *Diabetes and Metabolism* **25**, 459-476.

Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE & Saltiel AR (2001) Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* **410**, 944-948.

Chin SF, Liu W, Storkson JM, Ha YL & Pariza MW (1992) Dietary sources of conjugated dieonic isomers of linoleic acid, a newly recognised class of anti-carcinogens. *Journal of Food Composition Analysis* **5**, 185-197.

Chin SF, Storkson JM, Albright KJ, Cook ME & Pariza MW (1994) Conjugated linoleic acid is a growtg factor for rats as shown by enhanced weight gain and improved feed efficiency. *Journal of Nutrition* **124**, 2344-2349.

Chinetti G, Fruchart JC & Staels B (2003) Peroxisome proliferator-activated receptors and inflammation: from basic science to clinical applications. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S41-S45.

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI & Birnbaum MJ (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* **292**, 1728-1731.

Choi JS, Jung MH, Park HS & Song J (2004) Effect of conjugated linoleic acid isomers on insulin resistance and mRNA levels of genes regulating energy metabolism in high-fat-fed rats. *Nutrition* **20**, 1008-1017.

Choi Y, Kim YC, Han YB, Park Y, Pariza MW & Ntambi JM (2000) The trans-10, cis-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes. *Journal of Nutrition* **130**, 1920-1924.

Choi Y, Park Y, Pariza MW & Ntambi JM (2001) Regulation of stearoyl-CoA desaturase activity by the trans-10, cis-12 isomer of conjugated linoleic acid in HepG2 cells. *Biochemical and Biophysical Research Communications* **284**, 689-693.

Chomczynski & Sacchi (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**: 156-159.

Christ ER, Carroll PV, Albany E, Umplaby Am, Lumb PJ, Wierbicki AS, Simpson HL, Sonkson PH & Russel-Ones DL (2001) Normal VLDL metabolism despite altered lipoprotein composition in type I diabetes mellitus. *Clinical Endocrinology* **55**, 777-787.

Ciaraldi TP, Gilmore A, Olefsky JM, Goldberg M & Heidenreich KA (1990) In vitro studies on the action of CS-045, a new antidiabetic agent. *Metabolism* **39**, 1056-1062.

Cinti S (2001) The adipose organ: morphological perspectives of adipose tissues. *Proceedings of the Nutrition Society* **60**, 319-328.

Clark SF, Martin S, Carozzi AJ, Hill MM & James DE (1998) Intracellular localisation of phosphatidylinositide 3-kinase and insulin receptor substrate-1 in adipocytes: potential involvement of a membrane skeleton. *Journal of Cell Biology* **140**, 1211-1225.

Clarke SD (2001) Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome. *Journal of Nutrition* **131**, 1129-1132.

Clement L, Poirier H, Niot I, Bocher V, Guerre-Millo M, Krief S, Staels B & Besnard P (2002) Dietary trans-10, cis-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *Journal of Lipid Research* **43**, 1400-1409.

Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Le Marchand-Brustel Y, Erneux C, Hue L & Schurmans S (2001) The lipid phosphatase SHIP2 controls insulin sensitivity. *Nature* **409**, 92-97.

Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R & Stanton C (2003) Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species. *Journal of Applied Microbiology* **94**, 138-145.

Cockerill GW, Rye KA, Gamble JR, Vadas MA & Barter PJ (1995) High-density lipoprotein inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arteriosclerosis, Thrombosis, and Vascular Biology* **15,** 1987-1994.

Colombo C, Cutson JJ, Yamauchi T, Vinson C, Kadowaki T, Gavrilova O & Reitman ML (2002) Transplantation of adipose tissue lacking leptin is unable to reverse the metabolic abnormalities associated with lipoatrophy. *Diabetes* **51**, 2727-2733.

Combs TP, Berg AH, Obici S, Scherer PE & Rossetti L (2001) Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *Journal of Clinical Investigation* **108**, 1875-1881.

Combs TP, Berg AH, Rajala MW, Klebanov S, Iyengar P, Jimenez-Chillaron JC, Patti ME, Klein SL, Weinstein RS & Scherer PE (2003) Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes* **52**, 268-276.

Combs TP, Wagner JA, Berger J, Doebber T, Wang WJ, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DB, Chatterjee K, Weiss S, Larson PJ, Gottesdiener KM, Gertz BJ, Charron MJ, Scherer P & Moller DE (2002) Induction of adipocytes complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitisation. *Endocrinology* **143**, 998-1007.

Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI & Quon MJ (1997) Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Molecular Endocrinology* **11**, 1881-1890.

Cook ME, Miller CC, Park Y & Pariza M (1993) Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. *Poultry Science* **72**, 1301-1305.

Coppack SW (1997) Postprandial lipoproteins in non-insulin-dependent diabetes mellitus. *Diabetic Medicine* **14** (suppl 3), S67-S74.

Coppack SW (2001) Pro-inflammatory cytokines and adipose tissue. *Proceedings of the Nutrition Society* **60**, 349-356.

Corl BA, Baumgard LH, Griinari JM, Delmonte P, Morehouse KM, Yurawecz MP & Bauman DE (2002) Trans-7, cis-9 CLA is synthesised endogenously by delta9-desaturase in dairy cows. *Lipids* **37**, 681-688.

Cortright RN & Dohm GL (1997) Mechanisms by which insulin and muscle contraction stimulate glucose transport. *Canadian Journal of Applied Physiology* **22**, 519-530.

Cowherd RM, Lyle RE & McGehee RE, Jr. (1999) Molecular regulation of adipocyte differentiation. *Seminars in Cell and Developmental Biology* **10**, 3-10.

Cullen P (2000) Evidence that triglycerides are an independent coronary heart disease risk factor. *American Journal of Cardiology* **86**, 943-999.

Cummings MH, Cummings H, Watts GF, Umpleby AM, Hennessy TR, Naoumova R, Slavin BM, Thompson GR & Sönksen PH (1995) Increased hepatic secretion of very-low-density lipoprotein apolipoprotein B-100 in NIDDM. *Diabetologia.* **38**:959-967.

Czech MP (1995) Molecular actions of insulin on glucose transport. *Annual Review of Nutrition* **15**, 441-471.

D

Dalen KT, Ulven SM, Bamberg K, Gustafsson JA & Nebb HI (2003) Expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent on liver X receptor alpha. *Journal of Biological Chemistry* **278**, 48283-48291.

Das UN (2002) Is metabolic syndrome X an inflammatory condition? *Experimental Biology and Medicine* **227**, 989-997.

deBose-Boyd RA, Ou J, Goldstein JL & Brown MS (2001) Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proceedings of the National Academy of Sciences, USA* **98**, 1477-1482.

deCarvalho Papa P, Vargas AM, da Silva JL, Nunes MT & Machado UF (2002) GLUT4 protein is differently modulated during development of obesity in monosodium glutamate-treated mice. *Life Sciences* **71**, 1917-1928.

deFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J & Felber JP (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterisation. *Diabetes* **30**, 1000-1007.

deLany JP, Blohm F, Truett AA, Scimeca JA & West DB (1999) Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *American Journal of Physiology* **276**, R1172-R1179.

Delerive P, Fruchart JC & Staels B (2001) Peroxisome proliferator-activated receptors in inflammation control. *Journal of Endocrinology* **169**, 453-459.

deMarco R, Locatelli F, Zoppini G, Verlato G, Bonora E & Muggeo M (1999) Cause-specific mortality in type 2 diabetes. The Verona Diabetes Study. *Diabetes Care* **22**, 756-761.

Demaree SR, Gilbert CD, Mersmann HJ & Smith SB (2002) Conjugated linoleic acid differentially modifies fatty acid composition in subcellular fractions of muscle and adipose tissue but not adiposity of postweaning pigs. *Journal of Nutrition* **132**, 3272-3279.

Despres JP (2003) Inflammation and cardiovascular disease: is abdominal obesity the missing link? *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S22-S24.

Despres JP, Lamarche B, Mauriege P, Cantin B, Dagenais GR, Moorjani S & Lupien PJ (1996) Hyperinsulinemia as an independent risk factor for ischemic heart disease. *New England Journal of Medicine* **334**, 952-957.

Diabetes Care, Securing the Future (2002) Report of the Diabetes Service Development Group, editors Barragry J, Forde R, Shelley E, Mc Kenna TJ, Quirke R.

Doria A (2002) Adipokine genes and the insulin-resistance syndrome. *International Congress Series* **1253**, 63-71.

Drake PG & Posner BI (1998) Insulin receptor-associated protein tyrosine phosphatase(s): role in insulin action. *Molecular and Cellular Biochemistry* **182**, 79-89.

Ducluzeau PH, Fletcher LM, Vidal H, Laville M & Tavare JM (2002) Molecular mechanisms of insulin-stimulated glucose uptake in adipocytes. *Diabetes & Metabolism* **28**, 85-92.

E

Ebihara K, Ogawa Y, Masuzaki H, Shintani M, Miyanaga F, Aizawa-Abe M, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Gavrilova O, Reitman ML & Nakao K (2001) Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. *Diabetes* **50**, 1440-1448.

Eckel RH, Grundy SM & Zimmet PZ (2005) The metabolic syndrome. *Lancet* **365**, 1415-1428.

Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML & Kennedy BP (1999) Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* **283**, 1544-1548.

Ellis KJ, Bell SJ, Chertow GM, Chumlea VC, Knox TA, Kotler DP, Lukaski HC & Schoeller SA (1999) Bioelectrical impedance methods in clinical research: a follow-up to the NIH technology assessment conference. *Nutrition* **15**, 874-880.

Elmendorf JS (2002) Signals that regulate GLUT4 translocation. *Journal of Membrane Biology* **190**, 167-174.

Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D & Van Obberghen E (2000) SOCS-3 is an insulin-induced negative regulator of insulin signaling. *Journal of Biological Chemistry* **275**, 15985-15991.

Emoto M, Nishizawa Y, Maekawa K, Hiura Y, Kanda H, Kawagishi T, Shoji T, Okuno Y & Morii H (1999) Homeostasis model assessment as a clinical index of insulin resistance in type 2 diabetic patients treated with sulfonylureas. *Diabetes Care* **22**, 818-822.

Ens JG, Ma DW, Cole KS, Field CJ & Clandinin MT (2001) An assessment of c9, t11 linoleic acid intake in a small group of young Canadians. *Nutrition Research* **21**, 955-960.

Erkelens DW (2001) Insulin resistance syndrome and type II diabetes mellitus. *American Journal of Cardiology* **88**, J38-J42.

Esposito K, Pontillo A, Ciotola M, Di Palo C, Grella E, Nicoletti G & Giugliano D (2002) Weight loss reduces interleukin-18 levels in obese women. *Journal of Clinical Endocrinology and Metabolism* **87**, 3864-3866.

Etgen GJ, Valasek KM, Broderick CL & Miller AR (1999) In vivo adenoviral delivery of recombinant human protein kinase C-zeta stimulates glucose transport activity in rat skeletal muscle. *Journal of Biological Chemistry* **274**, 22139-22142.

Evans M, Brown J & McIntosh M (2002a) Isomer-specific effects of conjugated linoleic acid on adiposity and lipid metabolism. *Journal of Nutritional Biochemistry* **13**, 508-516.

Evans M, Geigerman C, Cook J, Curtis L, Kuebler B & McIntosh M (2000) Conjugated linoleic acid suppresses triglyceride accumulation and induces apoptosis in 3T3-L1 preadipocytes. *Lipids* **35**, 899-910.

Evans M, Lin X, Odle J & McIntosh M (2002b) Trans-10, cis-12 conjugated linoleic acid increases fatty acid oxidation in 3T3-L1 preadipocytes. *Journal of Nutrition* **132**, 450-455.

Eyjolfson V, Spriet LL & Dyck DJ (2004) Conjugated linoleic acid improves insulin sensitivity in young, sedentary humans. *Medicine & Science in Sports & Exercise* **36**, 814-820.

F

Fain JN, Bahouth SW & Madan AK (2004) Haptoglobin release by human adipose tissue in primary culture. *Journal of Lipid Research* **4**, 536-542

Fantin VR, Wang Q, Lienhard GE & Keller SR (2000) Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. *American Journal of Physiology. Endocrinology and Metabolism* **278**, E127-E133.

Fasching P, Ratheiser K, Waldhausl W, Rohac M, Osterrode W, Nowotny P & Vierhapper H (1991) Metabolic effects of fish-oil supplementation in patients with impaired glucose tolerance. *Diabetes* **40**, 583-589.

Fasshauer M, Klein J, Neumann S, Eszlinger M & Paschke R (2002) Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications* **290, 1**084-1089.

Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J & Paschke R (2003) Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communications* **301**, 1045-1050.

Faulconnier Y, Arnal MA, Patureau Mirand P, Chardigny JM & Chilliard Y (2004) Isomers of conjugated linoleic acid decrease plasma lipids and stimulate adipose tissue lipogenesis without changing adipose weight in post-prandial adult sedentary or trained Wistar rat. *Journal of Nutritional Biochemistry* **15**, 741-748.

Fawcett J, Tsui BT, Kruer MC & Duckworth WC (2004) Reduced action of insulin glargine on protein and lipid metabolism: possible relationship to cellular hormone metabolism. *Metabolism* **53**, 1037-1044.

Feingold KR & Grunfeld C (1992) Role of cytokines in inducing hyperlipidemia. *Diabetes* **41** (suppl 2), S97-S101.

Feinstein R, Kanety H, Papa MZ, Lunenfeld B & Karasik A (1993) Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *Journal of Biological Chemistry* **268**, 26055-26058.

Ferrannini E & Balkau B (2002) Insulin: in search of a syndrome. *Diabetic Medicine* **19**, 724-729.

Ferrannini E & Mari A (1998) How to measure insulin sensitivity. *Journal of Hypertension* **16**, 895-906.

Ferre P (2004) The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* **53** (suppl 1), S43-S50.

Feskens EJ, Bowles CH & Kromhout D (1991) Inverse association between fish intake and risk of glucose intolerance in normoglycemic elderly men and women. *Diabetes Care* **14**, 935-941.

Feskens EJ, Loeber JG & Kromhout D (1994) Diet and physical activity as determinants of hyperinsulinemia: the Zutphen Elderly Study. *American Journal of Epidemiology* **140**, 350-360.

Finegood DT, Hramiak IM & Dupre J (1990) A modified protocol for estimation of insulin sensitivity with the minimal model of glucose kinetics in patients with insulin-dependent diabetes. *Journal of Clinical Endocrinology and Metabolism* **70**, 1538-1549.

Flatt PF (2003) The hormonal and neural control of endocrine pancreatic function. In *Textbook of Diabetes Volume 1,* eds. Pickup JC & Williams G, pp 11.1-11.21. Blackwell Publishing, Massachusetts, USA.

Flier JS (1998) What's in a name? In search of leptin's physiologic role. *Journal of Clinical Endocrinology and Metabolism* **83,** 1407-1413.

Folch J, Lees M & Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry* **226**, 497-509.

Francis GA, Fayard E, Picard F & Auwerx J (2003) Nuclear receptors and the control of metabolism. *Annual Review of Physiology* **65**, 261-311.

Frayn KN (2001) Adipose tissue and the insulin resistance syndrome. *Proceedings of the Nutrition Society* **60**, 375-380.

Frevert EU & Kahn BB (1996) Protein kinase C isoforms epsilon, eta, delta and zeta in murine adipocytes: expression, subcellular localisation and tissue-specific regulation in insulin-resistant states. *Biochemical Journal* **316**, 865-871.

Fritsche A, Stefan N, Hardt E, Schützenauer S, Häring H & Stumvoll M (2000). A novel hyperglycemic clamp for characterization of islet function in humans: assessment of three

different secretagogues, maximal insulin response and reproducibility. *European Journal of Clinical Investigation* **30,** 411-418.

Frohlich J & Steiner G (2000) Dyslipidaemia and coagulation defects of insulin resistance. *International Journal of Clinical Practice* **113**, (suppl), S14-S22.

Fujimoto WY (2000) the importance of insulin resistance in the pathogenesis of type 2 diabetes mellitus. *American Journal of Medicine* **108**, 9S-14S.

Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y & Shimomura I (2005) Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* **307**, 426-430.

G

Galvin P, Ward G, Walters J, Pestell R, Koschmann M, Vaag A, Martin I, Best JD & Alford F A simple method for quantitation of insulin sensitivity and insulin release from an intravenous glucose tolerance test. *Diabetic Medicine* **9**, 921-928.

Garner DM (1991) Confronting the failure of behavioural and dietary treatments for obesity. *Clinical Psychology Review* **11**, 729-780

Garvey WT, Maianu L, Hancock JA, Golichowski AM & Baron A (2002) Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM. *Diabetes* **41**, 465-475.

Garvey WT, Maianu L, Hueckstead TP, Birnbaum MJ, Molina JM & Ciaraldi TP (1991) Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. *Journal of Clinical Investigation* **87**, 1072-1081.

Gaullier JM, Halse J, Hoye K, Kristiansen K, Fagertun H, Vik H & Gudmundsen O (2004) Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. *American Journal of Clinical Nutrition* **79**, 1118-1125.

Gavino VC, Gavino G, Leblanc MJ & Tuchweber B (2000) An isomeric mixture of conjugated linoleic acids but not pure cis-9, trans-11-octadecadienoic acid affects body weight gain and plasma lipids in hamsters. *Journal of Nutrition* **130**, 27-29.

Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, Leber TM, Mangan M, Miller K, Nayee P, Owen K, Patel S, Thomas W, Wells G, Wood LM & Wooley K (1994) Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* **370**, 555-557.

Gelding SV, Robinson S, Lowe S, Niththyananthan R & Johnston DG (1994) Validation of the low dose short insulin tolerance test for evaluation of insulin sensitivity. *Clinical Endocrinology* **40**, 611-615.

Gibbons GF, Wiggins D, Brown AM & Hebbachi AM (2004) Synthesis and function of hepatic very-low-density lipoprotein. *Biochemical Society Transactions* **32**, 59-64.

Gil-Campos M, Canete RR & Gil A (2004) Adiponectin, the missing link in insulin resistance and obesity. *Clinical Nutrition* **23**, 963-974.

Ginsberg HN, Zhang YL & Hernandez-Ono A (2005) Regulation of plasma triglycerides in insulin resistance and diabetes. *Archives of Medical Research* **36**, 232-240.

Giusti V, Verdumo C, Suter M, Gaillard RC, Burckhardt P & Pralong F (2003) Expression of peroxisome proliferator-activated receptor-gamma1 and peroxisome proliferator-activated receptor-gamma2 in visceral and subcutaneous adipose tissue of obese women. *Diabetes* **52**, 1673-1676.

Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe (1999) *Lancet* **354**, 617-621.

Goldstein BJ, Ahmad F, Ding W, Li PM & Zhang WR (1998) Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. *Molecular and Cellular Biochemistry* **182**, 91-99.

Gonzalez-Ortiz M, Martinez-Abundis E & Pascoe-Gonzalez S (1999) Comparison between usual and low doses of insulin in the assessment of insulin sensitivity with a short insulin tolerance test in obese women. *Archives of Medical Research* **30**, 385-387.

Goodrick GK & Foreyt JP (1991) Why treatments for obesity don't last. *Journal of the American Dietetic Association* **91**, 1243-1247.

Granlund L, Juvet LK, Pedersen JI & Nebb HI (2003) Trans 10, cis 12-conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by acting as a PPARgamma modulator. *Journal of Lipid Research* **44**, 1441-1452.

Green H & Kehinde O (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* **5**, 19-27.

Green H & Kehinde O (1979) Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *Journal of Cellular Physiology* **101**, 169-171.

Greene MW & Garofalo RS (2002) Positive and negative regulatory role of insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) serine/threonine phosphorylation. *Biochemistry* **41**, 7082-7091.

Greene MW, Sakaue H, Wang L, Alessi DR & Roth RA (2003) Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by Serine 312 phosphorylation. *Journal of Biological Chemistry* **278**, 8199-8211.

Gregory J, Foster K, Tyler H & Wiseman M (1990) The dietary and nutritional survey of British adults. London: Her Majesty's Stationery Office.

Griinari JM & Bauman DE (1999) Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In *Advances in Conjugated Linoleic Acid Research, Volume 1*, eds. Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW & Nelson GJ, pp 180-200. AOCS Press, Champaign, Illinois.

Griinari JM, Dwyer DA, McGuire MA, Bauman DE, Palmquist DL, Nurmela KV (1998) Trans-octadecenoic acids and milk fat depression in lactating dairy cows. *Journal of Dairy Science* **81**, 1251-1261.

Groff JL, Gropper SS & Hunt SM (1995a) Lipids. In *Advanced Nutrition & Human Metabolism*, pp123-132. West Publishing Company, New York.

Groff JL, Gropper SS & Hunt SM (1995b) Integration and Regulation of metabolism. In *Advanced Nutrition & Human Metabolism*, pp208-210. West Publishing Company, New York.

Groop LC, Bonadonna RC, DelPrato S, Ratheiser K, Zyck K, Ferrannini E & DeFronzo RA (1989) Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *Journal of Clinical Investigation* **84**, 205-213.

Grulet H, Durlach V, Hecart AC, Gross A & Leutenegger M (1993) Study of the rate of early glucose disappearance following insulin injection: insulin sensitivity index. *Diabetes Research and Clinical Practice* **20**, 201-207.

Guerre-Millo M (2003) Extending the glucose/fatty acid cycle: a glucose/adipose tissue cycle. *Biochemical Society Transactions* **31**, 1161-1164.

Guerre-Millo M (2004) Adipose tissue and adipokines: for better or worse. *Diabetes & Metabolism* **30**, 13-19.

Gutt M, Davis CL, Spitzer SB, Llabre MM, Kumar M, Czarnecki EM, Schneiderman N, Skyler JS & Marks JB (2000) Validation of the insulin sensitivity index (ISI(0,120)): comparison with other measures. *Diabetes Research and Clinical Practice* **47**, 177-184.

H

Ha YL, Grimm NK & Pariza MW (1987) Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. *Carcinogenesis* **8**, 1881-1887.

Ha YL, Storkson J & Pariza MW (1990) Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Research* **50**, 1097-1101.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK & Friedman JM (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* **269**, 543-546.

Halvorsen YD, Lea-Currie R, Geigerman C, McIntosh M (2000) Obesity Research 8 (suppl 1), 121S.

Hamann A, Benecke H, Le Marchand-Brustel Y, Susulic VS, Lowell BB & Flier JS (1995) Characterisation of insulin resistance and NIDDM in transgenic mice with reduced brown fat. *Diabetes* **44**, 1266-1273.

Hamura M, Yamatoya H & Kudo S (2004) *Journal of Oleo Science* **50**, 889-894.

Hanley AJ, Williams K, Stern MP & Haffner SM (2002) Homeostasis model assessment of insulin resistance in relation to the incidence of cardiovascular disease: the San Antonio Heart Study. *Diabetes Care* **25**, 1177-1184.

Harano Y, Hidaka H, Takatsuki K, Ohgaku S, Haneda M, Motoi S, Kawagoe K, Shigeta Y & Abe H (1978) Glucose, insulin, and somatostatin infusion for the determination of insulin sensitivity in vivo. *Metabolism* **27**, (suppl 1), S1449-S1452.

Harano Y, Hidaka H, Takatsuki K, Ohgaku S, Haneda M, Motoi S, Kawagoe K, Shigeta Y & Abe H (1978) Glucose, insulin, and somatostatin infusion for the determination of insulin sensitivity in vivo. *Metabolism* **27** (suppl 1), S1449-S1452.

Harano Y, Ohgaku S, Hidaka H, Haneda K, Kikkawa R, Shigeta Y & Abe H (1977) Glucose, insulin and somatostatin infusion for the determination of insulin sensitivity. *Journal of Clinical Endocrinology and Metabolism* **45**, 1124-1127.

Hardardottir I, Grunfeld C & Feingold KR (1994) Effects of endotoxin and cytokines on lipid metabolism. *Current Opinion in Lipidology* **5**, 207-215.

Harrington K, Robson PJ, Kiely M, Livingston MBE, Lambe J & Gibney MJ (2001) The North/South Ireland food consumption survey: survey design and methodology. *Public Health Nutrition* **4**, 1037-1042.

Haruta T, Morris AJ, Rose DW, Nelson JG, Mueckler M & Olefsky JM (1995) Insulin-stimulated GLUT4 translocation is mediated by a divergent intracellular signaling pathway. *Journal of Biological Chemistry* **270**, 27991-27994.

Hauner H (2005) Secretory factors from human adipose tissue and their functional role. *Proceedings of the Nutrition Society* **64**, 163-169.

Hauner H, Skurk T & Wabitsch M (2001) Cultures of human adipose precursor cells. *Methods in Molecular Biology* **155**, 239-247.

Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ & Martin RJ (2001) The biology of white adipocyte proliferation. *Obesity Reviews* **2**, 239-254.

Henkin L, Bergman RN, Bowden DW, Ellsworth DL, Haffner SM, Langefeld CD, Mitchell BD, Norris JM, Rewers M, Saad MF, Stamm E, Wagenknecht LE & Rich SS (2003) Genetic epidemiology of insulin resistance and visceral adiposity. The IRAS Family Study design and methods. *Annals of Epidemiology* **13**, 211-217.

Henriksen EJ, Teachey MK, Taylor ZC, Jacob S, Ptock A, Kramer K & Hasselwander O (2003) Isomer-specific actions of conjugated linoleic acid on muscle glucose transport in the obese Zucker rat. *American Journal of Physiology, Endocrinology and Metabolism* **285**, E98-E105.

Herbel BK, McGuire MK, McGuire MA & Shultz TD (1998) Safflower oil consumption does not increase plasma conjugated linoleic acid concentrations in humans. *American Journal of Clinical Nutrition* **67**, 332-337.

Herberg L & Coleman DL (1977) Laboratory animals exhibiting obesity and diabetes syndromes. *Metabolism* **26**, 59-99.

Hermans MP, Levy JC, Morris RJ & Turner RC (1999) Comparison of tests of β-cell function across a range of glucose tolerance from normal to diabetes. *Diabetes* **48**, 1779-1786.

Hesketh JE, Vasconcelos MH & Bermano G (1998) Regulatory signals in messanger RNA: determinants of nutrient-gene interaction and metabolic compartmentation. *British Journal of Nutrition* **80**, 307-321.

Higuchi R, Fockler C, Dollinger G & Watson RM (1991) Kinetic PCR: real time monitoring of DNA amplification reactions. *Biotechnology* **11**, 1026-1030.

Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M & Hotamisligil GS (2002) A central role for JNK in obesity and insulin resistance. *Nature* **420**, 333-336.

Hirst S, Phillips DI, Vines SK, Clark PM & Hales CN (1993) Reproducibility of the short insulin tolerance test. *Diabetic Medicine* **10**, 839-842.

Hofmann C, Lorenz K, Braithwaite SS, Colca JR, Palazuk BJ, Hotamisligil GS & Spiegelman BM (1994) Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology* **134**, 264-270.

Holland PM, Abramson RD, Watson R & Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilising the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences, USA* **88**, 7276-7280.

Horgaard A & Thayssen THE (1929) Clinical investigation into the effect of the intravenous injection of insuluin. *Acta Medica Scand* **72**, 92-95.

Hotamisligil GS (2003) Inflammatory pathways and insulin action. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S53-S55.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL & Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *Journal of Clinical Investigation* **95**, 2409-2415.

Hotamisligil GS, Budavari A, Murray D & Spiegelman BM (1994) Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha. *Journal of Clinical Investigation* **94**, 1543-1549.

Hotamisligil GS, Johnson RS, Distel RJ, Ellis R, Papaioannou VE & Spiegelman BM (1996b) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* **274**, 1377-1379.

Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF & Spiegelman BM (1996a) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* **271**, 665-668.

Hotamisligil GS, Shargill NS & Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91.

Hotamisligil, GS, Shargill, NS & Spiegelman, BM (1993) Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. *Science* **259**, 665-668.

Hotta K, Gustafson TA, Yoshioka S, Ortmeyer HK, Bodkin NL & Hansen BC (1998) Relationships of PPARgamma and PPARgamma2 mRNA levels to obesity, diabetes and hyperinsulinaemia in rhesus monkeys. *International Journal of Obesity and Related Metabolic Disorders* **22**, 1000-1010.

Houseknecht KL, Vanden Heuvel JP, Moya-Camarena SY, Portocarrero CP, Peck LW, Nickel KP & Belury MA (1998) Dietary conjugated linoleic acid normalises impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. *Biochemical and Biophysical Research Communications* **244**, 678-682.

Howard BV (1999) Insulin resistance and lipid metabolism. *American Journal of Cardiology* **84**, 28J-32J.

Hrebicek J, Janout V, Malincikova J, Horakova D & Cizek L (2002) Detection of insulin resistance by simple quantitative insulin sensitivity check index QUICKI for epidemiological assessment and prevention. *Journal of Clinical Endocrinology and Metabolism* **87**, 144-147.

Hu FB, VanDam RM & Liu S (2001) Diet and risk of type II diabetes: the role of types of fat and carbohydrate. *Diabetologia* **44**, 805-817.

Huang YC, Luedecke Lo & Shultz TD (1994) Effect of cheddar cheese consumption on the plasma conjugated linoleic acid concentration in men. *Nutrition Research* **14**: 373-386.

Hube F, Birgel M, Lee YM & Hauner H (1999) Expression pattern of tumour necrosis factor receptors in subcutaneous and omental human adipose tissue: role of obesity and non-

insulin-dependent diabetes mellitus. *European Journal of Clinical Investigation* **29**, 672-678.

Hube F, Birgel M, Lee Y-M & Hauner H (1999a) Expression pattern of tumour necrosis factor receptors in subcutaneous and omental human adipose tissue: role of obesity and non-insulin dependent diabetes mellitus. *European Journal of Clinical Investigation* **29**, 672-678.

Hudson L & Hay FC (1976) *Practical Immunology*, Oxford, UK: Blackwell Scientific Publications.

Hundal RS, Petersen KF, Mayerson AB, Randhawa PS, Inzucchi S, Shoelson SE & Shulman GI (2002) Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *Journal of Clinical Investigation* **109**, 1321-1326.

I

Ide T, Shimano H, Yahagi N, Matsuzaka T, Nakakuki M, Yamamoto T, Nakagawa Y, Takahashi A, Suzuki H, Sone H, Toyoshima H, Fukamizu A & Yamada N (2004) SREBPs suppress IRS-2-mediated insulin signalling in the liver. *Nature Cell Biology* **6**, 351-357.

Ip C, Chin SF, Scimeca JA & Pariza MW (1991) Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Research* **51**, 6118-6124.

Ip C, Dong Y, Thompson HJ, Bauman DE & Ip MM (2001) Control of rat mammary epithelium proliferation by conjugated linoleic acid. *Nutrition and Cancer* **39**, 233-238.

Ip C, Jiang C, Thompson HJ & Scimeca JA (1997) Retention of conjugated linoleic acid in the mammary gland is associated with tumor inhibition during the post-initiation phase of carcinogenesis. *Carcinogenesis* **18**, 755-759.

Ip C, Scimeca JA & Thompson (1995) Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. *Nutrition Cancer* **24**, 241-247.

Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR & Groop L (2001) Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care* **24**, 683-689.

J

Jackson K, Robertson MD, Fielding BA, Frayn KN & Williams CM (2002) Olive oil increases the number of triacylglycerol-rich chylomicron particles compared with other oils: an effect retained when a second meal is fed. *American Journal of Clinical Nutrition* **76**, 942-949.

Janowski BA, Willy PJ, Devi TR, Falck JR & Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* **383**, 728-731.

Jiang C, Ting AT & Seed B (1998b) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* **391**, 82-86.

Jiang J, Wolk A & Vessby B (1999) Relation between the intake of milk fat and the occurrence of conjugated linoleic acid in human adipose tissue. *American Journal of Clinical Nutrition* **70**, 21-27.

Jiang T, Sweeney G, Rudolf MT, Klip A, Traynor-Kaplan A & Tsien RY (1998a) Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate. *Journal of Biological Chemistry* **273**, 11017-11024.

Johnston AM, Pirola L & Van Obberghen E (2003) Molecular mechanisms of insulin receptor substrate protein-mediated modulation of insulin signalling. *FEBS Letters* **546**, 32-36.

Jonsson B (1997) Health economic aspects. Diabetes Endocrinology and Metabolism 4 (suppl B), 135-137.

Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ & Tontonoz P (2003) Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nature Medicine* **9**, 213-219.

Joseph SB, Laffitte BA, Patel PH, Watson MA, Matsukuma KE, Walczak R, Collins JL, Osborne TF & Tontonoz P (2002) Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *Journal of Biological Chemistry* **277**, 11019-11025.

Joshi RL, Lamothe B, Cordonnier N, Mesbak K, Monthioux E, Jami J & Bucchini D (1996) Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. *EMBO Journal* **15**, 1542-1547.

Joyce C, Freeman L, Brewer HB Jr & Santamarina-Fojo S (2003) Study of ABCA1 function in transgenic mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23**, 965-971.

Juhan-Vague I, Alessi MC & Vague P (1996) Thrombogenic and fibrinolytic factors and cardiovascular risk in non-insulin-dependent diabetes mellitus. *Annals of Medicine* **28**, 371-380.

Jun H, Bae HY, Lee BR, Koh KS, Kim YS, Lee KW, Kim H &Yoon J (1999) Pathogenesis of non-insulin-dependent (type II) diabetes mellitus (NIDDM) - genetic predisposition and metabolic abnormalities. *Advanced Drug Delivery Reviews* **35**, 157-177.

K

Kahn BB & Flier JS (2000) Obesity and insulin resistance. *Journal of Clinical Investigation* **106**, 473-481.

Kahn BB & Rossetti L (1998) Type 2 diabetes - who is conducting the orchestra? *Nature Genetics* **20**, 223-225.

Kahn CR (1994) Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* **43**, 1066-1084.

Kamphuis MM, Lejeune MP, Saris WH & Westerterp-Plantenga MS (2003) The effect of conjugated linoleic acid supplementation after weight loss on body weight regain, body composition, and resting metabolic rate in overweight subjects. *International Journal of Obesity and Related Metabolic Disorders* **27**, 840-847.

Kanemaki T, Kitade H, Kaibori M, Sakitani K, Hiramatsu Y, Kamiyama Y, Ito S & Okumura T (1998) Interleukin 1beta and interleukin 6, but not tumor necrosis factor alpha, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. *Hepatology* **27**, 1296-1303.

Kang K, Liu W, Albright KJ, Park Y & Pariza MW (2003) trans-10, cis-12 CLA inhibits differentiation of 3T3-L1 adipocytes and decreases PPAR gamma expression. *Biochemical and Biophysical Research Communications* **303**, 795-799.

Kannel WB (1987) Status of risk factors and their consideration in antihypertensive therapy. *American Journal of Cardiology* **59**, 80A-90A.

Kannel WB (1994) Risk factors for atherosclerotic cardiovascular outcomes in differential arterial territories. *Journal of Cardiovascular Risk* **1**, 333-339.

Kannel WB (1997) Hazards, risks and threats of heart disease from the early stages to symptomatic coronary heart disease to cardiac failure. *Cardiovascular Drugs and Theraapy* **11**, 199-212.

Kao AW, Noda Y, Johnson JH, Pessin JE & Saltiel AR (1999) Aldolase mediates the association of F-actin with the insulin-responsive glucose transporter GLUT4. *Journal of Biological Chemistry* **274**, 17742-17747.

Karin M & Delhase M (2000) The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Seminars in Immunology* **12**, 85-98.

Karpe F, Steiner G, Uffelman K, Olivecrona T & Hamsten A (1994) Postprandial lipoproteins and progression of atherosclerosis. *Atherosclerosis* **106**, 83–97.

Katsuki A, Sumida Y, Gabazza EC, Murashima S, Furuta M, Araki-Sasaki R, Hori Y, Yano Y & Adachi Y (2001) Homeostasis model assessment is a reliable indicator of insulin resistance during follow-up of patients with type 2 diabetes. *Diabetes Care* **24**, 362-365.

Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G & Quon MJ (2000) Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *Journal of Clinical Endocrinology and Metabolism* **85**, 2402-2410.

Katz EB, Stenbit AE, Hatton K, DePinho R & Charron MJ (1995) Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4. *Nature* **377**, 151-155.

Keim NL (2003) Conjugated Linoleic Acid and Body Composition. In *Advances in Conjugated Linoleic Acid, Volume 2*. eds Sebedio JL, Christie WW, Adlof R. pp316-324. AOCS Press, Champaign, Illinois

Kelley DS, Simon VA, Taylor PC, Rudolph IL, Benito P, Nelson GJ, Mackey BE & Erickson KL (2001) Dietary supplementation with conjugated linoleic acid increased its concentration in human peripheral blood mononuclear cells, but did not alter their function. *Lipids* **36**, 669-674.

Kelly GS (2001) Conjugated linoleic acid: a review. *Alternative Medicine Review* **6**, 367-382.

Kepler CR, Hirons KP, McNeill JJ & Tove SB (1966) Intermediates and products of the biohydrogenation of linoleic acid by Butyrinvibrio fibrisolvens. *Journal of Biological Chemistry* **241**, 1350-1354.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R & Simsolo RB (1995) The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *Journal of Clinical Investigation* **95**, 2111-2119.

Khosla P & Fungwe TV (2001) Conjugated linoleic acid: effects on plasma lipids and cardiovascular function. *Current Opinion in Lipidology* **12**, 31-34.

Kim HJ, Higashimori T, Park SY, Choi H, Dong J, Kim YJ, Noh HL, Cho YR, Cline G, Kim YB & Kim JK (2004) Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes* **53**, 1060-1067.

Kim JK, Fillmore JJ, Chen Y, Yu C, Moore IK, Pypaert M, Lutz EP, Kako Y, Velez-Carrasco W, Goldberg IJ (2001) Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proceedings of the National Academy of Science, USA* **98**, 7522-7527.

Kim KH, Lee K, Moon YS & Sul HS (2001) A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. *Journal of Biological Chemistry* **276**, 11252-11256.

Kim YB, Kotani K, Ciaraldi TP, Henry RR & Kahn BB (2003) Insulin-stimulated protein kinase C lambda/zeta activity is reduced in skeletal muscle of humans with obesity and type 2 diabetes: reversal with weight reduction. *Diabetes* **52**, 1935-1942.

King H, Aubert RE & Herman WH (1998) Global burden of diabetes, 1995-2025 - Prevalence, numerical estimates, and projections. *Diabetes Care* **21**, 1414-1431.

Kintscher U & Law RE (2005) PPARgamma-mediated insulin sensitisation: the importance of fat versus muscle. *American Journal of Physiology. Endocrinology and Metabolism* **288**, E287-E291.

Klein S, Sheard NF, Pi-Sunyer X, Daly A, Wylie-Rosett J, Kulkarni K & Clark NG (2004) American Diabetes Association; North American Association for the Study of Obesity; American Society for Clinical Nutrition: Weight management through lifestyle

modification for the prevention and management of type 2 diabetes: rationale and strategies. A statement of the American Diabetes Association, the North American Association for the Study of Obesity, and the American Society for Clinical Nutrition. *American Journal of Clinical Nutrition* **80**, 257-263.

Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA & Nathan DM (2002) Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *New England Journal of Medicine* **346**, 393-403.

Kohn AD, Barthel A, Kovacina KS, Boge A, Wallach B, Summers SA, Birnbaum MJ, Scott PH, Lawrence JC, Jr. & Roth RA (1998) Construction and characterisation of a conditionally active version of the serine/threonine kinase Akt. *Journal of Biological Chemistry* **273**, 11937-11943.

Kohn AD, Summers SA, Birnbaum MJ & Roth RA (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *Journal of Biological Chemistry* **271**, 31372-31378.

Koopman R, Schaart G & Hesselink MK (2001) Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochemistry and Cell Biology* **116**, 63-68.

Korhonen M, Kastarinen M, Uusitupa M, Puska P & Nissinen A (2003) The effect of intensified diet counselling on the diet of hypertensive subjects in primary health care: a 2-year open randomised controlled trial of lifestyle intervention against hypertension in eastern Finland. *Preventive Medicine* **36**, 8-16.

Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, Miyake K, Sano W, Akimoto K, Ohno S & Kasuga M (1998) Requirement of atypical protein kinase clambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Molecular and Cellular Biology* **18**, 6971-6982.

Kramer JK, Parodi PW, Jensen RG, Mossoba MM, Yurawecz MP & Adlof RO (1998) Rumenic acid: a proposed common name for the major conjugated linoleic acid isomer found in natural products. *Lipids* **33**, 835-840.

Kritchevsky D, Tepper SA, Wright S, Tso P & Czarnecki SK (2000) Influence of conjugated linoleic acid (CLA) on establishment and progression of atherosclerosis in rabbits. *Journal of the American College of Nutrition* **19**, 472S-477S.

Kroder G, Bossenmaier B, Kellerer M, Capp E, Stoyanov B, Muhlhofer A, Berti L, Horikoshi H, Ullrich A & Haring H (1996) Tumor necrosis factor-alpha- and hyperglycemia-induced insulin resistance. Evidence for different mechanisms and different effects on insulin signaling. *Journal of Clinical Investigation* **97**, 1471-1477.

Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Jr., Wallberg-Henriksson H & Zierath JR (2000) Characterisation of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* **49**, 284-292.

Krssak M, Falk Petersen K, Dresner A, DiPietro L, Vogel SM, Rothman DL, Roden M & Shulman GI (1999) Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* **42**, 113-116.

Kruszynske (2003) Normal metabolism: the physiology of fuel homeostasis. In *Textbook of Diabetes Volume 1,* eds. Pickup JC & Williams G, pp 9.1-9.38. Blackwell Publishing, Massachusetts, USA.

Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S, Nagai R, Tobe K, Kimura S & Kadowaki T (1999) PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Molecular Cell* **4**, 597-609.

Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Froguel P, Nagai R, Kimura S, Kadowaki T & Noda T (2002) Disruption of adiponectin causes insulin resistance and neointimal formation. *Journal of Biological Chemistry* **277**, 25863-25866.

Kudo M, Sugawara A, Uruno A, Takeuchi K & Ito S (2004) Transcription suppression of peroxisome proliferator-activated receptor gamma2 gene expression by tumor necrosis factor alpha via an inhibition of CCAAT/ enhancer-binding protein delta during the early stage of adipocyte differentiation. *Endocrinology* **145**, 4948-4956.

Kwiterovich PO (2000) The metabolic pathways of high-density lipoprotein, low-density lipoprotein, and triglycerides: a current review. *American Journal of Cardiology* **86** (suppl), 5L-10L.

L

Laakso M (1993) How good a marker is insulin level for insulin resistance? *American Journal of Epidemiology* **137**, 959-965.

Laakso M (1993) How good a marker is insulin level for insulin resistance? American *Journal of Epidemiology* **137**, 959-965.

Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB, Castrillo A, Wilpitz DC, Mangelsdorf DJ, Collins JL, Saez E & Tontonoz P (2003) Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. *Proceedings of the National Academy of Sciences, USA* **100**, 5419-5424.

Laffitte BA, Repa JJ, Joseph SB, Wilpitz DC, Kast HR, Mangelsdorf DJ & Tontonoz P (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proceedings of the National Academy of Sciences, USA* **98**, 507-512.

Lang CH, Dobrescu C & Bagby GJ (1992) Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. *Endocrinology* **130**, 43-52.

Larsen TM, Toubro S & Astrup A (2003) Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies. *Journal of Lipid Research* **44**, 2234-2241.

Laws A, Jeppesen JL, Maheux PC, Schaaf P, Chen YDC & Reaven GM (1994) Resistance to insulin-stimulated glucose uptake and dyslipidameia in Asian Indians. *Arteriosclerosis and Thrombosis* **14**, 917-922.

Le Lay S, Boucher J, Rey A, Castan-Laurell I, Krief S, Ferre P, Valet P & Dugail I (2001) Decreased resistin expression in mice with different sensitivities to a high-fat diet. *Biochemical and Biophysical Research Communications* **289**, 564-567.

Le Marchand-Brustel Y, Gual P, Gremeaux T, Gonzalez T, Barres R & Tanti JF (2003) Fatty acid-induced insulin resistance: role of insulin receptor substrate 1 serine phosphorylation in the retroregulation of insulin signalling. *Biochemical Society Transactions* **31**, 1152-1156.

Lee KN, Kritchevsky D & Pariza MW (1994) Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis* **108**, 19-25.

Lee KN, Pariza MW & Ntambi JM (1998) Conjugated linoleic acid decreases hepatic stearoyl-CoA desaturase mRNA expression. *Biochemical and Biophysical Research Communication* **248**, 817-821.

Lee SK, Singh J & Taylor RB (1975) Subclasses of T cells with different sensitivities to cytotoxic antibody in the presence of anaesthetics. *European Journal of Immunology* **5**, 259-262.

Legler G, Muller-Platz CM, Mentges-Hettkamp M, Pflieger G & Julich E (1985) On the chemical basis of the Lowry protein determination. *Analytical Biochemistry* **150**, 278-287.

Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA & Willson TM (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *Journal of Biological Chemistry* **272**, 3137-3140.

Leinonen E, Hurt-Camejo E, Wiklund O, Hultén LM, Hiukka A & Taskinen MR (2003) Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes. *Atherosclerosis* **166**, 387-394.

Leiter (1992) Jax Notes no. 451 (http://jaxmice.jax.org/library/notes/451b.html)

Lerman I, Villa AR, Rios Torres JM, Tamez LE, Gomez Perez F, del Villar Velasco SL & Rull Rodrigo JA (2003) Correlations between surrogate measures of insulin resistance and cardiovascular risk factors in obese and overweight patients. *Journal of Diabetes and its Complications* **17**, 66-72.

Levy JC, Matthews DR & Hermans MP (1998) Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* **21**, 2191-2192.

Li AC & Glass CK (2004) PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis. *Journal of Lipid Research* **45**, 2161-2173.

Libby P (2003) Vascular biology of atherosclerosis: overview and state of the art. *American Journal of Cardiology* **91** (suppl), 3A-6A.

Liew C, Schut HA, Chin SF, Pariza MW & Dashwood RH (1995) Protection of conjugated linoleic acids against 2-amino-3-methylimidazo[4,5-f]quinoline-induced colon carcinogenesis in the F344 rat: a study of inhibitory mechanisms. *Carcinogenesis* **16**, 3037-3043.

Liew C, Schut HA, Chin SF, Pariza MW & Dashwood RH (1995) Protection of conjugated linoleic acids against 2-amino-3- methylimidazo[4,5-f]quinoline-induced colon carcinogenesis in the F344 rat: a study of inhibitory mechanisms. *Carcinogenesis* **16**, 3037-3043.

Lillie RD & Ashburn LL (1943) Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxheimer's technique. *Archives of Pathology* **36**, 432-440.

Lin Y, Kreeft A, Schuurbiers JA & Draijer R (2001) Different effects of conjugated linoleic acid isomers on lipoprotein lipase activity in 3T3-L1 adipocytes. *Journal of Nutritional Biochemistry* **12**, 183-189.

Linsel-Nitschke P & Tall AR (2005) HDL as a target in the treatment of atherosclerotic cardiovascular disease. *Nature Reviews* **4**, 193-205.

Linton MF & Fazio S (2003) Macrophages, inflammation, and atherosclerosis. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S35-S40.

Liu YF, Paz K, Herschkovitz A, Alt A, Tennenbaum T, Sampson SR, Ohba M, Kuroki T, LeRoith D & Zick Y (2001) Insulin stimulates PKCzeta-mediated phosphorylation of insulin receptor substrate-1 (IRS-1). A self-attenuated mechanism to negatively regulate the function of IRS proteins. *Journal of Biological Chemistry* **276**, 14459-14465.

Livak KJ, Flood SJA, Marmaro J, Giusti W & Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridisation. *PCR Methods and Applications* **4**, 357-362.

Lizcano JM & Alessi DR (2002) The insulin signalling pathway. *Current Biology* **12**, R236-R238.

Lovejoy JC, Smith SR, Champagne CM, Most MM, Lefevre M, DeLany JP, Denkins YM, Rood JC, Veldhuis J & Bray GA (2002) Effects of diets enriched in saturated (palmitic), monounsaturated (oleic), or trans (elaidic) fatty acids on insulin sensitivity and substrate oxidation in healthy adults. *Diabetes Care* **25**, 1283-1288.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.

Lucchi L, Banni S, Melis MP, Angioni E, Carta G, Casu V, Rapana R, Ciuffreda A, Corongiu FP & Albertazzi A (2000) Changes in conjugated linoleic acid and its metabolites in patients with chronic renal failure. *Kidney International* **58**, 1695-1702.

Lund S, Holman GD, Schmitz O & Pedersen O (1995) Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proceedings of the National Academy of Sciences, USA* **92**, 5817-5821.

Lusis AJ (2000) Atherosclerosis. *Nature* **407**, 233-241.

M

MacDonald HB (2000) Conjugated linoleic acid and disease prevention: a review of current knowledge. *Journal of the American College of Nutrition* **19**, 111S-118S.

Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T & Matsuzawa Y (2002) Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nature Medicine* **8**, 731-737.

Maffucci T, Brancaccio A, Piccolo E, Stein RC & Falasca M (2003) Insulin induces phosphatidylinositol-3-phosphate formation through TC10 activation. *EMBO Journal* **22**, 4178-4189.

Mahadev K, Zilbering A, Zhu L & Goldstein BJ (2001) Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *Journal of Biological Chemistry* **276**, 21938-21942.

Malpuech-Brugere C, Verboeket-van de Venne WP, Mensink RP, Arnal MA, Morio B, Brandolini M, Saebo A, Lassel TS, Chardigny JM, Sebedio JL & Beaufrere B (2004) Effects of two conjugated linoleic acid isomers on body fat mass in overweight humans. *Obesity Research* **12**, 591-598.

Mannucci E, Bardini G, Ognibene A & Rotella CM (2000) Comparison between 2 insulin sensitivity indexes in obese patients. *Diabetes Care* **23**, 1042-1043.

Mannucci E, Bardini G, Rotella F & Rotella CM (2003) Comparison among different insulin sensitivity indices in obese patients. *Diabetic Medicine* **20**, 462-466.

Manson JE, Colditz GA, Stampfer MJ, Willett WC, Krolewski AS, Rosner B, Arky RA, Speizer FE & Hennekens CH (1991) A prospective study of maturity-onset diabetes

mellitus and risk of coronary heart disease and stroke in women. *Archives of Internal Medicine* **151**, 1141-1147.

Mari A, Pacini G, Murphy E, Ludvik B & Nolan JJ (2001) A model-based method for assessing insulin sensitivity from the oral glucose tolerance test. *Diabetes Care* **24**, 539-548.

Maron DJ, Fair JM & Haskell WL (1991) Saturated fat intake and insulin resistance in men with coronary artery disease. The Stanford Coronary Risk Intervention Project Investigators and Staff. *Circulation* **84**, 2020-2027.

Marrack J (1934) Nature of antibodies. *Nature* **133**, 292-293.

Marshall JA, Bessesen DH & Hamman RF (1997) High saturated fat and low starch and fibre are associated with hyperinsulinaemia in a non-diabetic population: the San Luis Valley Diabetes Study. *Diabetologia* **40**, 430-438.

Martin JC, Gregoire S, Siess MH, Genty M, Chardigny JM, Berdeaux O, Juaneda P & Sebedio JL (2000) Effects of conjugated linoleic acid isomers on lipid metabolising enzymes in male rats. *Lipids* **35**, 91-98.

Mater MK, Thelen AP, Pan DA & Jump DB (1999) Sterol response element-binding protein 1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription. *Journal of Biological Chemistry* **274**, 32725-32732.

Mather KJ, Hunt AE, Steinberg HO, Paradisi G, Hook G, Katz A, Quon MJ & Baron AD (2001) Repeatability characteristics of simple indices of insulin resistance: implications for research applications. *The Journal of Clinical Endocrinology and Metabolism* **86**, 5457-5464.

Matsuda M & DeFronzo RA (1999) Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycaemic insulin clamp. *Diabetes Care* **22**, 1462-1470.

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF & Turner RC (1985) Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**, 412-419.

Matthews JNS, Altman DG, Campbell MJ & Royston P (1990) Analysis of serial measurements in medical research. *British Medical Journal* **300**, 230-235.

Mauvais-Jarvis F, Kulkarni RN & Kahn CR (2002) Knockout models are useful tools to dissect the pathophysiology and genetics of insulin resistance. *Clinical Endocrinology* **57**, 1-9.

Mayer EJ, Newman B, Quesenberry CP, Jr. & Selby JV (1993) Usual dietary fat intake and insulin concentrations in healthy women twins. *Diabetes Care* **16**, 1459-1469.

McAuley KA, Williams SM, Mann JI, Goulding A, Chisholm A, Wilson N, Story G, McLay RT, Harper MJ & Jones IE (2002) Intensive lifestyle changes are necessary to improve insulin sensitivity: a randomised controlled trial. *Diabetes Care* **25**, 445-452.

McCance DR, Hanson RL, Charles MA, Jacobsson LT, Pettitt DJ, Bennett PH &Knowler WC (1994) Comparison of tests for glycated haemoglobin and fasting and two hour plasma glucose concentrations as diagnostic methods for diabetes. *British Medical Journal* **308**, 1323-1328.

McClenaghan NH & Flatt PR (1999) Physiological and pharmacological regulation of insulin release: insights offered through exploitation of insulin-secreting cell lines. *Diabetes Obesity and* Metabolism **1**, 137-150.

McDermott MF (2001) TNF and TNFR biology in health and disease. *Cellular & Molecular Biology (Noisy-le-grand)* **47**, 619-635.

McGarry JD (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* **51**, 7-18.

McGuire MA, McGuire MK, Parodi PW & Jensen RG (1999) Conjugated linoleic acids in human milk. In *Advances in Conjugated Linoleic Acid Research, Volume 1*, eds. Yucawecz MP, Mossoba MM, Kramer JKG, Pariza MW & Nelson GJ, pp296-306. AOCS Press, Champaign Illinois.

McKeown NM, Meigs JB, Liu S, Saltzman E, Wilson PW & Jacques PF (2004) Carbohydrate nutrition, insulin resistance, and the prevalence of the metabolic syndrome in the Framingham Offspring Cohort. *Diabetes Care* **27**, 538-546.

Medina EA, Horn WF, Keim NL, Havel PJ, Benito P, Kelley DS, Nelson GJ & Erickson KL (2000) Conjugated linoleic acid supplementation in humans: effects on circulating leptin concentrations and appetite. *Lipids* **35**, 783-788.

Medina-Gomez G, Virtue S, Lelliott C, Boiani R, Campbell M, Christodoulides C, Perrin C, Jimenez-Linan M, Blount M, Dixon J, Zahn D, Thresher RR, Aparicio S, Carlton M, Colledge WH, Kettunen MI, Seppanen-Laakso T, Sethi JK, O'Rahilly S, Brindle K, Cinti S, Oresic M, Burcelin R & Vidal-Puig A (2005) The link between nutritional status and insulin sensitivity is dependent on the adipocyte-specific peroxisome proliferator-activated receptor-gamma2 isoform. *Diabetes* **54**, 1706-1716.

Metzger S, Begleibter N, Barash V, Drize O, Peretz T, Shiloni E & Chajek-Shaul T (1997) Tumor necrosis factor inhibits the transcriptional rate of glucose-6-phosphatase in vivo and in vitro. *Metabolism* **46**, 579-583.

Miles PD, Barak Y, He W, Evans RM & Olefsky JM (2000) Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency. *Journal of Clinical Investigation* **105**, 287-292.

Miller CC, Park Y, Pariza MW & Cook ME (1994) Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection. *Biochemical and Biophysical Research Communications* **198**, 1107-1012.

Min J, Okada S, Kanzaki M, Elmendorf JS, Coker KJ, Ceresa BP, Syu LJ, Noda Y, Saltiel AR & Pessin JE (1999) Synip: a novel insulin-regulated syntaxin 4-binding protein mediating GLUT4 translocation in adipocytes. *Molecular Cell* **6**, 751-760.

Minamikawa J, Tanaka S, Yamauchi M, Inoue D & Koshiyama H (1998) Potent inhibitory effect of troglitazone on carotid arterial wall thickness in type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism* **83**, 1818-1820.

Miner JL (2004) The adipocyte as an endocrine cell. *Journal of Animal Science* **82**, 935-941.

Minokoshi Y, Kahn CR & Kahn BB (2003) Tissue-specific ablation of the GLUT4 glucose transporter or the insulin receptor challenges assumptions about insulin action and glucose homeostasis. *Journal of Biological Chemistry* **278**, 33609-33612.

Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D & Kahn BB (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* **415**, 339-343.

Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW (1997) Human subcutaneous adipose tissue secretes interleukin-6 but not TNF-α in vivo. *Journal of Clinical Endocrinology and Metabolism* **82,** 4196-4200.

Mohamed-Ali V, Pinkney JH, Coppack SW (1998). Adipose tissue as an endocrine and paracrine organ. *International Journal of Obesity and Related Metabolic Disorders* **22**, 1145-1158.

Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as an endocrine and paracrine organ (1998). *International Journal of Obesity* **22**, 1145-1158.

Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS & Marks JS (2003) Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *Journal of the American Medical Association* **289**, 76-79.

Moller DE & Berger JP (2003) Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S17-S21.

Moller DE (2000) Potential role of TNFα in the pathogenesis of insulin resistance and type 2 diabetes. *Trends in Endocrinology and Metabolism* **11**, 212-217.

Moller DE (2001) New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* **414**, 821-827.

Moloney F, Yeow TP, Mullen A, Nolan JJ & Roche HM (2004) Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. *American Journal of Clinical Nutrition* **80**, 887-895.

Monzillo LU & Hamdy O (2003) Evaluation of insulin sensitivity in clinical practice and in research settings. *Nutrition Reviews* **61**, 397-412.

Moon B, Kwan JJ, Duddy N, Sweeney G & Begum N (2003) Resistin inhibits glucose uptake in L6 cells independently of changes in insulin signaling and GLUT4 translocation. *American Journal of Physiology. Endocrinology and Metabolism* **285**, E106-E115.

Mooney RA, Senn J, Cameron S, Inamdar N, Boivin LM, Shang Y & Furlanetto RW (2001) Suppressors of cytokine signaling-1 and -6 associate with and inhibit the insulin receptor. A potential mechanism for cytokine-mediated insulin resistance. *Journal of Biological Chemistry* **276**, 25889-25893.

Morik KJ (1991) What we can say from in vitro culture work: limitation of interpretation. *Experimental Hematology* **19**, 851-853.

Morris AD, Ueda S, Petrie JR, Connell JM, Elliott HL, Donnelly R (1997) The euglycaemic hyperinsulinaemic clamp: an evaluation of current methodology. *Clinical and Experimental Pharmacolology* **24**, 513-518.

Mothe I & Van Obberghen E (1996) Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662, and 731, modulates insulin action. *Journal of Biological Chemistry* **271**, 11222-11227.

Mougios V, Matsakas A, Petridou A, Ring S, Sagredos A, Melissopoulou A, Tsigilis N & Nikolaidis M (2001) Effect of supplementation with conjugated linoleic acid on human serum lipids and body fat. *Journal of Nutritional Biochemistry* **12**, 585-594.

Moya-Camarena SY & Belury MA (1999) Species differences in the metabolism and regulation of gene expression by conjugated linoleic acid. *Nutrition Reviews* **57**, 336-340.

Muoio DM, Dohm GL, Fiedorek FT, Jr., Tapscott EB & Coleman RA (1997) Leptin directly alters lipid partitioning in skeletal muscle. *Diabetes* **46**, 1360-1363.

N

Nagao K, Inoue N, Wang YM & Yanagita T (2003) Conjugated linoleic acid enhances plasma adiponectin level and alleviates hyperinsulinemia and hypertension in Zucker diabetic fatty (fa/fa) rats. *Biochemical and Biophysical Research Communications* **310**, 562-566.

Nagao K, Inoue N, Wang YM, Hirata J, Shimada Y, Nagao T, Matsui T & Yanagita T (2003) The 10trans,12cis isomer of conjugated linoleic acid suppresses the development of hypertension in Otsuka Long-Evans Tokushima fatty rats. *Biochemical and Biophysical Research Communications* **306**, 134-138.

Nakashima N, Sharma PM, Imamura T, Bookstein R & Olefsky JM (2000) The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **275**, 12889-12895.

Nandi A, Kitamura Y, Kahn CR & Accili D (2004) Mouse models of insulin resistance. *Physiological Reviews* **84**, 623-647.

National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (2001) Executive Summary of the Third Report of the National Cholesterol education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III. *Journal of the American Medical Association* **285**, 2486-2497.

Nave BT, Haigh RJ, Hayward AC, Siddle K & Shepherd PR (1996) Compartment-specific regulation of phosphoinositide 3-kinase by platelet-derived growth factor and insulin in 3T3-L1 adipocytes. *Biochemical Journal* **318**, 55-60.

Nawrocki AR & Scherer PE (2004) The delicate balance between fat and muscle: adipokines in metabolic disease and musculoskeletal inflammation. *Current Opinion in Pharmacology* **4**, 281-289.

Newsholme P, Brennan L, Rubi B & Maechler P (2005) New insights into amino acid metabolism, beta-cell function and diabetes. *Clinical Science* **108**, 185-194.

Nicolosi RJ, Rogers EJ, Kritchevsky D, Scimeca JA & Huth PJ (1997) Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery* **22**, 266-277.

Nielsen JN, Jorgensen SB, Frosig C, Viollet B, Andreelli F, Vaulont S, Kiens B, Richter EA & Wojtaszewski JF (2003) A possible role for AMP-activated protein kinase in exercise-induced glucose utilization: insights from humans and transgenic animals. *Biochemical Society Transactions* **31**, 186-190.

Niesler CU, Siddle K & Prins JB (1998) Human preadipocytes display a depot-specific susceptibility to apoptosis. *Diabetes* **47**, 1365-1368.

NIH Consensus conference (1993) Triglyceride, high-density lipoprotein, and coronary heart disease. NIH Consensus Development Panel on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease. *Journal of the American Medical Association* **269**, 505-510.

Noone E, McBennett S, Gibney MJ & Roche HM (2001) The isomer-specific effects of conjugated linoleic acid on gene expression and lipid metabolism in male Ob/Ob mice. *Proceedings of the Nutrition Society* **60** (suppl), 149A

Noone EJ, Roche HM, Nugent AP & Gibney MJ (2002) The effect of dietary supplementation using isomeric blends of conjugated linoleic acid on lipid metabolism in healthy human subjects. *British Journal of Nutrition* **88**, 243-251.

Norris LA (2003) Blood coagulation. *Best Practice & Research. Clinical Obstetrics & Gynaecology* **17**, 369-383.

Norris LA, Joyce M, O'Keeffe N, Sheppard BL & Bonnar J (2002) Haemostatic risk factors in healthy postmenopausal women taking hormone replacement therapy. *Maturitas* **25**, 125-133.

Novikoff AB, Novikoff PM, Rosen OM & Rubin CS (1980) Organelle relationships in cultured 3T3-L1 preadipocytes. *Journal of Cell Biology* **87**, 180-196.

Nyomba BL, Ossowski VM, Bogardus C & Mott DM (1990) Insulin-sensitive tyrosine kinase: relationship with in vivo insulin action in humans. *American Journal of Physiology* **258**, E964-E974.

O

Ofei F, Hurel S, Newkirk J, Sopwith M & Taylor R (1996) Effects of an engineered human anti-TNF-α antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* **45**, 881-885.

Ohashi A, Matsushita Y, Kimura K, Miyashita K & Saito M (2004) Conjugated linoleic acid deteriorates insulin resistance in obese/diabetic mice in association with decreased production of adiponectin and leptin. *Journal of Nutritional Science and Vitaminology* **50**, 416-421.

Okada T, Kawano Y, Sakakibara T, Hazeki O & Ui M (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *Journal of Biological Chemistry* **269**, 3568-3573.

Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y & Kadowaki T (1998) Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *Journal of Clinical Investigation* **101**, 1354-1361.

Osborn L, Kunkel S & Nabel GJ (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proceedings of the National Academy of Sciences, USA* **86**, 2336-2340.

Osborne TF (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *Journal of Biological Chemistry* **275**, 32379-32382.

Osei K, Cottrell DA, Henry ML, Tesi RJ, Ferguson RM & O'Dorisio TM (1992) Minimal model analysis of insulin sensitivity and glucose-mediated glucose disposal in type 1 (insulin-dependent) diabetic pancreas allograft recipients. *Diabetologia* **35**, 676-680.

O'Shea M, Stanton C & Devery R (1999) Antioxidant enzyme defence responses of human MCF-7 and SW480 cancer cells to conjugated linoleic acid. *Anticancer Research* **19**, 1953-1959.

Ostrowska E, Muralitharan M, Cross RF, Bauman DE & Dunshea FR (1999) Dietary conjugated linoleic acids increase lean tissue and decrease fat deposition in growing pigs. *Journal of Nutrition* **129**, 2037-2042.

Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T & Matsuzawa Y (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* **100**, 2473-2476.

Ozyilkan O, Colak D, Akcali Z & Basturk B (2005) Olive: fruit of peace against cancer. *Asian Pacific Journal of Cancer Prevention* **6**, 77-82.

P

Pacini G & Bergman RN (1986) MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsivity from the frequently sampled intravenous glucose tolerance test. *Computer Methods and Programs in Biomedicine* **23**, 113-122.

Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M & Scherer PE (2003) Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications for metabolic regulation and bioactivity. *Journal of Biological Chemistry* **278**, 9073-9085.

Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M & Scherer PE (2003) Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin: Implications for metabolic regulation and bioactivity. *Journal of Biological Chemistry* **278**, 9073-9085.

Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA & Scherer PE (2004) Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione -mediated improvement in insulin sensitivity. *Journal of Biological Chemistry* **279**, 12152-12162.

Pan DA, Lillioja S, Milner MR, Kriketos AD, Baur LA, Bogardus C & Storlien LH (1995) Skeletal muscle membrane lipid composition is related to adiposity and insulin action. *Journal of Clinical Investigation* **96**, 2802-2808.

Pang G, Couch L, Batey R, Clancy R & Cripps A (1994) GM-CSF, IL-1α, IL-1β, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1α and TNF-α. *Clinical and Experimental Immunology* **96**, 437-443.

Papadakis JA, Milionis HJ, Press M & Mikhailidis DP (2001) Treating dyslipidaemia in non-insulin-dependent diabetes mellitus - a special reference to statins. *Journal of Diabetes and its Complications* **15**, 211-226.

Pariza MW & Hargraves WA (1985) A beef-derived mutagenesis modulator inhibits initiation of mouse epidermal tumors by 7,12-dimethylbenz[a]anthracene. *Carcinogenesis* **6**, 591-593.

Pariza MW, Park Y & Cook ME (2000) Mechanisms of action of conjugated linoleic acid: evidence and speculation. *Proceedings of the Society for Experimental Biology and Medicine* **223**, 8-13.

Park HS, Ryu JH, Ha YL & Park JH (2001) Dietary conjugated linoleic acid induces apoptosis of colonic mucosa in 1,2-dimethylhydrazine-treated rats: a possible mechanism of the anticarcinogenic effect by CLA. *British Journal of Nutrition* **86**, 549-555.

Park Y, Albright KJ, Liu W, Storkson JM, Cook ME & Pariza MW (1997) Effect of conjugated linoleic acid on body composition in mice. *Lipids* **32**, 853-858.

Park Y, Storkson JM, Albright KJ, Liu W & Pariza MW (1999) Evidence that the trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids* **34**, 235-241.

Parker DR, Weiss ST, Troisi R, Cassano PA, Vokonas PS & Landsberg L (1993) Relationship of dietary saturated fatty acids and body habitus to serum insulin concentrations: the Normative Aging Study. *American Journal of Clinical Nutrition* **58**, 129-136.

Parodi PW (1997) Cows' milk fat components as potential anticarcinogenic agents. *Journal of Nutrition* **127**, 1055-1060.

Parodi PW (2003) Conjugated Linoleic Acid and Body Composition. In *Advances in Conjugated Linoleic Acid Research*, Volume 2 eds. Sebedio JL, Christie WW & Adlof R, pp316-324 AOCS Press, Champaign Illinois.

Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH, Smith SA (2003) Resistin is expressed in human macrophages and directly regulated by PPARgamma activators. *Biochemical and Biophysical Research Communications* **300**, 472–476.

Paternostro G, Camici PG, Lammerstma AA, Marinho N, Baliga RR, Kooner JS, Radda GK & Ferrannini E (1996) Cardiac and skeletal muscle insulin resistance in patients with coronary heart disease. A study with positron emission tomography. *Journal of Clinical Investigation* **98**, 2094-2099.

Patti ME & Kahn CR (1998) The insulin receptor - a critical link in glucose homeostasis and insulin action. Journal of Basic and Clinical Physiology and Pharmacology **9**, 89-109.

Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H & Zick Y (1997) A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *Journal of Biological Chemistry* **272**, 29911-29918.

Paz K, Liu YF, Shorer H, Hemi R, LeRoith D, Quan M, Kanety H, Seger R & Zick Y (1999) Phosphorylation of insulin receptor substrate-1 (IRS-1) by protein kinase B positively regulates IRS-1 function. *Journal of Biological Chemistry* **274**, 28816-28822.

Pederson TM, Kramer DL & Rondinone CM (2001) Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. *Diabetes* **50**, 24-31.

Pekala PH, Kawakami M, Angus CW, Lane MD & Cerami A (1983) Selective inhibition of synthesis of enzymes for *de novo* fatty acid biosynthesis by an endotoxin-induced mediator from exudate cells. *Proceedings of the National Academy of Sciences USA* **80**, 2743-2747.

Peraldi P & Spiegelman B (1998) TNF-alpha and insulin resistance: summary and future prospects. *Molecular and Cellular Biochemistry* **182**, 169-175.

Perez-Jimenez F, Lopez-Miranda J, Pinillos MD, Gomez P, Paz-Rojas E, Montilla P, Marin C, Velasco MJ, Blanco-Molina A, Jimenez Pereperez JA & Ordovas JM (2001) A Mediterranean and a high-carbohydrate diet improve glucose metabolism in healthy young persons. *Diabetologia* **44**, 2038-2043.

Persaud SJ & Howell SL (2003) The biosynthesis and secretion of insulin. In *Textbook of Diabetes Third edition*, eds. Pickup JC & Williams, pp13.9. Blackwell Science, Oxford.

Persaud SJ & Howell SL (2003) The biosynthesis of insulin secretion. In *Textbook of Diabetes Volume 1,* eds. Pickup JC & Williams G, pp 13.1-13.17. Blackwell Publishing, Massachusetts, USA.

Perseghin G, Caumo A & Caloni M (2001) Incorporation of the fasting plasma FFA concentration into QUICKI improves its association with insulin sensitivity in non obese individuals. *Journal of Clinical Endocrinology and Metabolism* **86**, 4776-4781.

Perseghin G, Caumo A, Caloni M, Testolin G & Luzi L (2001) Incorporation of the fasting plasma FFA concentration into QUICKI improves its association with insulin sensitivity in nonobese individuals. *Journal of Clinical Endocrinology and Metabolism* 86, 4776-4781.

Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A & Luzi L (1999) Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* **48**, 1600-1606.

Pessin JE & Saltiel AR (2000) Signaling pathways in insulin action: molecular targets of insulin resistance. *Journal of Clinical Investigation* **106**, 165-169.

Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ & Okada S (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. *Journal of Biological Chemistry* **274**, 2593-2596.

Peters AL, Davidson MB, Schriger DL & Hasselblad V (1996) A clinical approach for the diagnosis of diabetes mellitus: an analysis using glycosylated hemoglobin levels. Meta-

analysis Research Group on the Diagnosis of Diabetes Using Glycated Hemoglobin Levels. *Journal of the American Medical Association* **276**, 1246-1252.

Petridou A, Mougios V & Sagredos A (2003) Supplementation with CLA: Isomer incorporation into serum lipids and effect on body fat of women. *Lipids* **38**, 805-811.

Petruschke T & Hauner H (1993) Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. *Journal of Clinical Endocrinology and Metabolism* **76**, 742-747.

Phillips DI, Clark PM, Hales CN & Osmond C (1994) Understanding oral glucose tolerance: comparison of glucose or insulin measurements during the oral glucose tolerance test with specific measurements of insulin resistance and insulin secretion. *Diabetic Medicine* **11**, 286-292.

Pickup JC (2003) Diabetic control and its measurement. In *Textbook of Diabetes Volume 1*, eds. Pickup JC & Williams G, pp 34.5-34.10. Blackwell Publishing, Massachusetts, USA.

Popp-Snijders C, Schouten JA, Heine RJ, van der Meer J & van der Veen EA (1987) Dietary supplementation of omega-3 polyunsaturated fatty acids improves insulin sensitivity in non-insulin-dependent diabetes. *Diabetes Research* **4**, 141-147.

Powell DJ, Hajduch E, Kular G & Hundal HS (2003) Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. *Molecular and Cellular Biology* **23**, 7794-7808.

Prins JB, Niesler CU, Winterford CM, Bright NA, Siddle K, O'Rahilly S, Walker NI & Cameron DP (1997) Tumor necrosis factor-□ induces apoptosis of human adipose cells. *Diabetes* **46**, 1939-1944.

Q

Not applicable

R

Rader DJ, Davidson MH, Caplan RJ & Pears JS (2003) Lipid and lipoprotein ratios: associations with coronary artery disease and effects of rosuvastatin compared with atorvastatin, pravastatin, and simvastatin. *American Journal of Cardiology* **91** (suppl), C20-C24.

Radziuk J (2000) Insulin sensitivity and its measurement: structural commonalities among the methods. *Journal of Clinical Endocrinology and Metabolism* **85**, 4426-4433.

Radziuk J (2000) Insulin sensitivity and its measurement: structural commonalities among the methods. *Journal of Clinical Endocrinology and Metabolism* **85**, 4426-4433.

Rahman SM, Wang Y, Yotsumoto H, Cha J, Han S, Inoue S & Yanagita T (2001) Effects of conjugated linoleic acid on serum leptin concentration, body-fat accumulation, and beta-oxidation of fatty acid in OLETF rats. *Nutrition* **17**, 385-390.

Rajala MW & Scherer PE (2003) Minireview: The adipocyte - at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* **144**, 3765-3773.

Randle PJ, Garland PB, Hales CN, Newsholme EA (1963) The glucose fatty-acid cycle, its role in insulin sensitivity and the metabolic disturbance of diabetes mellitus. *Lancet* **63**, 785-789.

Randle PJ, Newsholme EA & Garland PB (1964) Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. *Biochemical Journal* **93**, 652-665.

Rawadi G, Garcia J, Lemercier B & Roman-Roman S (1999) Signal transduction pathways involved in the activation of NF-kappa B, AP-1, and c-fos by Mycoplasma fermentans membrane lipoproteins in macrophages. *Journal of Immunology* **162**, 2193-2203.

Rea S & James DE (1997) Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. *Diabetes* **46**, 1667-1677.

Rea S, Martin LB, McIntosh S, Macaulay SL, Ramsdale T, Baldini G & James DE (1998) Syndet, an adipocyte target SNARE involved in the insulin-induced translocation of GLUT4 to the cell surface. *Journal of Biological Chemistry* **273**, 18784-18792.

Reaven GM (1995) Pathophysiology of insulin resistance in human disease. *Physiological Reviews* **75**, 473-486.

Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL & Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes & Development* **14**, 2819-2830.

Rett K (1999) The relation between insulin resistance and cardiovascular complications of the insulin resistance syndrome. *Diabetes, Obesity and Metabolism* **1** (suppl 1), S8-S16.

Revzin A (1989) Gel electrophoresis assays for DNA-protein interactions. *Biotechniques* **7**, 346-355.

Ribon V & Saltiel AR (1997) Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes. *Biochemical Journal* **324**, 839-845.

Ribon V, Herrera R, Kay BK & Saltiel AR (1998a) A role for CAP, a novel, multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions. *Journal of Biological Chemistry* **273**, 4073-4080.

Ribon V, Johnson JH, Camp HS & Saltiel AR (1998b) Thiazolidinediones and insulin resistance: peroxisome proliferatoractivated receptor gamma activation stimulates

expression of the CAP gene. *Proceedings of the National Academy of Sciences, USA* **95**, 14751-14756.

Ridker PM (1999) Evaluating novel cardiovascular risk factors: can we better predict heart attacks? *Annals of Internal Medicine* **130**, 933-937.

Ridker PM, Rifai N, Stampfer MJ & Hennekens CH (2000) Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* **101**, 1767-1772.

Rifai N, Albers JJ & Bachorik PS (2001) Lipids, Lipoproteins, and Apolipoproteins. In Tietz Fundamentals of Clinical Chemistry Fifth edition, eds. Burtis CA, Ashwood ER & Border BG, pp464-479. W.B. Saunders Company, London.

Riserus U, Arner P, Brismar K & Vessby B (2002a) Treatment with dietary trans-10, cis-12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. *Diabetes Care* **25**, 1516-1521.

Riserus U, Basu S, Jovinge S, Fredrikson GN, Arnlov J & Vessby B (2002b) Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: a potential link to fatty acid-induced insulin resistance. *Circulation* **106**, 1925-1929.

Riserus U, Berglund L & Vessby B (2001) Conjugated linoleic acid (CLA) reduced abdominal adipose tissue in obese middle-aged men with signs of the metabolic syndrome: a randomised controlled trial. *International Journal of Obesity and Related Metabolic Disorders* **25**, 1129-1135.

Riserus U, Vessby B, Arnlov J & Basu S (2004) Effects of cis-9, trans-11 conjugated linoleic acid supplementation on insulin sensitivity, lipid peroxidation, and proinflammatory markers in obese men. *American Journal of Clinical Nutrition* **80**, 279-283.

Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N & McGuire MA (2001) Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. *Journal of Nutrition* **131**, 1548-1554.

Rivellese AA, Maffettone A, Iovine C, Di Marino L, Annuzzi G, Mancini M & Riccardi G (1996) Long-term effects of fish oil on insulin resistance and plasma lipoproteins in NIDDM patients with hypertriglyceridemia. *Diabetes Care* **19**, 1207-1213.

Roche HM (1999) Unsaturated fatty acids. *Proceedings of the Nutrition Society* **58**, 397-401.

Roche HM (2005) Fatty acids and the metabolic syndrome. *Proceedings of the Nutrition Society* **64**, 23-29.

Roche HM, Noone E, Nugent A & Gibney (2001) Conjugated linoleic acid: a novel therapeutic nutrient? *Nutrition Research Reviews* **14**: 173-187.

Roche HM, Noone E, Sewter C, Mc Bennett S, Savage D, Gibney MJ, O'Rahilly S & Vidal-Puig AJ (2002) Isomer-dependent metabolic effects of conjugated linoleic acid: insights from molecular markers sterol regulatory element-binding protein-1c and LXRalpha. *Diabetes* **51**, 2037-2044.

Roche S, Downward J, Raynal P & Courtneidge SA (1998) A function for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in fibroblasts during mitogenesis: requirement for insulin- and lysophosphatidic acid-mediated signal transduction. *Molecular and Cellular Biology* **18**, 7119-7129.

Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI (1996) Mechanism of free fatty acid-induced insulin resistance in humans. *Journal of Clinical Investigation* **97**, 2859-2865.

Rodnick KJ, Slot JW, Studelska DR, Hanpeter DE, Robinson LJ, Geuze HJ & James DE (1992) Immunocytochemical and biochemical studies of GLUT4 in rat skeletal muscle. *Journal of Biological Chemistry* **267**, 6278-6285.

Rodriguez AM, Elabd C, Delteil F, Astier J, Vernochet C, Saint-Marc P, Guesnet J, Guezennec A, Amri EZ, Dani C & Ailhaud G (2004) Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochemical and Biophysical Research Communications* **315**, 255-263.

Rodriguez BL, Lau N, Burchfiel CM, Abbott RD, Sharp DS, Yano K & Curb JD (1999) Glucose intolerance and 23-year risk of coronary heart disease and total mortality: the Honolulu Heart Program. *Diabetes Care* **22**, 1262-1265.

Rondinone CM, Carvalho E, Wesslau C & Smith UP (1999) Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus. *Diabetologia* **42**, 819-825.

Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH & Smith U (1997) Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proceedings of the National Academy of Sciences, USA* **94**, 4171-4175.

Rosenbaum SE & Greenberg AS (1998) The short- and long-term effects of tumor necrosis factor-alpha and BRL 49653 on peroxisome proliferator-activated receptor (PPAR)gamma2 gene expression and other adipocyte genes. *Molecular Endocrinology* **12**, 1150-1160.

Ross R (1999) Atherosclerosis - an inflammatory disease. *New England Journal of Medicine* **340**, 115-126.

Rotter V, Nagaev I & Smith U (2003) Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *Journal of Biological Chemistry* **278**, 45777-45784.

Ruan H & Lodish HF (2003) Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine & Growth Factor Reviews* **14**, 447-455.

Ruan H, Hacohen N, Golub TR, Van Parijs L & Lodish HF (2002b) Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes* **51**, 1319-1336.

Ruan H, Miles PD, Ladd CM, Ross K, Golub TR, Olefsky JM & Lodish HF (2002a) Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. *Diabetes* **51**, 3176-3188.

Ruan H, Pownall HJ & Lodish HF (2003) Troglitazone antagonises tumor necrosis factor-alpha-induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-kappaB. *Journal of Biological Chemistry* **278**, 28181-28192.

Rudenski AS, Hosker JP, Burnett MA, Matthews DR& Turner RC (1988) The beta cell glucose stimulus-response curve in normal humans assessed by insulin and C-peptide secretion rates. *Metabolism* **37**, 526-534.

Rudenski AS, Matthews DR, Levy JC & Turner RC (1991) Understanding "insulin resistance": both glucose resistance and insulin resistance are required to model human diabetes. *Metabolism* **40**, 908-917.

Ruderman NB, Cacicedo JM, Itani S, Yagihashi N, Saha AK, Ye JM, Chen K, Zou M, Carling D, Boden G, Cohen RA, Keaney J, Kraegen EW & Ido Y (2003) Malonyl-CoA and AMP-activated protein kinase (AMPK): possible links between insulin resistance in muscle and early endothelial cell damage in diabetes. *Biochemical Society Transactions* **31**, 202-206.

Ruderman NB, Saha AK, Vavvas D & Witters LA (1999) Malonyl-CoA, fuel sensing, and insulin resistance. *American Journal of Physiology* **276**, E1-E18.

Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A & White MF (2001) Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *Journal of Clinical Investigation* **107**, 181-189.

Rui L, Yuan M, Frantz D, Shoelson S & White MF (2002) SOCS-1 and SOCS-3 block insulin signalling by ubiquitin-mediated degradation of IRS1 and IRS2. *Journal of Biological Chemistry* **277**, 42394-42398.

Rutter GA, Da Silva Xavier G &Leclerc I (2003) Roles of 5'-AMP-activated protein kinase (AMPK) in mammalian glucose homoeostasis. *Biochemical Journal* **375**, 1-16.

Ryder JW, Portocarrero CP, Song XM, Cui L, Yu M, Combatsiaris T, Galuska D, Bauman DE, Barbano DM, Charron MJ, Zierath JR & Houseknecht KL (2001) Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes* **50**, 1149-1157.

S

Saad MF, Anderson RL, Laws A, Watanabe RM, Kades WW, Chen YD, Sands RE, Pei D, Savage PJ & Bergman RN (1994) A comparison between the minimal model and the glucose clamp in the assessment of insulin sensitivity across the spectrum of glucose tolerance. Insulin Resistance Atherosclerosis Study. *Diabetes* **43**, 1114-1121.

Saladin R, Fajas L, Dana S, Halvorsen YD, Auwerx J & Briggs M (1999) Differential regulation of peroxisome proliferator activated receptor gamma1 (PPARgamma1) and PPARgamma2 messenger RNA expression in the early stages of adipogenesis. *Cell Growth & Differentiation* **10**, 43-48.

Salminen I, Mutanen M, Jauhiainen M & Aro A (1998) Dietary *trans* fatty acids increase conjugated linoleic acid levels in human serum. *Journal of Nutritional Biochemistry* **9**, 93-98.

Saltiel AR & Kahn CR (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799-806.

Saltiel AR & Pessin JE (2002) Insulin signaling pathways in time and space. *Trends in Cell Biology* **12**, 65-71.

Saltiel AR (2001) New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. *Cell* **104** 517-529.

Sartipy P & Loskutoff DJ (2003a) Expression profiling identifies genes that continue to respond to insulin in adipocytes made insulin-resistant by treatment with tumor necrosis factor-alpha. *Journal of Biological Chemistry* **278**, 52298-52306.

Sartipy R & Loskutoff (2003b) Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences, USA* **100**, 7265-7270.

Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, O'Rahilly S (2001) Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes* **50**, 2199–2202.

Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV & O'Rahilly S (2001) Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes* **50**, 2199-2202.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. *Journal of Biological Chemistry* **270**, 26746-26749.

Schmitz ML, Bacher S & Kracht M (2001) I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. *Trends in Biochemical Sciences* **26**, 186-190.

Schmitz-Peiffer C & Whitehead JP (2003) IRS-1 regulation in health and disease. *IUBMB Life* **55**, 367-374.

Schmitz-Peiffer C (2002) Protein kinase C and lipid-induced insulin resistance in skeletal muscle. *Annals of the New York Academy of Sciences* **967** 146-157.

Schoenherr W & Jewell D (1999) Effect of conjugated linoleic acid on body composition of mature obese beagles. FASEB Journal **13**, A262.

Schonberg S & Krokan HE (1995) The inhibitory effect of conjugated dienoic derivatives (CLA) of linoleic acid on the growth of human tumor cell lines is in part due to increased lipid peroxidation. *AntiCancer Research* **15**, 1241-1246.

Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwender S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B (2000) Role of LXRs in control of lipogenesis. *Genes and Development* 14, 2831-2838.

Sebedio JL, Gnaedig S & Chardigny JM (1999) Recent advances in conjugated linoleic acid research. *Current Opinion in Clinical Nutrition and Metabolic Care* **2**, 499-506.

Sebedio JL, Juaneda P, Dobson G, Ramilison I, Martin JC, Chardigny JM & Christie WW (1997) Metabolites of conjugated isomers of linoleic acid in the rat. *Biochimica et Biophysica Acta* **1345**, 5-10.

Sell H, Deshaies Y & Richard D (2004) The brown adipocyte: update on its metabolic role. *International Journal of Biochemistry & Cell Biology* **36**, 2098-2104.

Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW & Mooney RA (2003) Suppressor of cytokine signaling-3, a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *Journal of Biological Chemistry* **278**, 13740-13746.

Sesti G, Federici M, Hribal ML, Lauro D, Sbraccia P & Lauro R (2001) Defects of the insulin receptor substrate system in human metabolic disorders. *FASEB Journal* **15**, 2099-2111.

Sewter C & Vidal-Puig A (2002) PPARgamma and the thiazolidinediones: molecular basis for a treatment of 'Syndrome X'? *Diabetes, Obesity & Metabolism* **4**, 239-248.

Shantha NC, Crum AD & Decker EA (1994) Evaluation of conjugated linoleic acid concentrations in cooked beef. *Journal of Agricultural and Food Chemistry* **42**, 1757-1760.

Shepherd PR & Kahn BB (1999) Glucose transporters and insulin action - implications for insulin resistance and diabetes mellitus. *New England Journal of Medicine* **341**, 248-257.

Shepherd PR (2005) Mechanisms regulating phosphoinositide 3-kinase signalling in insulin-sensitive tissues. *Acta Physiologica Scandinavica* **183**, 3-12.

Shepherd PR, Nave BT & Siddle K (1995) Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase. *Biochemical Journal* **305,** 25-28.

Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB & Unger RH (1997) Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proceedings of the National Academy of Sciences, USA* **94**, 4637-4641.

Shimabukuro M, Zhou YT, Levi M & Unger RH (1998) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proceedings of the National Academy of Science, USA* **95**, 2498-2502.

Shimomura I, Hammer RE, Ikemoto S, Brown MS & Goldstein JL (1999) Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* **401**, 73-76.

Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS & Goldstein JL (2000) Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Molecular Cell* **6**, 77-86.

Shimomura I, Shimano H, Horton JD, Goldstein JL & Brown MS (1997) Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *Journal of Clinical Investigation* **99**, 838-845.

Shoelson SE, Lee J & Yuan M (2003) Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S49-S52.

Shulman GI (2000) Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation* **106**, 171-176.

Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA & Shulman RG (1990) Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. *New England Journal of Medicine* **322**, 223-228.

Siegel RD, Cupples A, Schaefer EJ & Wilson PW (1996) Lipoproteins, apolipoproteins, and low-density lipoprotein size among diabetics in the Framingham offspring study. *Metabolism* **45**, 1267-1272.

Sievenpiper JL, Jenkins DJ, Josse RG & Vuksan V (2001) Dilution of the 75-g oral glucose tolerance test improves overall tolerability but not reproducibility in subjects with different body compositions. *Diabetes Research and Clinical Practice* **51**, 87-95.

Smedman A & Vessby B (2001) Conjugated linoleic acid supplementation in humans - metabolic effects. *Lipids* **36**, 773-781.

Smith SA (2003) Central role of the adipocyte in the insulin-sensitising and cardiovascular risk modifying actions of the thiazolidediones. *Biochemie* **85**, 1219-1230.

Smith U (2002) Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance - is insulin resistance initiated in the adipose tissue? *International Journal of Obesity and Related Metabolic Disorders* **26**, 897-904.

Smith U, Axelsen M, Carvalho E, Eliasson B, Jansson PA & Wesslau C (1999) Insulin signaling and action in fat cells: associations with insulin resistance and type 2 diabetes. *Annals of the New York Academy of Sciences* **892**, 119-126.

Smith U, Gogg S, Johansson A, Olausson T, Rotter V & Svalstedt B (2001) Thiazolidinediones (PPARgamma agonists) but not PPARalpha agonists increase IRS-2 gene expression in 3T3-L1 and human adipocytes. *FASEB Journal* **15**, 215-220.

Sone H, Takahashi A, Iida K & Yamada N (2001) Disease model: hyperinsulinemia and insulin resistance. Part B-polygenic and other animal models. *Trends in Molecular Medicine* **7**, 373-376.

Sonnenberg GE, Krakower GR & Kissebah AH (2004) A novel pathway to the manifestations of metabolic syndrome. *Obesity Research* **12**, 180-186.

Spanger J, Kroke A, Mohlig M, Hoffman K, Bergman MM, Ristow M, Boeing H & Pfeiffer AF (2003) Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective investigation into cancer and nutrition (EPIC)-Potsdam Study. *Diabetes* **52**, 812-817.

Stamler J, Vaccaro O, Neaton JD & Wentworth D (1993) Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. *Diabetes Care* **16**, 434-444.

Stampfer MJ, Krauss RM, Ma J, Blanche PJ, Holl LG, Sacks FM & Hennekens CH (1996) A prospective study of triglyceride level, low-density lipoprotein particle diameter, and risk of myocardial infarction. *Journal of the American Medical Association* **276**, 882-888.

Stangl GI, Muller H & Kirchgessner M (1999) Conjugated linoleic acid effects on circulating hormones, metabolites and lipoproteins, and its proportion in fasting serum and erythrocyte membranes of swine. *European Journal of Nutrition* **38**, 271-277.

Stears AJ & Byrne CD (2001) Adipocyte metabolism and the metabolic syndrome. *Diabetes, Obesity & Metabolism* **3**, 129-142.

Stefan N, Wahl HG, Fritsche A, Haring H & Stumvoll (2001) Effect of the pattern of elevated free fatty acids on insulin sensitivity and insulin secretion in healthy humans. *Hormone Metabolism Research* **33**, 432-438.

Steffensen KR & Gustafsson JA (2004) Putative metabolic effects of the liver X receptor. *Diabetes* **53** (suppl 1), S36-S42.

Steinberg D (2002) Atherogenesis in perspective: hypercholesterolaemia and inflammation as partners in crime. *Nature Medicine* **8**, 1211-1217.

Stephens JM & Pekala PH (1991) Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. *Journal of Biological Chemistry* **266**, 21839-21845.

Stephens JM & Pekala PH (1992) Transcriptional repression of the C/EBP-alpha and GLUT4 genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. Regulations is coordinate and independent of protein synthesis. *Journal of Biological Chemistry* **267**, 13580-13584.

Steppan CM & Lazar MA (2004) The current biology of resistin. *Journal of Internal Medicine* **255**, 439-447.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS & Lazar MA (2001) The hormone resistin links obesity to diabetes. *Nature* **409**, 307-312.

Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG & Pascoe WS (1987) Fish oil prevents insulin resistance induced by high-fat feeding in rats. *Science* **237**, 885-888.

Straczkowski M, Kowalska I, Nikolajuk A, Dzienis-Straczkowska S, Szelachowska M & Kinalska I (2003) Plasma interleukin 8 concentrations in obese subjects with impaired glucose tolerance. *Cardiovascular Diabetology* **2**, 5.

Stumvoll M, Fritsche A & Haring H (2001) The OGTT as test for beta cell function? *European Journal of Clinical Investigation* **31**, 380-381.

Stumvoll M, Mitrakou A, Pimenta W, Jenssen T, Yki-Jarvinen H, Van Haeften T, Renn W & Gerich J (2000) Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes Care* **23**, 295-301.

Sul HS, Latasa MJ, Moon Y & Kim KH (2000) Regulation of the fatty acid synthase promoter by insulin. *Journal of Nutrition* **130**, 315S-320S.

Sumida M, Shiosaka T, Nagar A, Isshikimasuda M, Okuda H & Hamada M (1997) Suppressive effect of tumor necrosis factor-α on adipogenic cell differentiation and on gene expression of hormone-sensitive lipase. *Journal of Clinical Biochemistry and Nutrition* **22**, 1-11

Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD & Stein DT (1999) Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *American Journal of Physiology* **276**, E977-E989.

T

Takahashi Y, Kushiro M, Shinohara K & Ide T (2002) Dietary conjugated linoleic acid reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **133**, 395-404.

Takemori H, Katoh Y, Horike N, Doi J & Okamoto M (2002) ACTH-induced nucleocytoplasmic translocation of salt-inducible kinase. Implication in the protein kinase A-activated gene transcription in mouse adrenocortical tumor cells. *Journal of Biological Chemistry* **277**, 42334-42343.

Tall AR, Jiang X, Luo Y & Silver D (2000) 1999 George Duff memorial lecture: Lipid transfer proteins, HDL metabolism, and atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **20**, 1185-1188.

Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, Sekihara H, Yoshioka S, Horikoshi H, Furuta Y, Ikawa Y, Kasuga M, Yazaki Y & Aizawa S (1994) Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* **372**, 182-186.

Tamori Y, Kawanishi M, Niki T, Shinoda H, Araki S, Okazawa H & Kasuga M (1998) Inhibition of insulin-induced GLUT4 translocation by Munc18c through interaction with syntaxin4 in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **273**, 19740-19746.

Taniguchi A, Nakai Y, Doi K, Fukuzawa H, Fukushima M, Kawamura H, Tokuyama K, Suzuki M, Fujitani J, Tanaka H, et al (1995) Insulin sensitivity, insulin secretion, and glucose effectiveness in obese subjects: a minimal model analysis. *Metabolism* **44**, 1397-1400.

Taniguchi CM, Ueki K & Kahn R (2005) Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *Journal of Clinical Investigation* **115**, 718-727.

Taylor CG & Zahradka P (2004) Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. *American Journal of Clinical Nutrition* **79**, 1164S-1168S.

Taylor RW, Keil D, Gold EJ, Williams SM & Goulding A (1998) Body mass index, waist girth, and waist-to-hip ratio as indexes of total and regional adiposity in women: evaluation in women: evaluation using receiver operating characteristic curves. *American Journal of Clinical Nutrition* **67**, 44-49.

Terauchi Y, Tsuji Y, Satoh S, Minoura H, Murakami K, Okuno A, Inukai K, Asano T, Kaburagi Y, Ueki K, Nakajima H, Hanafusa T, Matsuzawa Y, Sekihara H, Yin Y, Barrett JC, Oda H, Ishikawa T, Akanuma Y, Komuro I, Suzuki M, Yamamura K, Kodama T, Suzuki H, Koyasu S, Aizawa S, Tobe K, Fukui Y, Yazaki Y & Kadowaki T (1999) Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. *Nature Genetics* **21**, 230-235.

Terpstra AH (2004) Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. *American Journal of Clinical Nutrition* **79**, 352-361.

The Jackson Laboratory, Research models for Diabetes and Obesity Research (http://jaxmice.jax.org/models/diabetes_obesity)

Thurmond DC, Ceresa BP, Okada S, Elmendorf JS, Coker K & Pessin JE (1998) Regulation of insulin-stimulated GLUT4 translocation by Munc18c in 3T3L1 adipocytes. *Journal of Biological Chemistry* **273**, 33876-33883.

Tirosh A, Potashnik R, Bashan N & Rudich A (1999) Oxidative stress disrupts insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. A putative cellular mechanism for impaired protein kinase B activation and GLUT4 translocation. *Journal of Biological Chemistry* **274**, 10595-10602.

Tischendorf F, Mockel P, Schone F, Plonne M & Jahreis G (2002) Effect of dietary conjugated linoleic acids on the distribution of fatty acids in serum lipoprotein fractions and different tissues of growing pigs. *Journal of Animal Physiology and Animal Nutrition* **86**, 313-325.

Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, Lodish HF & Ruderman NB (2002) Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proceedings of the National Academy of Sciences, USA* **99**, 16309-16313.

Toomey S, Roche H, Fitzgerald D & Belton O (2003) Regression of pre-established atherosclerosis in the apoE-/- mouse by conjugated linoleic acid. *Biochemical Society Transactions* **31**, 1075-1079.

Torti FM, Torti SV, Larrick JW & Ringold GM (1989) Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor. *Journal of Cell Biology* **108**, 1105-1113.

Towbin H, Staehelin T & Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76**: 4350-4354.

Tracy RP (2003) Emerging relationships of inflammation, cardiovascular disease and chronic diseases of aging. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S29-S34.

Trayhurn P & Beattie JH (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proceedings of the Nutrition Society* **60,** 329-339.

Trayhurn P & Wood IS (2004) Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition* **92**, 347-355.

Tricon S, Burdge GC, Kew S, Banerjee T, Russell JJ, Jones EL, Grimble RF, Williams CM, Yaqoob P & Calder PC (2004) Opposing effects of cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid on blood lipids in healthy humans. *American Journal of Clinical Nutrition* **80**, 614-620.

Trujillo ME & Scherer PE (2005) Adiponectin - journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. *Journal of Internal Medicine* **257**, 167-175.

Tsakiridis T, Tong P, Matthews B, Tsiani E, Bilan PJ, Klip A & Downey GP (1999) Role of the actin cytoskeleton in insulin action. *Microscopy Research and Technique* **47**, 79-92.

Tsao TS, Lodish HF & Fruebis J (2002) ACRP30, a new hormone controlling fat and glucose metabolism. *European Journal of Pharmacology* **440**, 213-221.

Tsao TS, Lodish HF & Fruebis J (2002) ACRP30, a new hormone controlling fat and glucose metabolism. *European Journal of Pharmacology* **440**, 213-221.

Tsuboyama-Kasaoka N, Miyazaki H, Kasaoka S & Ezaki O (2000) Increasing the amount of fat ina conjugated linoleic acid-supplemented diet reduces lipodystrophy in mice. *Journal of Nutrition* **133**, 1793-1799.

Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Kim HJ, Tange T, Okuyama H, Kasai M, Ikemoto S & Ezaki O (2000) Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes* **49**, 1534-1542.

Tushuizen ME, Diamant M & Heine RJ (2005) Postprandial dysmetabolism and cardiovascular disease in type 2 diabetes. *Postgraduate Medical Journal* **81**, 1-6.

U

Uysal KT, Wiesbrock SM, Marino MW & Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* **389**, 610-614.

V

Vague P & Nguten L (2001) Rationale and methods for the estimation of insulin secretion in a given patient: from research to clinical practice. *Diabetes* **51**, (suppl 1), S240-S244.

Valeille K, Gripois D, Blouquit MF, Souidi M, Riottot M, Bouthegourd JC, Serougne C & Martin JC (2004) Lipid atherogenic risk markers can be more favourably influenced by the cis-9, trans-11-octadecadienoate isomer than a conjugated linoleic acid mixture or fish oil in hamsters. *British Journal of Nutrition* **91**, 191-199.

Valverde AM, Burks DJ, Fabregat I, Fisher TL, Carretero J, White MF & Benito M (2003) Molecular mechanisms of insulin resistance in IRS-2-deficient hepatocytes. *Diabetes* **52**, 2239-2248.

Van Obberghen, Baron V, Delahaye L, Emanuelli B, Filippa N, Giorgetti-Peraldi S, Lebrun P, Mothe-Satney I, Peraldi P, Rocchi S, Sawka-Verhelle D, Tartare-Deckert S & Giudicelli J (2001) Surfing the insulin signalling web. *European Journal of Clinical Investigation* **31**, 966-977.

Ventre J, Doebber T, Wu M, MacNaul K, Stevens K, Pasparakis M, Kollias G & Moller DE (1997) Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice. *Diabetes* **46**, 1526-1531.

Vessby B, Unsitupa M, Hermansen K, Riccardi G, Rivellese AA, Tapsell LC, Nalsen C, Berglund L, Louheranta A, Rasmussen BM, Calvert GD, Maffetone A, Pedersen E, Gustafsson IB & Storlien LH (2001) Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia* **44**, 312-319.

Vidal-Puig A & O'Rahilly S (2001) Resistin: a new link between obesity and insulin resistance? *Clinical Endocrinology* **55**, 437-438.

Vidal-Puig AJ, Considine RV, Jimenez-Linan M, Werman A, Pories WJ, Caro JF & Flier JS (1997) Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *Journal of Clinical Investigation* **99**, 2416-2422.

Villegas R, Perry IJ, Creagh D, Hinchion R & O'Halloran D (2003) Prevalence of the metabolic syndrome in middle-aged men and women. *Diabetes Care* **26**, 3198-3199.

Volchuk A, Wang Q, Ewart HS, Liu Z, He L, Bennett MK & Klip A (1996) Syntaxin 4 in 3T3-L1 adipocytes: regulation by insulin and participation in insulin-dependent glucose transport. *Molecular and Cellular Biology* 7, 1075-1082.

W

Wada T, Sasaoka T, Funaki M, Hori H, Murakami S, Ishiki M, Haruta T, Asano T, Ogawa W, Ishihara H & Kobayashi M (2001) Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes via its 5'-phosphatase catalytic activity. *Molecular and Cellular Biology* **21**, 1633-1646.

Wahle KW, Heys SD & Rotondo D (2004) Conjugated linoleic acids: are they beneficial or detrimental to health? *Progress in Lipid Research* **43**, 553-587.

Wallace AJ, McCarthy SN & Gibney MJ (2002) Intakes of saturated, monosaturated and polyunsaturated fat in Irish adults: findings of the North/South Ireland food consumption survey. *Proceedings of the Nutrition Society* **61** (suppl), 85A.

Wallace TM & Matthews DR (2002) The assessment of insulin resistance in man. *Diabetic Medicine* **19**, 527-534.

Wallace TM, Levy JC & Matthews (2004) Use and abuse of HOMA modeling. *Diabetes Care* **27**, 1487-1495.

Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, Ohlsson C & Jansson JO (2002) Interleukin-6-deficient mice develop mature-onset obesity. *Nature Medicine* **8,** 75-79.

Wang M & Tafuri S (2003) Modulation of PPARgamma activity with pharmaceutical agents: treatment of insulin resistance and atherosclerosis. *Journal of Cellular Biochemistry* **89**, 38-47.

Wang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR & Klip A (1999) Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Molecular and Cellular Biology* **19**, 4008-4018.

Wang Y & Jones PJ (2004) Dietary conjugated linoleic acid and body composition. *American Journal of Clinical Nutrition* **79**, 1153S-1158S.

Wargent E, Sennitt MV, Stocker C, Mayes AE, Brown L, O'Dowd J, Wang S, Einerhand AW, Mohede I, Arch JR & Cawthorne MA (2005) Prolonged treatment of genetically obese

mice with conjugated linoleic acid improves glucose tolerance and lowers plasma insulin concentration: possible involvement of PPAR activation. *Lipids in Health and Disease* **4**, 3.

Warren JM, Simon VA, Bartolini G, Erickson KL, Mackey BE & Kelley DS (2003) Trans-10, cis-12 CLA increases liver and decreases adipose tissue lipids in mice: possible roles of specific lipid metabolism genes. *Lipids* **38**, 497-504.

Waters SB, D'Auria M, Martin SS, Nguyen C, Kozma LM & Luskey KL (1997) The amino terminus of insulin-responsive aminopeptidase causes Glut4 translocation in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **272**, 23323-23327.

Watson RT & Pessin JE (2001) Intracellular organisation of insulin signaling and GLUT4 translocation. *Recent Progress in Hormone Research* **56**, 175-193.

Way JM, Gorgun CZ, Tong Q, Uysal KT, Brown KK, Harrington WW, Oliver WR Jr, Willson TM, Kliewer SA & Hotamisligil GS (2001) Adipose tissue resistin expression is severely suppressed in obesity and stimulated by peroxisome proliferator-activated receptor gamma agonists. *Journal of Biological Chemistry* **276**, 25651-25653.

Weir GC, Bonner-Weir S & Sharma A (2000) Regulation of Insulin secretion and islet cell function. In *Atlas of Clinical Endocrinology Volume 2*, eds. Korenman SG & Kahn CR, pp1-13. Blackwell Science, Philadelphia.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL & Ferrante AW, Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation* **112**, 1796-1808.

Welch S, Gebhart SS, Bergman RN & Phillips LS (1990) Minimal model analysis of intravenous glucose tolerance test-derived insulin sensitivity in diabetic subjects. *Journal of Clinical Endocrinology and Metabolism* **71**, 1508-1518.

Wellen KE & Hotamisligil GS (2003) Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation* **112**, 1785-1788.

Werner ED, Lee J, Hansen L, Yuan M & Shoelson SE (2004) Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302. *Journal of Biological Chemistry* **279**, 35298-35305.

West DB, Blohm FY, Truett AA & DeLany JP (2000) Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. *Journal of Nutrition* **130**, 2471-2477.

West DB, Delany JP, Camet PM, Blohm F, Truett AA & Scimeca J (1998) Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. *American Journal of Physiology* **275**, R667-R672.

Whigham LD, O'Shea M, Mohede IC, Walaski HP & Atkinson RL (2004) Safety profile of conjugated linoleic acid in a 12-month trial in obese humans. *Food and Chemical Toxicology* **42**, 1701-1709.

White MF (2002) IRS proteins and the common path to diabetes. *American Journal of Physiology. Endocrinology and Metabolism* **283**, E413-E422.

Willy PJ & Mangelsdorf DJ (1997) Unique requirements for retinoid-dependent transcriptional activation by the orphan receptor LXR. *Genes & Development* **11**, 289-298.

Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA & Mangelsdorf DJ (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes & Development* **9**, 1033-1045.

Wilson TA, Nicolosi RJ, Chrysam M & Kritchevsky D (2000) Conjugated linoleic acid reduces early aortic atherosclerosis greater than linoleic acid in hypercholesterolemic hamsters. *Nutrition Research* **20**, 1795-1805

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S & White MF (1998) Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391**, 900-904.

Wolever TM, Chiasson JL, Csima A, Hunt JA, Palmason C, Ross SA & Ryan EA (1998) Variation of postprandial plasma glucose, palatability, and symptoms associated with a standardized mixed test meal versus 75 g oral glucose. *Diabetes Care* **21**, 336-340.

Woods A, Brull DJ, Humphries SE & Montgomery HE (2000) Genetics of inflammation and risk of coronary artery disease: the central role of interleukin-6. *European Heart Journal* **21**, 1574-1583.

Worgall TS, Sturley SL, Seo T, Osborne TF & Deckelbaum RJ (1998) Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *Journal of Biological Chemistry* **273**, 25537-25540.

X

Xing H, Northrop JP, Grove JR, Kilpatrick KE, Su JL & Ringold GM (1997) TNF alpha-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPARgamma without effects on Pref-1 expression. *Endocrinology* **138**, 2776-2783.

Xing L & Remick DG (2005) Mechanisms of dimethyl sulfoxide augmentation of IL-1 beta production. *Journal of Immunology* **174**, 6195-6202.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA & Chen H (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *Journal of Clinical Investigation* **112**, 1821-1830.

Xu J, Nakamura MT, Cho HP & Clarke SD (1999) Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *Journal of Biological Chemistry* **274**, 23577-23583.

Y

Yahagi N, Shimano H, Matsuzaka T, Sekiya M, Najima Y, Okazaki S, Okazaki H, Tamura Y, Iizuka Y, Inoue N, Nakagawa Y, Takeuchi Y, Ohashi K, Harada K, Gotoda T, Nagai R, Kadowaki T, Ishibashi S, Osuga J & Yamada N (2004) p53 involvement in the pathogenesis of fatty liver disease. *Journal of Biological Chemistry* **279**, 20571-20575.

Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R & Kadowaki T (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423**, 762-769.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB & Kadowaki T (2002) Adiponectin stimulates glucose utilisation and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine* **8**, 1288-1295.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB & Kadowaki T (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine* **8**, 1288-1295.

Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R & Kadowaki T (2002) Globular adiponectin protected ob/ob mice from diabetes and apoE deficient mice from atherosclerosis. *Journal of Biological Chemistry* **278**, 2461-2468.

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P & Kadowaki T (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine* **7**, 941-946.

Yang C, Coker KJ, Kim JK, Mora S, Thurmond DC, Davis AC, Yang B, Williamson RA, Shulman GI & Pessin JE (2001) Syntaxin 4 heterozygous knockout mice develop muscle insulin resistance. *Journal of Clinical Investigation* **107**, 1311-1318.

Yeni-Komshian H, Carantoni M, Abbasi F & Reaven GM (2000) Relationship between several surrogate estimates of insulin resistance and quantification of insulin-mediated glucose disposal in 490 healthy nondiabetic volunteers. *Diabetes Care* **23**, 171-175.

Yoshikawa T, Ide T, Shimano H, Yahagi N, Amemiya-Kudo M, Matsuzaka T, Yatoh S, Kitamine T, Okazaki H, Tamura Y, Sekiya M, Takahashi A, Hasty AH, Sato R, Sone H, Osuga J, Ishibashi S & Yamada N (2003) Cross-talk between peroxisome proliferator-activated receptor alpha and liver X receptor in nutritional regulation of fatty acid metabolism. I. PPARs suppress sterol regulatory element binding protein-1c promoter through inhibition of LXR signaling. *Molecular Endocrinology* **17**, 1240-1254.

Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Kimura S, Ishibashi S & Yamada N (2001) Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Molecular and Cellular Biology* **21**, 2991-3000.

Yoshizaki T, Maegawa H, Egawa K, Ugi S, Nishio Y, Imamura T, Kobayashi T, Tamura S, Olefsky JM & Kashiwagi A (2003) Protein phosphatase-2Cα as a positive regulator of insulin sensitivity through direct activation of phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **279**, 22715-22726.

Young PW, Cawthorne MA, Coyle PJ, Holder JC, Holman GD, Kozka IJ, Kirkham DM, Lister CA & Smith SA (1995) Repeat treatment of obese mice with BRL 49653, a new potent insulin sensitiser, enhances insulin action in white adipocytes. Association with increased insulin binding and cell-surface GLUT4 as measured by photoaffinity labelling. *Diabetes* **44**, 1087-1092.

Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW & Shulman GI (2002b) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 associated phosphatidylinositol 3-kinase activity in muscle. *Journal of Biological Chemistry* **277**, 50230-50236.

Yu JG, Javorschi S, Hevener AL, Kruszynska YT, Norman RA, Sinha M & Olefsky JM (2002a) The effect of thiazolidinediones on plasma adiponectin levels in normal, obese and type 2 diabetic subjects. *Diabetes* **51**, 2968-2974.

Yu Y, Correll PH & Vanden Heuvel JP (2002c) Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPARγ-dependent mechanism. *Biochimica et Biophysica Acta* **1581**: 89-99.

Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M & Shoelson SE (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* **293**, 1673-1677.

Yudkin JS, Kumari M, Humphries SE & Mohamed-Ali V (2000) Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* **148**, 209-214.

Yudkin JS, Stehouwer CDA, Emeis JJ & Coppack SW (1999) C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue. *Arteriosclerosis, Thrombosis, and Vascular Biology* **19**, 972-978.

Z

Zarowitz BJ & Pilla AM (1989) bioelectrical impedance in clinical practice. *Annals of Pharmacology* **23**, 548-555.

Zeng YH, Wu YM, Zhang WB, Yu MJ, Zhu CM & Tan LZ (2004) Activation of nuclear factor kappaB and induction of inducible nitric oxide synthase by lipid-associated membrane proteins isolated from Mycoplasma penetrans. *Chinese Medical Journal* **117**, 997-1001.

Zhande R, Mitchell JJ, Wu J & Sun XJ (2002) Molecular mechanism of insulin-induced degradation of insulin receptor substrate 1. *Molecular and Cellular Biology* **22**, 1016-1026.

Zhang B, Berger J, Hu E, Szalkowski D, White-Carrington S, Spiegelman BM & Moller DE (1996) Negative regulation of peroxisome proliferator-activated receptor-gamma gene expression contributes to the antiadipogenic effects of tumor necrosis factor-alpha. *Molecular Endocrinology* **10**, 1457-1466.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432.

Zhou QL, Park JG, Jiang ZY, Holik JJ, Mitra P, Semiz S, Guilherme A, Powelka AM, Tang X, Virbasius J & Czech MP (2004) Analysis of insulin signalling by RNAi-based gene silencing. *Biochemical Society Transactions* **32**, 817-821.

Zick Y (2003) Role of Ser/Thr kinases in the uncoupling of insulin signaling. *International Journal of Obesity and Related Metabolic Disorders* **27** (suppl 3), S56-S60.

Zick Y (2004) Uncoupling insulin signalling by serine/threonine phosphorylation: a molecular basis for insulin resistance. *Biochemical Society Transactions* **32**, 812-816.

Zick Y (2005) Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. *Science's Signal Transduction Knowledge Environment* **268**.

Zimmet P, Shaw J & Alberti KGMM (2003) Preventing Type 2 diabetes and the dysmetabolic syndrome in the real world: a realistic view. *Diabetic Medicine* **20**, 693-702.

Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, Wojtaszewski JF, Hirshman MF, Virkamaki A, Goodyear LJ, Kahn CR & Kahn BB (2000) Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nature Medicine* **6**, 924-928.

Zucker LM & Zucker TF (1962) Hereditary obesity in the rat associated with high senam fat and cholesterol. *Proceedings of the Society for Experimental Biology and Medicine* **110**, 165-171.

Zucker TF & Zucker LM (1963) Fat accretion and growth in the rat. *Journal of Nutrition* **80**, 6-19.

Zucker, LM & Antoniades HN (1972) Insulin and obesity in the Zucker genetically obese rat "fatty". *Endocrinology* **90**, 1320-1330.