



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.

An investigation into the role and regulation of uncoupling protein 1 (UCP 1)

A thesis submitted for the degree of Doctor of Philosophy at the University of Dublin

2011

By

Kieran John Clarke B.A.(Mod), PG.Dip.

School of Biochemistry and Immunology
Trinity College Dublin

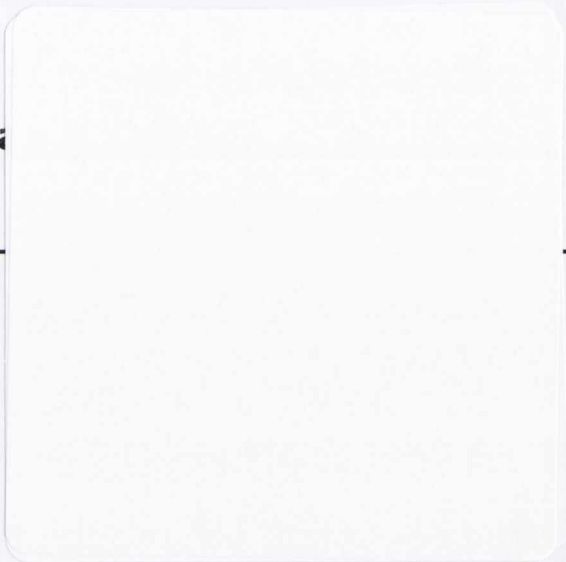


Thesis 9713

Declaration

This thesis is the result of my own work and does not include any work done in collaboration with others. It has not been submitted for any other degree at this or any other university. The library may lend or copy this thesis upon request.

Kiera



Summary

The use of the uncoupling protein inhibitor GDP was used to investigate UCP 1 dependent ROS production in BAT and thymus mitochondria. The effect of UCP 1 inhibition by GDP was then corroborated using UCP 1 ablated mice and their wild-type controls.

It was shown that UCP 1 was capable of attenuating ROS production in isolated BAT mitochondria. ROS due to reverse electron transport was shown to be particularly sensitive to UCP 1 attenuation. We have shown for the first time that BAT mitochondria from UCP 1 knock-out mice have significantly less complex I activity and abundance, thereby reducing ROS production due to reverse electron transport, and possibly explaining previous reports of no difference in oxidative damage in BAT mitochondria from UCP 1 knock-out and wild-type mice. We also show for the first time that BAT mitochondria from UCP 1 knock-out mice, surprisingly, have significantly greater glycerol-3-phosphate dehydrogenase activity.

The ability of UCP 1 in isolated BAT mitochondria to attenuate ROS production during initial cold exposure and following chronic cold-acclimation was also investigated.

Initial cold exposure of an animal was tested in vitro by activating mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) in isolated BAT mitochondria by calcium. Initial adrenergic stimulation of brown adipocytes has been shown to increase calcium levels in mitochondria which could activate mGPDH.

It was shown for the first time that mGPDH in BAT mitochondria can be activated by calcium, which in turn significantly increases UCP 1 dependent oxygen consumption by BAT mitochondria. It was also shown that activation of mGPDH by calcium can increase ROS production by BAT mitochondria but this increase is attenuated by UCP 1. It was concluded that UCP 1 activity is indirectly activated through

calcium mediated activation of the mGPDH which significantly increases respiratory activity of BAT mitochondria.

It was shown that UCP 1 can attenuate the increased capacity of BAT mitochondria to produce ROS following 4 weeks chronic cold-exposure, thereby allowing increased respiratory activity of BAT mitochondria without increasing oxidative damage.

ROS production by thymus mitochondria was also shown to be attenuated by UCP 1. However, this attenuation was not significant. The low level of attenuation was concluded to be due to the low expression of UCP 1 in thymus mitochondria compared to BAT mitochondria (10 fold less). ROS attenuation by UCP 1 in thymus mitochondria was proposed to be only physiologically relevant in proliferating thymocytes where oxidative phosphorylation increases and where the protein expression levels of UCP 1 have been shown to increase 2 fold.

The regulation of UCP 1 turnover in BAT and thymus mitochondria was also investigated. It was shown for the first time that UCP 1 in both BAT and thymus mitochondria can be immunoprecipitated using an anti-ubiquitin antibody. It was also shown that UCP 1 in isolated BAT and thymus mitochondria can be degraded by the cytosolic proteasome in an in vitro reconstituted ubiquitin-proteasome degradation system with a half-life that correlates with the half-lives of UCP 1 in intact brown adipocytes and thymocytes, ~48 hours and ~4 hours respectively. The cytosolic proteasome was also shown for the first time to be involved in the degradation of UCP 1 in intact mature brown adipocytes. Mass spectrometric analysis also identified that the higher molecular weight bands (45-100 kDa) immunoprecipitated by an anti-UCP 1 antibody in BAT mitochondria are in fact UCP 1.

Aims

The aims of the work presented in this thesis were to 1) investigate UCP 1 dependent ROS production by BAT mitochondria and try to resolve apparent contradictions in the literature. 2) Compare UCP 1 dependent ROS production by BAT mitochondria isolated from cold-acclimated and rats housed at room temperature and under conditions where ROS production by the mitochondrial glycerol-3-phosphate dehydrogenase is increased. 3) Investigate UCP 1 dependent ROS production by thymus mitochondria. 4) Determine whether the ubiquitin-proteasome degradation system is involved in the degradation of UCP 1.

Acknowledgments

I would first of all like to thank my supervisor Richie for all his support and guidance over the last three years. Your endless enthusiasm and energy for research have made the last three years so much more enjoyable than they otherwise could have been.

Thanks to all past and present members of lab 0.1:

Alison, thanks for getting me into this mess. I might forgive you someday, but don't hold your breath. I can't believe we are actually handing this in. The country must be in some state if they are letting us finish a PhD. Thanks for being there to vent at every time something shit happened, which let's be honest was a lot of the time. You have brought sarcasm to a whole new level, and I fear that I will never meet anyone else who will ever match it. I hope in a few years the only thing you will be stressing about is wedding cakes and not western blots.

Orlagh, I can't believe you abandoned ship on us. Why did you have to be the sensible one and get out. Thanks for all the laughs over the years, from all the dancing on nights out to impromptu stopovers in Madrid to watch ladies of the night in McDonalds. Most of all, thanks for blocking the view of Alison's head. It has been bad enough looking at her every day for the last few months. Three years could have irreversibly damaged me.

Lars, thank you for bringing your version of German efficiency to the lab! You have definitely done your best to kill that stereotype! I am really glad that you came over to us in Ireland. It has been a pleasure

to get to know you (and your better half, Anna, of course) .Look after the oxygraph for me and I might make one of your classes some time.

Audrey 'mother hen' Carroll, you made the transition from clueless undergrad to clueless postgrad as smooth as any human being could possibly have. Thanks for all the advice and laughs along the way , both when you were in the lab and when you moved across the road to the prep room.

Andrea, what can I say to the person who introduced me to the oxygraph but thank you. My life has been forever changed.

Eamon, thank you for arriving at the lab just in time to save us from self-destruction and to put us on the right track, before heading off on your adventures down under. Kids should not be left to run a lab!

To the new residents of lab 0.1, Belinda ,Alison and Rachel. It has been a pleasure to get to know all of you. You can have the rest of the lab now. I have no use for it.

Of course I have to thank all my fellow postgrad students in the department. How could I have got to the end without the wine receptions, nights in Kennedys, laughing at the undergrads and evenings in The Pav.

Thank you to all members of bio resources who have helped over the last three years, especially Anne, Sylvie and Ciaran.

Thank you to all the technical staff in the department who have made doing this PhD possible: Liam Mc Carthy, Liam and Noel, Dave and Kieran, Ronnie, Roisin, Brian, Glynis and of course Mary-Pat and Miriam in the office for your help

To Kate and all the part-time residents of 4 Clyde Court, ye know who ye are. I really wouldn't have made it this far without all the nights out and laughs along the way. I would also like to thank all my friends in Dublin and down home for all the support during the long absences.

Last but by no means least thank you to my family for standing by me for yet another few years of college! I am finishing now, I promise.

I acknowledge funding from science foundation Ireland and the trinity award.

Abbreviations

ADP	Adenosine 5' -diphosphate
ATP	Adenosine 5' -triphosphate
BAT	Brown adipose tissue
BSA	Bovine serum albumin
CAMP	cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum associated degradation
FADH ₂	Flavin adenine dinucleotide reduced form
FCCP	Carbonyl cyanide p trifluoromethoxyphenylhydrazine
GDP	Guanosine-5'-diphosphate
HRP	Horse radish peroxidase
HSL	Hormone sensitive lipase
HTP	Hydroxyl apatite
I.P.	Immunoprecipitation
kDa	Kilo Dalton
NADH	Nicotinamide adenine dinucleotide reduced form
NE	Norepinephrine
nm	nano metre
Octyl-POE	octylpentaoctylethylene
PAGE	Polyacrylamide gel electrophoresis
PDH	Pyruvate dehydrogenase
PKA	Protein kinase A
PVDF	polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.E.M.	Standard error of the mean
STE	Sucrose, Trizma and EGTA isolation buffer
SUMO	small ubiquitin-like degradation pathwa
UCP1	Uncoupling protein 1
VDAC	Voltage dependent anion channel
v/v	volume per unit volume
w/v	weight per unit volume

Table of contents

Declaration

Summary

Aims

Acknowledgments

Abbreviations

Chapter 1 Introduction

[1.1] The mitochondrion.....1

[1.2] Oxidative phosphorylation.....2

[1.3] Mitochondrial proton leak.....4

[1.4] Protein mediated proton leak.....4

[1.5] Brown adipose tissue (BAT).....5

[1.6] Uncoupling protein 1 (UCP1).....6

[1.7] Activation and inhibition of UCP1.....7

[1.8] Expression of UCP110

[1.9] Thymus.....10

[1.10] Uncoupling protein homologues.....11

[1.11]Physiological relevance of uncoupling proteins.....14

[1.12] Reactive oxygen species (ROS).....16

[1.13] Mitochondrial generation of reactive oxygen species.....18

[1.14]Mitochondrial glycerophosphate dehydrogenase (mGPDH).20

[1.15] Ubiquitination.....23

[1.14] Ubiquitination in the mitochondrion.....25

[1.15] Cold-acclimation.....27

Chapter 2 Materials and Methods

[2.1] Materials.....	29
[2.2] Mitochondrial protein determination using the Bicinchoninic Acid Assay.....	30
[2.3] Isolation of BAT mitochondria.....	30
[2.4] Isolation of thymus mitochondria.....	31
[2.5] Immunoprecipitation of ubiquitinated proteins.....	32
[2.6] Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	33
[2.7] Western blotting.....	34
[2.8] Western blotting for ubiquitinated proteins.....	34
[2.9] Immunodetection of proteins.....	35
[2.10] Coomassie Blue R-250 staining of SDS-PAGE gels.....	36
[2.11] In Gel Trypsin digestion.....	36
[2.12] LC-MS/MS analysis.....	36
[2.13] Data Analysis Parameters.....	38
[2.14] Measurement of O ₂ consumption rates by mitochondria.....	38
[2.15] Measurement of mitochondrial ROS generation.....	39
[2.16] Examination of mitochondrial membrane potential using the probe safranin.....	40
[2.17] Complex I activity assay.....	41
[2.18] Complex II activity assay.....	41
[2.19] Glycerol-3-phosphate dehydrogenase activity assay	42
[2.20] In vitro UCP1 degradation assay.....	42
[2.21] Isolation of thymocytes.....	43
[2.22] Isolation of brown pre-adipocytes and differentiation into mature brown adipocytes.....	43
[2.23] Wild-type and UCP1 knock-out mice.....	44
[2.24] Temperature acclimation.....	45
[2.25] Calculation of [Ca ²⁺] _{free}	45
[2.26] Statistical analysis.....	45

Chapter 3 UCP 1 dependent ROS production in BAT mitochondria

[3.1] Introduction.....46

[3.2] Results.....49

[3.3] Discussion.....87

Chapter 4 UCP 1 dependent ROS production in cold-acclimated BAT mitochondria

[4.1] Introduction.....107

[4.2] Results.....110

[4.3] Discussion.....128

Chapter 5 UCP 1 dependent ROS production in thymus mitochondria

[5.1] Introduction.....136

[5.2] Results.....138

[5.3] Discussion.....163

**Chapter 6 A role for the ubiquitin-proteasome
degradation system in the turnover of UCP 1**

[6.1] Introduction.....177

[6.2] Results.....179

[6.3] Discussion.....193

Chapter 7 General Discussion.....202

References.....210

Chapter 1 Introduction

[1.1] The Mitochondrion

A mitochondrion (plural mitochondria) is a membrane enclosed organelle ranging in size from 0.5-1.0 micrometer (μm) in diameter. Its structure consists of two membranes, the smooth outer membrane and the extensively folded inner membrane. The extensive folding of the inner membrane form cristae a feature which substantially increases the surface area available for mitochondrial functions. The outer membrane is permeable to most small molecules including protons, whereas the inner membrane is not. Together the two membranes form two compartments within the mitochondrion, the intermembrane space and the matrix. The matrix holds high concentrations of the soluble enzymes of oxidative metabolism, as well as substrates, nucleotide cofactors and inorganic ions. The matrix also contains mitochondrial genetic machinery, DNA, RNA and ribosomes. The structure of the mitochondrion is critical to its function with each compartment carrying out specific enzymatic reactions and having characteristic properties. The main function of the mitochondrion is to produce energy in the form of ATP for the cell. The mitochondrion is also involved in other functions which include programmed cell death (Apoptosis), calcium signalling and regulation of cellular metabolism. Some functions of the mitochondrion are tissue specific which reflect the function of that particular cell.

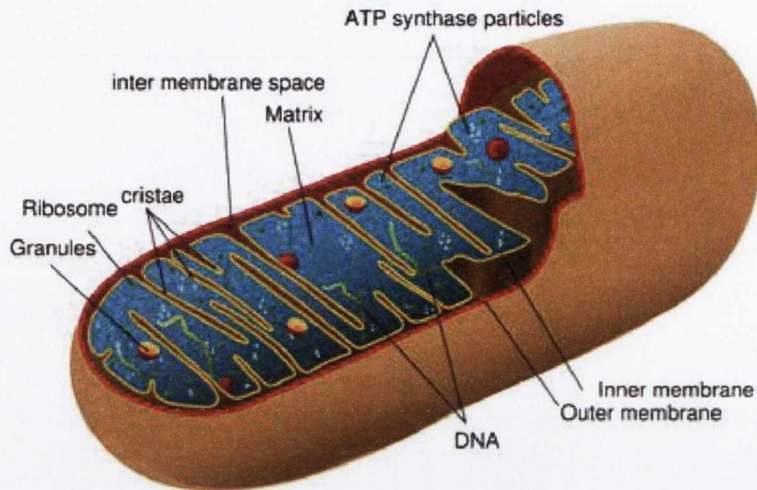


Figure 1.1 The Mitochondrion

(Sourced from:

commons.wikimedia.org/wiki/File:Animalmitochondrion_diagram_en.svg)

[1.2] Oxidative Phosphorylation

The synthesis of ATP from ADP and P_i is known as oxidative phosphorylation. It is a complex metabolic pathway that utilizes the energy released from the oxidation of nutrients to create an electrochemical gradient which drives the ATP synthase, the enzyme which catalyzes the synthesis of ATP. This process is best summarized by the chemiosmotic theory proposed by Mitchell in 1961. Oxidative phosphorylation is the culmination of a series of energy transformations that are called cellular respiration in their entirety. First metabolism of carbon fuels from either dietary or stored sources are oxidized in the citric acid cycle to yield reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). This electron-motive force is converted to a proton-motive force by three of the complexes of the electron transport chain, namely NADH-Q oxidoreductase (Complex I), Q-cytochrome c oxidoreductase (Complex III) and cytochrome c

oxidase (Complex IV). This is achieved as electrons pass through large membrane bound enzymes, such as glycerol phosphate dehydrogenase, the dehydrogenases of β -oxidation, Complex I or Complex II to the mobile lipid-soluble carrier, ubiquinone (Q). From Q the electrons pass down through Complex III, the water-soluble carrier cytochrome c and Complex IV to the final acceptor, oxygen. As electrons move through the electron transport chain Complexes I, III and IV translocate protons across the mitochondrial inner membrane from the matrix to the intermembrane space and in doing so create an electrochemical gradient (Δp). The final phase of oxidative phosphorylation is carried out by ATP synthase, which is driven by the flow of protons back into the matrix from the intermembrane space. Assuming all protons re-enter the matrix through ATP synthase then substrate oxidation and ATP phosphorylation are said to be coupled. The inner membrane contains an ATP/ADP translocator which exchanges ADP for the ATP synthesized during oxidative phosphorylation.

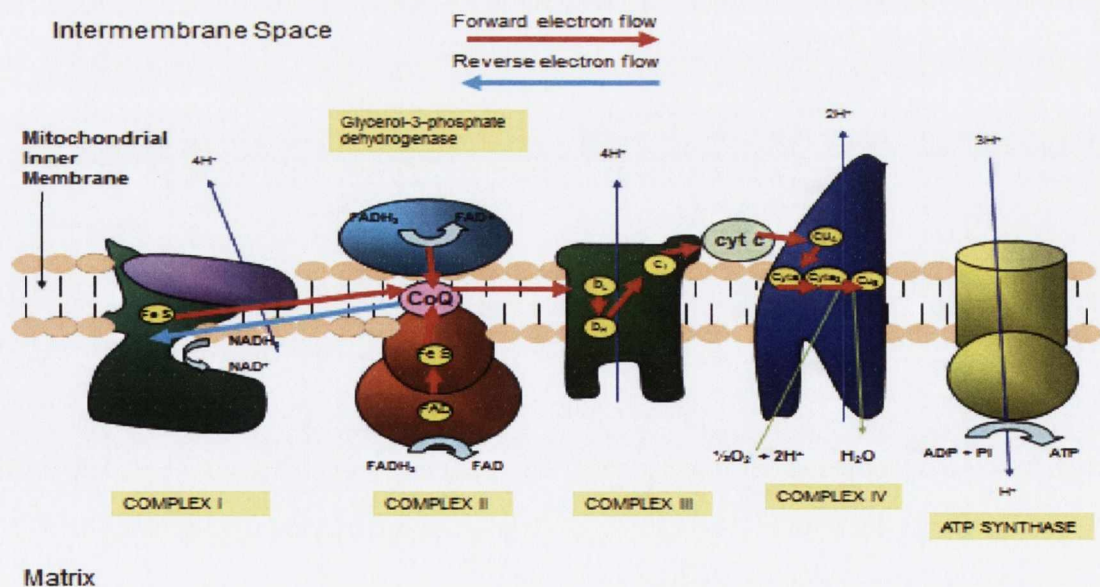


Figure 1.2 Forward and reverse electron flow through the respiratory chain

[1.3] Mitochondrial Proton Leak

Oxidative phosphorylation is never fully coupled and protons may re-enter the matrix through means other than the ATP synthase molecule in a process termed proton leak (Nicholls 1974b). Oxidative phosphorylation is never fully coupled in either isolated mitochondria or mitochondria *in situ* (Brand *et al.* 1994). When the ATP synthase is not working, either because of the presence of oligomycin (an ATP synthase inhibitor) or due to the absence of ADP, the mitochondria will still consume oxygen (Brand 1977). Proton leak is maximal when mitochondria are not making ATP (State 4 respiration) and lowest when ATP is being synthesized (State 3 respiration) (Brown and Brand 1986; Nicholls and Rial 1989; Porter and Andrews 1998)

[1.4] Protein Mediated Proton Leak

Uncoupling proteins are ~32kDa membrane proteins found in the mitochondrial inner membrane that belong to the mitochondrial carrier family. The first uncoupling protein to be discovered was UCP 1, then called thermogenin, in the 1970s (Heaton *et al.* 1978). Upon the discovery of several homologues in 1997 it was renamed uncoupling Protein 1 (UCP 1) (Fleury *et al.* 1997). UCP 1 is expressed in BAT and Thymus. In brown adipose tissue it exerts its thermogenic effect by allowing re-entry of protons into the matrix without ATP synthesis (Klaus *et al.* 1991). This tissue is able to greatly increase the oxidation of substrate, and the dissipation as heat, of the liberated energy with UCP 1 catalyzing the dissipation of the resulting electrochemical gradient.

[1.5] Brown Adipose Tissue

Brown adipose tissue (BAT) is found in numerous defined areas throughout the body of different animals or strains of animals. The function of BAT is to dissipate energy from food into heat through non-shivering thermogenesis (Cannon and Nedergaard 2004).

Briefly, in response to stimuli such as cold acclimation or overeating BAT shows considerable adaptive change including dense adrenergic innervation, vast mitochondrial content and it is highly vascular for rapid deployment of generated heat (Nechad 1986). The adaptive capabilities of BAT are under the control of adrenergic stimulation. Noradrenaline can promote brown preadipocytes to proliferate (Bronnikov *et al.* 1992) and mature brown adipocytes to differentiate (Gomez-Ambrosi *et al.* 2001; Puigserver *et al.* 1998). It also directly regulates the expression of the UCP 1 gene (Ricquier *et al.* 1984) which is the primary molecule involved in the thermogenic response of BAT. Noradrenaline also negatively regulates apoptosis in BAT (Lindquist *et al.* 2000). Although BAT is an adipose tissue, white adipose tissue and brown adipose tissue have two independent lineages (Timmons *et al.* 2007). A bidirectional switch exists between BAT and skeletal muscle which is controlled by the transcriptional regulator PRDM16 (Seale *et al.* 2008).

Until recently it was believed that BAT is only found in newborn mammals incapable of shivering, hibernators and throughout the life of rodents (Himms-Hagen 1990). Evidence of brown adipose tissue in adult humans is now appearing in the literature (Nedergaard *et al.* 2007). This unexpected finding came originally from the area of nuclear medicine where glucose uptake is used to identify tumors. This process of tumor identification was known to detect false positives, like the brain, which also uptake the labeled glucose. Many of these areas were expected and could be

discounted. Unexpected symmetrical areas of uptake however were commonly found in the neck and shoulder areas (Barrington and Maisey 1996). Further studies on these areas of uptake, identified them as brown adipose tissue depots, which contained UCP1 (Bar-Shalom *et al.* 2004; Garruti and Ricquier 1992; Kortelainen *et al.* 1993; Lean *et al.* 1986).

[1.6] Uncoupling Protein 1 (UCP 1)

UCP 1 is a 32kDa tripartite mitochondrial carrier protein of 306 amino acids (Heaton *et al.* 1978). UCP 1 has been successfully purified from BAT mitochondria (Lin and Klingenberg 1980). The sequence of UCP 1 is highly conserved between species (Klaus *et al.* 1991). It has been proposed that UCP 1 contains 6 transmembrane α -helices with the C- and N- termini protruding into the cytosolic side of the mitochondrial inner membrane (Klingenberg 1990). The amino acid sequence of UCP 1 is highly homologous to that of several ubiquitous mitochondrial inner-membrane carriers, including the ADP/ATP carrier, the phosphate carrier and the oxoglutarate carrier (Klaus *et al.* 1991). As a tripartite structure the structure can be divided into three similar repeat domains of about 100 residues, each containing two transmembrane helices (Palou *et al.* 1998). Within each domain the two transmembrane helices are separated on the matrix side by a stretch of approximately 40 residues. In studies involving UCP 1 from hamster it was shown that UCP 1 contains 28 positively and 19 negatively charged residues. This results in an excess of nine positive charges which are mainly localized to the hydrophilic matrix region (Klingenberg and Huang 1999). A well conserved motif KXR is found in all three domains in the matrix region, which is characteristic for the mitochondrial carrier family.

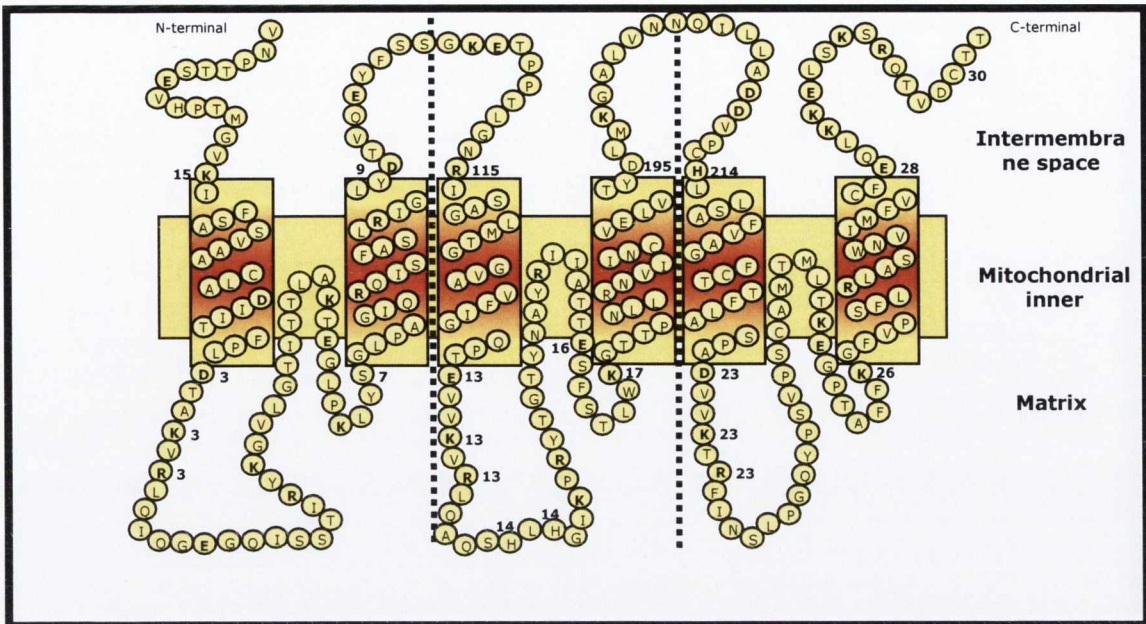


Figure 1.3: Tripartite structural model of hamster uncoupling protein 1 (adapted from Klingenberg *et al.*, 1999a). Kindly supplied by Dr. Audrey Carroll.

[1.7] Activation and Inhibition of UCP 1

In response to cold exposure or chronic overeating noradrenaline activates BAT UCP 1 (Ricquier *et al.* 1986). In BAT noradrenaline signals through the β_3 -adrenoreceptor in order to activate UCP 1, and also with the other two types of adrenoreceptors α_1 and α_2 which activate other signalling pathways (Cannon and Nedergaard 2004). The β -adrenoreceptors in BAT have been indirectly shown to be coupled to adenylyl cyclase via G proteins of the G_s subtype, which lead to an increase in cyclic adenosine monophosphate (cAMP) (Granneman 1988). As in other tissues cAMP interacts with protein kinase A to activate it. The activated protein kinase A further phosphorylates a series of targets to carry on the adrenergic signal. These targets are involved in both the expression and activation of UCP 1. The transcription factor CREB is phosphorylated which stimulates transcription from the UCP 1 gene (Thonberg *et al.* 2002). Along with the phosphorylation of nuclear related proteins, protein kinase A phosphorylates cytosolic proteins, in particular the

hormone sensitive lipase (HSL) which hydrolyzes triacylglycerol stores, releasing stores of free fatty acids which 'activate' UCP 1 (Shih and Taberner 1995). It also phosphorylates perilipin. Perilipin protects triacylglycerides from HSL activity and upon phosphorylation is deactivated, which leads to its dissociation from the triglyceride droplets and ultimately leaves them exposed to attack by HSL (Martinez-Botas *et al.* 2000) .

The mechanism by which fatty acids activate UCP 1 has been extensively studied, but as of yet a model has not been agreed upon. Currently there are two proposed models; the fatty acid buffering model and the fatty acid protonophore model.

The fatty acid buffering model suggests protons are directly transported by UCP 1 (Winkler and Klingenberg 1994). In this model it is proposed that the fatty acids act as cofactors by localizing to binding sites along the aqueous channel of UCP 1, where their acid moieties pass along protons from one fatty acid to the next until the proton is through the membrane into the matrix. As of yet no definite binding sites have been identified but in support of this model it has been shown that mutation of two histidine residues (His 145 and His 147) cause a loss of proton transport (Bienengraeber *et al.* 1998).

In the fatty acid protonophore model it is proposed that UCP 1 catalyzes not the transport of protons across the membrane but of the fatty acid anionic head group (Garlid *et al.* 1996). Transport of the anion is driven by the high, inside negative membrane potential. Once the head group reaches the intermembrane space it becomes protonated and spontaneously flops back to the matrix side where the cycle restarts.

Purine nucleotides can bind to UCP 1 and inhibit its activity (Nicholls 1974a). Binding of nucleotides is probably the best defined characteristic of UCP 1, and was key in identifying and isolating this protein (Heaton *et al.* 1978). Unlike the fatty acid binding site,

sequences relating to the nucleotide binding site have been identified on the outer leaflet of UCP 1 (Ledesma *et al.* 2002; Mayinger and Klingenberg 1992), and nucleotides can only bind to the cytosolic side of UCP 1 (Klingenberg and Huang 1999). UCP 1 accepts with strong preference the purine ribose di- and tri-nucleotides: GDP, GTP, ATP, and ADP, whereas the monophosphates GMP and AMP are poor ligands. Only the free forms of nucleotides, not the Mg^{2+} complexes are capable of binding to UCP 1 (Klingenberg 1988). UCP 1 is predicted to behave as a dimer in the mitochondrial inner membrane (Klingenberg 1984) and only one nucleotide binds per UCP 1 dimer (Klingenberg and Huang 1999).

Nucleotide binding to UCP 1 is pH dependent. The affinity for purine and pyrimidine nucleotides decreases upon increasing pH, notably above pH 7.2 (Klingenberg and Huang 1999).

GDP binding has been shown to be altered with acute temperature changes. In a phenomenon termed masking, the number of nucleotide binding sites is altered after the animal undergoes an acute temperature change (Desautels *et al.* 1978; Swick and Swick 1986). When animals housed at thermo neutrality (28°C) were exposed to cold (4°C), the GDP binding, as determined by radiolabelled GDP, increased immediately. Conversely when rats housed in the cold for two hours were returned to thermo neutrality the GDP binding decreased immediately. It has been proposed that this masking/unmasking phenomenon is due to swelling of the mitochondria due to the activity of thermogenesis (Nedergaard and Cannon 1987). It was also shown that the nucleotide binding site of UCP 1 from rats and hamsters kept at room temperature or from warm acclimated animals, is occupied by endogenous ATP and is therefore masked (Klingenberg and Huang 1999). In isolated mitochondria, UCP 1 can be completely unmasked by treatment with Dowex 21K anion exchanger at high pH.

BAT mitochondria are largely coupled since UCP 1 activity is mainly inhibited by endogenous ATP as basal free fatty-acid concentrations are low. When BAT is stimulated by either cold exposure or noradrenaline stimulation, fatty acids are released by the mechanism explained earlier and in doing so deplete endogenous ATP by fatty acid activation. The depletion of the endogenous ATP concentration results in dissociation of prebound ATP from UCP 1. This dissociation coupled with the liberation of fatty acids increase proton transport activity through UCP 1 activity (Huang and Klingenberg 1995).

[1.8] Expression of UCP 1

Up until recently UCP 1 has been thought to be a unique feature of BAT. However reports of UCP 1 expression have been made in various other tissues. Many reports on the expression of UCP 1 in white adipose tissue exist (Commins *et al.* 1999; Garruti and Ricquier 1992; Koza *et al.* 2000). These reports however, have been disputed and alternatively some believe that UCP 1 is not actually expressed in white adipose depots, but rather brown adipose depots can occur sporadically in white adipose tissue (Cannon and Nedergaard 2004). Recently in our lab it has been shown that UCP 1 is present in the thymus. It has been illustrated that mRNA and protein of UCP 1 exist in the thymus cell (thymocyte) mitochondria (Adams *et al.* 2008; Carroll *et al.* 2004). It has also been shown recently that UCP 1 is detected in skin (Mori *et al.* 2008).

[1.9] Thymus

The thymus is a primary lymphoid organ which plays a central role in the creation of a fully functional immune system. Its major

function is to provide the appropriate milieu within which cells of the T-lymphocyte lineage can develop, proliferate, mature and generate their receptor repertoire (Ritter 1992). The thymus is a bi-lobed greyish organ located above the heart and behind the sternum. It is largest and most active between late gestation and adolescence, but begins to atrophy during adolescence whereupon size and activity are significantly reduced. Anatomically the thymus is divided into an outer cortex and an inner medulla. Differentiation of T-cells occurs in the cortex, while final maturation before exiting the thymus occurs in the medulla. Apoptosis plays a critical role in selecting the thymocyte pool, removing cells expressing an unproductive T cell receptor (TCR), or exhibiting either hypo- or hyper-responsiveness upon encountering MHC/self-peptide complexes (Zacharchuk *et al.* 1991).

[1.10] Uncoupling protein homologues

Since the discovery of UCP 1 in the 1970s, other mitochondrial uncoupling proteins have been discovered. These new uncoupling proteins share high sequence homology with UCP1. These proteins are not confined to BAT and are distributed throughout the body in various tissues in both humans and rodents (Boss *et al.* 1997b; Fleury *et al.* 1997; Gong *et al.* 1997; Vidal-Puig *et al.* 1997).

Screening of a skeletal muscle library using cDNA as a probe led to the discovery of uncoupling protein 2 (UCP2) (Fleury *et al.* 1997). Further analysis revealed that this novel uncoupling protein shared 59% homology with UCP 1 and had an approximate molecular mass of 30kDa. Many studies have shown that UCP 2 mRNA expression is widespread in tissues including WAT, BAT, skeletal muscle, heart, placenta, brain, stomach, kidney, lung,

myocytes, lymphocytes, macrophages and the Kupffer cells of liver (Boss *et al.* 1997b; Fleury *et al.* 1997; Gimeno *et al.* 1997; Gong *et al.* 1997; Hodny *et al.* 1998; Horvath *et al.* 2003; Krauss *et al.* 2002; Larrouy *et al.* 1997; Negre-Salvayre *et al.* 1997; Pecqueur *et al.* 2001; Pedraza *et al.* 2001; Ricquier and Bouillaud 2000; Rousset *et al.* 2003). However UCP 2 protein expression is only found in mouse and rat spleen, lung, stomach, kidney, thymus, WAT, pancreatic islets and macrophages (Echtay and Brand 2001; Krauss *et al.* 2002; Pecqueur *et al.* 2001; Ricquier and Bouillaud 2000; Zhang *et al.* 2001). UCP 2 mRNA levels have been shown to increase under various physiological states including cold exposure, 48 hours starvation (Boss *et al.* 1997a) and leptin administration (Zhou *et al.* 1997).

A third uncoupling protein was found in skeletal muscle which was termed uncoupling protein 3 (UCP 3). Like the other two uncoupling proteins found UCP 3 was found to be a mitochondrial inner membrane protein. It was initially cloned by Boss *et al.* (1997) and shortly thereafter by Vidal-Puig *et al.* (1997) and Gong *et al.* (1997). Existing as two isoforms in humans, the long form (UCP 3_L) and the short form (UCP 3_S), it differs from UCP 1 and UCP 2 which have no known isomers. Along with the other uncoupling proteins and mitochondrial carriers, UCP 3 has six transmembrane domains (Boss *et al.* 1997b). However the UCP 3_S lacks the sixth potential transmembrane region and putative nucleotide binding site which is implicated in the control of UCP activity. UCP 3 mRNA is expressed in skeletal muscle of humans (Vidal-Puig *et al.* 1997). Rodents express UCP 3 in skeletal muscle, BAT, spleen and thymus (Carroll and Porter 2004; Gong *et al.* 1997). UCP 3 protein has been detected in mouse and rat skeletal muscle (Cadenas *et al.* 2002; Cunningham *et al.* 2003; Gong *et al.* 2000; Harper *et al.* 2002;

Hesselink *et al.* 2003; Jezek *et al.* 1999; Li *et al.* 2000; Vidal-Puig *et al.* 2000; Zhou *et al.* 2000), BAT mitochondria (Cunningham *et al.* 2003; Gong *et al.* 2000; Harper *et al.* 2002; Jezek *et al.* 1999; Li *et al.* 2000) and rat spleen and thymus mitochondria (Carroll and Porter 2004).

Further uncoupling proteins have been found. These include uncoupling protein 4 (UCP 4), which is a 36kDa protein of 323 amino acids. UCP 4 is 29%, 33% and 34% homologous to UCP 1, UCP 2 and UCP 3 respectively. UCP 4 transcripts have been found to be expressed in both fetal and adult brain tissues.

Uncoupling protein 5 (UCP 5) also termed brain-specific mitochondrial carrier protein (BMPC-1) has been identified. UCP 5 mRNA has been found in brain, testis, heart, kidney and uterus (Yu *et al.* 2000). UCP 5 has three isoforms; a short form (UCP 5_s), a short form with insert (UCP 5_{SI}) and a long form (UCP 5_L).

Plant uncoupling proteins (PUMP) have also been discovered in potato tubers and in tomatoes (Costa *et al.* 1999) and it has been shown that the non-photosynthetic soil amoeboid protozoan *Acanthamoeba catellanii* expresses an uncoupling protein (Jarmuszkiewicz *et al.* 1999). Also a new protein has been identified in the skeletal muscle of chickens, hummingbirds, ducklings and penguins (avUCP) which shares 70% amino acid identity with both UCP 2 and UCP 3 (Raimbault *et al.* 2001; Toyomizu *et al.* 2002; Vianna *et al.* 2001)

HOMOLOGUE	STRUCTURE	LOCATION
UCP 1	32kDa protein	BAT, Thymus, Skin
UCP 2	30kDa protein	Macrophages, Spleen, Gut, Lung, WAT, Pancreatic islets, Thymus, Kidney
UCP 3	34kDa protein	Thymus, Spleen, BAT of rodents, Skeletal muscle
UCP 4	36kDa protein	Brain
UCP 5	36kDa	Brain, mouse and human Heart, Kidney, skeletal muscle

Figure 1.4: Uncoupling proteins

[1.11] Physiological relevance of uncoupling proteins

Apart from UCP1, the function of many of the uncoupling proteins is not clear and is still under investigation. Initially when UCP 2 and UCP 3 were discovered it was proposed that they may have an uncoupling role like UCP 1, and are involved in energy expenditure. This was due to the similarities in their amino acid sequences to that of UCP 1. The residues involved in nucleotide binding and pH regulation are well conserved in these proteins (Boss *et al.* 1997b; Fleury *et al.* 1997). However one of the histidine residues vital for H⁺ transport in UCP 1 is not conserved (fatty acid protonophore model) (Bienengraeber *et al.* 1998). It is now generally agreed that these uncoupling protein homologues do not transport protons in the absence of specific activators. Studies utilising UCP 2 and UCP 3 knock-out mice showed no difference in basal proton conductance in isolated mitochondria compared to wild-types (Cadenas *et al.* 2002; Couplan *et al.* 2002; Echtay *et al.* 2003; Echtay *et al.* 2002; Krauss *et al.* 2003), but differences were noted when specific activators

such as hydroxynonenal were used (Brand *et al.* 2004a; Brand *et al.* 2004b; Considine *et al.* 2003; Talbot *et al.* 2003). However the levels of UCP 2 and UCP 3 are tiny compared to UCP 1 levels in BAT, 0.1% to 1% (Harper *et al.* 2002; Pecqueur *et al.* 2001), so any uncoupling effect may be only mild compared to the uncoupling observed in BAT.

This has led to the proposal that UCPs can attenuate mitochondrial production of free radicals and therefore protect against oxidative damage, degenerative diseases and aging. Mitochondrial reactive oxygen species (ROS) production is very sensitive to the proton motive force set up across the inner membrane by electron transport. Any putative mild uncoupling by UCP 2 or UCP 3 may reduce this proton motive force and thereby reduce ROS production by mitochondria, and protect against ROS-related cellular damage (Brand *et al.* 2002; Casteilla *et al.* 2001; Echtay *et al.* 2002).

A role for UCP 2 in signalling is also proposed. This model proposes that activation of UCP 2 may attenuate glucose-stimulated insulin secretion by pancreatic β cells (Rutter 2001). UCP 2 knock-out mice have higher islet ATP levels and increased glucose-stimulated insulin secretion suggesting that UCP 2 negatively regulates insulin secretion (Zhang *et al.* 2001).

The proposed roles for UCP 2 and UCP 3 make them attractive targets for obesity and inefficient insulin signalling in pancreatic β cells. Their proposed role in ROS attenuation and thus protection from oxidative damage, also suggests a role in normal aging and neurodegenerative disorders, and therefore make them attractive targets in this field also.

Although UCP 1 is normally studied in the context of adaptive thermogenesis in newborns and rodents, the study of its regulation and mechanism of uncoupling is still of physiological significance, due to its homology to the other uncoupling proteins. Any covalent

modifications of UCP 1 or methods of regulation may also apply to the other uncoupling proteins. Due to the abundance of UCP 1 in BAT compared to the abundance of the other uncoupling proteins in the tissues they are expressed in, it makes the study of UCP 1 function and regulation a good model for the study of the other more widely expressed uncoupling proteins.

[1.12] Reactive oxygen species (ROS):

Reactive oxygen species (ROS) is a collective term that describes the chemical species that are formed upon incomplete reduction of molecular oxygen. The term ROS includes the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$), all of which have one unpaired electron. Molecular oxygen is a bi-radical which contains two unpaired electrons with the same spin in its outer shell, which reduces reactivity of the molecule. The species formed by partial reduction of oxygen, however, are powerful oxidants. Upon partial reduction of oxygen, one of the two unpaired electrons is excited and changes its spin, allowing it to quickly react with other pairs of electrons.

Superoxide, the parental form of ROS, is formed by enzymes such as xanthine oxidase or NAD(P)H oxidases. It can also be non-enzymically formed by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. Superoxide can be enzymically converted to hydrogen peroxide by enzymes known as superoxide dismutases (SODs). This process can also occur non-enzymically (Deby and Goutier 1990). Hydrogen peroxide may be converted to water by catalase or glutathione peroxidase. Hydrogen peroxide can also be converted into the

hydroxyl radical ($\text{HO}\cdot$) in the presence of reduced transition metals such as ferrous or cuprous ions (Chance *et al.* 1979).

For many years ROS have been thought of as deleterious by-products of respiratory energy production, which only damage biological systems. However it is now accepted that ROS generation is not always a harmful process, and in many cases is an essential component of many biological processes (Buetler *et al.* 2004; Finkel 2003).

ROS are important regulatory mediators of many physiological responses including redox-dependent transcriptional regulation (Arrigo 1999; Liu *et al.* 2005), ion transport (Kourie 1998), as well as protein phosphorylation (Finkel 1998; Rhee *et al.* 2005).

It has been proposed that ROS formation is a sensor for changes in oxygen concentration. Generally carotid bodies are the sensory organs that detect changes in arterial blood oxygen concentrations, however, it has also been shown that several ROS-producing proteins are capable of sensing changes in oxygen concentration (Acker 1994). ROS is also involved in oxygen sensing by controlling the production of the hormone erythropoietin, which regulates red blood cell mass (Fandrey *et al.* 1994; Neumcke *et al.* 1999). The erythropoietin gene is controlled by the transcription factor hypoxia-inducible factor 1 (HIF-1) (Wang *et al.* 1995) which is a heterodimeric protein consisting of subunits HIF-1 α and HIF-1 β . Under normoxic conditions, HIF-1 α is rapidly degraded by the proteasome in a ROS-dependent manner. Hypoxia decreases the ROS-dependent degradation of HIF-1 α which enhances the formation of the heterodimeric complex (Huang *et al.* 1996; Huang *et al.* 1998).

ROS is also implicated in apoptosis, where high ROS levels have been shown to induce apoptosis (Dumont *et al.* 1999; Slater *et al.* 1995). Exposure of lymphocytes to moderate concentrations

of hydrogen peroxide was shown to induce apoptosis that requires mitochondrial ROS production and the activation of NF- κ B.

T lymphocyte activation can be enhanced by ROS. Superoxide and hydrogen peroxide were shown to enhance the production of interleukin-2 (IL-2) in stimulated T lymphocytes, as was the expression of the IL-2 receptor by micro molar concentrations of hydrogen peroxide (Los *et al.* 1995; Roth and Droge 1987).

ROS levels are increased during many signaling cascades including those of nerve growth factor (NGF) and epidermal growth factor (EGF) signaling (Bae *et al.* 1997; Suzukawa *et al.* 2000). Removal of hydrogen peroxide by the addition of catalase was shown to inhibit the NGF- and EGF-induced phosphorylation of downstream cellular proteins.

The role of ROS in insulin signaling has also been widely studied (Pi *et al.*, 2010) Physiologically relevant concentrations of hydrogen peroxide ($<0.1\text{mM}$) are able to enhance the response of the insulin receptor to insulin (Schmid *et al.* 1999). This co-regulatory function has been shown to be part of a positive-feedback regulatory mechanism, whereby insulin induces hydrogen peroxide production, which in turn enhances insulin signaling (Krieger-Brauer *et al.* 1997).

[1.13] Mitochondrial generation of reactive oxygen species:

Excluding phagocytes, the principal site of intracellular reactive oxygen species generation is the mitochondrial respiratory chain. Under conditions in which complexes I and III are highly reduced, a significant proportion of electrons are diverted directly to molecular oxygen, whereupon superoxide is produced (Lambert and Brand 2009). Backflow of electrons from the reduction of flavoprotein-dependent substrates to complex I have also been shown to

generate ROS at complex I (Lambert and Brand 2004; Miwa *et al.* 2003). Other mitochondrial proteins have also been shown to contribute to ROS generation including complex II (succinate:ubiquinone oxidoreductase) (McLennan and Degli Esposti 2000), the 2-oxoglutarate dehydrogenase complex (Starkov *et al.* 2004) and the mitochondrial glycerophosphate dehydrogenase (Mracek *et al.* 2009; Vrbacky *et al.* 2007).

Using isolated mitochondria, it has been shown that generation of ROS is greatly increased at times when the proton electrochemical gradient is high (Korshunov *et al.* 1997). Such conditions occur during State 4 respiration when ADP is unavailable. Addition of either ADP, which induces proton transport via the ATP synthase, or an uncoupling agent, which directly decreases the proton electrochemical gradient, strongly suppresses superoxide formation (Skulachev 1996).

The explanation put forward for this effect is that partial uncoupling stimulates oxygen consumption, thereby shortening the lifetime of the semi-ubiquinone compound, and in doing so reduces ROS formation (Miwa and Brand 2003; Skulachev 1997). Reverse electron transport to complex I from the Q pool has been reported to be particularly sensitive to 'mild uncoupling' (Boveris and Chance 1973; Hansford *et al.* 1997; Korshunov *et al.* 1997; Miwa and Brand 2003). The protonmotive force created when succinate or alpha-glycerophosphate are the electron sources is sufficient to drive some electrons in reverse (Hinkle *et al.* 1967). Depolarization of a few mV results in a decrease in the proton motive force which can prevent reverse electron transport, and thus the associated ROS generation (Hansford *et al.* 1997; Korshunov *et al.* 1997).

Therefore, uncoupling represents a powerful system for the limitation of ROS generation. This suggests that one of the functions of proteins that uncouple respiration (UCPs) could be the limitation of ROS production.

Negre-Salvayre *et al.* (1997) showed that H₂O₂ generation was stimulated by the addition of the UCP inhibitor GDP to BAT, spleen and thymus mitochondria, thereby indicating that UCP's may play a role in the regulation of ROS generation in BAT, spleen and thymus mitochondria. In other studies UCP 2 expression in spleen has been associated with a limitation of ROS generation (Arsenijevic *et al.* 2000; Vidal-Puig *et al.* 2000), thereby supporting a role for UCP 2 in the limitation of ROS generation in spleen mitochondria. UCP 3 ablated mitochondria have shown an increase in ROS generation (Gong *et al.* 2000; Vidal-Puig *et al.* 2000). More recently it has been shown by our lab that UCP 1 in BAT mitochondria plays a role in the regulation of ROS production (Dlaskova *et al.* 2010).

In this study it was shown that either ablation of UCP1, or inhibition by GDP, significantly increased ROS production by BAT mitochondria. The use of UCP 1^{-/-} in this study proved that it was in fact UCP 1 which was regulating ROS production, and not UCP 2 or 3, which are also present in BAT mitochondria. An increase in ROS generation by UCP 1 ablation or inhibition was mirrored by an increase in membrane potential.

[1.14] Mitochondrial glycerophosphate dehydrogenase (mGPDH):

The mitochondrial glycerophosphate dehydrogenase (mGPDH) is a 74kDa, FAD-dependent dehydrogenase localized on the outer side of the inner mitochondrial membrane donating electrons to the respiratory chain via co-enzyme Q (Garrib and McMurray 1986; Klingenberg 1970) mGPDH along with the NAD-dependent cytosolic glycerophosphate dehydrogenase (cGPDH), is part of the glycerophosphate shuttle, with a function of transporting reducing equivalents from the cytosol to the mitochondria and in doing so connecting glycolysis with the mitochondrial respiratory chain

(Klingenberg and Buecher 1961). It is proposed that the glycerophosphate shuttle catalyzes the interconversion of dihydroxyacetone phosphate and glycerol phosphate with the net oxidation of cytosolic NADH (Estabrook and Sacktor 1958).

The cGPDH has functions other than in the glycerophosphate shuttle, such as forming glycerol phosphate for triacylglycerol synthesis. The only known function of the mGPDH, however, is in the glycerophosphate shuttle. cGPDH is present in almost all tissues whereas the expression of mGPDH varies widely among different tissues. High levels of the mitochondrial dehydrogenase are found in BAT, placenta, testes, pancreatic islets, brain, fibroblasts and amniocytes, and low levels found in heart, muscle and liver (Chowdhury *et al.* 2000; Chretien *et al.* 1994; Honzik *et al.* 2006; Houstek *et al.* 1975; Jung and Wells 1997; Koza *et al.* 1996; MacDonald 1981; MacDonald *et al.* 1996; Scholz *et al.* 1997). This variation suggests that the need for the glycerophosphate shuttle varies between tissues.

mGPDH expression is also controlled by hormonal regulation with 3,5-tri-iodothyronine (T₃) capable of dramatically increasing mGPDH levels in heart, kidney and liver mitochondria (Dummler *et al.* 1996). It has also been shown that mGPDH levels in BAT are sensitive to cold acclimation (Koza *et al.* 1996) which led to the idea that the shuttle contributes to maintaining body temperature. However mGPDH knock-out mice display normal cold-acclimation and have an unaltered temperature increase in response to thyroid hormone, but have a reduced body mass index and in particular a reduction in the weight of white adipose tissue (Brown *et al.* 2002). Loss of the mGPDH was expected to affect glucose metabolism through the effect of the NADH/NAD⁺ ratio on the reaction catalyzed by glyceraldehyde phosphate dehydrogenase. No differences however were observed between these ratios in mGPDH knock-outs and controls, which the authors concluded was due to redundancy

between the various NADH shuttles (e.g. the malate-aspartate shuttle).

mGPDH is a major site of ROS production in mitochondria (Drahota *et al.* 2002; Miwa and Brand 2005; Miwa *et al.* 2003; Mracek *et al.* 2009). The mechanism of ROS production by mGPDH has been shown to be different from ROS production by other components of the respiratory chain, and in particular from succinate supported ROS production, which also donates electrons to co-enzyme Q (Vrbacky *et al.* 2007). The mGPDH itself was shown to be a significant site of ROS production in glycerol phosphate supported ROS production (~75%), facilitated by one-electron transfer from the dehydrogenase. Little ROS production was located at complex III. Succinate supported ROS production, however, was located mainly at complex III with little ROS produced by succinate dehydrogenase itself (SDH). ROS due to reverse electron transfer to complex I has been reported in both succinate and glycerol phosphate supported mitochondria (Miwa and Brand 2005; St-Pierre *et al.* 2002; Tretter *et al.* 2007a) but shown to be negligible in other studies (Mracek *et al.* 2009).

mGPDH possesses a calmodulin-like Ca^{2+} binding site (Brown *et al.*, 1994) which increases the activity of the enzyme by lowering the K_m for glycerol phosphate (Idahl and Lember 1995; Rutter *et al.* 1992). ROS production by glycerol-phosphate supported brain mitochondria at the mGPDH and due to reverse electron transfer to complex I, has been shown to increase in the presence of increasing amounts of calcium (Tretter *et al.* 2007b).

[1.15] Ubiquitination

Ubiquitination is a versatile post-translational modification that controls virtually all types of cellular events. It involves the covalent attachment of a ubiquitin molecule or a chain of ubiquitin molecules to a lysine residue of a target protein. Ubiquitin is an evolutionary highly conserved 76 amino acid polypeptide that is abundant in all eukaryotic cells. The attached molecule or chain can affect the localization, activity, structure or interaction partners of the target protein. One of the main functions of ubiquitination is to target the protein for degradation by the 26S proteasome (Ciechanover *et al.* 1980; Hershko and Ciechanover 1998). Like phosphorylation, ubiquitination is a reversible process due to the activity of deubiquitinating enzymes (Millard and Wood 2006).

Ubiquitin conjugation occurs through a three-step process involving three enzymes E1-3. E1 is a ubiquitin-activating enzyme, which through an ATP-dependent reaction is linked by a high-energy thioester bond to ubiquitin. The ubiquitin is then transferred in a second thioester linkage to a ubiquitin-conjugation enzyme (E2) which catalyzes the transfer of ubiquitin to the substrate protein in a covalent bond. A ubiquitin ligase (E3) is required to transfer the activated ubiquitin to the substrate protein. Ubiquitin transfer to the target protein occurs by the α -carboxyl group glycine of ubiquitin to a lysine ϵ -amino group on the target protein. (Hershko and Ciechanover, 1998). It has been shown that Lys48-linked poly-ubiquitination is a signal for proteasomal degradation by the 26S proteasome, whereas other types of ubiquitination like Lys63-linked mono-ubiquitination are involved in non-degradation regulation of proteins.

The 26S proteasome consists of the 20S core particle and two 19S regulatory subunits. The core which is hollow provides an enclosed cavity in which targeted proteins are degraded. Each end

of the core particle associates with one 19S regulatory subunit which contains the ubiquitin binding sites and ATPase sites for unfolding proteins and threading them into the 20S core (Braun et al., 1999).

The mechanism of proteolysis by the β subunits of the 20S core particle is through a threonine-dependent nucleophilic attack (Seemuller et al., 1995). The 20S core ensures all peptide bonds within a substrate by possessing multiple proteolytic activities in one proteolytic internal chamber, i.e. chymotrypsin-like, trypsin-like and post-glutamyl peptide hydrolyzing activities (Cordoza, 1993; Orlowski et al., 1993; Dick et al., 1998; McCormack et al., 1998).

In the last ten years the small ubiquitin-like protein modifier (SUMO) has emerged as a key regulator of proteins (Saitoh *et al.* 1997). Unlike ubiquitination which can target proteins for degradation, sumoylation regulates substrate function mainly by altering the intracellular localization, protein-protein interactions or other types of post-translational modifications. Initially studies indicated that sumoylation occurred mainly in the nucleus, but recently non-nuclear targets have been reported. Like ubiquitination, sumoylation can be reversed by dissimulation (Cheng *et al.* 2006). Unlike ubiquitination which has only one form of ubiquitin, with 2 single subunit E1s, a number of E2s and several hundred substrate-specific E3s, the sumoylation pathway involves more than one SUMO, a heterodimeric E1, a single E2 and just a few E3s which have a broad specificity (Zhao 2007). Other conjugatable human ubiquitins have been reported including NEDD8 which is involved in the regulation of p53 (Xirodimas *et al.* 2004).

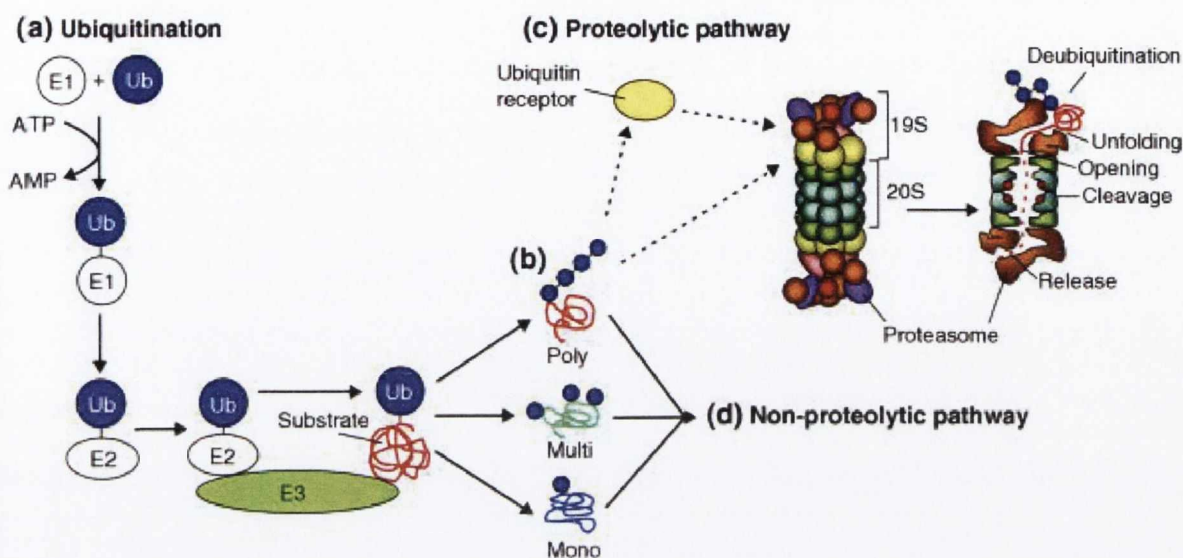


Figure 1.5 The ubiquitin-proteasome degradation system
(Taken from Kaiser and Huang, (2005), *Genome Biology*, Vol:6,)

[1.16] Ubiquitination in the mitochondrion

Until recently the mitochondrion was considered a ubiquitination-free organelle, and ATP-dependent proteases such as the Lon and the Clp proteases were considered to be the main player in the degradation of mitochondrial proteins (Bota and Davies 2002). However recent reports published suggest that ubiquitination may play an important role in mitochondrial protein quality control (Margineantu *et al.* 2007; Radke *et al.* 2008). Over 60 ubiquitinated proteins including prohibitin of mammalian sperm (Thompson *et al.* 2003), and the OSCP subunit of mitochondrial F_1F_0 ATPase (Margineantu *et al.* 2007) have been identified.

Indirect evidence using proteasome inhibitors have also supported a role for a ubiquitin dependent protein quality control system in mitochondria (Margineantu *et al.* 2007). In this study

many proteins critical to mitochondrial function such as COXI, III and IV accumulated upon proteasome treatment, and with no increase in transcript levels of the proteins. Advances in the sensitivity of techniques to detect sites of ubiquitination without using proteasome inhibition or ubiquitin over-expression have allowed the identification of ubiquitination sites in mitochondrial proteins with increased accuracy (Shi *et al.* 2010).

Drp1, the mitochondrial fission protein is also believed to be regulated by ubiquitination. Inhibition of MARCH5/MITOL, a RING finger ubiquitin ligase located on the outer membrane of the mitochondria, leads to excessive recruitment of Drp1 to the mitochondria and an elongated mitochondrial morphology (Karbowski *et al.* 2007; Nakamura *et al.* 2006; Yonashiro *et al.* 2006). Drp1 is also modified by the ubiquitin-like protein SUMO which leads to increased mitochondrial fragmentation, which can be reversed by over expression of the SUMO-protease SENP5 (Harder *et al.* 2004; Wasiaak *et al.* 2007; Zunino *et al.* 2007). Ubiquitination of anti-apoptotic proteins has also been reported to play a role in up regulation of apoptosis (Azad *et al.* 2006; Tait *et al.* 2007; Warr *et al.* 2005).

The mechanism by which ubiquitinated mitochondrial proteins are exported to the cytosol where the bulk of the proteasome machinery is located is still under debate. The process whereby targeted proteins are retrotranslocated through one or two mitochondrial membranes is unknown. However a similar pathway through one membrane occurs in the ER (endoplasmic reticulum) called the ERAD pathway (ER-associated degradation pathway) (Meusser *et al.* 2005). This pathway involves ubiquitination of target proteins followed by retro translocation across the membrane and subsequent degradation by the proteasome in the cytosol. However inner-mitochondrial and matrix proteins would require to retrotranslocate through two membranes, so extra steps in any

putative mitochondrial associated degradative pathway would be required.

Recently the cytosolic proteasome has been implicated in the turnover of uncoupling proteins 2 and 3 (UCP 2 and 3) (Azzu and Brand, 2010; Azzu *et al.* 2010). In these reports by the Brand lab it was shown that inhibition of the cytosolic proteasome blocked UCP 2 and UCP 3 degradation. A reconstituted, cell-free system to study proteasome dependent degradation of UCP 2 and 3 was also used to show that the cytosolic proteasome is capable of degrading both proteins with a half-life similar to the half-life observed in cyclohexamide treated cells. Furthermore a role for mitochondrial membrane potential was implicated in the unfolding and degradation of inner-membrane mitochondrial proteins in these studies.

The implication of a role for ubiquitin-dependent regulation in mitochondrial fusion, and the findings which show that defects in the proteasome-dependent protein degradation pathway as well as in mitochondrial function are associated with neurodegenerative disorders like Parkinsons (Ding *et al.* 2006) makes the elucidation of a model for ubiquitin-dependent signalling in mitochondria an exciting area of future research.

[1.17] Cold acclimation

During cold acclimation the thermogenic activity of BAT is known to rise, as animals require heat production to maintain their body temperature. This adaptation is through a process called non-shivering thermogenesis (Nicholls and Locke 1984). This adaptation occurs through an increase in the expression of UCP1 in BAT mitochondria, an increase in mitochondrial content of brown adipocytes, (Nicholls and Locke, 1984) an increase in the number of mature brown adipocytes in the tissue as well as an increase in

mitochondrial enzymes which contribute to the enhanced oxidative capacity of BAT (Bukowiecki *et al.* 1982). Upon onset of cold acclimation, shivering-derived thermogenesis as well as non-shivering thermogenesis is initiated. Shivering thermogenesis occurs through constant nerve stimulation of the muscles, where the muscle fibers constantly contract and relax (Behrens and Himms-Hagen 1977; Suzuki *et al.* 1997). As the period of cold acclimation increases and the oxidative capacity of BAT increases non-shivering thermogenesis replaces shivering thermogenesis as the major source of heat production in the body (Cannon and Nedergaard, 2004). Studies have shown that upon cold exposure, changes in the transcription levels of genes involved in cold acclimation are upregulated or in some cases down regulated (Watanabe *et al.* 2008), reflecting the role of that protein in the adaptation to cold acclimation.

Chapter 2 Materials and Methods

[2.1] Materials

All materials were sourced from Sigma unless otherwise stated.

Protogel	National Diagnostics
Protein G	Calbiochem
PVDF	Millipore
Anti- UCP1	Sigma
Anti-ubiquitin	Santa Cruz
Anti-PDH	Mitosciences

Addresses of suppliers

Sigma-Aldrich: Vale road, Arklow, Co. Wicklow

Millipore: Tullagreen, Carrigtwohill, Co. Cork

Santa Cruz: Bergheimer Str. 89-2, 69115 Heidelberg, Germany

National Diagnostics: Unit 4 Fleet Business Park, Itlings lane
Hessle Hull, HU13 9LX, England

Mitosciences: 1850 Millrace Drive, Suite 3A, Eugene, Oregon
97403

[2.2] Mitochondrial protein determination using the Bicinchoninic Acid Assay

Quantification of protein concentrations in tissue samples was carried out using the Bicinchoninic Acid Assay described by (Smith *et al.* 1985). All of the following solutions (standards or samples) were directly prepared in a 96-well plate. A standard protein solution of BSA was suitably diluted in deionised water from 0 to 0.25 mg.ml⁻¹. The amount of mitochondrial protein present in the samples was determined by reference to a standard curve derived from the above known concentrations of BSA. Isolated mitochondria were generally diluted in H₂O before analysis so the final concentration of the samples would fall within the range of the standard curve. Once the samples and the standards were prepared, 200µl of a working solution of BCA (1:49(v/v) BCA:Cu₂SO₄) was added to each well. The plate was then incubated for 30 minutes in an incubator set at 37°C. The absorbance of each sample was measured spectrophotometrically at 550nm.

[2.3] Isolation of Brown Adipose Tissue mitochondria

BAT mitochondria were prepared by the method of (Scarpace *et al.* 1991). BAT was removed from the interscapular region of the rat between the head and the shoulder blades. BAT was weighed and washed several times in ice cold (0-4°C) STE buffer (250mM Sucrose, 1mM EGTA, 5mM Trizma-base, pH 7.4). BAT was then placed into a pre-weighed 50ml beaker containing 30ml of ice cold STE buffer. BAT was chopped carefully in the beaker and poured into a potter homogeniser tube. The tissue was then homogenised by hand with four passes using a pestle of 0.26 inch (loose) clearance, followed by homogenisation by hand with 6 passes using a pestle of 0.12 inch (tight) clearance. The homogenate was then filtered through four layers of muslin cloth and the filtrate was

centrifuged at 8,600 x g for 10 minutes at 4°C. The supernatant was discarded and the sides of the centrifuge tube were wiped with tissue to remove any fat deposits. The pellet was re-suspended in approximately 20 ml STE buffer containing 2% (w/v) de-fatted BSA and centrifuged at 750 x g for 10 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 8,600 x g for 10 minutes at 4°C. The pellet was then re-suspended in STE buffer and centrifuged as above. The resulting mitochondrial pellet was re-suspended gently and thoroughly with 0.05ml of STE buffer per gram of original tissue and used within 6-8 hours of isolation and subsequently stored at -20°C.

[2.4] Isolation of Thymus mitochondria

Thymus mitochondria were prepared by the method of (Chappell 1972). The Thymus was removed from the abdominal cavity, trimmed of connective tissue and fat and placed into a beaker containing ice cold (0- 4 °C) STE buffer (250 mM Sucrose, 5mM Trizma-base, 1mM EGTA, pH7.4). Any BAT present in the vicinity of the Thymus was clearly visible and distinguishable from the Thymus and was removed prior to the Thymus being weighed. The Thymus was chopped finely using a scissors and washed several times using ice-cold STE buffer. The tissue was poured into a Potter homogeniser tube to a final volume of approximately 40 mls. The tissue was then homogenised by hand with four passes using a pestle of 0.26 inch (loose) clearance followed by homogenisation by hand with six passes using a pestle of 0.12 inch (tight) clearance. The homogenate was filtered through 4 layers of muslin and the filtrate was centrifuged at 800 x g for 3 minutes at 4 °C. The pellet was discarded and the supernatant was centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was

discarded and the pellet was re-suspended in STE buffer containing 2%(w/v) de-fatted BSA and centrifuged at 12,000 x g for 10 minutes at 4 °C. This pellet was subsequently re-suspended in STE buffer and centrifuged as above. The resulting mitochondrial pellet was re-suspended in 0.05ml STE buffer per gram of original tissue and used within 6-8 hours of isolation and subsequently stored at - 20°C.

[2.5] Immunoprecipitation of ubiquitinated proteins

2mg of mitochondrial protein were lysed in modified RIPA buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA) containing 1% NP-40, 25mM 2-chloroacetamide and freshly added protease inhibitors, at 4°C for 20 minutes with gentle rotation. Lysates were centrifuged at 14,000 g in a pre-cooled centrifuge for 15 minutes. Lysates were next pre-cleared with protein A or G-conjugated-beads (as appropriate) for 15 minutes at 4°C with gentle rotation. The beads were removed by centrifugation at 4,000 g for 3 minutes in a pre-cooled centrifuge. Pre-cleared lysates were incubated with the appropriate primary antibody overnight at 4°C with gentle rotation. The next morning the lysate was added to pre-equilibrated protein A or G-conjugated beads for 2 hours at 4 °C. The beads were pelleted and washed 3 x 10 minutes in RIPA buffer containing protease inhibitors and 0.1% NP-40. The bound material was eluted by heating the beads in 2X sample buffer for 5 minutes at 95°C and the samples were subjected to SDS-PAGE.

[2.6] Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of (Laemmli 1970), using a mini-PROTEAN® 3 CELL (Bio-Rad) with a 5% stacking and a 12% resolving gel. The stacking and resolving gels consisted of the following:

RESOLVING GEL	per gel	STACKING GEL	per gel
H ₂ O	3.35ml	H ₂ O	6.1ml
1.5M Tris pH8.8	2.5ml	0.5M Tris pH.6.8	2.5ml
10%SDS	100µl	10% SDS	100µl
PROTOGEL	4.0ml	PROTOGEL	1.33ml
10%APS	50µl	10% APS	50µl
TEMED	5µl	TEMED	10µl

Pre-stained molecular weight marker standards (Fisher cat No:BP3601)) were used. They consisted of β-galactosidase (116kDa), bovine serum albumin (66.2kDa), ovalbumin (45kDa), Carbonic Anhydrase (36kDa), β -lactoglobulin (26kDa), lysozyme (20kDa).

The sample buffer was made up as a 4 X strength buffer and was added to the mitochondrial protein so that the final concentration of sample buffer was 1 X. 5% β-mercaptoethanol (v/v) was added to the sample buffer immediately prior to use. Once the sample buffer and mitochondrial protein were added together, the samples were vortexed briefly and boiled for 5 minutes at 100°C on a heating block. The samples were then loaded into separate wells and electrophoresed at a constant current (200V) for 45 minutes. A glycine based running buffer (0.38M glycine, 0.05M Tris, 0.1% (w/v) SDS) was used for electrophoresis.

4 x sample buffer	
H ₂ O	3.8ml
0.5M Tris-HCl, pH6.8	1.0ml
glycerol	0.8ml
10% SDS	1.6ml
1% Bromophenol blue	0.4ml

[2.7] Western Blotting

Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Transfer was achieved using a Hoeffer semi-dry transfer apparatus at 110mA for 90 minutes. Firstly the stacking gel was removed and the gel was briefly rinsed in semi-dry transfer buffer (0.192M glycine, 0.025M Tris-HCl pH 8.3, 0.013M SDS, 15%(V/V) Methanol). One sheet of PVDF membrane was pre-soaked in methanol for 30 seconds and then in distilled water for 1 minute. Six sheets of filter paper cut to the appropriate size of the gel were pre-soaked along with the sheet of PVDF for 15 minutes in transfer buffer. A layered sandwich was then prepared in which the PVDF was placed on top of three layers of filter paper at the anode. The gel, followed by three layers of filter paper was then placed on top of this. Air bubbles were removed and the lid containing the cathode was placed on top.

[2.8] Western Blotting for Ubiquitinated proteins

Following SDS-PAGE, resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Transfer was achieved using a Bio-Rad wet transfer apparatus at 20V for 5 hours at room temperature. Firstly the stacking gel was removed and the gel, methanol activated PVDF, 2 sheets of filter paper and 2 foam pads were soaked for 15 minutes

in transfer buffer (25mM Tris, 186mM Glycine pH8.0). A sandwich consisting of one sheet of filter paper and one sheet of PVDF on the bottom was prepared with the gel and one sheet of filter paper on top. This sandwich was loaded into the cassette with a foam pad on either side. The cassette was loaded into the tank along with an ice-pack and submerged in transfer buffer. The anode and cathode were attached to the power supply. Following the transfer the membrane was soaked in fixation buffer (7% Acetic Acid, 40% Methanol, 3% Glycerol, 50% H₂O) for 30 minutes. This was followed by washing in TBS-Tween (TBS containing 0.1% (w/v) Tween) for 3 x 5 minutes.

[2.9] Immunodetection of proteins

Blocking of the membrane was performed by incubating the blot in TBS-Tween (0.1%) containing either 5% Marvel milk powder (anti UCP1, anti-PDH) or 5% Bovine Serum Albumin (anti-ubiquitin) for 1 hour at room temperature. Blots were then incubated in primary antibody (in TBS-Tween containing 5% Marvel milk powder or 5% Bovine Serum Albumin as appropriate) overnight at 4°C. Following this primary antibody incubation, the blots were washed 3 x 10 minutes in TBS-Tween. The blots were then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody in TBS-Tween containing either 5% Marvel milk powder or 5% Bovine Serum Albumin, for 1 hour at room temperature. Following this blots were further washed 3x 10 minutes in TBS - Tween. Blots were developed using an enhanced chemiluminescence (ECL) detection system (Pierce) for detecting horseradish peroxidase labelled antibody, by means of the HRP catalysed oxidation of luminol under alkaline conditions and the results were visualised by exposure to Kodak X-Omat LS film.

[2.10] Coomassie Blue R-250 staining of SDS-PAGE gels

Following SDS-PAGE, the Laemmli gels were stained using Coomassie Blue R-250 according to the method of Laemmli (1970). Laemmli gels were stained using 0.5% (w/v) Coomassie Blue R-250 in 50% methanol and 10% acetic acid, for 1 hour on a shaking platform. The Coomassie Blue R-250 stain was then discarded and the gel was destained using 50% methanol and 10% acetic acid until background staining was eliminated, and only specific staining of bands was visible.

[2.11] In Gel Trypsin digestion

Briefly, gel slices were manually cut into 1mm cubes and de-stained (50/45/5 v/v methanol/water/acetic acid) prior to reduction (10 mM dithiothreitol, Sigma) for 30 minutes at room temperature and alkylation (100 mM 2-chloroacetamide, Sigma) for 30 minutes at room temperature. Modified sequencing grade porcine trypsin solution 30 μ L (20 ng/ μ L, Promega, Madison, WI) was added to the gel slice enzyme/protein ratio 1:50 and then digested for 16 h at 37°C. The peptides were extracted out of the gel slices with 400 μ L (50/40/10 v/v acetonitrile/water/formic acid). The sample was then speed vac centrifuged to dryness and stored at -80°C until analysis.

[2.12] LC-MS/MS analysis

An Ultimate Nano-HPLC (LC-Packings/Dionex Canada, Oakville, ON, Canada) coupled to an Applied Biosystems/MDS Sciex QSTAR Pulsar I Hybrid Quadrupole-TOF LC-MS/MS Mass Spectrometer (Concord, ON, Canada) was used to perform the LC-MS/MS analyses.

The sample was rehydrated in 40 μ l (2% v/v acetonitrile, 0.1% formic acid) and 1 μ l was injected via a FAMOS Autosampler (LC-Packings/ Dionex Canada, Oakville, ON, Canada) for each analysis. The samples were directly injected onto an in-house prepared nano-analytical column Magic C₁₈AQ resin, 150 mm x 75 μ m column diameter (Michrom Bioresources, Inc, Auburn, CA, USA). The nano-column was connected to a 20 μ m I.D. emitter tip (New Objective Inc., Woburn, MA, USA) positioned at the orifice plate of the mass spectrometer with spray established by applying a tip voltage of 1900V pre-nano column via a platinum wire nano-tee high voltage connection. The following 1hr HPLC analytical separation was performed at 300 nl/min (5 min 100% Solvent A (98% v/v water/2% acetonitrile, 0.1% formic acid) , 25 min linear gradient from 5-28% solvent B (98% v/v acetonitrile, 0.1% formic acid), 11min linear gradient from 28-43% solvent B, 80% solvent B over 2 minutes and held for 2 minutes at 80% solvent B. The gradient was then ramped down over 2 minutes and re-equilibrated at 100% A solvent for 13minutes before the next injection.

Mass spectrum analyses were acquired collecting a 1sec TOFMS survey scan 400-1600 m/z followed by two 2.5sec product ion scans 100-1500 m/z . MS/MS spectra were acquired in a data dependent manner selecting the top 2 most intense eluting ions in the 400-1,600 m/z range with a 2+ to 5+ charge state. Following selection for MS/MS analysis, precursor ions were excluded from selection for MS/MS analysis for 180 sec. A rolling collision energy for fragmentation was selected based on the precursor ion mass using the following formula $0.05m/z + 5V$. Mass spectrometer parameters used were as follows: Declustering potential setting of 50, Focusing Potential setting of 220, Curtain gas setting of 25 and CAD gas setting of 8 with Nitrogen in the collision cell.

[2.13] Data Analysis Parameters

MSMS data was searched by centroiding with Analyst QS 1.1 Mascot script 1.6 b25 (MDS Sciex) to create the Mascot generic format file (mgf) for data base searching. The mgf files were searched against the Uniprot-Swissprot 20090225 (410518 sequences; 148080998 residues) allspecies, Rodentia and Rat IPI databases with the following parameters: Fixed: Carbamidomethyl (C) Variable: Deamidated (NQ), oxidation (M), propionamide (C), GlyGly (K), LeuArgGlyGly (K) as variable modifications, peptide mass tolerance ± 0.3 amu, fragment mass tolerance ± 0.15 amu and trypsin enzyme specificity with 2 missed cleavages.

[2.14] Measurement of Oxygen consumption rates of mitochondria

All measurements of respiration were made in duplicate, simultaneously using an Oxygraph-2k respirometer (Oroboros Instruments). The oxygraph-2k is a two chamber titration-injection respirometer with a limit of oxygen flux detection of $1\text{pmol s}^{-1}\text{ml}^{-1}$. The instrumentation allows for O_2 flux measurements with only 0.04mg of mitochondrial protein. Standardised instrumental and chemical calibrations were performed to correct for back diffusion of O_2 into the chamber from the various components; leak from the exterior, O_2 consumption by the chemical medium, and sensor O_2 consumption. O_2 flux was resolved by software capable of converting nonlinear changes in the negative time derivative of the oxygen concentration signal. The mitochondrial suspension was stirred using a PVDF magnetic stirrer and thermostatically maintained at 37°C . Mitochondria were incubated in $5\text{ }\mu\text{M}$ atractyloside, $2\text{ }\mu\text{g/ml}$ oligomycin, 120mM KCl, 5mM Hepes, 1mM EGTA and 0.1% defatted bovine serum albumin, pH7.4 (KOH).

Before each experiment the medium was equilibrated for 30-40 minutes with air in the oxygraph chambers until a stable signal was achieved to calibrate for oxygen saturation. Mitochondria were added and a basal rate was measured for 5 minutes. Steady state oxygen consumption rates (state 4) were then measured in the presence of, 10mM succinate, 10mM glycerol-3-phosphate, 10mM pyruvate plus 6mM malate or 10 mM glutamate plus 6 mM malate. Where indicated 1 μ M rotenone was present. Subsequent additions of 1 mM GDP and 0.25 μ M FCCP were added.

[2.15] Measurement of mitochondrial ROS generation

H₂O₂ generation, detected by the Amplex Red II assay, was essentially as described by (Dlaskova *et al.* 2006). The assay medium contained mitochondria (0.05mg/ml), 5 μ M Amplex Red, 10 U/ml of horseradish peroxidase, 30 U/ml superoxide dismutase (SOD), 5 μ M atractyloside, 2 μ g/ml oligomycin, 120mM KCl, 5mM Hepes, 1mM EGTA and 0.1% defatted bovine serum albumin, pH7.4 (KOH). Fluorescence was detected by a Perkin Elmer LS 55 Fluorometer with excitation set at 570 $\pm$ 8nm and emission at 585 $\pm$ 4nm. The temperature throughout the experiment was maintained at 37°C. Substrates used were 10mM succinate, 10mM glycerol-3-phosphate, 10mM pyruvate plus 6mM malate or 10 mM glutamate plus 6 mM malate. Where indicated 1 μ M rotenone was present. 1mM GDP was added where indicated. 0.5 μ M of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was also added where indicated. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day.

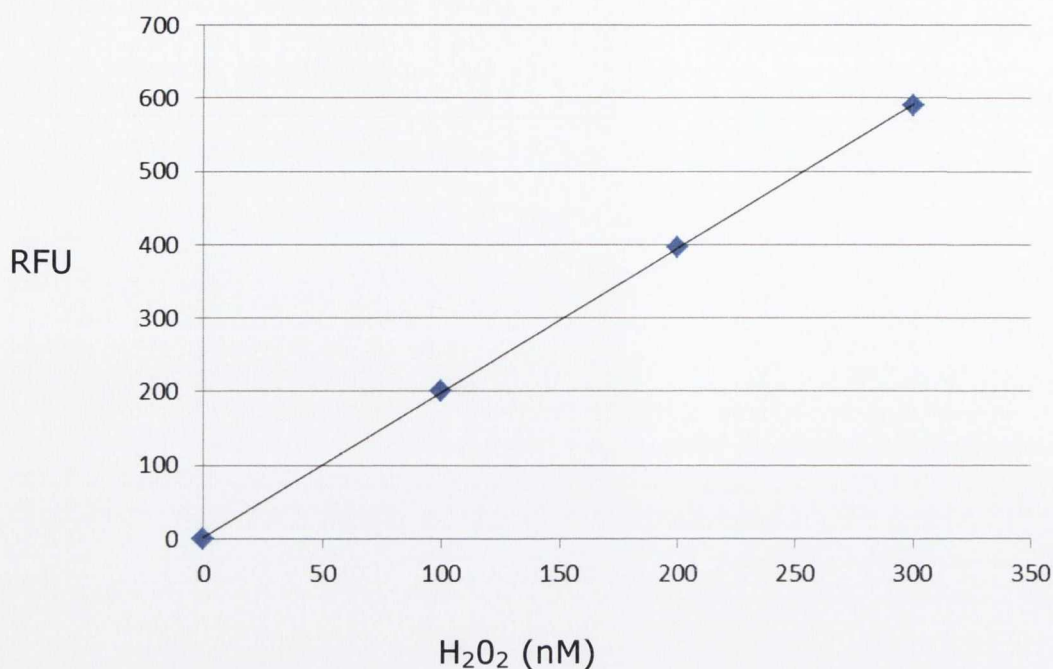


Figure 2.1 Standard curve of fluorescent measurements of H₂O₂ detection

[2.16] Examination of mitochondrial membrane potential using the probe safranin

Membrane potential of isolated mitochondria was examined by the fluorescent dye safranin, which accumulates and quenches in polarized mitochondria (Akerman and Wikstrom 1976). The assay medium consisted of mitochondria (0.125mg/ml), 5 μ M safranin, 5 μ M atractyloside, 2 μ g/ml oligomycin, 120mM KCl, 5mM Hepes, 1mM EGTA and 0.1% defatted bovine serum albumin (BSA), pH 7.4 (KOH). Fluorescence was detected by a Perkin Elmer LS 55 Fluorimeter with excitation set at 570 $\pm$ 5nm and emission at 585 $\pm$ 5 nm. The temperature of the medium was maintained at 37°C. Substrates used were 10mM succinate, 10mM glycerol-3-phosphate, 10mM pyruvate plus 6mM malate or 10 mM glutamate plus 6 mM malate. Where indicated 1 μ M rotenone was present. 1mM GDP was added where indicated. 0.5 μ M of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was also added where indicated.

[2.17] Complex I activity assay

The activity of Complex I (NADH:ubiquinone oxidoreductase) was determined by monitoring the oxidation of NADH at 340nm. The assay medium consisted of 25 mM potassium phosphate, 5 mM MgCl_2 , 2.5 mg/ml bovine serum albumin and 2 mM KCN, pH7.2.

Absorbance changes were monitored on a Shimadzu UVmini-1240 uv-vis spectrophotometer at 30°C. Baseline activity was established for 3 minutes after the addition of 0.2 mM NADH, 65 μM decylubiquinone and 2.7 $\mu\text{g/ml}$ antimycin A. The reaction was initiated by the addition of mitochondria (75 μg). The rate of oxidation of NADH was monitored by the decrease in absorbance at 340 nm for 3 minutes. 2 $\mu\text{g/ml}$ rotenone was then added, and the rate of change in absorbance was measured for an additional 3 minutes. Complex I activity was determined by subtracting the rotenone insensitive activity from the total activity.

[2.18] Complex II activity assay

The activity of Complex II (succinate:ubiquinone oxidoreductase) was determined by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. Absorbance changes were monitored on a Shimadzu UVmini-1240 uv-vis spectrophotometer at 30°C. Mitochondria (25 μg) were incubated at 30°C for 20 minutes in assay buffer containing 25 mM potassium phosphate, 5 mM MgCl_2 , 2.5 mg/ml bovine serum albumin 20mM succinate and 0.2mM ATP, pH 7.2. The reaction was initiated by the addition of 2 $\mu\text{g/ml}$ antimycin A, 2 $\mu\text{g/ml}$ rotenone, 2mM KCN, 50 μM 2,6-dichloroindophenolate and 65 μM decylubiquinone. The rate of reduction of 2,6-dichloroindophenolate was monitored for 3 minutes. 10 mM malonate was then added to inhibit the enzymatic

activity and the rate of change in absorbance was measured for an additional 3 minutes. Complex II activity was determined by subtracting the malonate insensitive activity from the total activity.

[2.19] Glycerol-3-phosphate dehydrogenase activity assay

The activity of glycerol-3-phosphate dehydrogenase was determined by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. Absorbance changes were monitored on a Shimadzu UVmini-1240 uv-vis spectrophotometer at 30°C. Mitochondria (25 µg) were incubated in assay buffer containing 25 mM potassium phosphate, 5 mM MgCl₂, 2.5 mg/ml bovine serum albumin, 2 µg/ml antimycin A, 2 µg/ml rotenone, 2mM KCN, 50 µM 2,6-dichloroindophenolate and 65 µM decylubiquinone, pH 7.2. Baseline activity was monitored for 3 minutes. The reaction was initiated by the addition of 20mM glycerol-3-phosphate. The rate of reduction of 2,6-dichloroindophenolate was monitored for 3 minutes.

[2.20] In vitro UCP1 degradation assay

An in vitro degradation assay for UCP1 was carried out essentially as described by Azzu and Brand, (2010). Mitochondria were isolated as described in sections 2.3 and 2.4. Mitochondria were resuspended in STE buffer (250mM Sucrose, 1mM EGTA, 5mM Trizma-base, pH 7.4). For each assay mitochondria were incubated with an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine and 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. Where indicated 20 mM succinate, 20 µM FCCP, ubiquitin mix (35 µg ubiquitin, 1.4 µg fraction 1, 1.4 µg fraction 2 from Calbiochem cat. #662096) and 5

µg 26S proteasome fraction were added to the assay. Mitochondria were incubated at 37 °C and samples were removed at the relevant time point, resuspended in 2X SDS-PAGE sample buffer and boiled at 95°C for 5 minutes.

[2.21] Isolation of thymocytes

Thymocytes were isolated from the thymus as described by (Buttgereit *et al.* 1994). The thymus was removed from the rat, trimmed clean of connective tissue and brown fat and transferred to RPMI-1640 medium. The thymus and medium were poured onto a nylon mesh (70µm) in a petri-dish and using a plunger of a 5 ml syringe, the thymus was disaggregated into a suspension of thymus cells. This thymus suspension was transferred to a 15ml centrifuge tube and centrifuged at 300 x g for 5 minutes. Any fat droplets present on the surface of the supernatant were removed. The supernatant was discarded and the thymocyte pellet resuspended in RPMI-1640 and spun again at 300 x g. Cells were counted. Oxygen consumption of thymocytes was measured at 37° C in an Oroboros oxygraph with constant stirring. Measurements were carried out in RPMI-1640 medium with 5×10^6 cells/ml.

[2.22] Isolation of brown pre-adipocytes and differentiation into mature brown adipocytes

Primary cultures of brown adipocytes were prepared essentially as described by (Petrovic *et al.* 2008). Male C57/BL6 mice were used for the preparation of primary cultures of brown adipocytes. Brown adipose tissue was isolated from the interscapular depots under sterile conditions. White fat and connective tissue was carefully trimmed from the brown fat. The trimmed brown fat was minced

and transferred to a solution containing 100 mM Hepes, 120mM NaCl, 4.8 mM KCl, 1mM CaCl₂, 4.5 mM glucose, 1.5%(w/v) BSA and 1.5 mg/ml collagenase (type II). The digestion was performed at 37 °C with constant mixing for 30 minutes. The resulting cell suspension was filtered through 250-µM pore size nylon filter. The filtered suspension was placed on ice for 30 minutes. The mature adipocytes floated to the top. This top layer was removed and the remaining suspension was filtered through a 40 µm sterile filter and centrifuged at 700 x g for 10 minutes. The pellet was resuspended in DMEM culture medium with 10% fetal bovine serum, 4 mM insulin, 10 mM HEPES, 4 mM glutamine, 50 U penicillin and 50 µg streptomycin and centrifuged at 700 x g for 10 minutes. The pellet was then resuspended in culture medium (0.5 ml/animal). The cells were cultured in 6- well Corning plates. 0.2 ml of cell suspension was added to 1.8 ml of culture medium in each well. The cells were grown at 37°C. The PPAR agonist rosiglitazone maleate (1 µM) was added to each well to initiate differentiation of pre-adipocytes into mature brown adipocytes. Cells were washed in DMEM and the medium was changed on day 1 after isolation, and then every second day. 1 µM rosiglitazone was also added with each change of medium. On day ten after isolation, experiments/harvesting were performed.

[2.23] Wild Type (WT) and UCP1 Knock-out (KO) mice

Wild Type (C57BL/6J) and UCP1 Knock-out (C57BL.6J) mice were used in this study. Animals were housed in groups of 6 in a specific pathogen free (SPF) environment. Wild-Type and UCP1 Knock-out mice were housed in individually ventilated cages at 25±1°C. All animals were allowed free access to food and water and a 12-hour light/dark cycle was in place. All mice were killed by CO₂ asphyxiation.

[2.24] Temperature acclimation

3 week old female Wistar rats were used for the cold acclimation study. Animals were randomly assigned to either a control group or to a cold acclimated group. Cold acclimated rats were housed in pairs and held in a temperature controlled room set at 5 ± 1 °C for 4 weeks. Control rats were housed in groups of 6 at 25°C for 4 weeks. All animals were allowed free access to laboratory chow and water. A normal 12-hour light/dark cycle was adhered to. All rats were killed by cervical dislocation.

[2.25] Calculation of $[Ca^{2+}]_{free}$

$[Ca^{2+}]_{free}$ was calculated using a Ca-EGTA calculator from www.stanford.edu/~cpatton/CaEGTA using the appropriate buffer concentrations and EGTA and BSA concentrations.

[2.26] Statistical analysis

All results are expressed as mean $\pm$ SEM unless otherwise indicated. Unless otherwise stated mean values were compared using an unpaired student t-test test, one-way ANOVA analysis and repeated measures ANOVA with Dunnett's post-testing. A p value of < 0.05 was taken to indicate significance.

Chapter 3 UCP 1 dependent ROS generation in BAT mitochondria

[3.1] Introduction

Excluding phagocytes, mitochondria are the major site of reactive oxygen species (ROS) generation. ROS are a byproduct formed when free electrons react in an enzyme free manner with oxygen. When this occurs the superoxide radical is formed, which in turn can be converted to various other reactive oxygen species. Complexes I and III are major sites of ROS production, as is the glycerol-3-phosphate dehydrogenase (Murphy 2009; Vrbacky *et al.* 2007).

Using isolated mitochondria, it has been shown that generation of ROS is greatly increased when the proton electrochemical gradient is high (Korshunov *et al.* 1997). Such conditions occur during non-phosphorylating conditions, that is to say state 4 respiration when ADP is unavailable. Addition of either ADP, which induces proton transport via the ATP synthase, or addition of an uncoupling agent, which directly increases proton leak and thus decreases the proton electrochemical gradient, strongly suppresses superoxide formation (Skulachev 1996).

These observations have led to the idea that 'mild uncoupling' could be a potential physiological mechanism of alleviating ROS production. Mitochondrial uncoupling proteins have been implicated in the alleviation of ROS production by mitochondria (Brand *et al.* 2004b). In particular UCP 2 and 3 have been shown to alleviate ROS production by mitochondria (Bai *et al.* 2005; McLeod *et al.* 2005). Previous studies have however questioned whether UCP1 plays a role in the regulation of ROS production. Shabalina *et al.* (2006) showed no difference in superoxide production by BAT mitochondria from UCP 1 knock-out and wild-type mice and also

reported no difference in the anti-oxidant mechanisms in BAT mitochondria from UCP 1 knock-out compared to wild-type mice, therefore questioning whether UCP 1 in BAT mitochondria played a role in ROS attenuation.

However recently it has been shown by our laboratory that UCP1 has the potential to attenuate ROS production by BAT mitochondria (Dlaskova et al., 2010) and we were able to resolve the difference in observations between our findings and that of Shablina et al., (2006). We were able to show that the UCP 1 ligand, GDP, could increase ROS production by isolated BAT mitochondria from rats or wild-type mice respiring on a range of substrates (pyruvate plus malate, succinate plus rotenone and glycerol-3-phosphate plus rotenone). Furthermore, we were able to demonstrate that this GDP sensitive ROS production was UCP 1 dependent.

The discrepancy between not detecting a difference in ROS production by mitochondria from brown adipose tissue from wild-type mice and UCP1 knock-out mice by Shabalina et al. (2006) and the demonstration of a difference in ROS production by mitochondria from brown adipose tissue from wild-type mice and UCP1 knock-out mice by Dlaskova et al. (2010) centres on the ROS detecting probes used. Dlaskova et al. (2010) used amplex red while Shabalina et al. (2006) used reduction of dihydroethidium.

Dlaskova et al. (2010) were able to show that Amplex red which measures H_2O_2 and indirectly measures superoxide by the addition of excess superoxide dismutase was more sensitive for ROS production by mitochondria than dihydroethidium which only measures superoxide production.

However, in the course of that investigation, we noted that when BAT mitochondria were respiring on succinate alone (without rotenone) plus the UCP1 ligand GDP, the rate of reactive oxygen

species production detected in mitochondria from wild-type mice was greater than in mitochondria from UCP1 knock-out mice, plus or minus GDP (A. Dlaskova, K.J. Clark and R.K. Porter, unpublished observation). In addition, we noted that the rate of oxygen consumption by BAT mitochondria respiring on complex I substrates ($\pm$ uncoupler) was greater in mitochondria from wild-type mice compared to those from UCP1 knock-out mice (Dlaskova et al., 2010).

The aims of the project covered in this chapter were to investigate further the aforementioned anomalies associated with reverse electron flow and apparent differences in complex I activity, in a comparison of BAT mitochondria from wild-type and UCP1 knock-out mice

[3.2] Results

Figure 3.1 Illustrates representative traces of H_2O_2 production by brown adipose tissue mitochondria respiring on succinate (red), succinate plus GDP (blue), succinate plus rotenone (orange) and succinate plus GDP plus rotenone (green). FCCP addition is indicated at the 250 second mark.

Figure 3.2 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria respiring on succinate, succinate plus GDP, succinate plus rotenone and succinate plus GDP plus rotenone. The effect of FCCP on H_2O_2 production in the presence of both substrates is also illustrated. Significantly higher H_2O_2 production was observed by brown adipose tissue mitochondria respiring on succinate plus GDP compared to H_2O_2 production by brown adipose tissue mitochondria respiring on succinate alone (-GDP= 0.47 ± 0.083 ; +GDP= 11.14 ± 1.07 , $p=0.0006$). Significantly higher H_2O_2 production was also observed by brown adipose tissue mitochondria respiring on succinate plus GDP plus rotenone compared to H_2O_2 production by brown adipose tissue mitochondria respiring on succinate plus rotenone alone (-GDP= 0.59 ± 0.14 ; +GDP= 4.36 ± 0.71 , $p=0.0064$). No significant differences in H_2O_2 production was observed upon FCCP addition to brown adipose tissue mitochondria respiring on succinate (-FCCP= 0.47 ± 0.083 ; +FCCP= 0.37 ± 0.06) or succinate plus rotenone (-FCCP= 0.59 ± 0.14 ; +FCCP= 0.55 ± 0.13). Significantly lower H_2O_2 production was observed upon FCCP addition to brown adipose tissue mitochondria respiring on succinate plus GDP (-FCCP= 11.14 ± 1.07 ; +FCCP= 0.52 ± 0.12 , $p=0.0006$) and succinate plus GDP plus rotenone (-FCCP= 4.36 ± 0.71 ; +FCCP= 0.66 ± 0.17 , $p=0.0072$).

Figure 3.3 illustrates state 4 oxygen consumption rates (nmol O₂/min/mg mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats respiring on succinate and succinate plus rotenone. A significant increase in O₂ consumption was observed upon the addition of GDP to mitochondria respiring on succinate (-GDP=71.95±5.09; +GDP=136.21±10.76, p=0.0057), while a significant decrease in oxygen consumption was observed upon the addition of GDP to mitochondria respiring on succinate plus rotenone (-GDP=278.64±12.97; +GDP=117.25±9.17, p=0.0005). O₂ consumption in the presence of FCCP was 176.39±5.5 nmol O₂/min/mg mitochondria by mitochondria respiring on succinate and 259.8±36 nmol O₂/min/mg mitochondria by mitochondria respiring on succinate plus rotenone.

Figure 3.4 illustrates the profile of mitochondrial membrane potential in isolated brown adipose tissue mitochondria respiring on succinate (red) and succinate plus rotenone (blue). Where indicated substrate (100 s mark), GDP (600 s mark) and FCCP (1000 s mark) were added.

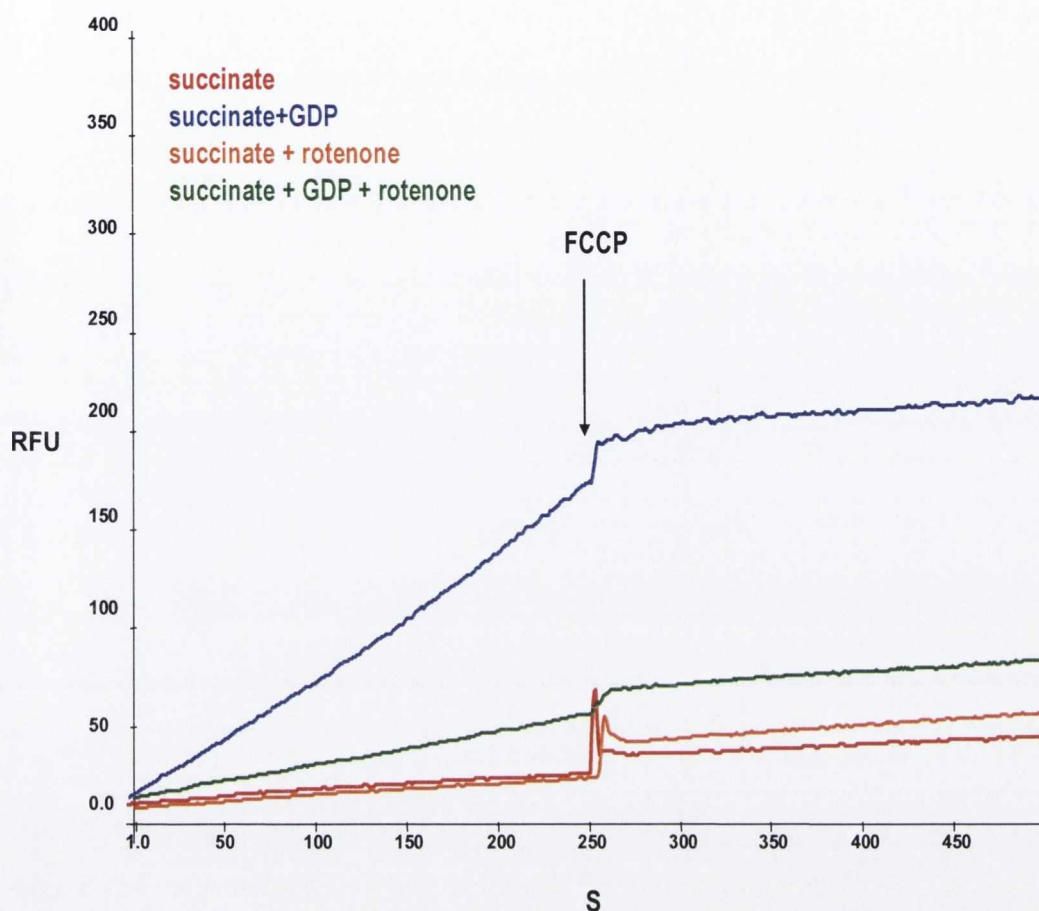


Figure 3.1 Representative trace of time course for the detection of H_2O_2 production by isolated brown adipose tissue mitochondria respiring on **succinate**, **succinate plus GDP**, **succinate plus rotenone** and **succinate plus GDP plus rotenone**.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H_2O_2 production rates by BAT mitochondria ($100\mu\text{g}/\text{ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, $1\mu\text{g}/\text{ml}$ oligomycin, $5\mu\text{M}$ atractyloside, 0.1% de-fatted BSA, $5\mu\text{M}$ Amplex Red, $10\text{ U}/\text{ml}$ horseradish peroxidase and $30\text{ U}/\text{ml}$ superoxide dismutase. Substrates used were 10 mM succinate-KOH, 10 mM succinate-KOH plus 1 mM GDP, 10 mM succinate-KOH plus $1\mu\text{M}$ rotenone and 10 mM succinate-KOH plus 1 mM GDP plus $1\mu\text{M}$ rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at $570 \pm 8\text{ nm}$ and emission at $585 \pm 4\text{ nm}$. H_2O_2 production was measured for 250 seconds, whereupon $0.5\mu\text{M}$ of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H_2O_2 production was measured for a further 250 seconds.

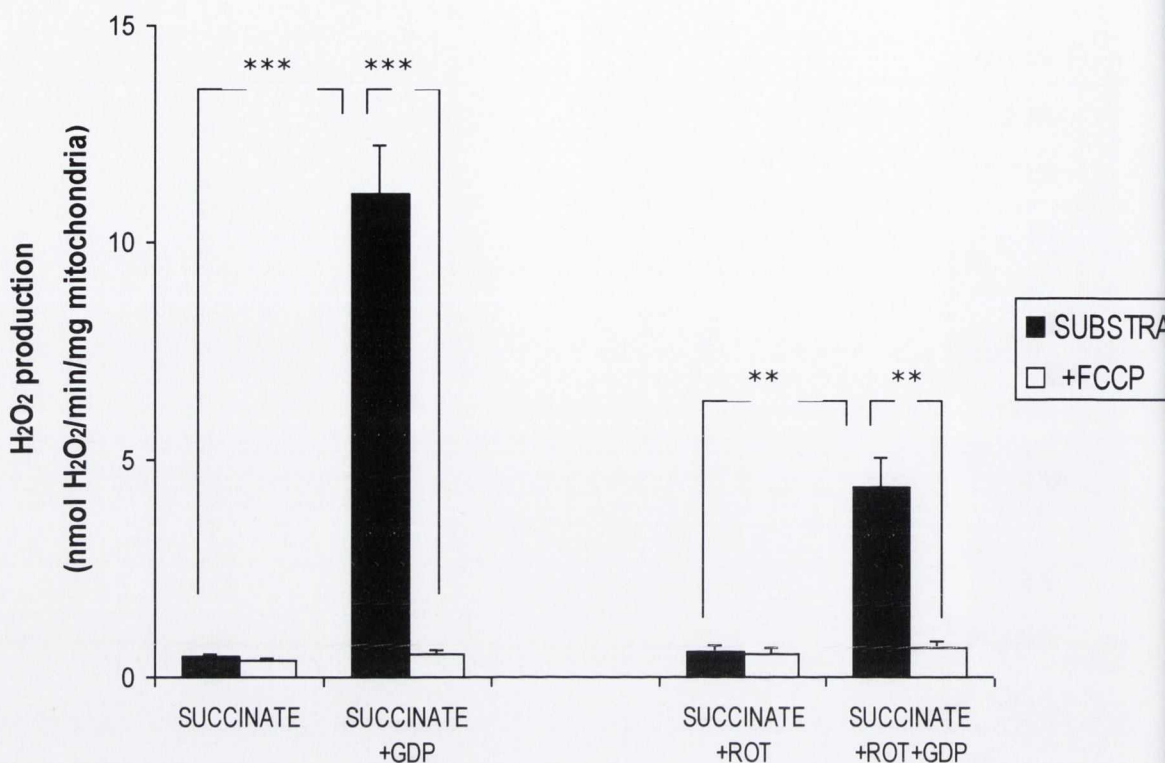


Figure 3.2 GDP sensitive H₂O₂ production in brown adipose tissue mitochondria respiring on succinate and succinate plus rotenone.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates used were 10 mM succinate-KOH, 10 mM succinate-KOH plus 1 mM GDP, 10 mM succinate-KOH plus 1 µM rotenone and 10 mM succinate-KOH plus 1 mM GDP plus 1 µM rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (**=p<0.01; ***=p<0.001).

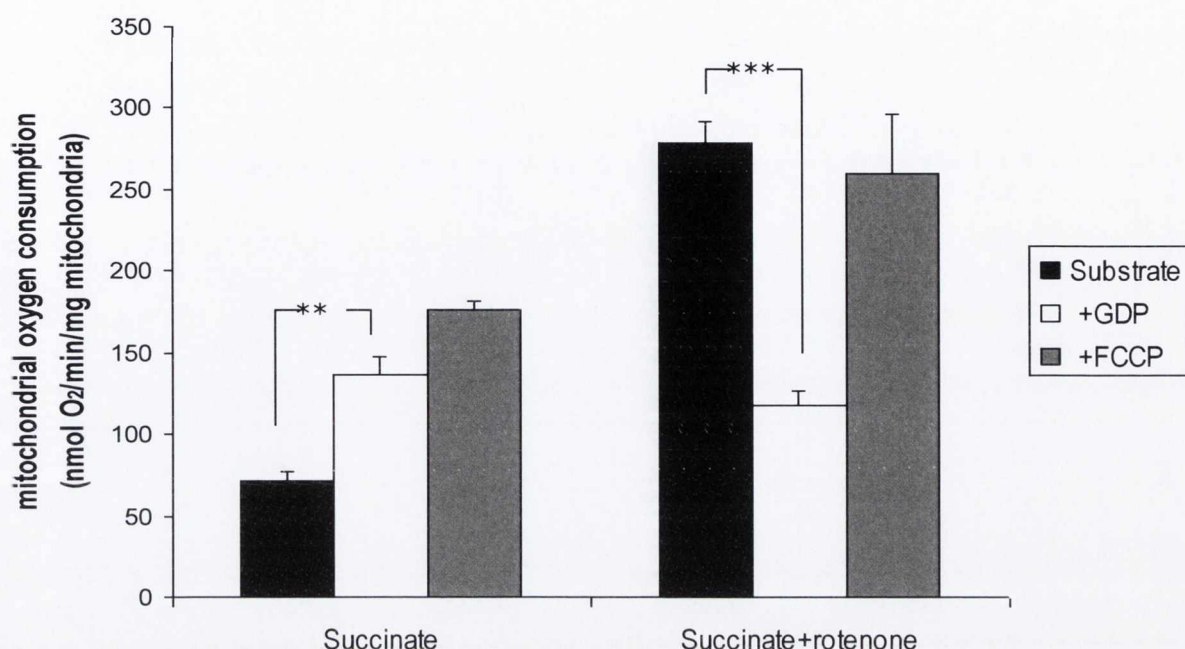


Figure 3.3 GDP sensitive non-phosphorylating oxygen consumption by isolated brown adipose tissue mitochondria respiring on succinate and succinate plus rotenone.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA and 10 mM succinate-KOH, plus or minus 1 µM rotenone in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (**=p<0.01; ***=p<0.001).

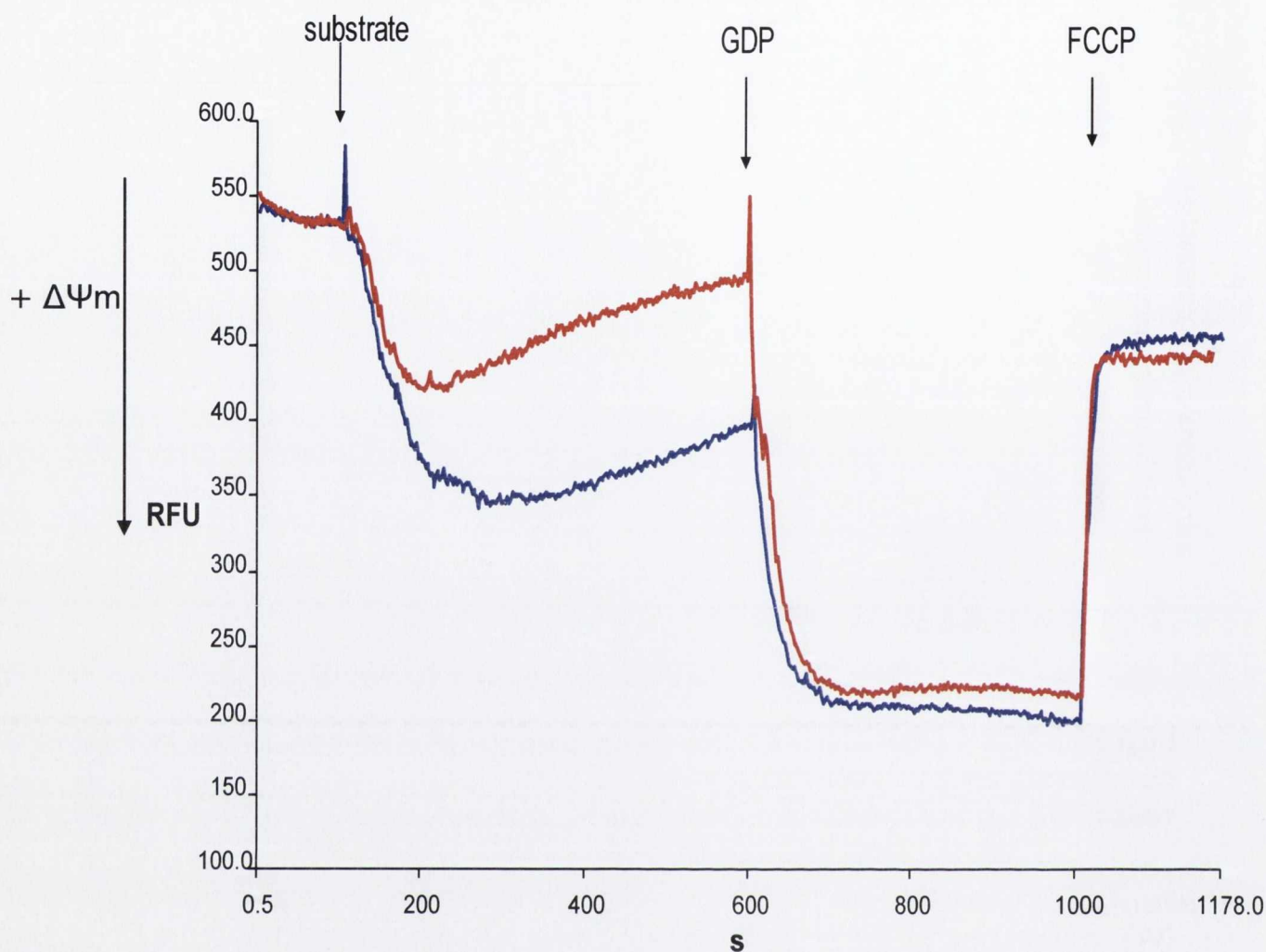


Figure 3.4 Profile of mitochondrial membrane potential in isolated brown adipose tissue mitochondria respiring on succinate and succinate plus rotenone.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. The membrane potential of BAT mitochondria (100 $\mu\text{g/ml}$) was monitored at 37 $^{\circ}\text{C}$ in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM safranin, 5 μM atractyloside, 0.1% de-fatted BSA and 10 mM succinate-KOH, plus or minus 1 μM rotenone. Where indicated 1 mM GDP and 2.5 μM FCCP were added.

Figure 3.5 Illustrates representative traces of H_2O_2 production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate (red), glycerol-3-phosphate plus GDP (blue), glycerol-3-phosphate plus rotenone (orange) and glycerol-3-phosphate plus GDP plus rotenone (green). FCCP addition is at the 250 second mark.

Figure 3.6 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria respiring on glycerol-3-phosphate, glycerol-3-phosphate plus GDP, glycerol-3-phosphate plus rotenone and glycerol-3-phosphate plus GDP plus rotenone. The effect of FCCP on H_2O_2 production is also illustrated. Significantly higher H_2O_2 production was observed by brown adipose tissue mitochondria respiring on glycerol-3-phosphate plus GDP compared to H_2O_2 production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate alone (-GDP= 9.18 ± 1.07 ; +GDP= 18.63 ± 2.4 , $p=0.0228$). Significantly higher H_2O_2 production was also observed by brown adipose tissue mitochondria respiring on glycerol-3-phosphate plus GDP plus rotenone compared to H_2O_2 production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate plus rotenone alone (-GDP= 11.49 ± 0.29 ; +GDP= 17.07 ± 2.06 , $p=0.05$). No significant differences in H_2O_2 production was observed upon FCCP addition to brown adipose tissue mitochondria respiring on glycerol-3-phosphate (-FCCP= 9.18 ± 1.07 ; +FCCP= 10.55 ± 0.86), glycerol-3-phosphate plus GDP (-FCCP= 18.63 ± 2.4 ; +FCCP= 13.83 ± 1.77), glycerol-3-phosphate plus rotenone (-FCCP= 11.49 ± 0.29 ; +FCCP= 13.89 ± 1.71) or glycerol-3-phosphate plus GDP plus rotenone (-FCCP= 17.07 ± 2.06 ; +FCCP= 14.09 ± 0.89).

Figure 3.7 illustrates state 4 oxygen consumption rates (nmol O₂/min/mg mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone. A significant decrease in O₂ consumption was observed upon the addition of GDP to mitochondria respiring on glycerol-3-phosphate (-GDP=100.298±3.9; +GDP=85.311±3.5, p=0.0459) and on glycerol-3-phosphate plus rotenone (-GDP=108.59±3.71; +GDP=89.73±3.56, p=0.0216). O₂ consumption in the presence of FCCP was 123.407±12.6 nmol O₂/min/mg mitochondria by mitochondria respiring on glycerol-3-phosphate and 114.91±11.5 nmol O₂/min/mg mitochondria by mitochondria respiring on glycerol-3-phosphate plus rotenone.

Figure 3.8 illustrates the profile of mitochondrial membrane potential in isolated brown adipose tissue mitochondria respiring on glycerol-3-phosphate (red) and glycerol-3-phosphate plus rotenone (blue). Where indicated substrate (100 s mark), GDP (600 s mark) and FCCP (1000 s mark) were added.

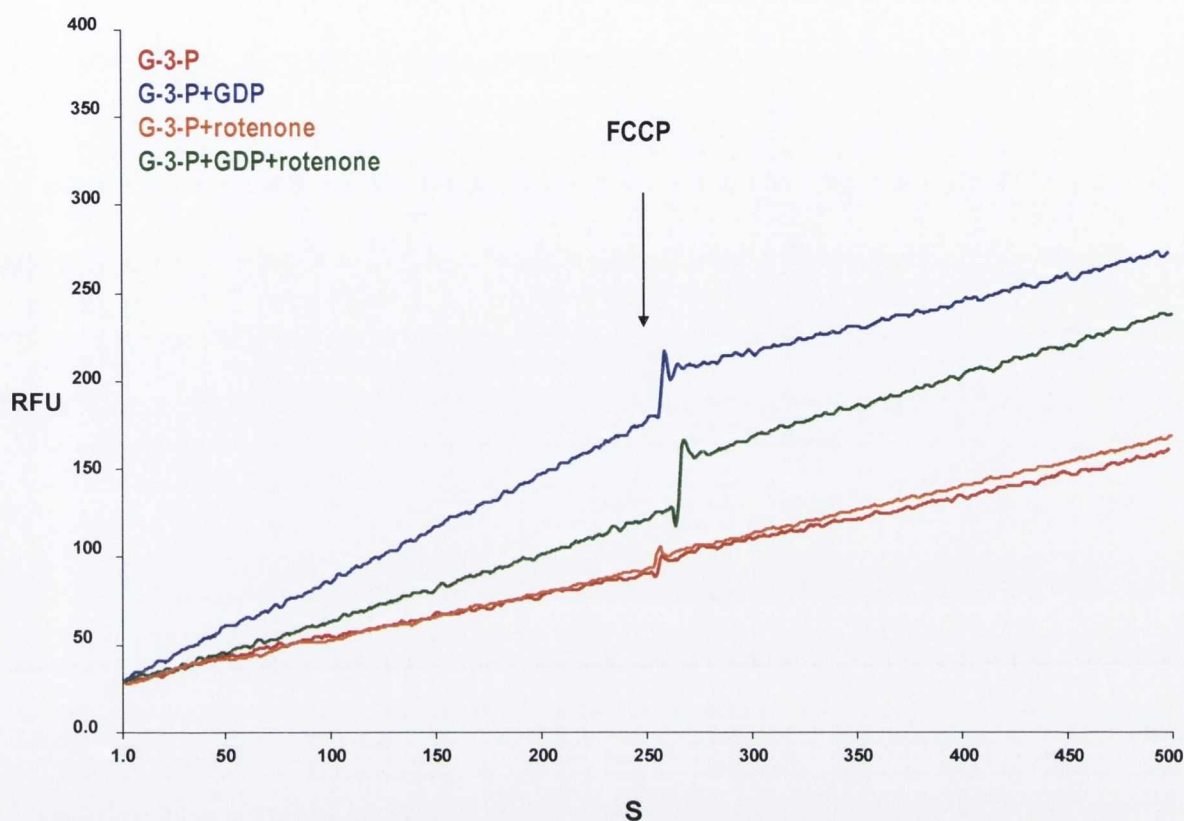


Figure 3.5 Representative trace of time course for the detection of H_2O_2 production by isolated brown adipose tissue mitochondria respiring on **glycerol-3-phosphate, **glycerol-3-phosphate plus GDP**, **glycerol-3-phosphate+rotenone** and **glycerol-3-phosphate +GDP+rotenone**.**

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H_2O_2 production rates by BAT mitochondria ($100\mu\text{g}/\text{ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, $1\mu\text{g}/\text{ml}$ oligomycin, $5\mu\text{M}$ atractyloside, 0.1% de-fatted BSA, $5\mu\text{M}$ Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate, 10 mM glycerol-3-phosphate plus 1 mM GDP, 10 mM glycerol-3-phosphate plus $1\mu\text{M}$ rotenone and 10 mM glycerol-3-phosphate plus 1 mM GDP plus $1\mu\text{M}$ rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at $570 \pm 8\text{ nm}$ and emission at $585 \pm 4\text{ nm}$. H_2O_2 production was measured for 250 seconds, whereupon $0.5\mu\text{M}$ of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H_2O_2 production was measured for a further 250 seconds

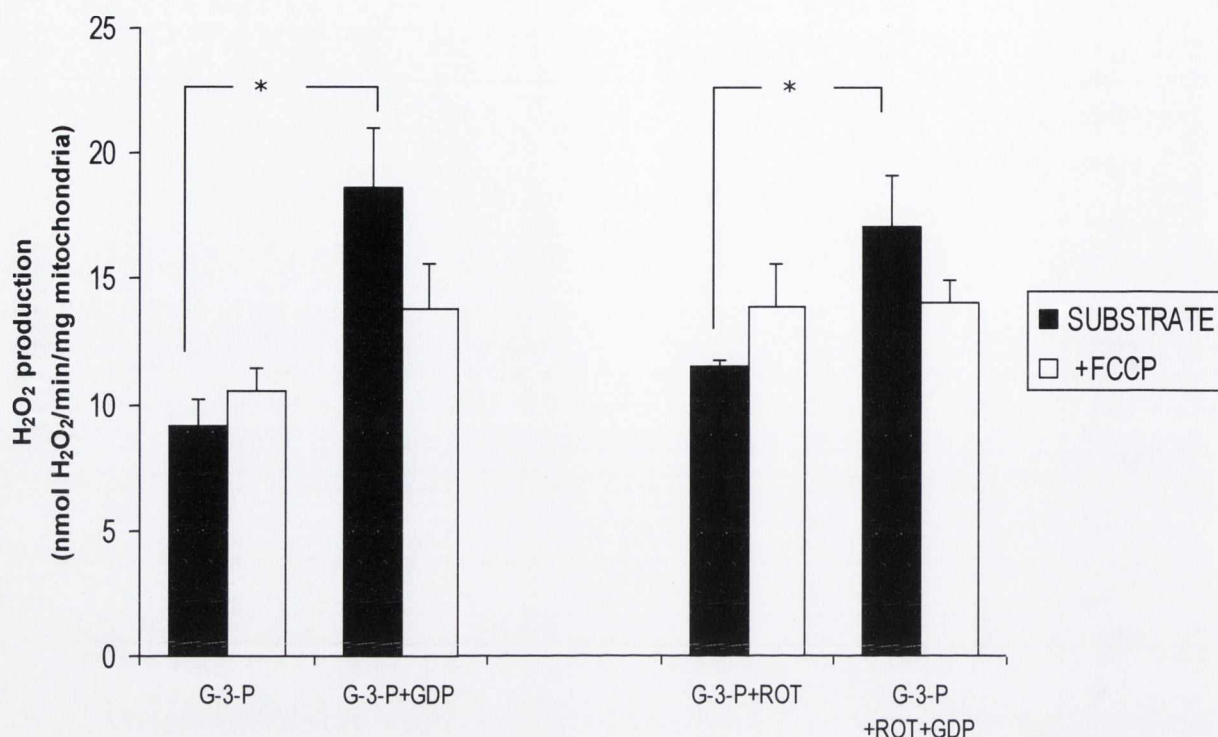


Figure 3.6 GDP sensitive H₂O₂ production in brown adipose tissue mitochondria respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate, 10 mM glycerol-3-phosphate plus 1 mM GDP, 10 mM glycerol-3-phosphate plus 1 µM rotenone and 10 mM glycerol-3-phosphate plus 1 mM GDP plus 1 µM rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05).

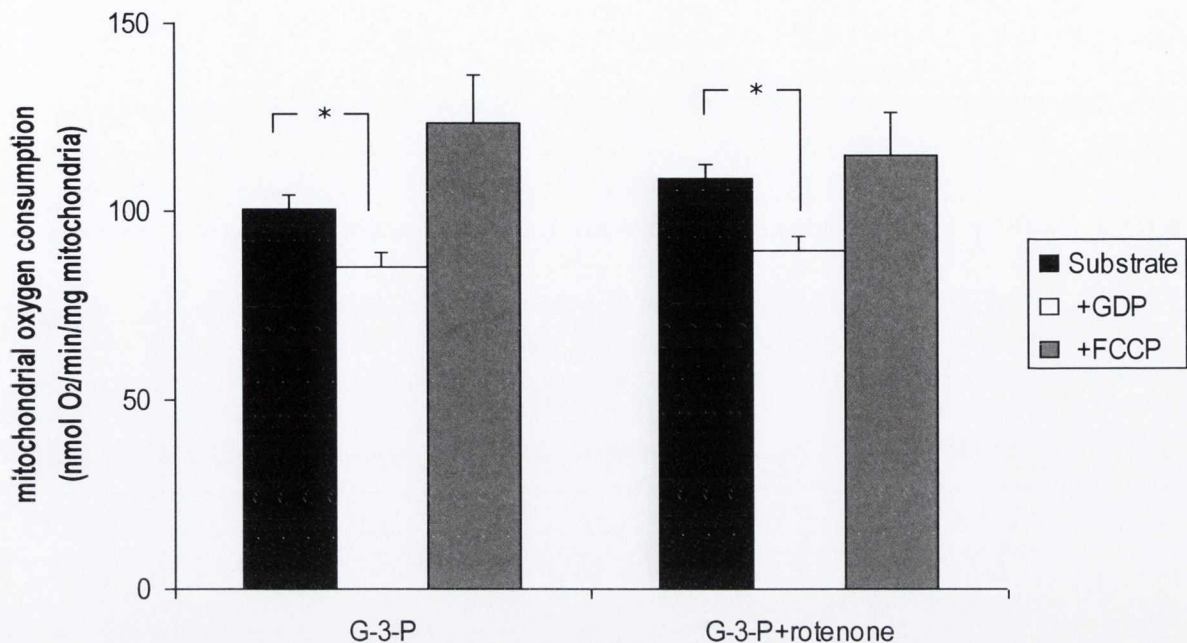


Figure 3.7 GDP sensitive non-phosphorylating oxygen consumption by isolated brown adipose tissue mitochondria respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA and 10 mM glycerol-3-phosphate, plus or minus 1 µM rotenone in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05).

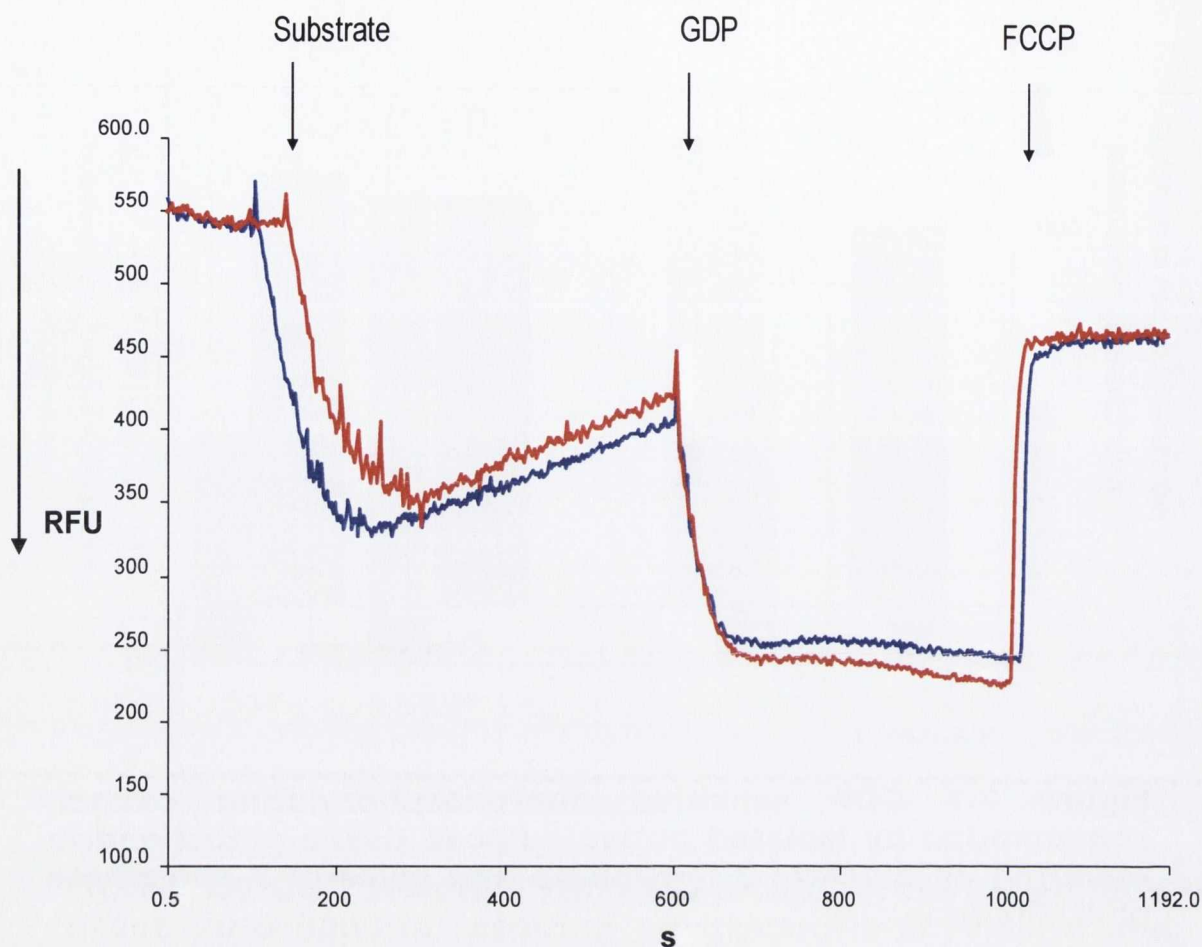


Figure 3.8 Profile of mitochondrial membrane potential in isolated brown adipose tissue mitochondria respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. The membrane potential of BAT mitochondria (100 μ g/ml) was monitored at 37 $^{\circ}$ C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 μ g/ml oligomycin, 5 μ M safranin, 5 μ M atractyloside, 0.1% de-fatted BSA and 10 mM glycerol-3-phosphate, plus or minus 1 μ M rotenone. Where indicated 1 mM GDP and 2.5 μ M FCCP were added.

Figure 3.9 Illustrates representative traces of H_2O_2 production by brown adipose tissue mitochondria respiring on pyruvate plus malate (red) and pyruvate plus malate plus GDP plus (blue). FCCP addition is at the 250 second mark.

Figure 3.10 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria respiring on pyruvate plus malate and pyruvate plus malate plus GDP. The effect of FCCP on H_2O_2 production in the presence of both substrates is also illustrated. No significant difference in H_2O_2 production by brown adipose tissue mitochondria was observed by mitochondria respiring on pyruvate plus malate plus GDP compared to H_2O_2 production by brown adipose tissue mitochondria respiring on pyruvate plus malate alone (-GDP= 0.52 ± 0.1 ; +GDP= 0.63 ± 0.084). No significant differences in H_2O_2 production was observed upon FCCP addition to brown adipose tissue mitochondria respiring on pyruvate plus malate (-FCCP= 0.52 ± 0.1 ; +FCCP= 0.391 ± 0.07). Significantly lower H_2O_2 production was observed upon FCCP addition to brown adipose tissue mitochondria respiring on pyruvate plus malate plus GDP (-FCCP= 0.63 ± 0.084 ; +FCCP= 0.322 ± 0.05 , $p=0.0355$).

Figure 3.11 illustrates state 4 oxygen consumption rates (nmol O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats. A significant decrease in O_2 consumption was observed upon the addition of GDP to mitochondria respiring on pyruvate plus malate (-GDP= 374.71 ± 27 ; +GDP= 72.64 ± 6.66 , $p=0.0004$). O_2 consumption in the presence of FCCP was 385.79 ± 19.44 nmol O_2 /min/mg mitochondria.

Figure 3.12 illustrates the profile of mitochondrial membrane potential in isolated brown adipose tissue mitochondria respiring on pyruvate plus malate . Where indicated substrate (100 s mark), GDP (600 s mark) and FCCP (1000 s mark) were added.

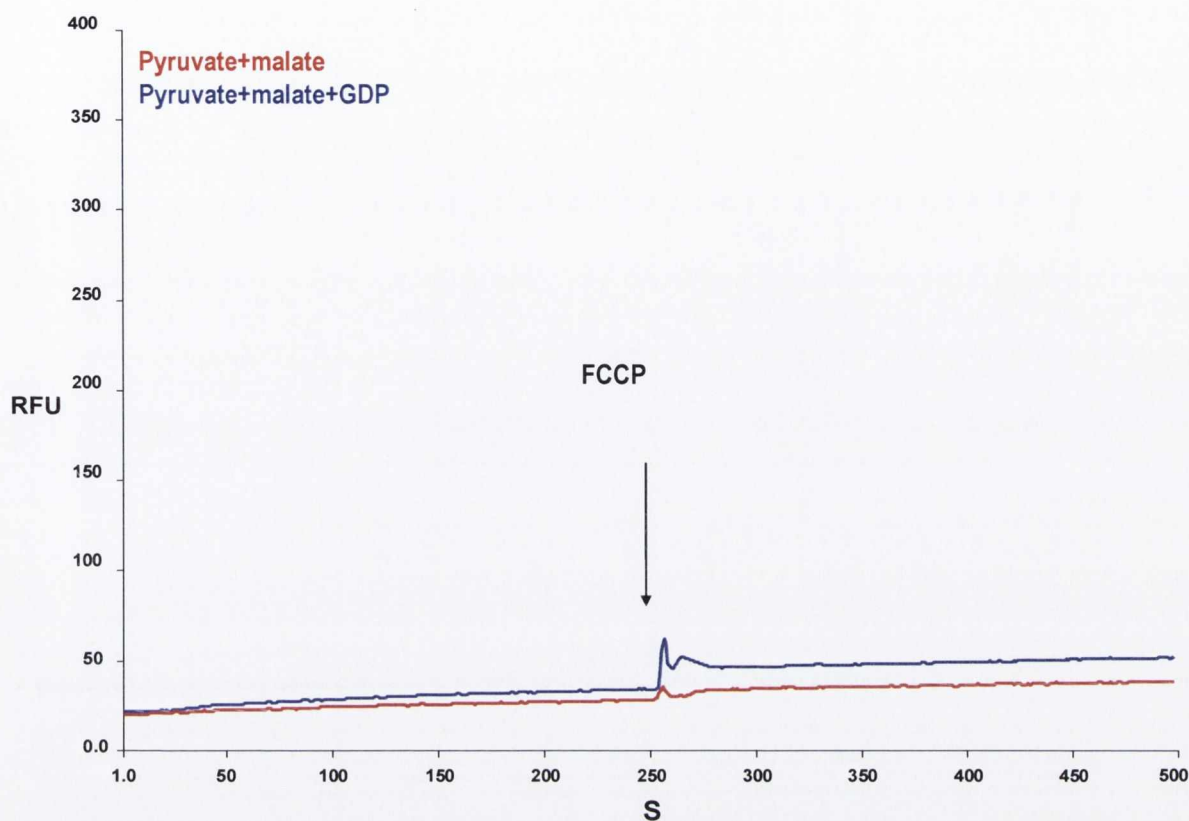


Figure 3.9 Representative trace of time course for the detection of H_2O_2 production by isolated brown adipose tissue mitochondria respiring on **pyruvate plus malate and **pyruvate plus malate plus GDP**.**

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H_2O_2 production rates by BAT mitochondria ($100\mu\text{g}/\text{ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g}/\text{ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA, 5 μM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM pyruvate+6 mM malate or 10 mM pyruvate+6 mM malate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H_2O_2 production was measured for 250 seconds, whereupon 0.5 μM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H_2O_2 production was measured for a further 250 seconds.

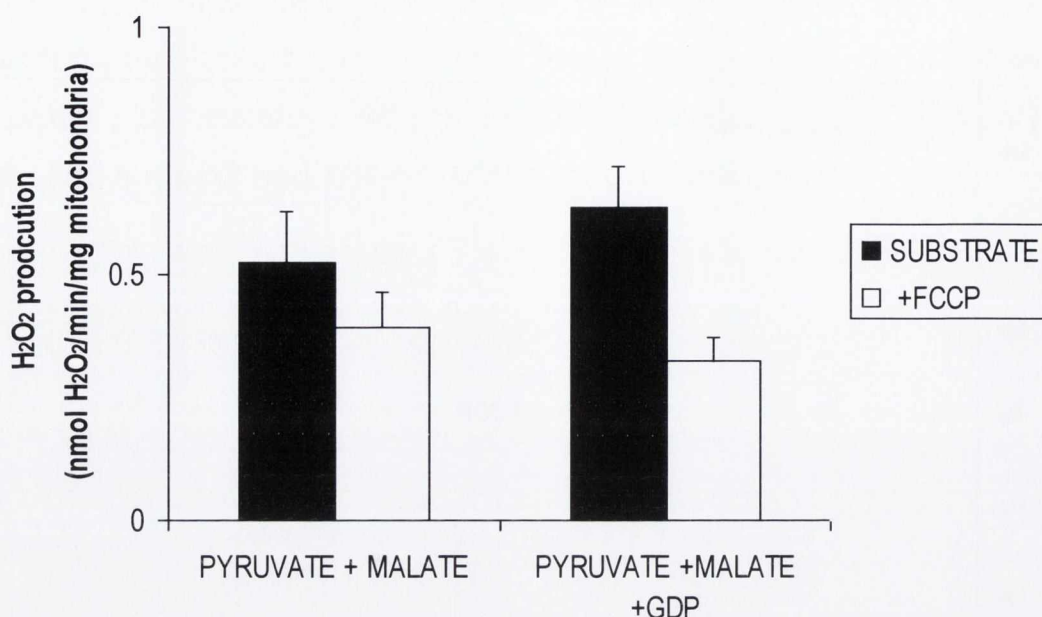


Figure 3.10 GDP insensitive H₂O₂ production in brown adipose tissue mitochondria respiring on pyruvate plus malate.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM pyruvate+6 mM malate or 10 mM pyruvate+6 mM malate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

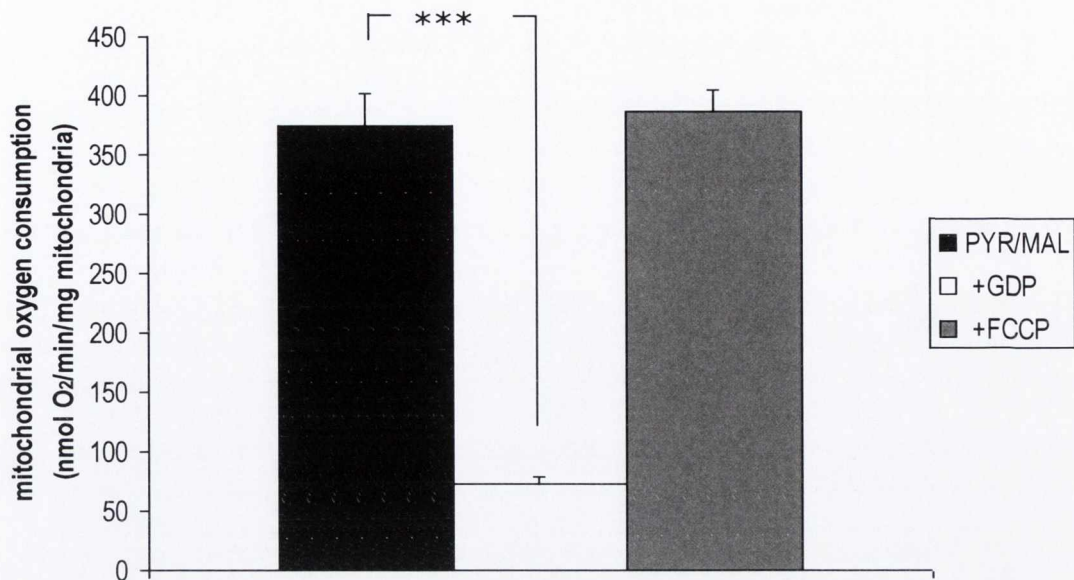


Figure 3.11 GDP sensitive non-phosphorylating oxygen consumption by isolated brown adipose tissue mitochondria respiring on pyruvate plus malate.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA and 10 mM pyruvate + 6 mM malate in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (***= $p < 0.001$).

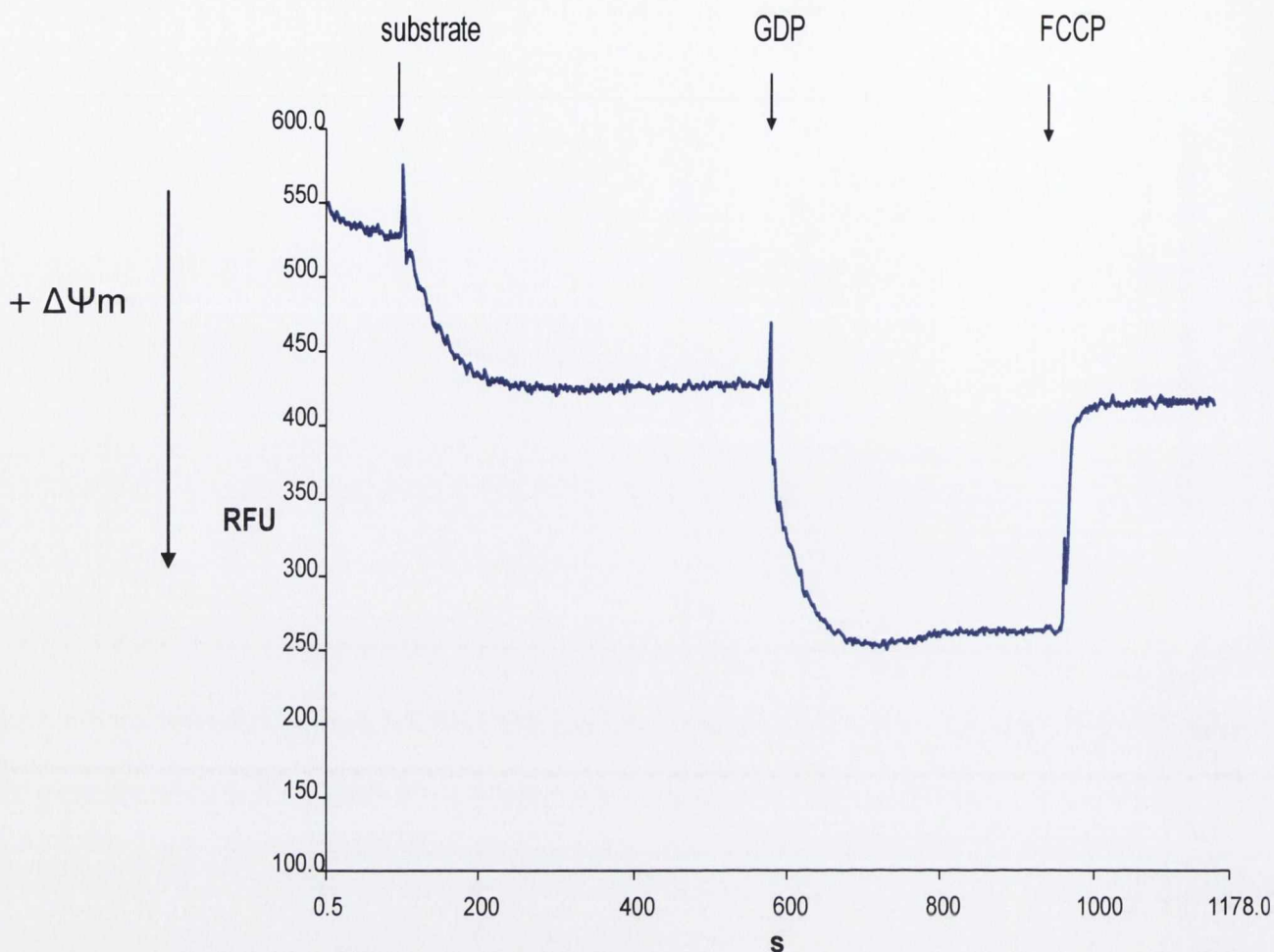


Figure 3.12 Profile of mitochondrial membrane potential in isolated brown adipose tissue mitochondria respiring on pyruvate plus malate

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. The membrane potential of BAT mitochondria (100 $\mu\text{g/ml}$) was monitored at 37 $^{\circ}\text{C}$ in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM safranin, 5 μM atractyloside, 0.1% de-fatted BSA and 10 mM pyruvate plus 6 mM malate. Where indicated 1 mM GDP and 2.5 μM FCCP were added.

From the data generated with brown adipose tissue mitochondria from Wistar rats, it can be seen that H_2O_2 production in brown adipose tissue mitochondria respiring on succinate, glycerol-3-phosphate and pyruvate plus malate increases upon GDP addition (Figures 3.1, 3.4, 3.7, 3.13). This suggests that inhibition of UCP 1 by GDP causes an increase in H_2O_2 production in brown adipose tissue mitochondria. In parallel oxygen consumption measurements were carried out using the same substrates. In these experiments oxygen consumption was also inhibited by GDP, verifying that GDP was indeed inhibiting UCP 1 (Figures 3.3, 3.6, 3.9, 3.12).

GDP is a well established inhibitor of UCP 1, however it may have a UCP 1 independent effect on H_2O_2 production. To verify that the increase in H_2O_2 production upon GDP addition is due to the inhibition of UCP 1, and thus its uncoupling activity, brown adipose tissue mitochondria from UCP 1 knock-out and wild-type mice were used and the experiments performed with brown adipose tissue mitochondria from rats were repeated with brown adipose tissue mitochondria from the UCP 1 knock-out and wild-type mice.

Figure 3.13 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock out mice respiring on succinate, succinate plus GDP, succinate plus rotenone and succinate plus rotenone plus GDP. H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock out mice was significantly higher compared to H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on succinate (WT=1.03 $\pm$ 0.0009; KO=3.71 $\pm$ 0.89, p =0.0398) and succinate plus rotenone (WT=1.22 $\pm$ 0.06; KO=2.68 $\pm$ 0.78, p =0.0001). GDP significantly increased H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on succinate (-GDP=1.03 $\pm$ 0.0009; +GDP=5.97 $\pm$ 1.53, p =0.0322) and succinate plus rotenone (-GDP=1.22 $\pm$ 0.06; +GDP=1.85 $\pm$ 0.02, p =0.0426) but does not significantly affect H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock-out mice respiring on succinate (-GDP=3.71 $\pm$ 0.89; +GDP=4.65 $\pm$ 0.86) or succinate plus rotenone (-GDP=2.68 $\pm$ 0.78; +GDP=2.17 $\pm$ 0.29).

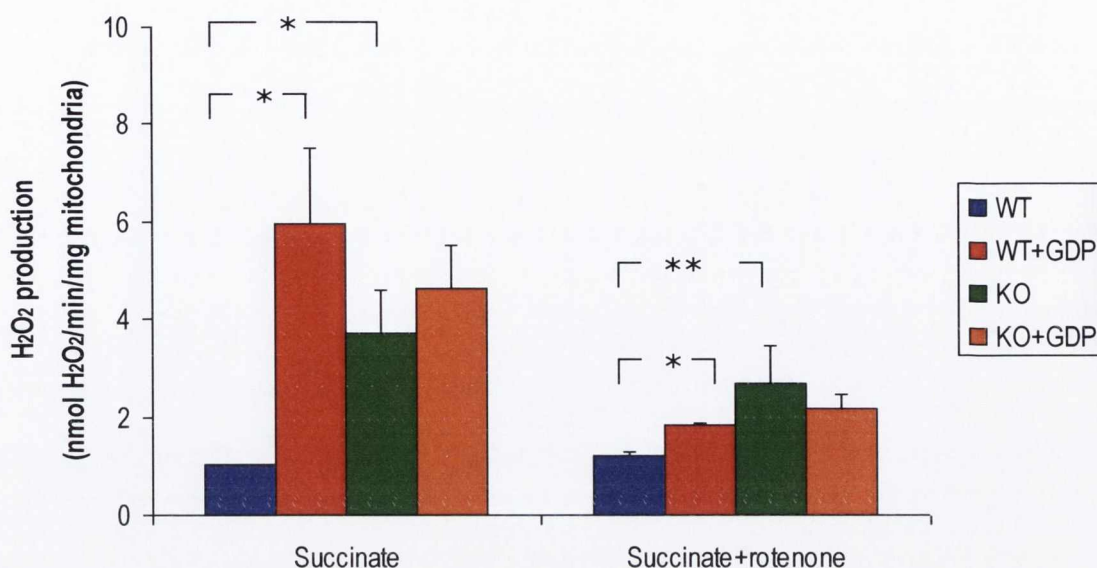


Figure 3.13 GDP sensitive H_2O_2 production by brown adipose tissue mitochondria respiring on succinate and succinate plus rotenone is UCP 1 dependent

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Subsequent H_2O_2 production rates by BAT mitochondria (100 μ g/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 μ g/ml oligomycin, 5 μ M atractyloside, 0.1% de-fatted BSA, 5 μ M Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM succinate, 10 mM succinate + 1 μ M rotenone, 10 mM succinate + 1 mM GDP and 10 mM succinate + 1 μ M rotenone + 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H_2O_2 production was measured for 250 seconds, whereupon 0.5 μ M of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H_2O_2 production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H_2O_2 each experimental day. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate (*= $p < 0.05$, **= $p < 0.01$).

Figure 3.14 compares state 4 non-phosphorylating O₂ consumption (nmol O₂/min/mg mitochondria) by brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock-out mice respiring on succinate and succinate plus rotenone. Succinate supported state 2 oxygen consumption was significantly higher in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice compared to brown adipose tissue mitochondria isolated from wild-type mice (WT=69.118±5.08; KO=95.19±1.54, p=0.008). However succinate plus rotenone supported state 2 oxygen consumption was significantly higher in brown adipose tissue mitochondria isolated from wild-type mice, compared to brown adipose tissue mitochondria isolated from UCP 1 knock-out mice (WT=267.79±25.34; KO=103.71±2.02, p=0.003). No significant differences were observed in a comparison of GDP sensitive oxygen consumption by mitochondria respiring on succinate (WT=78.24±13.79; KO=99.21±2.91) or succinate plus rotenone (WT=152.10±25.74; KO=111.98±1.98). Significantly higher state 4 uncoupled rates were observed by mitochondria isolated from UCP 1 knock-out mice respiring on succinate compared to mitochondria isolated from wild-type mice respiring on succinate (WT=65.901±10.45; KO=154.105±24.52; p=0.0297). No differences in state 4 uncoupled rates were observed by mitochondria respiring on succinate plus rotenone (WT=196.22±29.5; KO=204.94±6.91). GDP significantly decreased oxygen consumption by brown adipose tissue mitochondria isolated from wild-type mice respiring on succinate plus rotenone (-GDP=267.79±25.34; +GDP=152.10±25.74, p=0.0328), but not on succinate alone (-GDP=69.118±5.08; +GDP=78.239±13.79). GDP did not significantly affect oxygen consumption by brown adipose tissue isolated from UCP 1 knock-out mice respiring on succinate (-GDP=95.19±1.54; +GDP=99.21±2.91) or succinate plus rotenone (-GDP=103.71±2.02; +GDP=111.98±1.98).

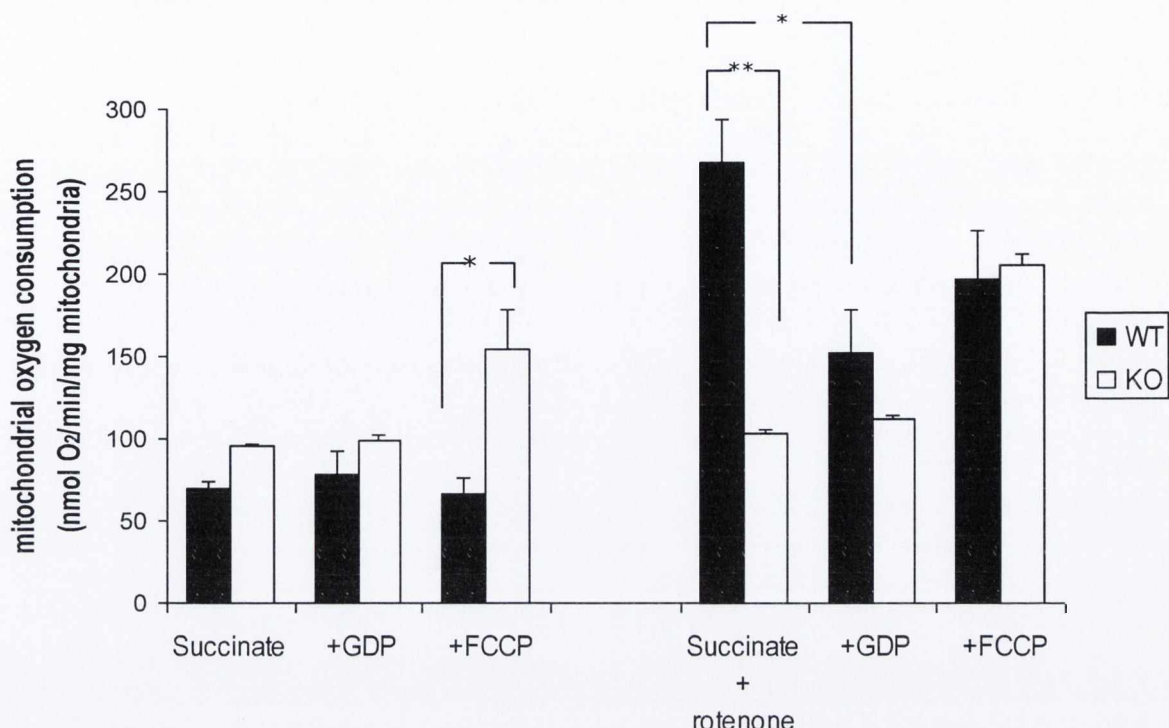


Figure 3.14 GDP inhibition of oxygen consumption by mitochondria respiring on succinate plus rotenone is UCP 1 dependent.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Subsequent oxygen consumption rates by BAT mitochondria (100 μ g/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 μ g/ml oligomycin, 5 μ M atractyloside, 1 μ M rotenone, 0.1% de-fatted BSA and 10 mM succinate-KOH in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μ M FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate (*= p <0.05, **= p <0.01).

Figure 3.15 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock out mice respiring on glycerol-3-phosphate, glycerol-3-phosphate plus GDP, glycerol-3-phosphate plus rotenone and glycerol-3-phosphate plus rotenone plus GDP. H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock out mice was significantly higher compared to H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate (WT=14.16 $\pm$ 0.45; KO=35.35 $\pm$ 7.13, $p=0.0414$). H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock out mice was consistently higher compared to H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate plus rotenone, although not significantly higher (WT=16.35 $\pm$ 1.03; KO=30.91 $\pm$ 6.97). GDP significantly increased H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate (-GDP=14.16 $\pm$ 0.45; +GDP=17.65 $\pm$ 0.94, $p=0.0285$). GDP consistently increased H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate plus rotenone, although not significantly (-GDP=16.35 $\pm$ 1.03; +GDP=19.98 $\pm$ 3.57). GDP does not significantly affect H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock-out mice respiring on glycerol-3-phosphate (-GDP=35.35 $\pm$ 7.13; +GDP=32.65 $\pm$ 3.4) or glycerol-3-phosphate plus rotenone (-GDP=30.91 $\pm$ 6.97; +GDP=27.09 $\pm$ 5.06).

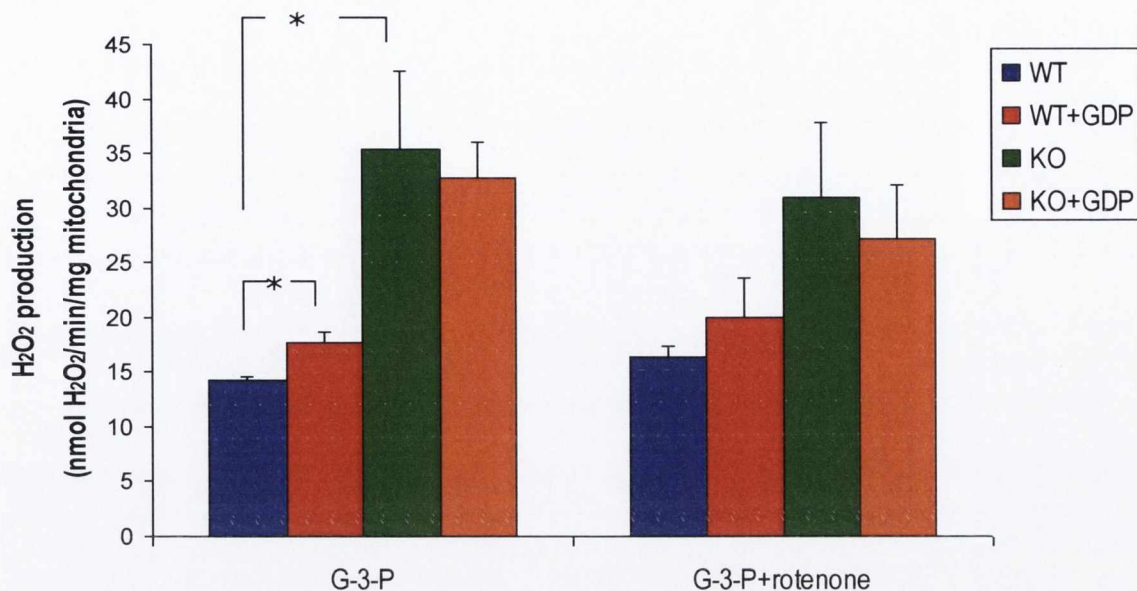


Figure 3.15 GDP sensitive H₂O₂ production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate is UCP 1 dependent.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate, 10 mM glycerol-3-phosphate + 1 µM rotenone, 10mM glycerol-3-phosphate + 1 mM GDP and 10 mM glycerol-3-phosphate + 1 µM rotenone + 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05).

Figure 3.16 compares state 4 non-phosphorylating O₂ consumption (nmol O₂/min/mg mitochondria) by brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock-out mice respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone. State 2 oxygen consumption was higher in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice, compared to brown adipose tissue mitochondria isolated from wild-type mice in the presence of glycerol-3-phosphate (WT=31.15±6.8; KO=56.22±9.5) and glycerol-3-phosphate plus rotenone (WT=29.45±3.5; KO=53.53±4.14, p=0.0113). GDP sensitive oxygen consumption was also higher in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice compared to brown adipose tissue mitochondria isolated from wild-type mice in the presence of glycerol-3-phosphate (WT=30.54±4.73; KO=56.25±11.2) and glycerol-3-phosphate plus rotenone (WT=30.56±3.47; KO=55.21±4.35, p=0.0114), as was state 4 uncoupled rates by mitochondria respiring on glycerol-3-phosphate (WT=29.94±4.98; KO=56.74±17.7) and glycerol-3-phosphate plus rotenone (WT=32.5±5.64; KO=66.45±4.71, p=0.0099).

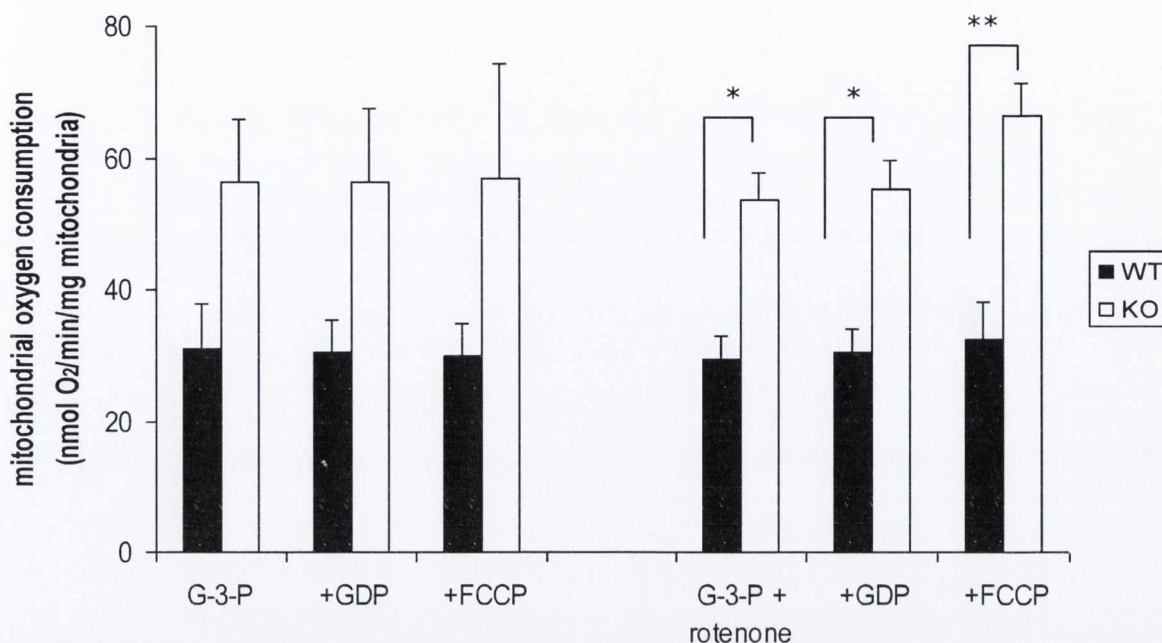


Figure 3.16 Glycerol-3-phosphate supported oxygen consumption by brown adipose tissue mitochondria isolated from UCP 1 knock-out mice is higher compared to oxygen consumption by brown adipose tissue mitochondria isolated from wild-type mice.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 1 µM rotenone, 0.1% de-fatted BSA and 10 mM glycerol-3-phosphate in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*= $p < 0.05$; **= $p < 0.01$).

Figure 3.17 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock out mice respiring on pyruvate plus malate and pyruvate plus malate plus GDP. H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock out mice was consistently higher compared to H_2O_2 production by brown adipose tissue mitochondria isolated from wild-type mice respiring on pyruvate plus malate, although not significantly higher (WT=1.57 $\pm$ 0.3; KO=1.92 $\pm$ 0.5). GDP consistently increased H_2O_2 production by brown adipose tissue mitochondria respiring on pyruvate plus malate, although not significantly (-GDP=1.57 $\pm$ 0.3; +GDP=1.8 $\pm$ 0.19). GDP did not significantly affect H_2O_2 production by brown adipose tissue mitochondria isolated from UCP 1 knock-out mice respiring on pyruvate plus malate(-GDP=1.92 $\pm$ 0.5; +GDP=1.5 $\pm$ 0.57).

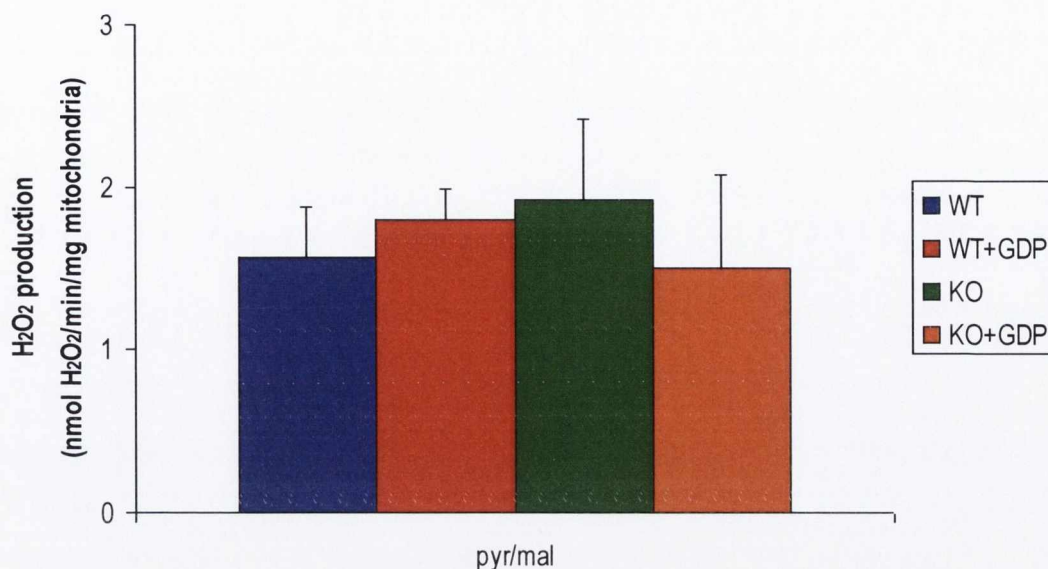


Figure 3.17 GDP sensitive H₂O₂ production by brown adipose tissue mitochondria respiring on pyruvate plus malate is UCP 1 dependent.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM pyruvate + 6 mM malate and 10 mM pyruvate + 6 mM malate + 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

Figure 3.18 compares state 4 non-phosphorylating O₂ consumption (nmol O₂/min/mg mitochondria) by brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock-out mice respiring on pyruvate plus malate. State 2 oxygen consumption was significantly higher in brown adipose tissue mitochondria isolated from wild-type mice, compared to brown adipose tissue mitochondria isolated from UCP 1 knock-out mice (WT=121.91±16.65; KO=20.163±1.72, p=0.0037). No significant differences in GDP sensitive oxygen consumption was observed between brown adipose tissue mitochondria isolated from wild-type mice and brown adipose tissue mitochondria isolated from UCP 1 knock-out mice (WT=21.117±5.34; KO=22.68±3.1). State 4 uncoupled oxygen consumption rates were significantly higher by brown adipose tissue mitochondria isolated from wild-type mice compared to brown adipose tissue mitochondria isolated from UCP 1 knock-out mice (WT=115.61±23.55; KO=35.61±0.041, p=0.0274). GDP significantly decreased oxygen consumption by brown adipose tissue mitochondria isolated from wild-type mice (-GDP=121.91±16.65; +GDP=21.117±5.34, p=0.0045), but did not significantly affect oxygen consumption by brown adipose tissue isolated from UCP 1 knock-out mice(-GDP=20.163±1.72; +GDP=22.68±3.1).

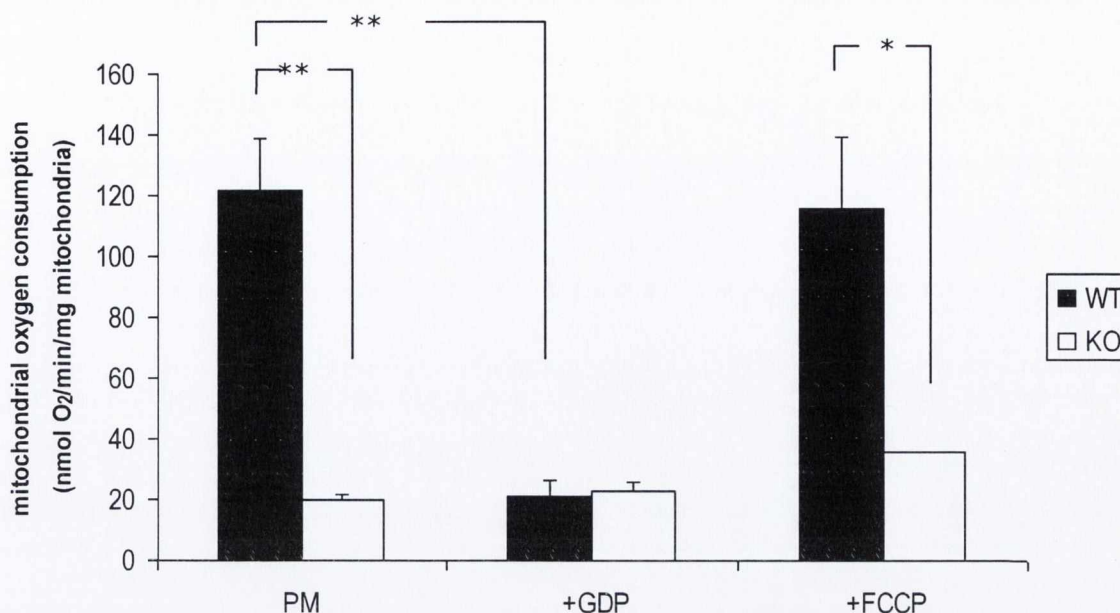


Figure 3.18 GDP inhibition of oxygen consumption by mitochondria respiring on pyruvate plus malate is UCP 1 dependent.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA and 10 mM pyruvate + 6 mM malate in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*= $p < 0.05$, **= $p < 0.01$).

Apparent differences in uncoupled electron transport activity between brown adipose tissue mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on the complex I substrate pyruvate/malate and respiring on glycerol-3 -phosphate suggest that differences in the activity or abundance of complex I and glycerol-3-phosphate dehydrogenase may exist in brown adipose tissue mitochondria from UCP 1 knock-out and wild-type mice. Uncoupled electron transport activity between brown adipose tissue mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on the complex II substrate succinate were equal.

These observations are further supported by comparing H_2O_2 production rates by mitochondria from wild-type mice respiring on succinate plus GDP and mitochondria from knock-out mice respiring on succinate. Under these conditions H_2O_2 production by reverse electron transport through complex I is active. H_2O_2 production rates should be equal under these conditions because UCP 1 is either inhibited or ablated. However H_2O_2 production rates by mitochondria from wild-type mice respiring on succinate plus GDP are greater than H_2O_2 production rates by mitochondria from UCP 1 knock-out mice respiring on succinate alone. This suggests that Complex I levels in brown adipose tissue mitochondria from UCP 1 knock-outs may be lower. In the presence of rotenone however H_2O_2 production rates by mitochondria from wild-type mice respiring on succinate plus GDP were equal to H_2O_2 production rates by mitochondria from UCP 1 knock-out mice suggesting that it was not differences in complex II activity or abundance that was affecting H_2O_2 production, but actually the activity of Complex I or its abundance.

Glycerol-3-phosphate supported H_2O_2 production rates in brown adipose tissue mitochondria from UCP 1 knock-out mice were significantly higher than in brown adipose tissue mitochondria from

wild-type mice. GDP addition to brown adipose tissue mitochondria from wild-type mice did not increase H_2O_2 production rates to equal H_2O_2 production rates in brown adipose tissue mitochondria from UCP 1 knock-out mice. This suggests that glycerol-3-phosphate activity or abundance is higher in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice compared to brown adipose tissue mitochondria from wild-type mice.

For the above mentioned reasons Complex I,II and glycerol-3-phosphate dehydrogenase activity rates were measured in brown adipose tissue mitochondria isolated from UCP 1 knock-out and wild-type mice.

Figure 3.19 compares complex I activity (nmol NADH/min/mg mitochondria) in brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock-out mice. Complex I activity in brown adipose tissue isolated from wild-type mice was significantly higher than complex I activity in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice (WT=8.05 $\pm$ 0.93; KO=2.86 $\pm$ 0.36, $p=0.0065$)

Figure 3.20 compares the expression of complex I subunit NDUF5 in isolated brown adipose tissue mitochondria isolated from UCP 1 knock-out and wild-type mice, as detected by SDS-PAGE and western blotting. The immunoblot illustrates that the expression of NDUF5 is lower in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice compared to brown adipose tissue mitochondria isolated from wild-type mice. The loading control, pyruvate dehydrogenase- α -E1, shows that equal amounts of BAT mitochondria were used in the blot.

Figure 3.21 compares complex II activity (nmol 2,6-dichloroindophenolate/min/mg mitochondria) in brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock-out mice. No significant differences in Complex II activity were observed (WT=7.41 $\pm$ 0.21; KO=7.3 $\pm$ 0.18).

Figure 3.22 compares glycerol-3-phosphate dehydrogenase activity (nmol 2,6-dichloroindophenolate/min/mg mitochondria) in brown adipose tissue mitochondria isolated from wild-type and UCP 1 knock-out mice. Glycerol-3-phosphate dehydrogenase activity is significantly higher in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice compared to brown adipose tissue mitochondria isolated from wild-type mice (WT=33.22 $\pm$ 1.24; KO=52.56 $\pm$ 2.29, $p=0.0017$).

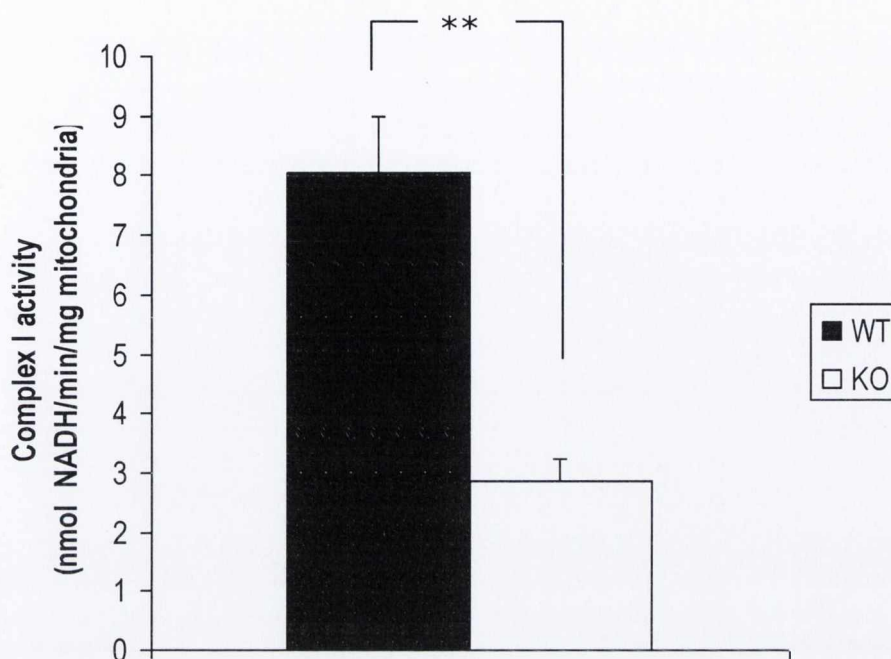


Figure 3.19 Complex I activity in brown adipose tissue mitochondria isolated from wild-type mice is significantly greater than complex I activity in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. The activity of Complex I was determined by monitoring the oxidation of NADH at 340nm. The assay medium consisted of 25 mM potassium phosphate, 5 mM MgCl_2 , 2.5 mg/ml bovine serum albumin and 2 mM KCN, pH7.2. Absorbance changes were monitored on a Shimadzu UV mini-1240 uv-vis spectrophotometer at 30°C. Baseline activity was established after the addition of 0.2 mM NADH, 65 μM decylubiquinone and 2.7 $\mu\text{g/ml}$ antimycin A. The reaction was initiated by the addition of mitochondria (75 $\mu\text{g/ml}$). The rate of oxidation of NADH was monitored by the decrease in absorbance at 340 nm. 2 $\mu\text{g/ml}$ rotenone was then added, and the rate of change in absorbance was measured. Complex I activity was determined by subtracting the rotenone insensitive activity from the total activity. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate (**=p<0.01)

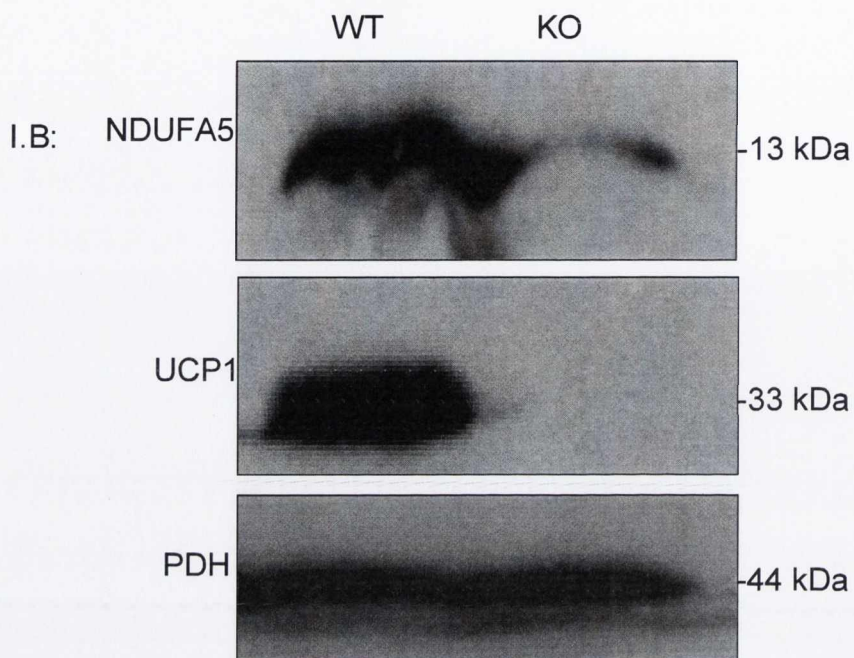


Figure 3.20 Complex I subunit NDUFA5 expression is lower in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. Mitochondria were subjected to SDS-PAGE as described in section 2.6 followed by western blotting as described in section 2.7 Using anti-UCP 1 (1/500, Sigma) and anti-PDH-E1-alpha (1/500, Mitosciences, cat#MSPO3) and anti-NDUFA5 for immunodetection as indicated.

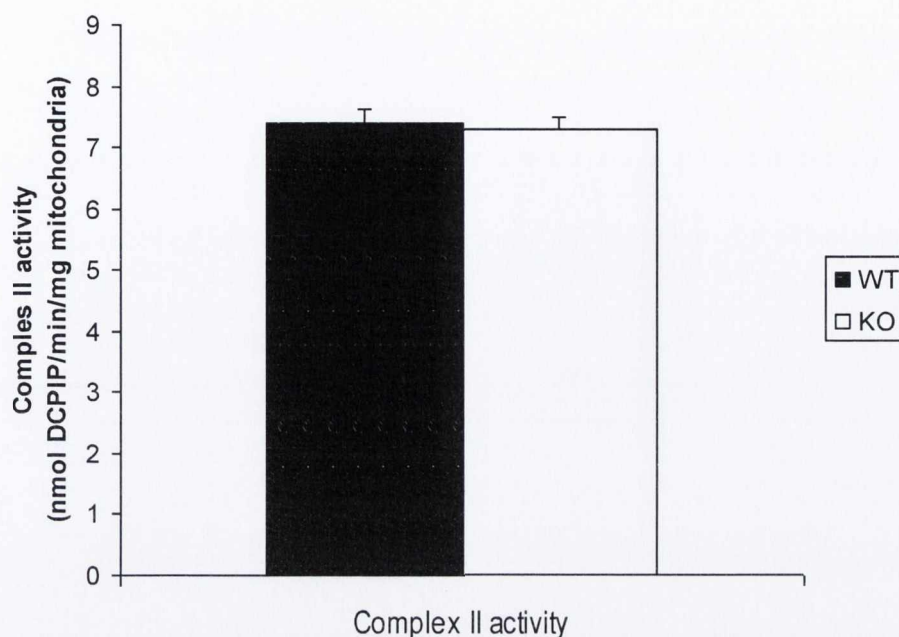


Figure 3.21 Equal complex II activity in brown adipose tissue mitochondria isolated from UCP 1 knock-out and wild-type mice

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. The activity of Complex II was determined by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. Absorbance changes were monitored on a Shimadzu UVmini-1240 uv-vis spectrophotometer at 30°C. Mitochondria (25 µg) were incubated at 30°C for 20 minutes in assay buffer containing 25 mM potassium phosphate, 5 mM MgCl₂, 2.5 mg/ml bovine serum albumin 20mM succinate and 0.2mM ATP, pH 7.2. The reaction was initiated by the addition of 2 µg/ml antimycin A, 2 µg/ml rotenone, 2mM KCN, 50 µM 2,6-dichloroindophenolate and 65 µM decylubiquinone. The rate of reduction of 2,6-dichloroindophenolate was monitored. 10 mM malonate was then added to inhibit the enzymatic activity and the rate of change in absorbance was measured. Complex II activity was determined by subtracting the malonate insensitive activity from the total activity. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

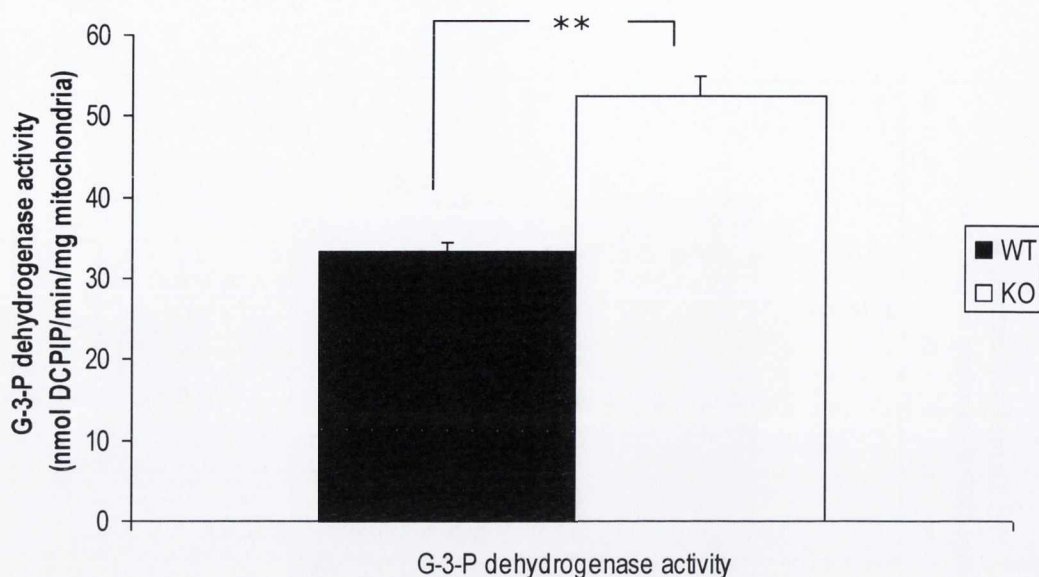


Figure 3.22 glycerol-3-phosphate dehydrogenase activity in brown adipose tissue mitochondria isolated from UCP 1 knock-out mice is greater than glycerol-3-phosphate dehydrogenase activity in brown adipose tissue mitochondria isolated from wild-type mice.

BAT mitochondria were isolated as described in section 2.3 from female wild-type and UCP 1 knock-out mice. The activity of glycerol-3-phosphate dehydrogenase was determined by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. Absorbance changes were monitored on a Shimadzu UVmini-1240 uv-vis spectrophotometer at 30°C. Mitochondria (25 µg) were incubated in assay buffer containing 25 mM potassium phosphate, 5 mM MgCl₂, 2.5 mg/ml bovine serum albumin and 0.2mM ATP 2 µg/ml antimycin A, 2 µg/ml rotenone, 2mM KCN, 50 µM 2,6-dichloroindophenolate and 65 µM decylubiquinone, pH 7.2. Baseline activity was monitored for 3 minutes. The reaction was initiated by the addition of 20mM glycerol-3-phosphate The rate of reduction of 2,6-dichloroindophenolate was monitored. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (**=p<0.01)

[3.3] Discussion

The effect of UCP 1 activity on ROS production due to reverse electron transport and forward electron transport through complex I was investigated using both complex I substrates (pyruvate plus malate) and FAD-dependent substrates. Succinate alone (without rotenone) was used as a substrate to facilitate any possible reverse electron transport through complex I and forward electron transport through the remainder of the electron transport chain or succinate plus rotenone was used to facilitate forward electron transport only. Glycerol-3-phosphate alone was also used as a substrate, without rotenone present to facilitate any possible reverse and forward electron transport, and plus rotenone to facilitate forward electron transport only.

[3.3.1] GDP sensitive H_2O_2 production by BAT mitochondria respiring on succinate $\pm$ rotenone.

Figures 3.1 (raw data) and 3.2 (collated data) illustrates H_2O_2 production by non-phosphorylating BAT mitochondria isolated from Wistar rats respiring on succinate alone (forward and reverse electron transport) and succinate plus rotenone (forward electron transport only). Addition of GDP significantly increased H_2O_2 production regardless of whether rotenone was present or not. However the increase in H_2O_2 production in the absence of rotenone was 2-fold greater than when rotenone was present. This data suggests that UCP 1 is capable of attenuating H_2O_2 production by both forward and reverse electron transport and also demonstrates that isolated BAT mitochondria respiring on succinate have a large capacity to generate H_2O_2 by reverse electron transport when UCP 1 is inhibited by GDP. The presence of rotenone however does not affect H_2O_2 production in BAT mitochondria respiring on succinate

when UCP 1 is active (i.e. in the absence of GDP) suggesting that succinate supported H_2O_2 production by reverse electron transport does not occur when UCP 1 is active.

The data presented in these figures also show that the increase in H_2O_2 production upon GDP addition to BAT mitochondria respiring on succinate alone and succinate plus rotenone can be abolished by addition of the protonophore FCCP, while FCCP addition has no effect on H_2O_2 production by BAT mitochondria respiring on succinate alone and succinate plus rotenone in the absence of GDP. This indicates that the increase in H_2O_2 production by BAT mitochondria upon GDP addition correlates with a coupled state of the mitochondria, while the low H_2O_2 production in the absence of GDP correlates with an uncoupled state.

Non-phosphorylating oxygen consumption rates were determined in parallel with the H_2O_2 production measurements presented in figure 3.1. Figure 3.3 shows that oxygen consumption by BAT mitochondria respiring on succinate alone is increased in the presence of rotenone, indicating increased forward electron flow to oxygen reduction in cytochrome oxidase. Addition of GDP, which inhibits UCP 1, should decrease oxygen consumption, due to coupling of the mitochondria. This is evident with BAT mitochondria respiring on succinate in the presence of rotenone, where a significant decrease in oxygen consumption was observed. However this was not observed by BAT mitochondria respiring on succinate alone in the absence of rotenone. In fact a significant increase in oxygen consumption was observed upon GDP addition.

If GDP is inhibiting UCP 1, a decrease in oxygen consumption would be expected, but as observed in figure 3.1, a large increase in H_2O_2 production also occurs in the presence of GDP. This large increase in H_2O_2 production would also register as oxygen consumption in an oxygraph, due to the initial conversion of O_2 to O_2^- , a species which is not detected as O_2 by the oxygraph sensor

and thus registers as a decrease in O_2 concentration. The decrease in O_2 consumption due to the decreased forward transport of electrons through the respiratory chain may be masked by the large increase in O_2 consumption due to superoxide formation.

The addition of the protonophore FCCP increases oxygen consumption by BAT mitochondria respiring on succinate both in the absence and the presence of rotenone, again indicating that the decrease in H_2O_2 production observed upon FCCP addition correlates with an uncoupled state and forward flow of electrons to cytochrome oxidase.

Our oxygen consumption measurements help us to verify that the observed GDP-sensitive H_2O_2 production correlates with coupling of the mitochondria, or uncoupling in the case of the decreased H_2O_2 production observed upon FCCP addition. However the effect of GDP on BAT mitochondria respiring on succinate alone in the absence of rotenone is anomalous in that inhibition of GDP should result in decreased oxygen consumption by the respiratory chain. This does not appear to be the case, and an increase in oxygen consumption due to increased superoxide formation is a credible explanation of such an anomaly.

To further clarify the effect of GDP on BAT mitochondria respiring on succinate in the absence of rotenone, the mitochondrial membrane potential was monitored in parallel to H_2O_2 production and oxygen consumption measurements.

Figure 3.4 illustrates the generation of membrane potential by BAT mitochondria respiring on succinate alone (red) and succinate plus rotenone (blue) as monitored by the fluorescent dye safranin, which accumulates and quenches in polarized mitochondria. It can be observed that less membrane potential is generated by BAT mitochondria respiring on succinate in the absence of rotenone compared to BAT mitochondria respiring on succinate in the presence of rotenone. The diminished membrane potential

correlates with less electrons traveling forward along the respiratory chain, most probably due to reverse flow of electrons through complex I, and thus reduced proton pumping across the membrane occurs, which results in the generation of less membrane potential.

It can also be observed that upon GDP addition, the membrane potential in BAT mitochondria respiring on succinate alone and succinate plus rotenone increases, which re-enforces our observation of increased H_2O_2 production by mitochondria with increased membrane potential. The increase in membrane potential upon GDP addition also illustrates that the BAT mitochondria are not completely coupled when respiring on substrate in the absence of the UCP 1 ligand GDP, and again re-enforces our observation of low membrane potential with low H_2O_2 production. It is also shown that FCCP addition dissipates the membrane potential generated upon GDP addition, which re-enforces the observation of decreased H_2O_2 production in mitochondria with low membrane potential.

We can conclude from our H_2O_2 measurements, oxygen consumption measurements and membrane potential profiles that UCP 1 is capable of attenuating H_2O_2 production, due to reverse and forward electron transport in isolated BAT mitochondria from rats respiring on succinate, through dissipation of the mitochondrial membrane potential.

[3.3.2] GDP sensitive H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate $\pm$ rotenone.

Figures 3.5 (raw data) and 3.6 (collated data) illustrates H_2O_2 production by non-phosphorylating BAT mitochondria isolated from Wistar rats respiring on glycerol-3-phosphate alone (forward and reverse electron transport) and glycerol-3-phosphate plus rotenone (forward electron transport).

Similar to BAT mitochondria respiring on succinate, addition of GDP significantly increased H_2O_2 production. Unlike BAT mitochondria respiring on succinate, rotenone did not significantly affect H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate. This data suggests that UCP 1 is capable of attenuating H_2O_2 production by forward electron transport by isolated BAT mitochondria respiring on glycerol-3-phosphate and to a lesser extent H_2O_2 production due to reverse electron transport. It also suggests that reverse electron transport does not occur in BAT mitochondria respiring on glycerol-3-phosphate to the same extent as it does in BAT mitochondria respiring on succinate.

The data presented here illustrates that the increase in H_2O_2 production observed upon GDP addition is not significantly decreased upon FCCP addition. H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone in the absence of GDP is not decreased by FCCP, in fact a consistent increase in H_2O_2 production was observed.

Non-phosphorylating oxygen consumption rates were determined in parallel with the H_2O_2 production measurements presented in figure 3.5. Figure 3.7 illustrates non-phosphorylating oxygen consumption by isolated BAT mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone.

It can be seen from this data that rotenone has no significant effect on non-phosphorylating oxygen consumption by isolated BAT mitochondria respiring on glycerol-3-phosphate. This suggests that there is a lack of a detectable level of reverse electron transport associated with BAT mitochondria respiring on glycerol-3-phosphate, which correlates with our H_2O_2 production data in figure 3.5, which shows that rotenone does not significantly affect H_2O_2 production by isolated BAT mitochondria respiring on glycerol-3-phosphate.

GDP addition significantly decreases oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate, and this decrease is rotenone independent. This decrease in oxygen consumption is due to inhibition of UCP 1 and thus coupling of the mitochondria and correlates with an increase in H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate (plus or minus rotenone) under the same conditions.

Addition of the protonophore FCCP increases oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate both in the absence and the presence of rotenone, indicating that the decrease in H_2O_2 production observed upon FCCP addition correlates with an uncoupled state.

The oxygen consumption data presented in figure 3.7 verifies that the observed increase in H_2O_2 production upon the addition of GDP correlates with coupling of the mitochondria, and the decrease in H_2O_2 production upon FCCP addition correlates with uncoupling of the mitochondria.

To further verify that the observed effect of GDP is due to coupling of the mitochondria and the effect of FCCP is due to uncoupling of the mitochondria the membrane potential of BAT mitochondria respiring on glycerol-3-phosphate plus or minus rotenone was monitored in parallel to H_2O_2 production and oxygen consumption measurements.

Figure 3.8 illustrates the generation of membrane potential by BAT mitochondria respiring on glycerol-3-phosphate alone (red) and glycerol-3-phosphate plus rotenone (blue) as monitored by the fluorescent dye safranin. It can be observed that the presence of rotenone does not affect membrane potential generation in BAT mitochondria respiring on glycerol-3-phosphate, which correlates with our H_2O_2 production and oxygen consumption data, where rotenone did not affect either measurement. GDP addition increases membrane potential generation, which re-enforces our observation

of increased H_2O_2 production in BAT mitochondria with increased membrane potential while respiring on glycerol-3-phosphate (plus or minus rotenone).

We can conclude from our H_2O_2 measurements, oxygen consumption measurements and membrane potential profiles that UCP 1 is capable of attenuating H_2O_2 production due to forward electron transport, and to a lesser extent reverse electron transport, in isolated rat BAT mitochondria respiring on glycerol-3-phosphate, through dissipation of the mitochondrial membrane potential. The lack of a detectable level of reverse electron transport associated with BAT mitochondria respiring on glycerol-3-phosphate and the negligible effect of FCCP agrees with previous reports by Mracek et al., (2009) which showed that membrane potential dependent glycerol-3-phosphate dependent ROS production was negligible.

[3.3.3] GDP sensitive H_2O_2 production by BAT mitochondria respiring on pyruvate plus malate.

The H_2O_2 production by isolated BAT mitochondria in the presence of pyruvate plus malate, providers of complex I substrate was measured to determine whether UCP 1 can play a role in the attenuation of H_2O_2 production due to forward electron transport from complex I.

Figures 3.9 and 3.10 illustrate that H_2O_2 production by isolated BAT mitochondria respiring on pyruvate plus malate is insensitive to GDP inhibition of UCP 1. In comparison to H_2O_2 production by isolated BAT mitochondria respiring on succinate or glycerol-3-phosphate, H_2O_2 production by isolated BAT mitochondria respiring on pyruvate plus malate is relatively low. It can also be

seen that addition of the protonophore FCCP does not significantly affect H_2O_2 production by BAT mitochondria respiring on pyruvate plus malate, but significantly decreases H_2O_2 production by isolated BAT mitochondria respiring on pyruvate plus malate plus GDP.

Non-phosphorylating oxygen consumption rates were determined in parallel with the H_2O_2 production measurements presented in figure 3.9. Figure 3.11 illustrates non-phosphorylating oxygen consumption by isolated BAT mitochondria respiring on pyruvate plus malate.

GDP addition significantly decreases state 4 non-phosphorylating oxygen consumption by isolated BAT mitochondria respiring on pyruvate plus malate, through coupling of the mitochondria, however this does not correlate with a significant increase in H_2O_2 production by BAT mitochondria respiring on pyruvate plus malate.

Addition of the protonophore FCCP increased GDP sensitive oxygen consumption which correlates with a significant decrease in H_2O_2 production upon the addition of FCCP to isolated BAT mitochondria respiring on pyruvate plus malate plus GDP.

The membrane potential of BAT mitochondria respiring on pyruvate plus malate was also monitored in parallel to H_2O_2 production and oxygen consumption measurements. Figure 3.12 illustrates that membrane potential generated upon substrate oxidation is increased in the presence of GDP and dissipated by the addition of FCCP.

From a comparison of our H_2O_2 production and O_2 consumption measurements along with the examination of the membrane potential profile of BAT mitochondria respiring on pyruvate plus malate, it can be seen that GDP addition inhibits UCP 1, which increases the membrane potential and FCCP dissipates this membrane potential while increasing oxygen consumption. However unlike our observations of a correlation between increased

membrane potential with increased H_2O_2 production by BAT mitochondria respiring on succinate or glycerol-3-phosphate, no such correlation can be deduced from the data presented here regarding BAT mitochondria respiring on pyruvate plus malate. H_2O_2 production by BAT mitochondria respiring on pyruvate plus malate appears to be insensitive to membrane potential.

The level of H_2O_2 production measured here is extremely low compared to the H_2O_2 production by BAT mitochondria respiring on succinate or glycerol-3-phosphate. This agrees with previous reports which have shown H_2O_2 production in the presence of Complex I substrates to be almost negligible (Hansford *et al.* 1997; Liu *et al.* 2002; St-Pierre *et al.* 2002). Due to this low generation of H_2O_2 production by isolated BAT mitochondria respiring on complex I substrate, any effect of uncoupling by UCP 1 may be negligible.

[3.3.4]Determination of UCP 1 dependent H_2O_2 production by comparing H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out and wild-type mice.

From our study using isolated rat mitochondria, it can be observed that H_2O_2 production by BAT mitochondria is maximal in the presence of GDP, with rotenone capable of reducing this H_2O_2 production to varying degrees, depending on which substrates are used. This indicates that H_2O_2 production due to reverse and forward electron transport can be attenuated by UCP 1 in rat BAT mitochondria.

To corroborate our data so far presented in this chapter using isolated BAT mitochondria from rats, H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice was compared to H_2O_2 production by BAT mitochondria isolated from wild-type mice.

Like our study using BAT mitochondria isolated from rats, ROS production due to reverse and forward electron transport was

examined using pyruvate plus malate, succinate plus and minus rotenone and glycerol-3-phosphate plus and minus rotenone as substrates. Any UCP 1-sensitive ROS production was investigated by comparing the ROS production in BAT mitochondria isolated from UCP 1 knock-out and wild-type mice. If the GDP-sensitive ROS production observed in BAT mitochondria isolated from rats is due to inhibition of UCP 1, then it would be expected that BAT mitochondria isolated from UCP 1 knock-out mice would have greater ROS production compared to BAT mitochondria isolated from wild-type mice. Furthermore in order to determine that the GDP-sensitive ROS production observed in BAT mitochondria isolated from rats was in fact due to UCP 1 inhibition, GDP-sensitive ROS production was investigated in BAT mitochondria isolated from UCP 1 knock-out and wild-type mice.

Figure 3.13 illustrates H_2O_2 production by non-phosphorylating BAT mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate alone and succinate plus rotenone. The data presented here illustrates that H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice is significantly greater than H_2O_2 production by BAT mitochondria isolated from wild-type mice respiring on succinate alone and succinate in the presence of rotenone. This confirms that ablation of UCP 1 significantly increases H_2O_2 production both by forward electron transport and reverse electron transport and thus supports a role for UCP 1 in the attenuation of H_2O_2 production by BAT mitochondria respiring on succinate.

GDP significantly increases H_2O_2 production by BAT mitochondria isolated from wild-type mice, but not from UCP 1 knock-out mice. This confirms that the GDP sensitive H_2O_2 production observed in BAT mitochondria isolated from wild-type mice and rats respiring on succinate alone and succinate plus rotenone is UCP 1 sensitive H_2O_2 production.

Non-phosphorylating oxygen consumption rates were determined in parallel with H_2O_2 production measurements presented in figure 3.13. Figure 3.14 shows that no difference in state 4 oxygen consumption is apparent between BAT mitochondria isolated from UCP1 knock-out and wild-type mice respiring on succinate in the absence of rotenone. However state 4 oxygen consumption by BAT mitochondria isolated from wild-type mice is significantly greater than state 4 oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out mice respiring on succinate in the presence of rotenone.

GDP addition decreases oxygen consumption by BAT mitochondria isolated from wild-type mice respiring on succinate in the presence of rotenone but not in the absence of rotenone. This was also observed in BAT mitochondria isolated from Wistar rats and reasons were previously given which might explain this. GDP addition did not affect oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out mice respiring on succinate in the absence or the presence of rotenone.

Significantly higher state 4 uncoupled oxygen consumption was observed by BAT mitochondria respiring on succinate alone compared to BAT mitochondria isolated from wild-type mice respiring on succinate alone. No significant difference in state 4 uncoupled oxygen consumption was observed between BAT mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate in the presence of rotenone.

From the data presented in figure 3.14 it can be seen that the presence of rotenone increases oxygen consumption by BAT mitochondria isolated from wild-type mice, which is consistent with more forward electron transport occurring due to inhibition of reverse electron transport by rotenone. However the presence of rotenone does not affect oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out mice, suggesting that reverse

electron transport does not occur to the same extent in BAT mitochondria isolated from UCP 1 knock-out mice as it does in BAT mitochondria isolated from rats and wild-type mice. Later in this chapter we discuss the outcome of our investigation into components of the respiratory chain which contribute to reverse electron transport, which will explain this apparent discrepancy between BAT mitochondria isolated from UCP 1 knock-out and wild-type mice.

Figure 3.15 illustrates H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glycerol-3-phosphate in the presence and absence of rotenone. The data presented here illustrates that H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice is higher than H_2O_2 production by BAT mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate alone and glycerol-3-phosphate in the presence of rotenone. This confirms that ablation of UCP1 increases H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate.

GDP increases H_2O_2 production by BAT mitochondria isolated from wild-type mice, but not from UCP 1 knock-out mice. This confirms that the GDP sensitive H_2O_2 production observed in BAT mitochondria isolated from rats respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone is UCP 1 sensitive H_2O_2 production.

It would be expected that H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice would be equal to H_2O_2 production by BAT mitochondria isolated from wild-type mice in the presence of GDP. However H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice is greater than H_2O_2 production by BAT mitochondria isolated from wild-type mice in the presence of GDP. This suggests that the absence of UCP 1 in

BAT mitochondria isolated from UCP 1 knock-out mice respiring on glycerol-3-phosphate is not the only contributing factor to the increased H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice.

Non-phosphorylating oxygen consumption rates by mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone were determined in parallel with H_2O_2 production measurements presented in figure 3.15. Figure 3.16 shows non-phosphorylating oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out and wild-type mice. Non-phosphorylating, GDP sensitive and uncoupled oxygen consumption rates were significantly greater by BAT mitochondria isolated from UCP 1 knock-out mice compared to BAT mitochondria isolated from wild-type mice.

This suggests that glycerol-3-phosphate respiration is higher in BAT mitochondria in UCP 1 knock-out mice either through increased activity of glycerol-3-phosphate dehydrogenase, and/or through increased expression of the dehydrogenase. Such an occurrence would also help explain the large difference in H_2O_2 production by BAT mitochondria from UCP 1 knock-out mice respiring on glycerol-3-phosphate and BAT mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate in the presence of GDP. Later in this chapter an investigation into glycerol-3-phosphate activity in BAT mitochondria isolated from UCP 1 knock-out and wild-type mice will be discussed.

The H_2O_2 production by isolated BAT mitochondria isolated from UCP 1 knock-out and wild-type mice in the presence of complex I substrates pyruvate plus malate was also measured and the data is presented in figure 3.17. No difference in H_2O_2 production was observed in H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on

pyruvate plus malate. GDP did not affect H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out or wild-type mice. The H_2O_2 data presented here confirms our observation using rat mitochondria that UCP 1 does not significantly affect H_2O_2 production by BAT mitochondria respiring on pyruvate plus malate.

Figure 3.18 illustrates non-phosphorylating oxygen consumption rates by BAT mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on pyruvate plus malate. State 4 oxygen consumption by BAT mitochondria isolated from wild-type mice is significantly higher than state 4 oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out mice. GDP significantly decreases oxygen consumption by BAT mitochondria isolated from wild-type mice but not in BAT mitochondria isolated from UCP 1 knock-out mice.

Unusually, state 4 uncoupled oxygen consumption is significantly higher by BAT mitochondria isolated from wild-type mice compared to BAT mitochondria isolated from UCP 1 knock-out mice. Ablation of UCP 1 should not affect the uncoupled rate. This suggests that complex I supported respiration is significantly lower in BAT mitochondria isolated from UCP1 knock-out mice compared to BAT mitochondria isolated from wild-type mice.

This is supported by examination of succinate (minus rotenone) supported oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out and wild-type mice. Succinate (minus rotenone) supported state 4 uncoupled oxygen consumption is significantly greater in BAT mitochondria isolated from UCP 1 knock-out mice compared to BAT mitochondria isolated from wild-type mice in the absence of rotenone, while no difference is observed in state 4 uncoupled oxygen consumption in the presence of rotenone. This indicates that there is less capacity for reverse electron transport in BAT mitochondria isolated from UCP 1 knock-out mice. A difference in complex II activity is unlikely because in

the presence of rotenone no difference in state 4 uncoupled oxygen consumption is observed.

[3.3.5] Differential expression of complex I and glycerol-3-phosphate dehydrogenase in BAT mitochondria isolated from UCP 1 knock-out and wild-type mice.

The use of UCP 1 knock out mice has allowed us to verify that the GDP sensitive H_2O_2 production observed in BAT mitochondria isolated from rats is due to inhibition of UCP 1, and thus can be termed UCP 1 sensitive H_2O_2 production.

The presence of UCP 1 sensitive H_2O_2 production in BAT mitochondria is further supported by the observation that H_2O_2 production in BAT mitochondria isolated from UCP 1 knock-out mice is higher than H_2O_2 production by BAT mitochondria isolated from wild-type mice respiring on succinate and glycerol-3-phosphate.

The H_2O_2 production and oxygen consumption measurement data just presented suggest that differences in complex I and glycerol-3-phosphate dehydrogenase expression/activity exist between BAT mitochondria isolated from UCP 1 knock-out and wild-type mice. To resolve this complex I, complex II and glycerol-3-phosphate dehydrogenase activity was measured directly in BAT mitochondria from UCP 1 and wild-type mice.

Figure 3.19 shows that complex I activity in BAT mitochondria isolated from UCP 1 knock-out mice is significantly lower compared to complex I activity in BAT mitochondria isolated from wild-type mice. This helps explain the significantly lower complex I supported state 4 uncoupled oxygen consumption by BAT mitochondria isolated from UCP 1 knock-out mice compared to BAT mitochondria isolated from wild-type mice.

It also explains why rotenone does not appear to affect

succinate supported oxygen consumption in BAT mitochondria isolated from UCP 1 knock-out mice and why state 4 uncoupled oxygen consumption is significantly higher in BAT mitochondria isolated from UCP 1 knock-out mice respiring on succinate in the absence of rotenone. Reduced complex I activity/expression would reduce the capacity for reverse electron transport and thus explain the higher oxygen consumption due to forward electron transport in BAT mitochondria isolated from UCP 1 knock-out mice compared to BAT mitochondria isolated from wild-type mice in the absence of rotenone.

Figure 3.20 illustrates an immunoblot showing that there is less complex I subunit NDUFA5 protein expressed in BAT mitochondria isolated from UCP 1 knock out mice compared to BAT mitochondria isolated from wild-type mice.

We observed that that H_2O_2 production by BAT mitochondria isolated from rats is maximal in the presence of GDP, with a large proportion of this GDP sensitive H_2O_2 production inhibited by rotenone. This indicates that H_2O_2 production due to reverse electron transport can be attenuated by UCP 1. We would therefore expect a large proportion of H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice to be produced due to reverse electron transport, if in fact UCP 1 is attenuating H_2O_2 production due to reverse electron transport.

This assumption was correct and we saw that H_2O_2 production by BAT mitochondria isolated from UCP 1 knock out mice is significantly higher than H_2O_2 production by BAT mitochondria isolated from wild-type mice respiring on succinate. However, upon addition of GDP to wild-type mitochondria H_2O_2 production is greater than H_2O_2 production by BAT mitochondria isolated from knock-out mice respiring on succinate alone (Fig 3.13). It would be expected that there would be no difference in such a comparison.

In the presence of rotenone H_2O_2 production by BAT

mitochondria isolated from wild-type mice respiring on succinate plus GDP is equal to H_2O_2 production by BAT mitochondria isolated from UCP 1 knock-out mice respiring on succinate alone (Fig 3.13).

This indicates that the expected production of H_2O_2 by reverse electron transport, which would be expected by BAT mitochondria isolated from UCP 1 knock-out mice, is prevented by a decrease in the amount of complex I present. Such a compensation would prevent an increase in oxidative damage to BAT mitochondria isolated from UCP 1 knock-out mice.

It has been questioned whether UCP 1 could play a physiological role in the defense against oxidative damage due to the observation that BAT mitochondria isolated from UCP 1 knock-out mice do not display an increase in oxidative damage compared to BAT mitochondria isolated from wild-type mice (Shabalina et al., 2006). This report also showed that there was no increase in the activity of antioxidant enzymes such as aconitase which may be compensating for an increase in ROS production.

Our observation of a decrease in the expression of Complex I in BAT mitochondria isolated from UCP 1 knock-out mice, and therefore a reduction in reverse electron mediated ROS production, may explain the lack of oxidative damage to BAT mitochondria isolated from UCP 1 knock-out mice.

A decrease in Complex I activity has previously been reported in superoxide dismutase 2 (SOD2) mutant mice (Melov *et al.* 1999). Here a 59% decrease in complex I activity was reported. It was concluded that complex I activity was decreased due to ROS mediated inactivation of the iron-sulphur clusters of complex I, of which there are six. Iron-sulphur cluster containing enzymes have been shown to be susceptible to ROS induced inactivation (Rotig *et al.* 1997). Only the activity of complex I in the SOD2 mutant mice was measured with no quantification of protein levels presented. We have shown however that protein expression levels of Complex I are

decreased along with activity levels.

ROS inactivation of Complex I in BAT of UCP 1 knock-outs may occur during the first few days following birth when BAT is most active, and hence more ROS are produced by BAT due to ablation of UCP 1. Our immunoblot analysis also shows that expression of Complex I subunit NDUFA5 is decreased in knock-out mice (Fig 3.20). Whether this is a result of developmental differences at the embryo stage or if long term exposure of Complex I to ROS results in a decrease in the expression of subunits of this complex cannot be determined from our results. A time course analysis of complex I activity and expression during development of neonates would allow clarification of this.

Very recently another report involving a role for UCP 1 in the attenuation of ROS has been published (Oelkrug *et al.* 2010) The effects of GDP on H₂O₂ production rates reported by this group confirm our observations. However the discrepancies in complex I and mGPDH activities between UCP 1 knock-out and wild-type mice were not explored in this report.

Figure 3.21 shows that there is no difference in complex II activity in BAT mitochondria isolated from UCP 1 knock-out and wild-type mice. This rules out any contribution of complex II to the observed differences in succinate supported oxygen consumption by BAT mitochondria in the absence of rotenone.

Figure 3.22 shows that glycerol-3-phosphate dehydrogenase activity in BAT mitochondria from UCP 1 knock-out mice is significantly higher compared to glycerol-3-phosphate dehydrogenase activity in BAT mitochondria isolated from wild-type mice. This explains the higher oxygen consumption and H₂O₂ production by BAT mitochondria isolated from UCP 1 knock-out mice.

A role for the higher levels of expression of mitochondrial

glycerol-3-phosphate dehydrogenase (mGPDH) in BAT mitochondria of UCP 1 knock-out mice compared to BAT mitochondria of wild-type mice is not readily assigned from our data or from the literature.

However a model involving activation of uncoupling proteins directly by superoxide production, thereby preventing further superoxide production has been proposed (Echtay *et al.* 2003; Echtay *et al.* 2002; Krauss *et al.* 2003). mGPDH is a major site of superoxide production in mitochondria (Mracek *et al.*, 2009; Drahota *et al.*, 2002; Miwa and Brand, 2005; Miwa *et al.*, 2003). From our results it is also clear that a significant proportion of ROS produced due to glycerol-3-phosphate supported respiration is insensitive to uncoupling (Fig 3.5). Under the proposed model of superoxide activation of UCPs, this membrane potential insensitive superoxide production could be an activator of UCP 1, thereby preventing further superoxide production by the respiratory chain.

However this model has been disputed. Shabalina *et al.*,(2006) disputed the physiological relevance of attenuation of ROS by UCP 1. In this chapter we have provided evidence which strongly suggests that a lack of oxidative damage in BAT mitochondria from UCP 1 knock-outs is due to the prevention of reverse electron transport mediated ROS production by decreased Complex I activity and expression, and not as suggested by Shablina *et al.*,(2006) due to a lack of attenuation by UCP 1.

Other reports have disputed the effect of superoxide on the activity of uncoupling proteins (Couplan *et al.* 2002; Silva *et al.* 2005). This area needs further work to clarify the reported discrepancies.

Conclusions:

Inhibition or ablation of UCP 1 increases ROS production by isolated BAT mitochondria. Therefore we can conclude that UCP 1 in isolated BAT mitochondria is capable of attenuating ROS production by mitochondria respiring on succinate and glycerol-3-phosphate. UCP 1 does not play a role in the attenuation of ROS produced by complex I substrates.

Low ROS production by BAT mitochondria occurs when membrane potential is low, while high ROS production occurs when membrane potential is high. This suggests that attenuation of ROS production by UCP 1 occurs through dissipation of membrane potential and thus increased transport of electrons through the respiratory chain, which shortens the life of species capable of forming superoxide with oxygen.

A significant proportion of ROS produced by succinate supported BAT mitochondria is due to reverse electron transport, but only when UCP 1 is inhibited. When UCP 1 is active it is capable of preventing ROS production due to reverse electron transport.

BAT mitochondria isolated from UCP 1 knock-out mice do not produce the expected levels of ROS as extrapolation from the results in rats and wild-type mice would suggest. Further investigation has shown that reduced expression levels of Complex I protein and thus the activity of the complex, prevent the expected ROS production due to reverse electron transport.

BAT mitochondria isolated from UCP 1 knock-outs have higher mGPDH activity compared to BAT mitochondria isolated from wild-type mice, but the physiological significance of this difference is not clear at present. Possible reasons have been discussed.

Chapter 4 UCP 1 dependent ROS production in cold-acclimated BAT mitochondria

[4.1] Introduction

During cold acclimation the thermogenic activity of BAT rises, as animals require heat production to maintain their body temperature (Nicholls and Locke, 1984). This adaptation occurs as a result of a sympathetic and thyroid hormone innervation of BAT resulting in an increase in mitochondrial content, an increase in the expression of UCP1 in BAT mitochondria (Nicholls and Locke, 1984), an increase in the number of mature brown adipocytes in the tissue as well as an increase in mitochondrial enzymes which contribute to the enhanced oxidative capacity of BAT (Bukowiecki et al., 1982).

In response to cold exposure noradrenaline is released (Ricquier *et al.* 1986) which activates thermogenesis in BAT. The noradrenaline signal in BAT is mediated via the β_3 , α_1 and α_2 adrenoreceptors (Cannon and Nedergaard, 2004). Signaling through the β_3 adrenoreceptor is probably the best characterized of the pathways leading to the activation of UCP 1 in BAT. The current evidence suggests that signaling through the β_3 receptor leads to the release of free fatty acids, which are capable of "activating" UCP 1 (Martinez-Botas *et al.* 2000).

Noradrenaline can also activate α_1 receptors on brown adipocytes which leads to a rapid increase in the intracellular Ca^{2+} levels through release from intracellular stores and plasma membrane fluxes (Koivisto *et al.* 2000; Lee *et al.* 1993). This response is one of the immediate responses of sympathetic stimulation by noradrenaline. It has been reported that the increase

in intracellular Ca^{2+} levels is involved in the thermogenic response (Zhao *et al.* 1997). Thus noradrenaline can stimulate thermogenesis via the α_1 adrenergic receptors as well as via the β adrenergic receptors.

An increase in mitochondrial Ca^{2+} levels can have a wide range of downstream consequences, one of which is the full activation of intramitochondrial substrate dehydrogenases, which require Ca^{2+} for full activation (McCormack *et al.* 1990).

One such dehydrogenase which is highly expressed in brown adipose tissue is the mitochondrial glycerophosphate dehydrogenase (mGPDH). mGPDH possesses a calmodulin-like Ca^{2+} binding site (Brown *et al.* 1994) which increases the sensitivity of the enzyme for substrates by lowering the K_m for glycerol phosphate (Idahl and Lembert 1995; Rutter *et al.* 1992). It has been shown that H_2O_2 production is significantly increased in the presence of increasing amounts of Ca^{2+} , as is membrane potential and oxygen consumption in isolated brain mitochondria (Tretter *et al.* 2007b).

In this chapter we set out to investigate whether calcium affects ROS production in BAT mitochondria respiring on glycerol-3-phosphate. In vivo calcium activation of mGPDH could occur during initial adrenergic stimulation of brown adipocytes through the increase in intracellular Ca^{2+} levels which occurs through adrenergic stimulation via the α_1 adrenergic receptor (Koivisto *et al.*, 2000).

The effect of increasing calcium concentrations on mGPDH activity, H_2O_2 production and oxygen consumption in BAT mitochondria respiring on glycerol-3-phosphate are unknown. Therefore in this chapter we investigated whether a) Ca^{2+} increases the activity of mGPDH in BAT mitochondria b) Ca^{2+} increases H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate c) UCP 1 is capable of attenuating any putative increase in H_2O_2 production in the presence of Ca^{2+} and d) oxygen consumption by

BAT mitochondria respiring on glycerol-3-phosphate is affected by Ca^{2+} activation of mGPDH.

Furthermore we investigated the ability of UCP 1 to attenuate ROS production under conditions where BAT mitochondria have increased oxidative capacity, i.e. post 4-weeks cold acclimation). Increased oxidative capacity could increase the capacity for H_2O_2 production. We have shown in chapter 3 that UCP 1 can attenuate H_2O_2 production, and in particular H_2O_2 production due to reverse electron transport.

In this chapter we investigated whether UCP 1 is capable of attenuating any putative increase in H_2O_2 production by BAT mitochondria following cold acclimation.

[4.2] Results

Figure 4.1 illustrates direct glycerol-3-phosphate dehydrogenase activity (nmol DCPIP/min/mg mitochondria) by brown adipose tissue mitochondria in the presence of increasing amounts of CaCl_2 . The data shows that increasing CaCl_2 concentration, increases glycerol-3-phosphate dehydrogenase activity 1.8 fold up to a concentration of 0.75 mM whereupon activity decreases. A one way ANOVA analysis of the data gives a p-value of 0.0146, confirming that increasing the concentration of CaCl_2 in the assay medium affects the glycerol-3-phosphate dehydrogenase activity.

Figure 4.2 illustrates H_2O_2 production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate and glycerol-3-phosphate plus GDP in the presence of 0, 0.1, 0.2, 0.5, 0.75 and 1.0 mM total CaCl_2 (10 nM, 20 nM, 50 nM, 140 nM and 6.66 μM free). The presence of CaCl_2 does not significantly affect H_2O_2 production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate alone. However the presence of 0.1 mM CaCl_2 significantly increases H_2O_2 production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate plus GDP (- $\text{CaCl}_2=18.63\pm2.4$; +0.1mM $\text{CaCl}_2=29.45\pm1.72$, $p=0.0215$) as does the presence of 0.2 mM CaCl_2 (- $\text{CaCl}_2=18.63\pm2.4$; +0.2 mM $\text{CaCl}_2=31.4\pm1.3$, $p=0.0095$).

Figure 4.3 illustrates O_2 consumption (nmol O_2 /min/mg mitochondria) by brown adipose tissue mitochondria respiring on glycerol-3-phosphate and 0, 0.1, 0.2, 0.5, 0.75 and 1.0 mM $CaCl_2$. State 4 oxygen consumption is significantly higher in the presence of all concentrations of $CaCl_2$ compared to oxygen consumption with no $CaCl_2$ present (+0.1 mM, $p=0.0155$; +0.2 mM, $p=0.0023$; +0.5 mM, $p=0.0013$; +0.75 mM, $p=0.0009$; +1.0 mM, $p=0.0004$). GDP sensitive oxygen consumption is also significantly increased in the presence of the following concentrations of $CaCl_2$ compared to GDP sensitive oxygen consumption in the absence of $CaCl_2$ +0.1 Mm ($p=0.0166$), +0.2 mM ($p=0.0042$), +0.5 mM ($p=0.011$), +0.75 mM ($p=0.011$). State 4 uncoupled oxygen consumption is significantly higher in the presence of 0.2 mM ($p=0.0188$), 0.5 mM ($p=0.0158$) and 0.75 mM $CaCl_2$ ($p=0.0051$).

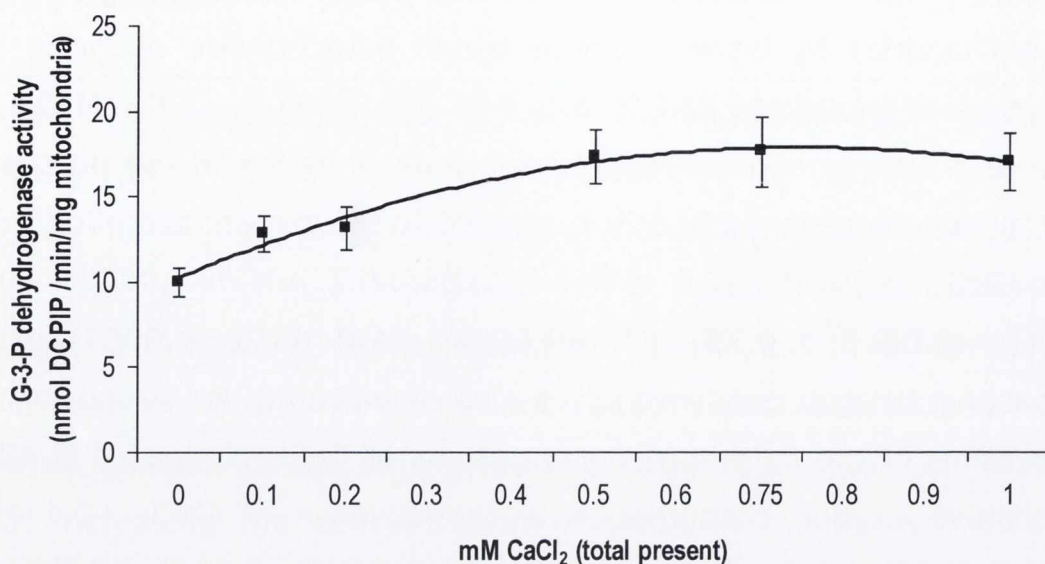


Figure 4.1 Glycerol-3-phosphate dehydrogenase activity in brown adipose tissue mitochondria increases in the presence of increasing amounts of CaCl₂.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. The activity of glycerol-3-phosphate dehydrogenase was determined by monitoring the reduction of 2,6-dichloroindophenolate at 600 nm. Absorbance changes were monitored on a Shimadzu UVmini-1240 uv-vis spectrophotometer at 30°C. Mitochondria (25 µg) were incubated in assay buffer containing 25 mM potassium phosphate, 5 mM MgCl₂, 2.5 mg/ml bovine serum albumin, 2 µg/ml antimycin A, 2 µg/ml rotenone, 2mM KCN, 1 mM EGTA, 50 µM 2,6-dichloroindophenolate and 65 µM decylubiquinone, pH 7.2. Baseline activity was monitored for 3 minutes. The reaction was initiated by the addition of 10mM glycerol-3-phosphate and the appropriate concentration of CaCl₂. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate

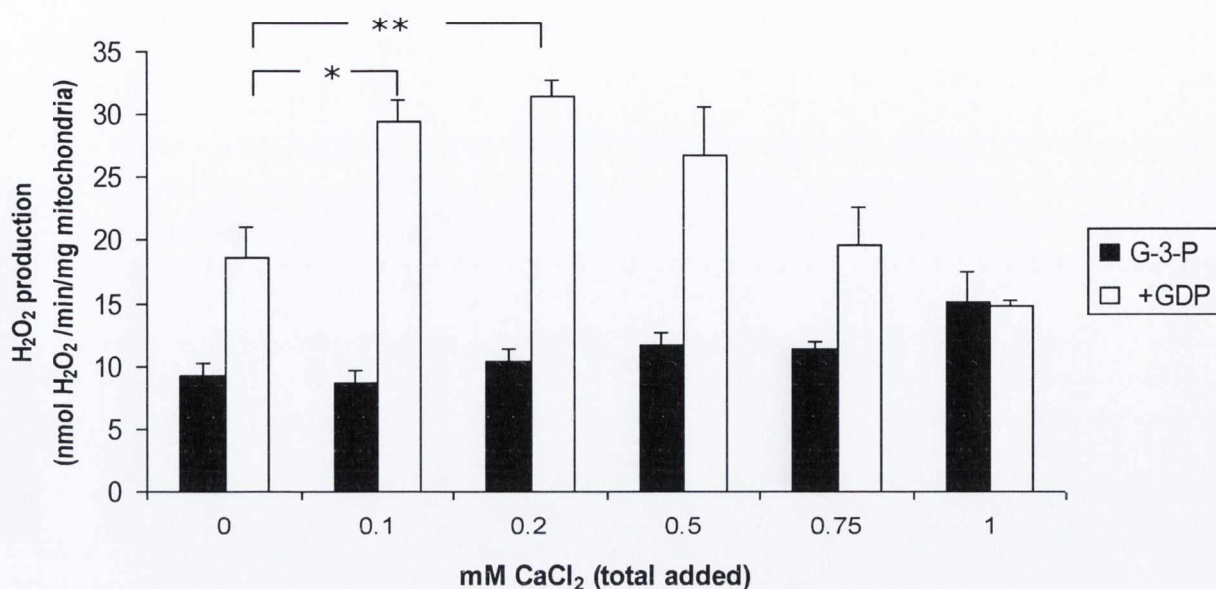


Figure 4.2 GDP sensitive H₂O₂ production by non-phosphorylating brown adipose tissue mitochondria respiring on glycerol-3-phosphate increases significantly upon addition of CaCl₂.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate or 10 mM glycerol-3-phosphate plus 1 mM GDP. Mitochondria were incubated with the desired concentration of CaCl₂ prior to addition of substrate. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05; **=p<0.01)

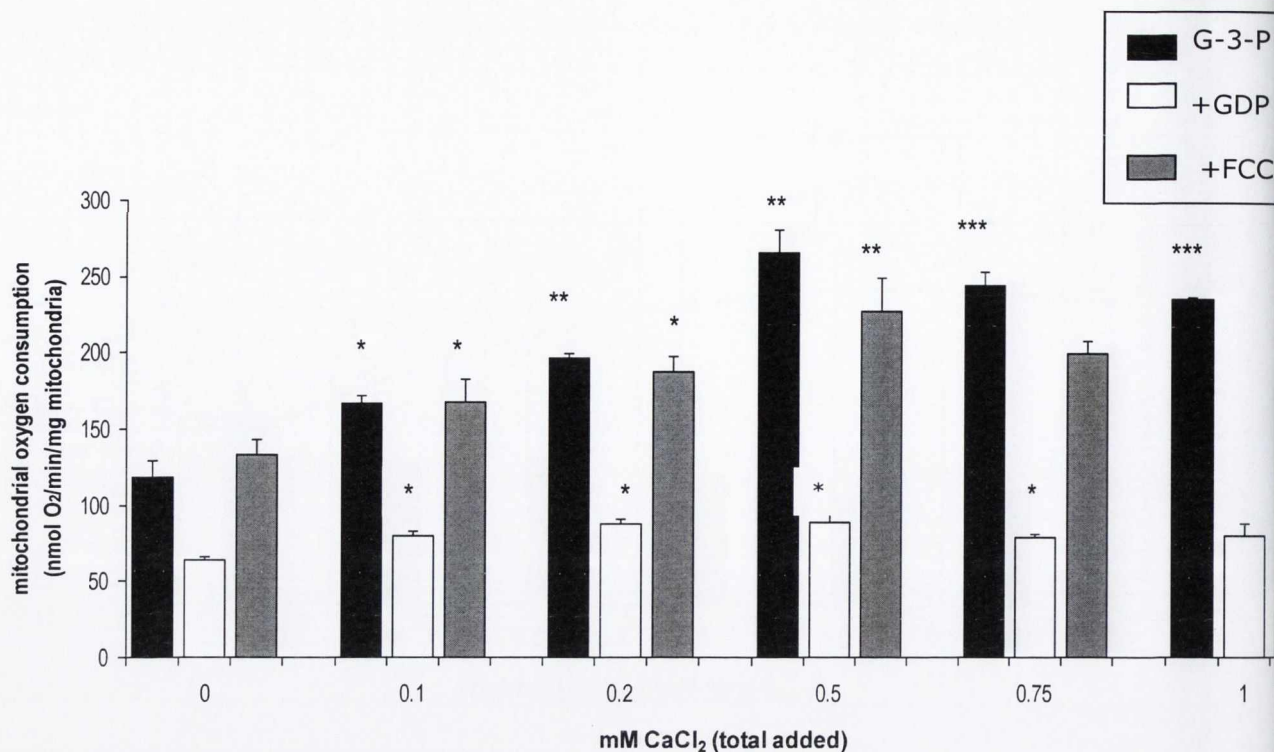


Figure 4.3 Oxygen consumption by non-phosphorylating brown adipose tissue mitochondria respiring on glycerol-3-phosphate upon activation of mGPDH by CaCl₂.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 10 mM glycerol-3-phosphate and the appropriate concentration of CaCl₂ in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05; **=p<0.01; ***=p<0.001)

The CaCl_2 induced increase in oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate, may solely be due to activation of the mGPDH but could also be due to uncoupling by calcium transport. To confirm that our observations were indeed due to activation of the mGPDH and not uncoupling, the oxygen consumption experiments presented in figure 4.3 were repeated in the presence of ruthenium red ($5\mu\text{M}$), an inhibitor of calcium transport into mitochondria.

To confirm that the increase in oxygen consumption is due to calcium activation of mGPDH and not activation of another dehydrogenase which may affect the activity of the respiratory chain, oxygen consumption by BAT mitochondria respiring on succinate was examined in the presence and absence of 0.2mM CaCl_2 . 0.2mM CaCl_2 was used because it significantly increased oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate.

Figure 4.4 compares oxygen consumption by brown adipose tissue mitochondria in the presence and absence of ruthenium red, respiring on glycerol-3-phosphate and increasing concentrations of CaCl_2 . The data shows that the presence of ruthenium red does not inhibit the CaCl_2 induced increase in oxygen consumption observed by brown adipose tissue mitochondria respiring on glycerol-3-phosphate.

Figure 4.5 compares oxygen consumption by brown adipose tissue mitochondria respiring on succinate plus or minus 0.2 mM CaCl_2 . No differences in state 4 oxygen consumption ($-\text{CaCl}_2=256.043\pm16.07$; $+\text{CaCl}_2=255.86\pm25.26$), GDP inhibitable oxygen consumption ($-\text{CaCl}_2=78.94\pm4.18$; $+\text{CaCl}_2=95.33\pm5.05$) or state 4 uncoupled oxygen consumption ($-\text{CaCl}_2=244.05\pm10.4$; $+\text{CaCl}_2=248.46\pm31.74$) were observed.

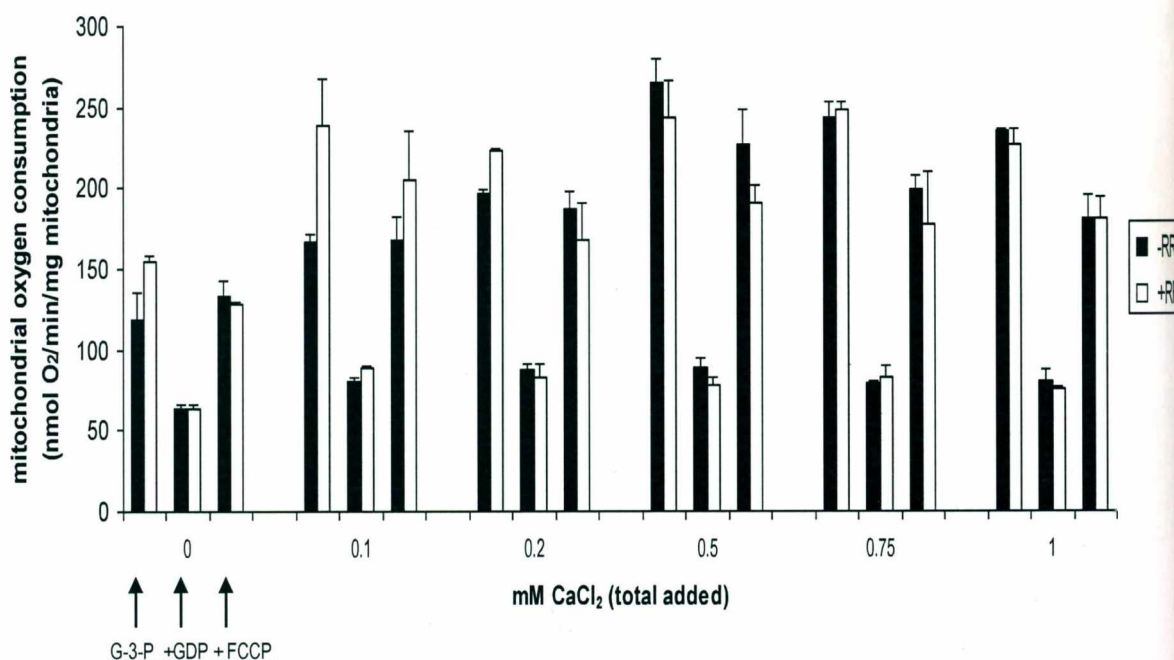


Figure 4.4 Ruthenium red does not inhibit the CaCl_2 induced increase in oxygen consumption by brown adipose tissue mitochondria respiring on glycerol-3-phosphate.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 $\mu\text{g}/\text{ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g}/\text{ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA, 10 mM glycerol-3-phosphate, the appropriate concentration of CaCl_2 and plus or minus 5 μM ruthenium red in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate.

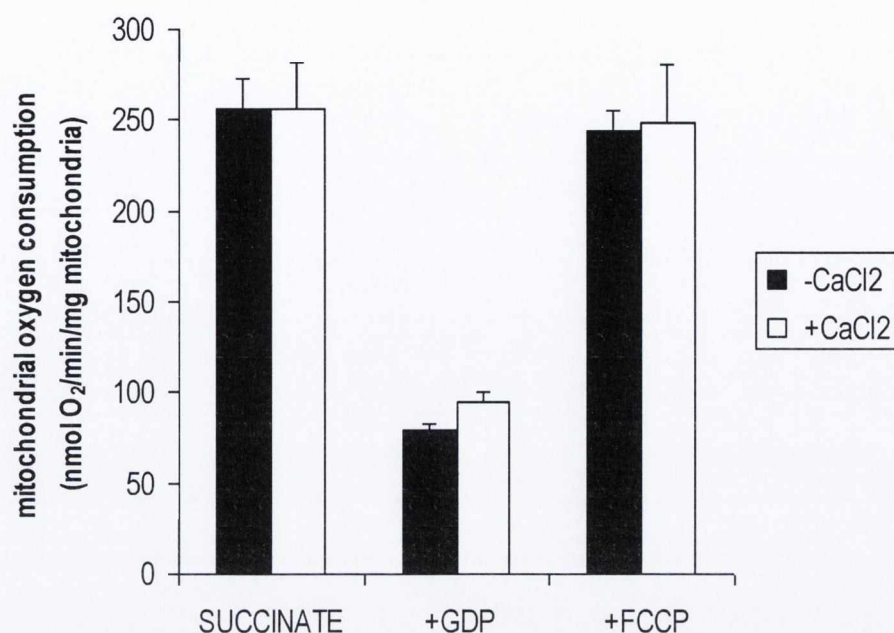


Figure 4.5 CaCl₂ addition to succinate supported brown adipose tissue mitochondria does not affect oxygen consumption.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 10 mM succinate, 1 µM rotenone and 0.2 mM CaCl₂ in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

The data in figures 4.4 and 4.5 show that the observed calcium induced increase in oxygen consumption observed by BAT mitochondria respiring on glycerol-3-phosphate is indeed due to calcium activation of mGPDH in BAT mitochondria and not due to calcium transport induced uncoupling, or activation of other dehydrogenases which may affect respiratory chain activity.

To determine whether oxygen consumption due to UCP 1 activity is increased in the presence of calcium activated mGPDH, we indirectly measured this oxygen consumption by subtracting the GDP sensitive oxygen consumption from the state 4 non-phosphorylating oxygen consumption.

Figure 4.6 illustrates O_2 consumption due to UCP 1 activity (nmol O_2 /min/mg mitochondria) by brown adipose tissue mitochondria respiring on glycerol-3-phosphate and 0, 0.1, 0.2, 0.5, 0.75 and 1.0 mM $CaCl_2$. Significantly greater UCP 1 dependent oxygen consumption was observed in the presence of all calcium concentrations examined (0 mM $CaCl_2$ =54.763 $\pm$ 6.39; +0.1 mM $CaCl_2$ = 86.6 $\pm$ 3.32, p = 0.0115; +0.2 mM $CaCl_2$ = 108.77 $\pm$ 2.6, p =0.0014; +0.5 mM $CaCl_2$ = 175.74 $\pm$ 9.08, p =0.0004; +0.75 mM $CaCl_2$ = 165.04 $\pm$ 5.46, p =0.0002; +1mM $CaCl_2$ = 155.22 $\pm$ 4.62, p =0.0002).

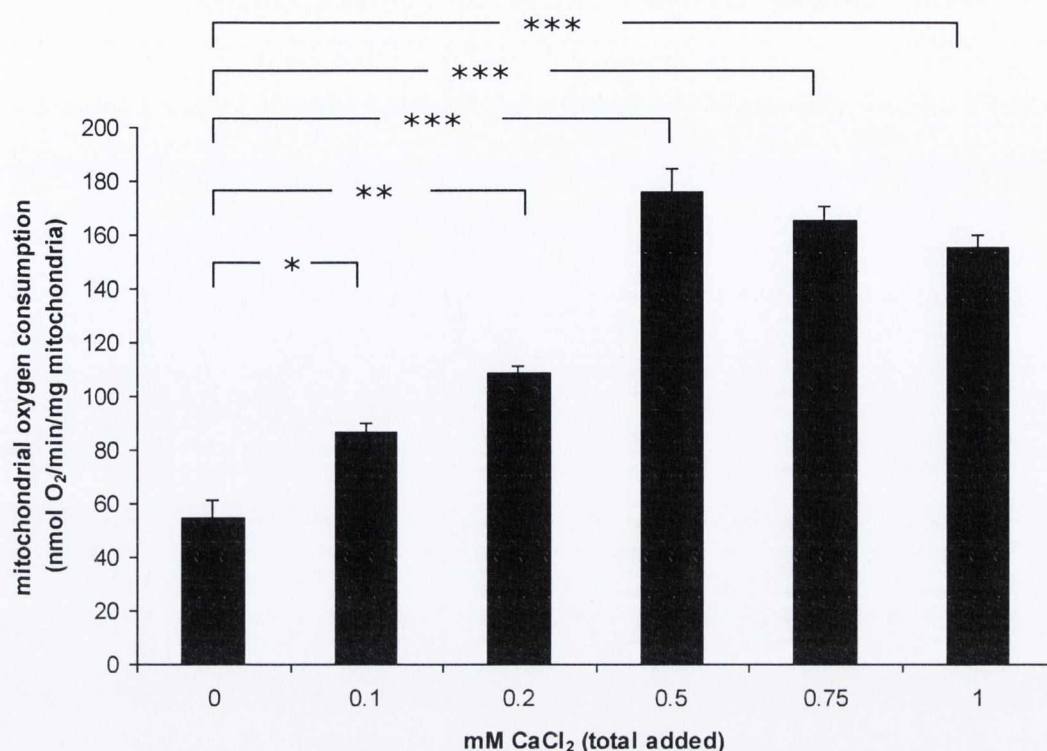


Figure 4.6 UCP 1 dependent oxygen consumption is increased by calcium activation of mGPDH.

BAT mitochondria were isolated as described in section 2.3 from female wistar rats. Subsequent oxygen consumption rates by BAT mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 10 mM glycerol-3-phosphate and the appropriate concentration of CaCl₂ in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate. (*=p<0.05; **=p<0.01; ***=p<0.001)

In chapter 3 we saw that UCP 1 in BAT mitochondria isolated from rats and mice housed at room temperature was capable of attenuating H_2O_2 production with a variety of substrates, and in particular H_2O_2 production due to reverse electron transport. In this chapter we investigated whether UCP 1 is capable of attenuating any putative increase in H_2O_2 production by BAT mitochondria isolated from rats following cold acclimation. In this chapter we present a comparison of H_2O_2 production by BAT mitochondria respiring on succinate, succinate plus rotenone, glycerol-3-phosphate, glycerol-3-phosphate plus rotenone and pyruvate plus malate from cold-acclimated rats and rats housed at room temperature.

Figure 4.7 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats housed at 4°C for 4 weeks and from female Wistar rats housed at room temperature for 4 weeks respiring on succinate and succinate plus GDP. Significantly higher H_2O_2 production by brown adipose tissue mitochondria isolated from cold-acclimated rats was observed by mitochondria respiring on succinate plus GDP compared to H_2O_2 production by brown adipose tissue mitochondria isolated from rats housed at ambient room temperature respiring on succinate plus GDP (CA= 22.42 ± 2.5 ; RT= 11.14 ± 1.07 , $p=0.0143$). No significant differences in H_2O_2 production were observed by brown adipose tissue mitochondria isolated from cold acclimated rats and rats housed at room temperature respiring on succinate alone (CA= 0.441 ± 0.07 ; RT= 0.467 ± 0.083).

Figure 4.8 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats housed at 4°C for 4 weeks and from female Wistar rats housed at room temperature for 4 weeks respiring on succinate plus rotenone and succinate plus GDP plus rotenone. Consistently higher H_2O_2 production by brown adipose tissue mitochondria isolated from rats housed at room temperature was observed by mitochondria respiring on succinate plus GDP plus rotenone compared to H_2O_2 production by brown adipose tissue mitochondria isolated from cold acclimated rats respiring on succinate plus GDP plus rotenone, although this difference was not statistically significant ($\text{CA}=2.46\pm0.44$; $\text{RT}=4.36 \pm 0.709$). Consistently higher H_2O_2 production was observed by brown adipose tissue mitochondria isolated from cold acclimated rats compared to H_2O_2 production by brown adipose tissue mitochondria isolated from rats housed at room temperature respiring on succinate alone, although this difference was not statistically significant ($\text{CA}=0.585\pm0.139$; $\text{RT}=1.07\pm0.177$).

Figure 4.9 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats housed at 4°C for 4 weeks and from female Wistar rats housed at room temperature for 4 weeks respiring on glycerol-3-phosphate and glycerol-3-phosphate plus GDP. Consistently higher H_2O_2 production by brown adipose tissue mitochondria isolated from cold-acclimated rats was observed by mitochondria respiring on glycerol-3-phosphate plus GDP compared to H_2O_2 production by brown adipose tissue mitochondria isolated from rats housed at ambient room temperature respiring on glycerol-3-phosphate plus GDP, although this difference was not statistically significant ($\text{CA}=24.28\pm3.21$; $\text{RT}=18.63 \pm 2.4$). No

significant differences in H_2O_2 production was observed by brown adipose tissue mitochondria isolated from cold acclimated rats and rats housed at room temperature respiring on glycerol-3-phosphate alone ($\text{CA}=9.81\pm0.7$; $\text{RT}=9.18\pm1.07$).

Figure 4.10 compares H_2O_2 production ($\text{nmol H}_2\text{O}_2/\text{min}/\text{mg}$ mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats housed at 4°C for 4 weeks and from female Wistar rats housed at room temperature for 4 weeks respiring on glycerol-3-phosphate plus rotenone and glycerol-3-phosphate plus GDP plus rotenone. No significant differences in H_2O_2 production by brown adipose tissue mitochondria isolated from cold-acclimated rats and rats housed at room temperature were observed by mitochondria respiring on glycerol-3-phosphate plus GDP ($\text{CA}=16.06\pm1.09$; $\text{RT}=17.07 \pm 2.06$) or respiring on glycerol-3-phosphate plus rotenone ($\text{CA}=10.78\pm0.704$; $\text{RT}=11.49\pm0.29$).

Figure 4.11 compares H_2O_2 production ($\text{nmol H}_2\text{O}_2/\text{min}/\text{mg}$ mitochondria) by brown adipose tissue mitochondria isolated from female Wistar rats housed at 4°C for 4 weeks and from female Wistar rats housed at room temperature for 4 weeks respiring on pyruvate plus malate and pyruvate plus malate plus GDP. No significant differences in H_2O_2 production by brown adipose tissue mitochondria isolated from cold-acclimated rats and rats housed at room temperature were observed by mitochondria respiring on pyruvate plus malate plus GDP ($\text{CA}=0.529\pm0.125$; $\text{RT}=0.633 \pm 0.084$) or respiring on pyruvate plus malate ($\text{CA}=0.557\pm0.0.154$; $\text{RT}=0.523\pm0.102$).

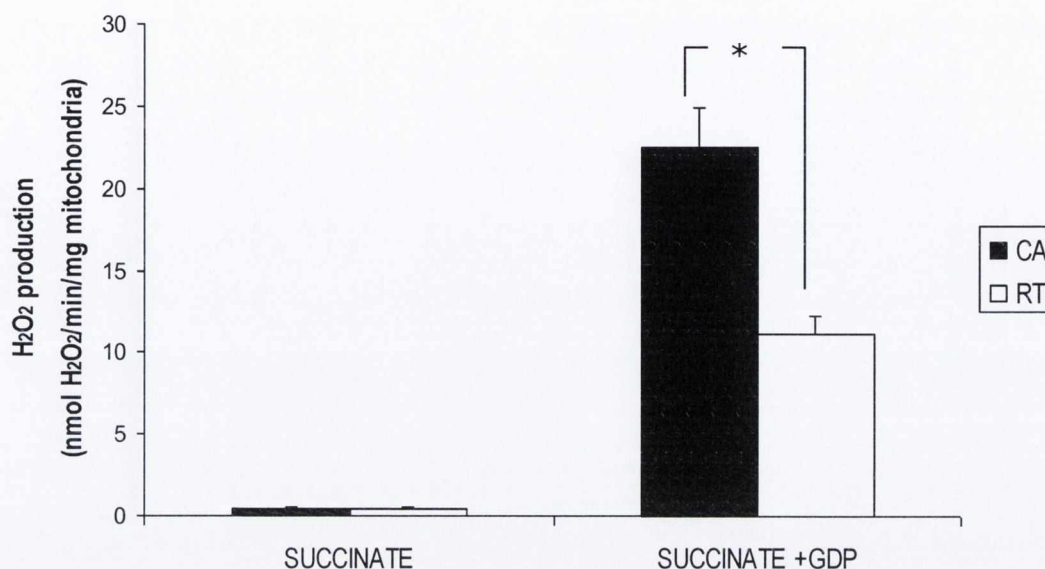


Figure 4.7 GDP sensitive H₂O₂ production increases significantly in succinate supported brown adipose tissue mitochondria following cold-acclimation.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats housed at 4° C for 4 weeks and from female Wistar rats housed at ambient room temperature for 4 weeks. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates used were 10 mM succinate-KOH or 10 mM succinate-KOH plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate. (*=p=<0.05)

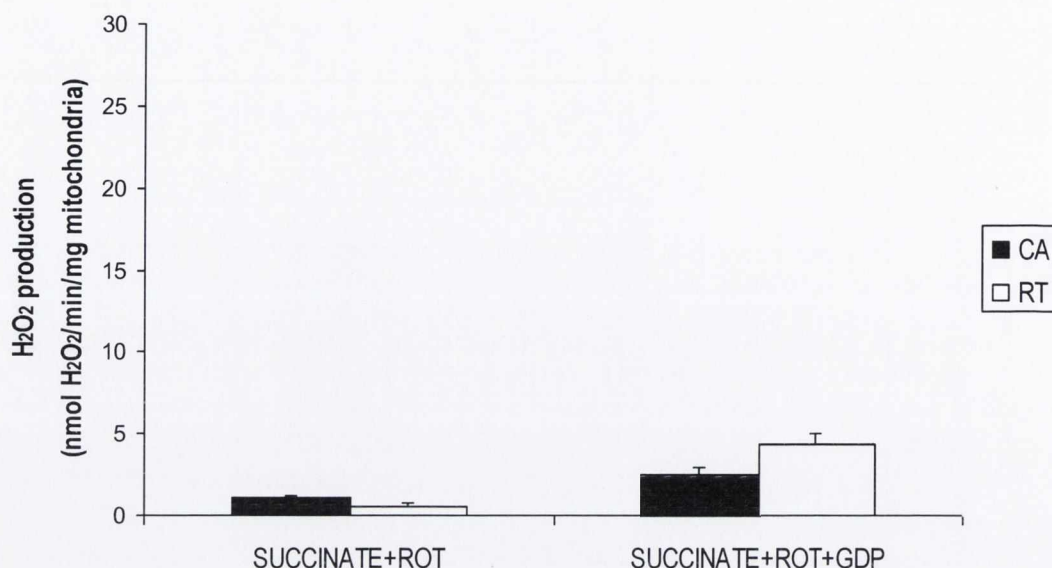


Figure 4.8 GDP sensitive H₂O₂ production decreases in succinate and rotenone supported brown adipose tissue mitochondria following cold-acclimation.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats housed at 4° C for 4 weeks and from female Wistar rats housed at ambient room temperature for 4 weeks. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 1µM rotenone, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM succinate-KOH or 10 mM succinate-KOH plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate

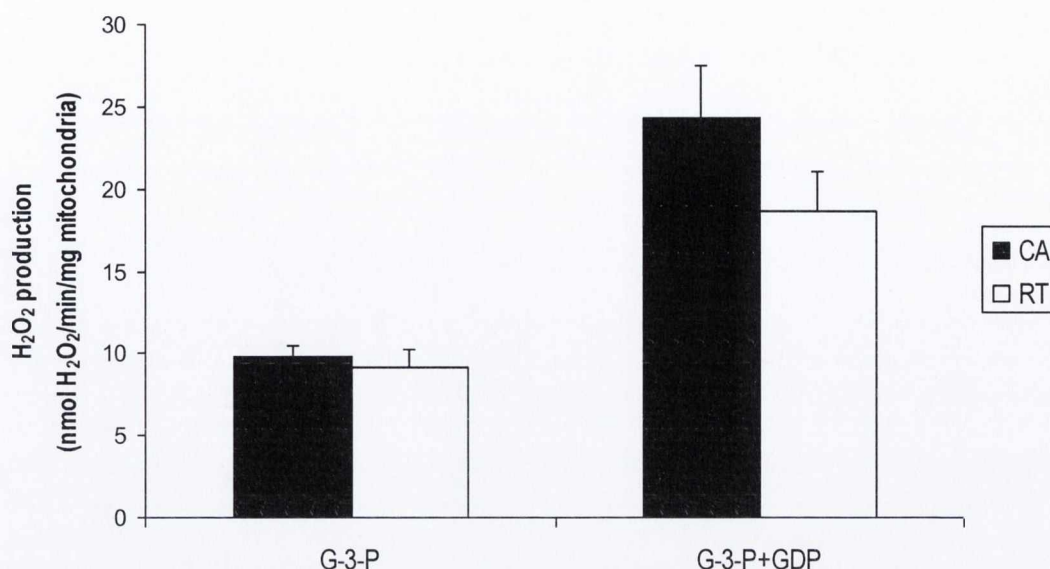


Figure 4.9 GDP sensitive H₂O₂ production increases in glycerol-3-phosphate supported brown adipose tissue mitochondria following cold-acclimation.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats housed at 4° C for 4 weeks and from female Wistar rats housed at ambient room temperature for 4 weeks. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate or 10 mM glycerol-3-phosphate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

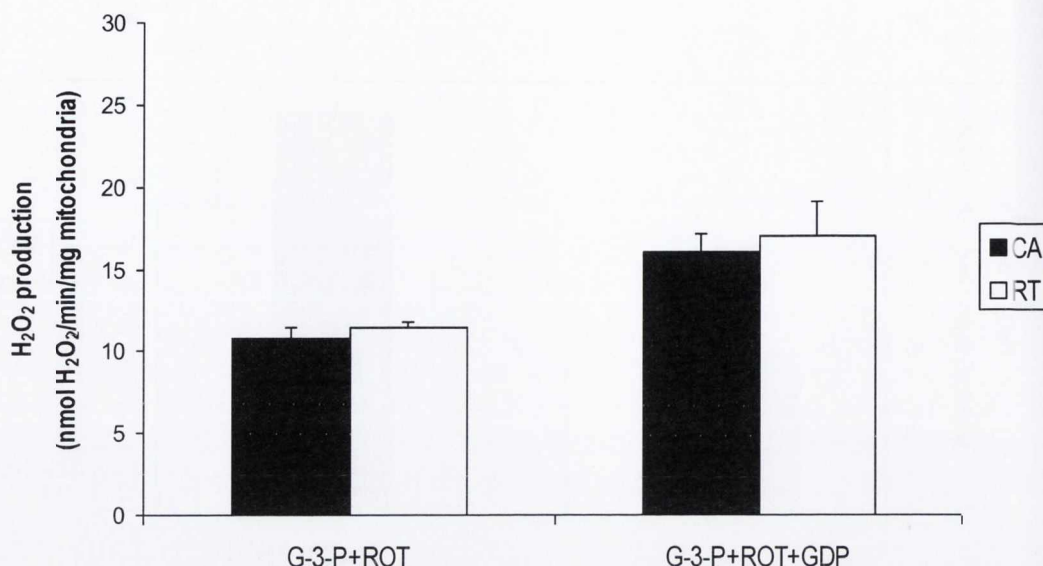


Figure 4.10 Cold acclimation does not affect H₂O₂ production by brown adipose tissue mitochondria respiring on glycerol-3-phosphate and rotenone.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats housed at 4° C for 4 weeks and from female Wistar rats housed at ambient room temperature for 4 weeks. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 1 µM rotenone, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate or 10 mM glycerol-3-phosphate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

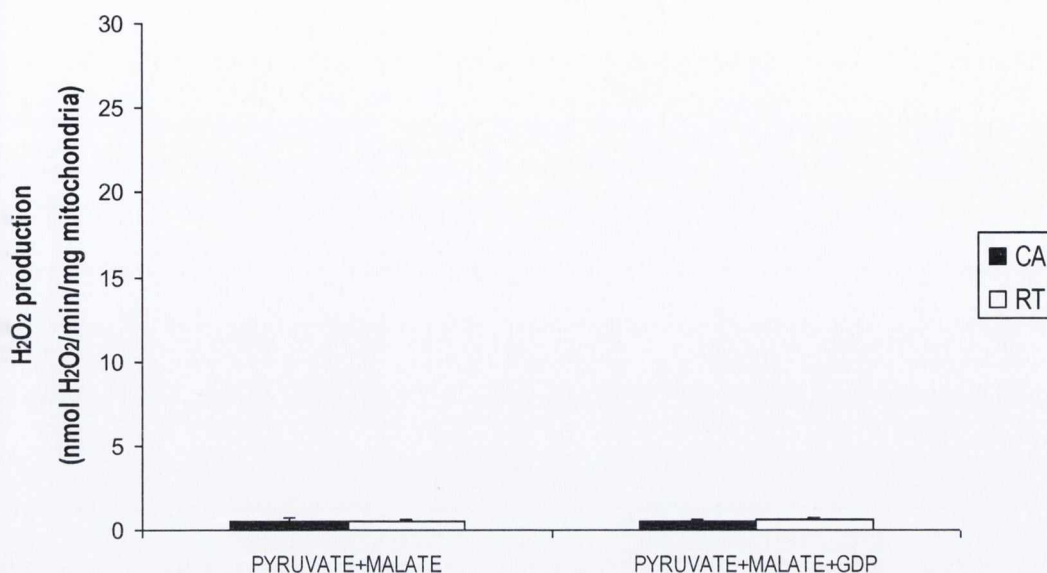


Figure 4.11 Cold acclimation does not affect H₂O₂ production by brown adipose tissue mitochondria respiring on pyruvate and malate.

BAT mitochondria were isolated as described in section 2.3 from female Wistar rats housed at 4° C for 4 weeks and from female Wistar rats housed at ambient room temperature for 4 weeks. Subsequent H₂O₂ production rates by BAT mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM pyruvate+6 mM malate or 10 mM pyruvate+6 mM malate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

[4.3] Discussion

The effect of increasing calcium concentrations on mGPDH activity, H_2O_2 production and oxygen consumption in BAT mitochondria respiring on glycerol-3-phosphate are unknown. Therefore in this chapter we investigated whether a) Ca^{2+} increases the activity of mGPDH in BAT mitochondria b) Ca^{2+} increases H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate c) UCP 1 is capable of attenuating any putative increase in H_2O_2 production in the presence of Ca^{2+} and d) oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate is affected by Ca^{2+} activation of mGPDH.

Figure 4.1 Illustrates that mitochondrial glycerol-3-phosphate dehydrogenase activity significantly increases in the presence of increasing amounts of calcium. Increasing the calcium concentration up to 0.75 mM total (140 nM free) has an activating effect on activity, but increasing the concentration above this does not further increase activity.

Figure 4.2. Illustrates that H_2O_2 production by non-phosphorylating BAT mitochondria respiring on glycerol-3-phosphate is significantly increased in the presence of 0.1 mM (10 nM free) and 0.2 mM (20 nM free) CaCl_2 , but only when UCP 1 is inhibited by GDP. In the absence of GDP (i.e. UCP 1 is active) no significant increase in H_2O_2 production was observed. This suggests that the increased activity of mGPDH by calcium activation also increases H_2O_2 production which can be attenuated by UCP 1.

Figure 4.3 illustrates that glycerol-3-phosphate supported state 4, GDP sensitive and state 4 uncoupled oxygen consumption rates are significantly increased by calcium activation of mGPDH. This shows that electron flow through the respiratory chain is

significantly increased by calcium activation of mGPDH in BAT mitochondria. Thus the Ca^{2+} activation of oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate could solely be due to activation of the mGPDH but could also be due to uncoupling by calcium transport. To investigate whether our observed increase in oxygen consumption was due to activation of the mGPDH and not calcium induced uncoupling, the oxygen consumption experiments presented in figure 4.3 were repeated in the presence of ruthenium red, an inhibitor of calcium transport into mitochondria.

Figure 4.4 shows that ruthenium red does not inhibit the increase in oxygen consumption observed upon calcium activation of mGPDH in BAT mitochondria respiring on glycerol-3-phosphate.

To confirm that the increase in oxygen consumption is due to calcium activation of mGPDH and not activation of another dehydrogenase which may affect the activity of the respiratory chain, oxygen consumption by BAT mitochondria respiring on succinate was examined in the presence and absence of 0.2mM CaCl_2 . 0.2mM CaCl_2 was used because it significantly increased oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate.

Figure 4.5 shows that calcium does not affect oxygen consumption by BAT mitochondria respiring on succinate. We can therefore conclude that the observed calcium induced increase in oxygen consumption observed by BAT mitochondria respiring on glycerol-3-phosphate is due to calcium activation of mGPDH in BAT mitochondria and not due to calcium transport induced uncoupling, or activation of other dehydrogenases which may affect respiratory chain activity.

In brain mitochondria an increase in mGPDH activity was associated with an increase in H_2O_2 production and an increase in membrane potential (Tretter et al., 2007). We have shown here

that an increase in mGPDH activity in BAT mitochondria does not lead to an increase in H_2O_2 production. Membrane potential was not measured in this study, but from our results in chapter 3 we can see that UCP 1 is capable of dissipating the membrane potential by BAT mitochondria respiring on glycerol-3-phosphate. We can therefore conclude that mGPDH activation by calcium does not cause an increase in H_2O_2 production by BAT mitochondria due to attenuation of H_2O_2 production by UCP 1 through dissipation of the membrane potential.

By indirectly calculating the oxygen consumption due to UCP 1 (state 4 oxygen consumption minus the GDP inhibited oxygen consumption), it can be seen that the apparent UCP 1 activity significantly increases upon calcium activation of mGPDH (Fig 4.6).

The significant increase in non-phosphorylating oxygen consumption due to UCP 1 activity presented in figure 4.6 suggests an acute mechanism for indirectly increasing thermogenesis in brown adipocytes without increasing the amount of UCP 1 present in BAT mitochondria or directly activating UCP 1. Such a mechanism of activation of thermogenesis would allow an immediate response to cold-exposure by brown adipocytes before recruitment of UCP 1 could begin.

Norepinephrine stimulation of cells through the α_1 -adrenergic pathway results in increased mitochondrial Ca^{2+} levels (Koivisto et al., 2000) and from the results presented in this chapter it can be seen that an increase in Ca^{2+} levels results in increased mGPDH activity (figure 4.1). We also show that this increase in mGPDH activity results in an increase in non-phosphorylating oxygen consumption (figure 4.3).

We can therefore extrapolate from our results that norepinephrine stimulation of brown adipocytes can promote thermogenesis by a fatty acid independent mechanism. This agrees with previous reports that norepinephrine promotes thermogenesis

via the α_1 -adrenergic pathway as well as through the β_3 -adrenergic pathway (Zhao *et al.* 1997).

In chapter 3 we presented data which showed that BAT mitochondria isolated from UCP 1 knock-out mice have significantly greater mGPDH activity compared to BAT mitochondria isolated from wild-type mice (Fig 3.22). It was also shown that non-phosphorylating oxygen consumption by glycerol-3-phosphate supported BAT mitochondria is significantly greater in BAT mitochondria isolated from UCP 1 knock-out mice compared to non-phosphorylating oxygen consumption by glycerol-3-phosphate supported BAT mitochondria isolated from wild-type mice (Fig 3.16). Such an increase in the respiration of glycerol-3-phosphate supported BAT mitochondria from UCP 1 knock-out mice could compensate for the decrease in respiration due to ablation of UCP 1 and therefore contribute toward the thermogenic capacity of brown adipocytes of UCP 1 knock-out mice.

In this chapter we show for the first time that calcium can significantly activate mGPDH activity in isolated BAT mitochondria. Given the high level of expression of mGPDH in BAT mitochondria, calcium activation of mGPDH is an attractive means of promoting thermogenesis in brown adipocytes upon initial adrenergic stimulation.

Future work in this area could involve investigating further a role for mGDPH in the activation of thermogenesis, particularly any possible role for the membrane potential independent superoxide production by mGDPH in the activation of UCP 1. Our data presented here does not allow such a conclusion to be reached but does not rule out such a conclusion.

We have shown that UCP 1 is capable of attenuating increased H_2O_2 production by BAT mitochondria when there is increased

supply of electrons to the respiratory chain due to increased activity of mGPDH due to calcium activation. To further investigate whether UCP 1 is capable of attenuating H_2O_2 production under conditions of increased oxidative capacity of the respiratory chain, we investigated H_2O_2 production by BAT mitochondria isolated from rats which have been cold-acclimated for 4 weeks at 4°C .

During cold acclimation the thermogenic activity of brown adipocytes rises (Nicholls and Locke 1984). An increase in oxidative capacity of BAT mitochondria is one of the factors contributing to the increased thermogenic activity of brown adipocytes (Bukowiecki et al, 1982). Increased oxidative capacity could increase the capacity for H_2O_2 production. We have shown in chapter 3 that UCP 1 can attenuate H_2O_2 production, and in particular H_2O_2 production due to reverse electron transport.

In this chapter we investigated whether UCP 1 is capable of attenuating any putative increase in H_2O_2 production by BAT mitochondria following cold acclimation by comparing H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats and rats housed at room temperature.

Figure 4.7 illustrates that there is no difference in H_2O_2 production by BAT mitochondria respiring on succinate isolated from cold-acclimated rats or rats housed at room temperature. However in the presence of GDP there is significantly greater H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats. This shows that when UCP 1 is inactive (i.e. in the presence of GDP) BAT mitochondria isolated from cold-acclimated rats have an increased capacity to produce H_2O_2 , but when UCP 1 is active (in the absence of GDP) this increase in H_2O_2 production is attenuated by UCP 1. In chapter 3 it was shown that BAT mitochondria have a large capacity to produce H_2O_2 by reverse electron transport when UCP 1 is inhibited. It appears that this capacity to produce H_2O_2 by reverse

electron transport is increased by the increased oxidative capacity of BAT mitochondria following chronic cold acclimation.

Figure 4.8 illustrates that there is no difference in H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats or rats housed at room temperature respiring on succinate plus rotenone. In the presence of GDP there is no significant difference in H_2O_2 production by BAT mitochondria isolated from cold acclimated rats or rats housed at room temperature, however H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats is lower than H_2O_2 production by BAT mitochondria isolated from rats housed at room temperature.

In the presence of rotenone reverse electron transport is prevented, so therefore any H_2O_2 production in the presence of rotenone is due to forward electron transport. Following cold acclimation there is an increase in the oxidative capacity in BAT mitochondria, which would result in an increase in the rate of respiration and therefore decrease the length of time that transients of the electron transport chain are capable of reducing O_2 to O_2^- , such as CoQH-.

Figure 4.9 illustrates that there is no difference in H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate isolated from cold-acclimated rats or rats housed at room temperature. In the presence of GDP greater H_2O_2 production is produced by BAT mitochondria isolated from cold-acclimated rats compared to H_2O_2 production by BAT mitochondria isolated from rats housed at room temperature, although the difference is not significant.

Figure 4.10 illustrates that the presence of rotenone abolishes the difference in H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats and rats housed at room temperature respiring on glycerol-3-phosphate. This again suggests that BAT mitochondria isolated from cold-acclimated rats have an increased capacity to

produce H_2O_2 by reverse electron transport compared to BAT mitochondria isolated from rats housed at room temperature which UCP 1 is capable of attenuating. It also illustrates that the increased capacity of the respiratory chain does not lead to an increase the production of H_2O_2 by forward electron transport.

Figure 4.11 illustrates that there is no difference in H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats or rats housed at room temperature respiring on pyruvate plus malate. GDP does not affect H_2O_2 production by BAT mitochondria isolated from either group. As seen in chapter 3, H_2O_2 production by BAT mitochondria respiring on pyruvate plus malate is relatively low compared to H_2O_2 production by BAT mitochondria respiring on succinate or glycerol-3-phosphate, and this does not change following cold-acclimation.

From our comparison of H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats and rats housed at room temperature it can be seen that BAT mitochondria isolated from cold-acclimated rats have an increased capacity to produce H_2O_2 by reverse electron transport when UCP 1 is inactive, due to dissipation of the membrane potential. However when UCP 1 is active no difference in H_2O_2 production by BAT mitochondria isolated from cold-acclimated rats or rats housed at room temperature is observed. We can conclude that the increased oxidative capacity of BAT mitochondria following chronic cold-acclimation does not result in increased H_2O_2 production due to attenuation of H_2O_2 production by UCP 1.

The data presented in this chapter show that increased activity of the respiratory chain in BAT mitochondria does not result in increased H_2O_2 production due to attenuation of H_2O_2 production by UCP 1. Both conditions investigated have physiological

relevance. The first condition investigated using calcium activation of mGPDH would likely occur during initial adrenergic stimulation of brown adipocytes. The second condition of cold-acclimation at 4°C is due to chronic adrenergic stimulation, both physiological relevant processes.

Attenuation of H₂O₂ production by UCP 1 allows increased substrate oxidation without a concomitant increase in ROS production, thereby protecting the mitochondria from oxidative damage during a physiological challenge.

Future work in this area could involve investigating ROS production in intact brown adipocytes from wild-type and UCP 1 knock-out mice. However, the reduction in complex I levels in BAT of UCP 1 knock-out mice would complicate interpretation of such a comparison. ROS production in intact brown adipocytes, and in particular in brown adipocytes stimulated with noradrenaline, would however allow the physiological significance of our findings to be confirmed.

Chapter 5 UCP 1 dependent ROS production in thymus mitochondria

[5.1] Introduction

Until relatively recently UCP 1 has only been associated with mitochondria of brown adipose tissue (Cannon *et al.* 1982; Klaus *et al.* 1991). However, recently in our laboratory it has been shown that UCP1 is present in the thymus. It has been demonstrated that mRNA is present in thymus tissue and cells (thymocytes) and that UCP1 protein is associated with thymus/thymocyte mitochondria (Adams *et al.* 2008; Carroll *et al.* 2004).

The function of UCP 1 in the thymus is not presently known. It is not believed to serve a thermogenic role, as cold acclimation does not increase oxygen consumption of thymocytes or increase UCP 1 expression in thymocytes (Brennan *et al.* 2006). Preliminary studies into the function of UCP 1 in thymus mitochondria have shown the absence of UCP 1 to correlate with a reduced apoptotic potential of thymocytes (Adams *et al.* 2010). Apoptosis plays a critical role in selecting the thymocyte pool, removing cells expressing an unproductive T cell receptor (TCR), or exhibiting either hypo- or hyper-responsiveness upon encountering MHC/self-peptide complexes (Zacharchuk *et al.* 1991). The observed reduction in apoptotic potential in UCP 1 knock-out mice correlated with a change in the cell profile of thymocytes both within the thymus and within the population of circulating thymocytes which leave the thymus (Adams *et al.*, 2010). However the mode by which UCP 1 affects apoptotic potential is unknown. ROS has been shown to induce apoptosis (Dumont *et al.* 1999; Slater *et al.* 1995) and it has also been linked to inhibition of apoptosis (Yousefi *et al.* 1994).

In chapter 3 it was shown that UCP 1 can regulate ROS production in brown adipose tissue mitochondria, a role previously disputed (Shabilina et al., 2006).

The aim of this chapter was to investigate whether UCP 1 in thymus mitochondria is also capable of attenuating ROS production. ROS production due to reverse and forward electron transport was examined using glutamate plus malate, succinate plus and minus rotenone and glycerol-3-phosphate plus and minus rotenone as substrates. Any UCP 1-sensitive ROS production was investigated by comparing the ROS production in thymus mitochondria isolated from UCP 1 knock-out and wild-type mice.

[5.2] Results:

Figure 5.1 Illustrates representative traces of H_2O_2 production by thymus mitochondria respiring on succinate (red), succinate plus GDP (blue), succinate plus rotenone (orange) and succinate plus GDP plus rotenone (green). FCCP addition is indicated at the 250 second mark.

Figure 5.2 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by thymus mitochondria respiring on succinate, succinate plus GDP, succinate plus rotenone and succinate plus GDP plus rotenone. The effect of FCCP on H_2O_2 production is also illustrated. No significant differences in H_2O_2 production by thymus mitochondria were observed by mitochondria respiring on succinate plus GDP compared to H_2O_2 production by thymus mitochondria respiring on succinate alone ($-\text{GDP}=2.51\pm0.13$; $+\text{GDP}=2.13\pm0.39$) or in a comparison of H_2O_2 production by mitochondria respiring on succinate plus GDP plus rotenone compared to H_2O_2 production by mitochondria respiring on succinate plus rotenone ($-\text{GDP}=0.77\pm0.075$; $+\text{GDP}=0.97\pm0.076$), although the addition of GDP consistently increased H_2O_2 production. Significantly lower H_2O_2 production was observed upon FCCP addition to thymus mitochondria respiring on succinate ($-\text{FCCP}=2.51\pm0.13$; $+\text{FCCP}=0.36\pm0.013$, $p=0.0001$), succinate plus GDP ($-\text{FCCP}=2.13\pm0.39$; $+\text{FCCP}=0.37\pm0.14$, $p=0.0128$), succinate plus rotenone ($-\text{FCCP}=0.77\pm0.075$; $+\text{FCCP}=0.513\pm0.067$, $p=0.0286$) and succinate plus GDP plus rotenone ($-\text{FCCP}=0.97\pm0.076$; $+\text{FCCP}=0.514\pm0.006$, $p=0.0039$).

Figure 5.3 illustrates state 4 oxygen consumption rates (nmol O₂/min/mg mitochondria) by thymus mitochondria isolated from female Wistar rats respiring on succinate and succinate plus rotenone. No significant differences in O₂ consumption were observed upon the addition of GDP to mitochondria respiring on succinate (-GDP=14.52±0.35; +GDP=15.117±0.82). A significant decrease in O₂ consumption was observed upon the addition of GDP to mitochondria respiring on succinate plus rotenone (-GDP=20.17±1.64; +GDP=14.41±0.93, p=0.0377). O₂ consumption in the presence of FCCP was 20.02±1.23 nmol O₂/min/mg mitochondria by mitochondria respiring on succinate and 28.55±2.02 nmol O₂/min/mg mitochondria by mitochondria respiring on succinate plus rotenone.

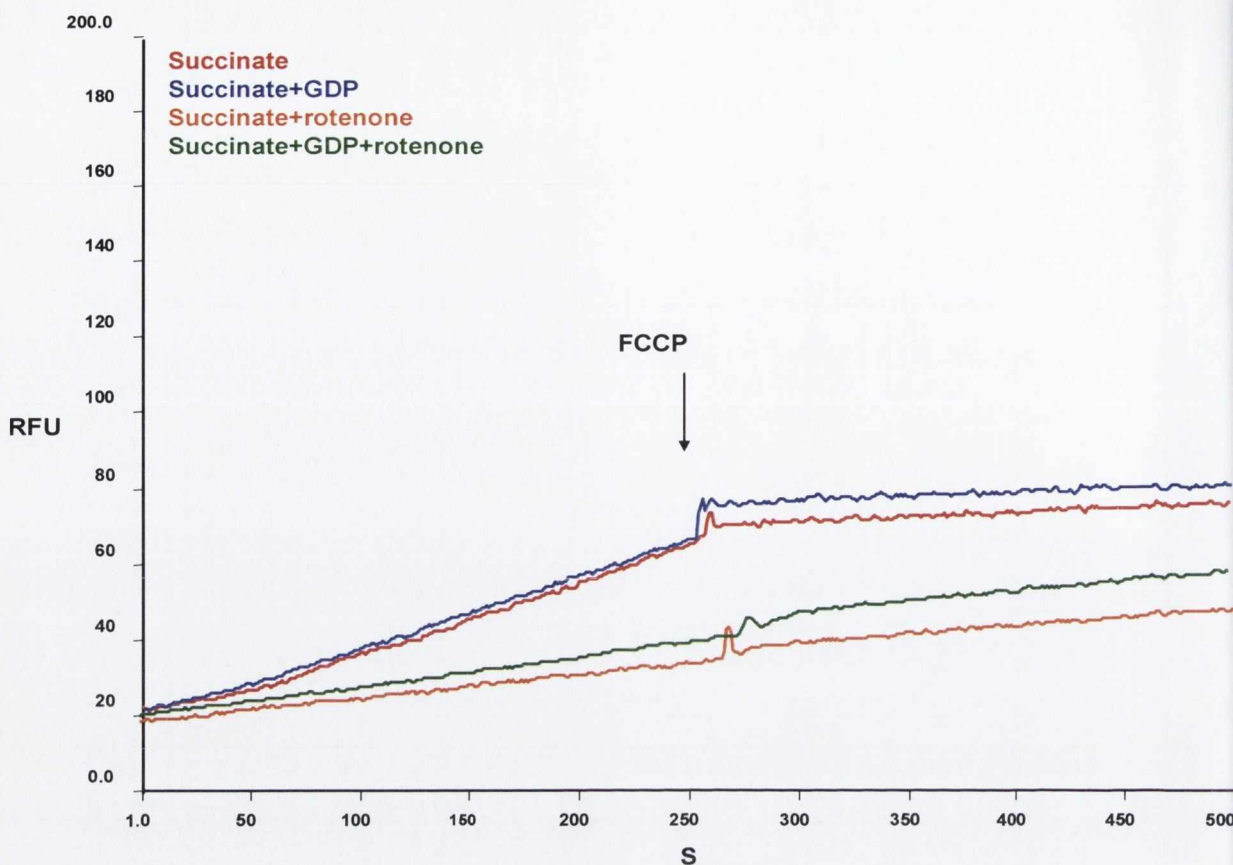


Figure 5.1 Representative trace of time course for the detection of H_2O_2 production by isolated thymus mitochondria respiring on **succinate**, **succinate plus GDP**, **succinate plus rotenone** and **succinate plus GDP plus rotenone**.

Thymus mitochondria were isolated as described in section 2.4 from female Wistar rats. Subsequent H_2O_2 production rates by thymus mitochondria ($100\mu\text{g}/\text{ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, $1\mu\text{g}/\text{ml}$ oligomycin, $5\mu\text{M}$ atractyloside, 0.1% de-fatted BSA, $5\mu\text{M}$ Amplex Red, $10\text{ U}/\text{ml}$ horseradish peroxidase and $30\text{ U}/\text{ml}$ superoxide dismutase. Substrates used were 10 mM succinate-KOH, 10 mM succinate-KOH plus 1 mM GDP, 10 mM succinate-KOH plus $1\mu\text{M}$ rotenone and 10 mM succinate-KOH plus 1 mM GDP plus $1\mu\text{M}$ rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at $570 \pm 8\text{ nm}$ and emission at $585 \pm 4\text{ nm}$. H_2O_2 production was measured for 250 seconds, whereupon $0.5\mu\text{M}$ of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H_2O_2 production was measured for a further 250 seconds.

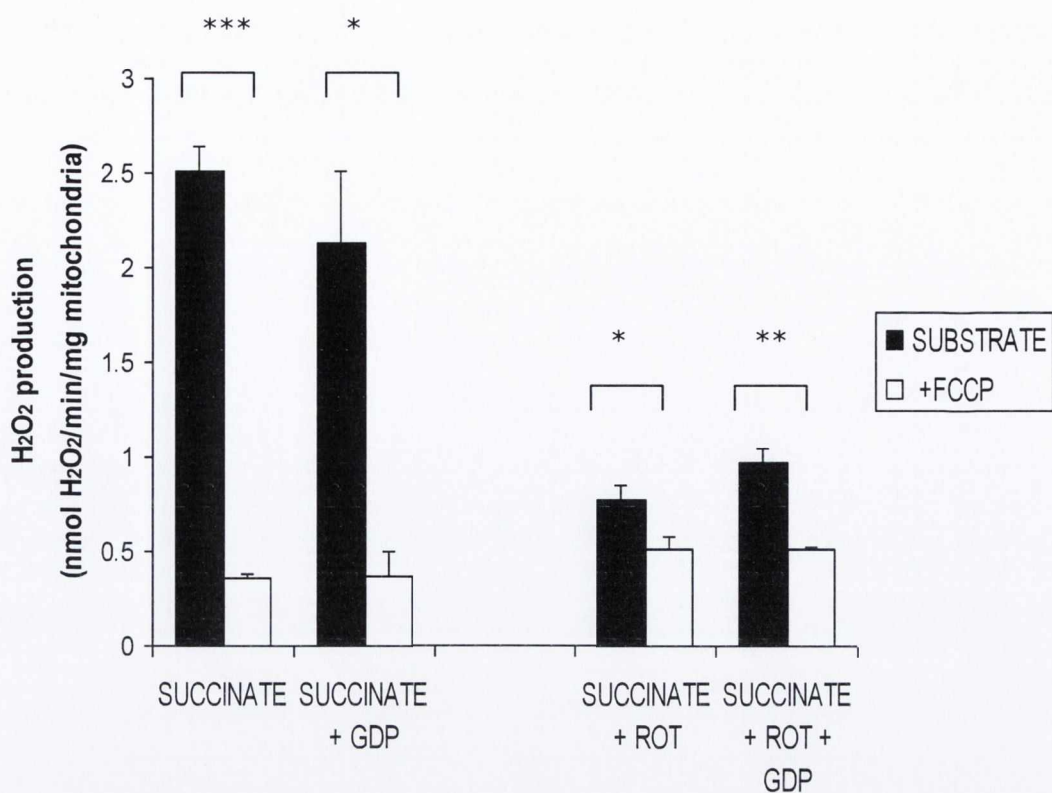


Figure 5.2 H₂O₂ production in isolated thymus mitochondria respiring on succinate is GDP insensitive.

Thymus mitochondria were isolated as described in section 2.4 from female Wistar rats. Subsequent H₂O₂ production rates by thymus mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates used were 10 mM succinate-KOH, 10 mM succinate-KOH plus 1 mM GDP, 10 mM succinate-KOH plus 1 µM rotenone and 10 mM succinate-KOH plus 1 mM GDP plus 1 µM rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05; **=p<0.01; ***=p<0.001)..

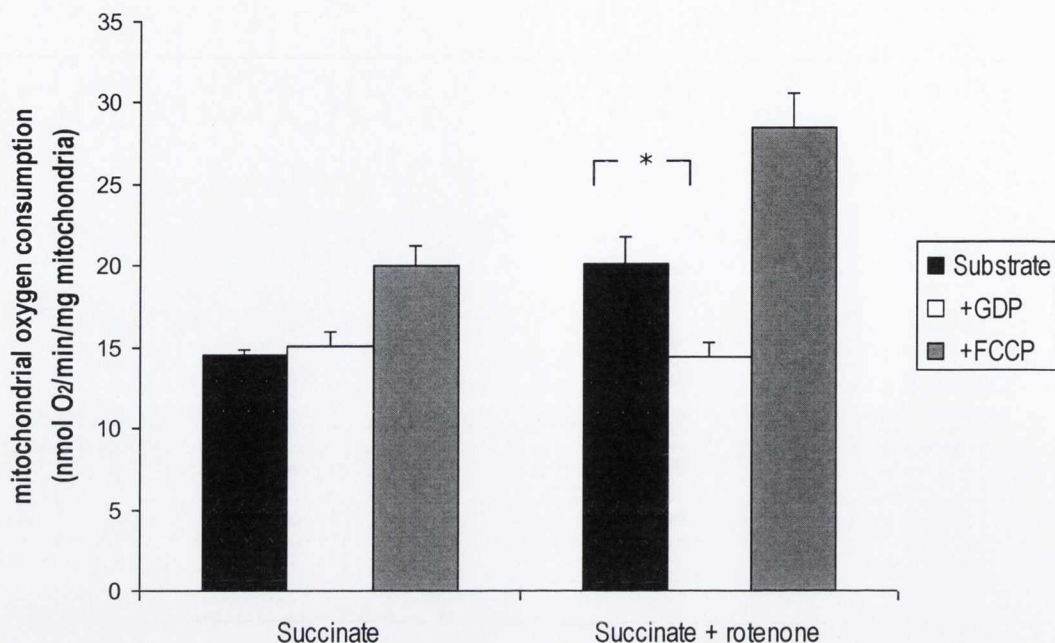


Figure 5.3 Non-phosphorylating oxygen consumption by isolated thymus mitochondria respiring on succinate is GDP sensitive in the presence of rotenone.

Thymus mitochondria were isolated as described in section 2.4 from female wistar rats. Subsequent oxygen consumption rates by thymus mitochondria (100 $\mu\text{g/ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA, 10 mM succinate-KOH plus or minus 1 μM rotenone in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate (*= $p < 0.05$).

Figure 5.4 Illustrates representative traces of H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate (red), glycerol-3-phosphate plus GDP (blue), glycerol-3-phosphate plus rotenone (orange) and glycerol-3-phosphate plus GDP plus rotenone (green). FCCP addition is at the 250 second mark.

Figure 5.5 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by thymus mitochondria respiring on glycerol-3-phosphate, glycerol-3-phosphate plus GDP, glycerol-3-phosphate plus rotenone and glycerol-3-phosphate plus GDP plus rotenone. The effect of FCCP on H_2O_2 production in the presence of both substrates is also illustrated. Significantly higher H_2O_2 production by thymus mitochondria was observed by mitochondria respiring on glycerol-3-phosphate plus GDP compared to H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate alone (-GDP=1.15±0.25; +GDP=2.32 ±0.32, p=0.028). No significant differences in H_2O_2 production by thymus mitochondria were observed by mitochondria respiring on glycerol-3-phosphate plus GDP plus rotenone compared to H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate plus rotenone (-GDP=0.79±0.14; +GDP=1.04 ±0.021). No significant differences in H_2O_2 production were observed upon FCCP addition to thymus mitochondria respiring on glycerol-3-phosphate (-FCCP=1.15±0.25; +FCCP=0.935±0.336) or glycerol-3-phosphate plus rotenone (-FCCP=0.79±0.14; +FCCP=0.65±0.16). Significantly lower H_2O_2 production was however observed upon FCCP addition to thymus mitochondria respiring on glycerol-3-phosphate plus GDP (-FCCP=2.32±0.32; +FCCP=0.81±0.095,p=0.0105) and on glycerol-3-phosphate plus GDP plus rotenone (-FCCP=1.04±0.021; +FCCP=0.554±0.033).

Figure 5.6 illustrates state 4 oxygen consumption rates (nmol O₂/min/mg mitochondria) by thymus mitochondria isolated from female Wistar rats respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone. A significant decrease in O₂ consumption was observed upon the addition of GDP to mitochondria respiring on glycerol-3-phosphate (-GDP=9.3±0.07; +GDP=7.44±0.32, p=0.0044) and on glycerol-3-phosphate plus rotenone (-GDP=8.8±0.29; +GDP=7.4±0.08, p=0.0103). O₂ consumption in the presence of FCCP was 11.55±1.8 nmol O₂/min/mg mitochondria by mitochondria respiring on glycerol-3-phosphate and 11.35±1.05 nmol O₂/min/mg mitochondria by mitochondria respiring on glycerol-3-phosphate plus rotenone.

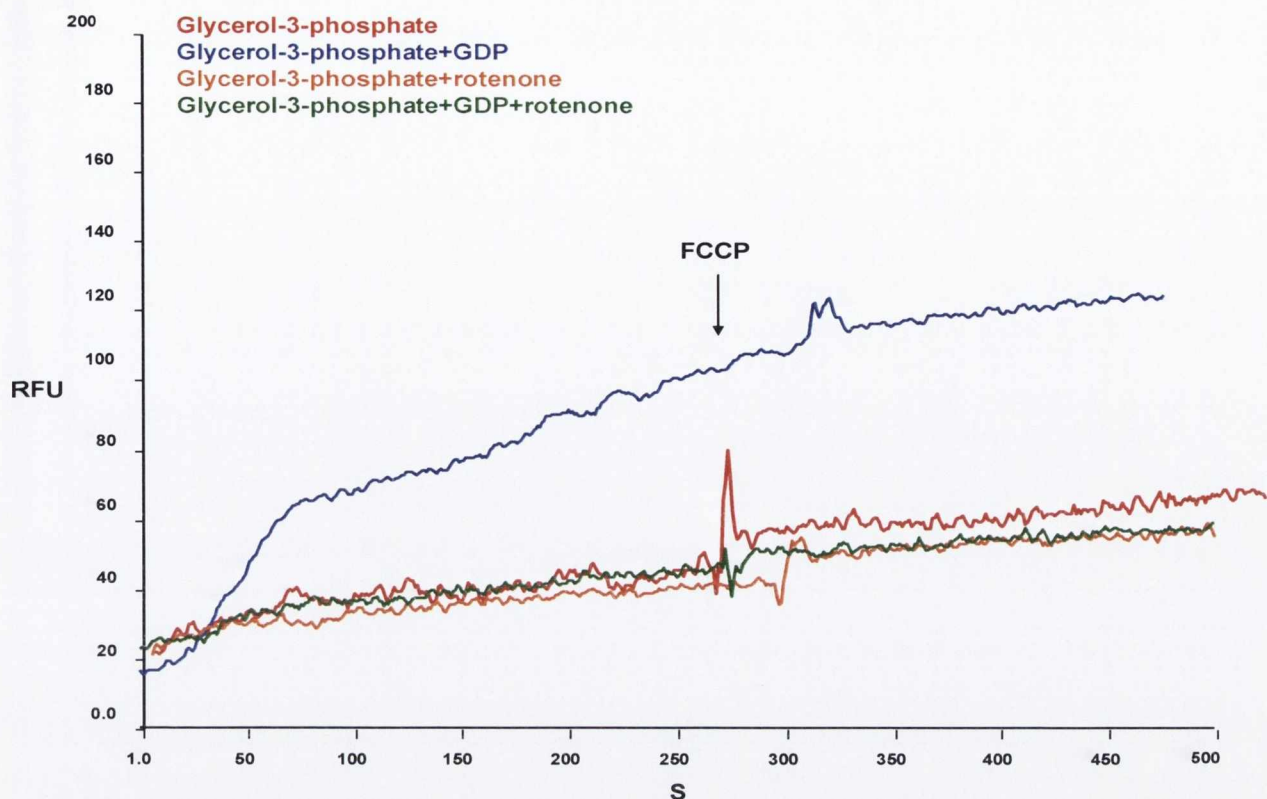


Figure 5.4 Representative trace of time course for the detection of H_2O_2 production by isolated thymus mitochondria respiring on **glycerol-3-phosphate**, **glycerol-3-phosphate plus GDP**, **glycerol-3-phosphate+rotenone** and **glycerol-3-phosphate +GDP+rotenone**.

Thymus mitochondria were isolated as described in section 2.4 from female Wistar rats. Subsequent H_2O_2 production rates by thymus mitochondria ($100\mu\text{g/ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA, 5 μM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate, 10 mM glycerol-3-phosphate plus 1 mM GDP, 10 mM glycerol-3-phosphate plus 1 μM rotenone and 10 mM glycerol-3-phosphate plus 1 mM GDP plus 1 μM rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H_2O_2 production was measured for 250 seconds, whereupon 0.5 μM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H_2O_2 production was measured for a further 250 seconds.

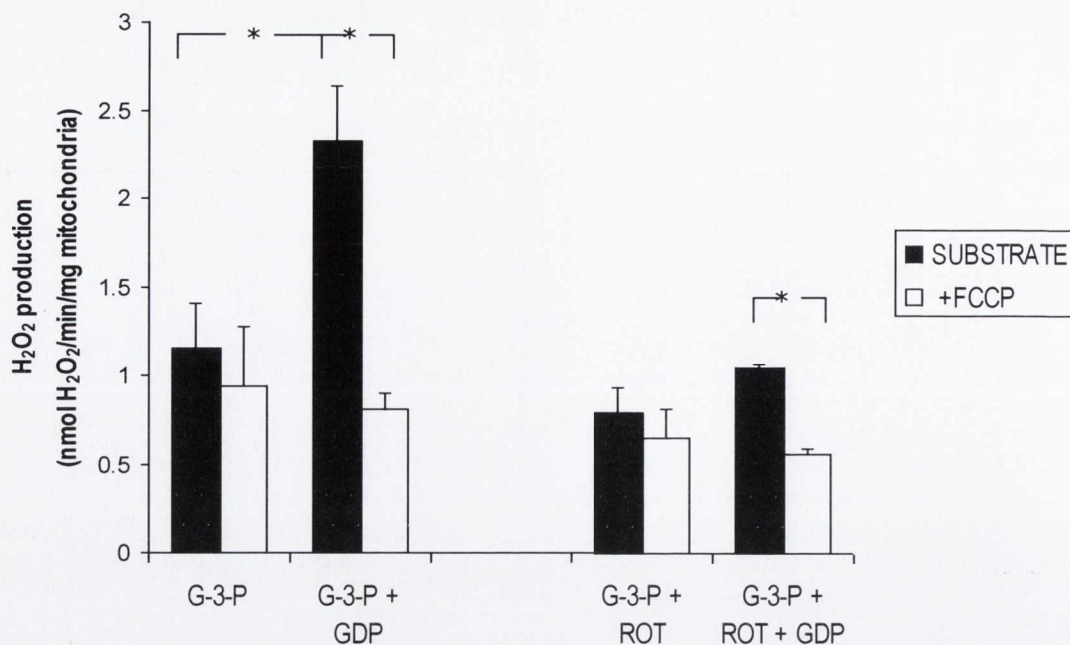


Figure 5.5 H₂O₂ production in thymus mitochondria respiring on glycerol-3-phosphate is GDP sensitive.

Thymus mitochondria were isolated as described in section 2.4 from female Wistar rats. Subsequent H₂O₂ production rates by thymus mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate, 10 mM glycerol-3-phosphate plus 1 mM GDP, 10 mM glycerol-3-phosphate plus 1 µM rotenone and 10 mM glycerol-3-phosphate plus 1 mM GDP plus 1 µM rotenone. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05).

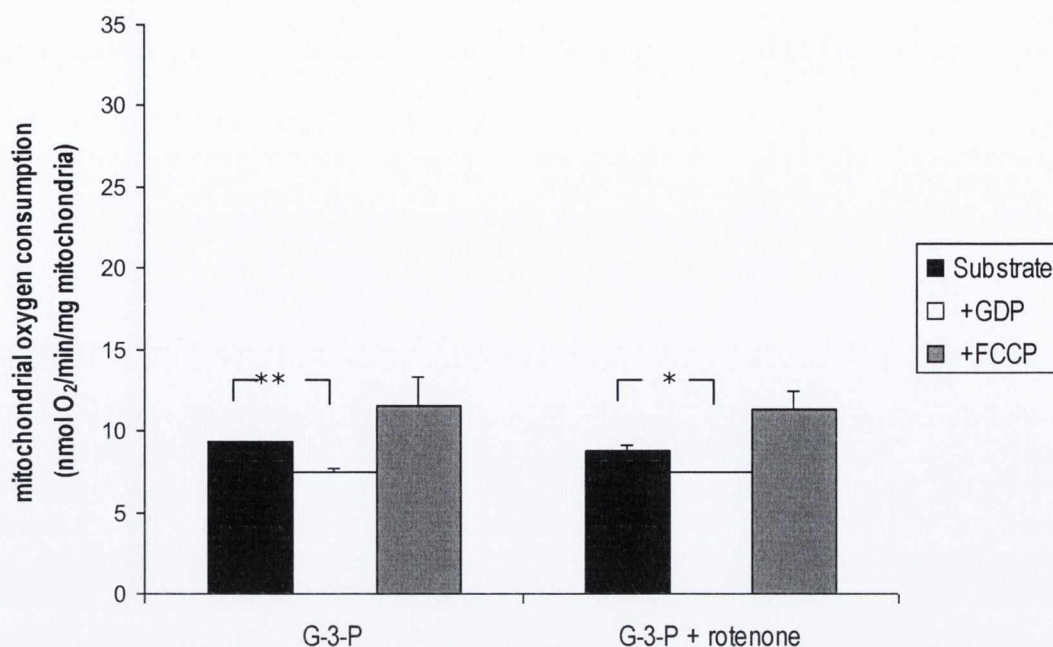


Figure 5.6 Non-phosphorylating oxygen consumption by isolated thymus mitochondria respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone is GDP sensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wistar rats. Subsequent oxygen consumption rates by thymus mitochondria (100 $\mu\text{g/ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA, 10 mM glycerol-3-phosphate plus or minus 1 μM rotenone in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate (*= $p < 0.05$; **= $p < 0.01$).

Figure 5.7 Illustrates representative traces of H_2O_2 production by thymus mitochondria respiring on glutamate plus malate (red) and glutamate plus malate plus GDP plus (blue). FCCP addition is at the 250 second mark.

Figure 5.8 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by thymus mitochondria respiring on glutamate plus malate and glutamate plus malate plus GDP. The effect of FCCP on H_2O_2 production in the presence of both substrates is also illustrated. Significantly higher H_2O_2 production by thymus mitochondria was observed by mitochondria respiring on glutamate plus malate plus GDP compared to H_2O_2 production by thymus mitochondria respiring on glutamate plus malate alone (-GDP= 0.742 ± 0.04 ; +GDP= 1.8 ± 0.232 , $p=0.0109$). Significantly lower H_2O_2 production was observed upon FCCP addition to thymus mitochondria respiring on glutamate plus malate (-FCCP= 0.742 ± 0.04 ; +FCCP= 0.29 ± 0.029 , $p=0.0008$) and respiring on glutamate plus malate plus GDP (-FCCP= 1.8 ± 0.232 ; +FCCP= 0.7 ± 0.293 , $p=0.0423$).

Figure 5.9 illustrates state 4 oxygen consumption rates (nmol O_2 /min/mg mitochondria) by thymus mitochondria isolated from female Wistar rats. No significant differences in O_2 consumption were observed upon the addition of GDP to mitochondria respiring on glutamate plus malate (-GDP= 8.3 ± 0.41 ; +GDP= 8.03 ± 0.025). O_2 consumption in the presence of FCCP was 13.381 ± 0.2 nmol O_2 /min/mg mitochondria.

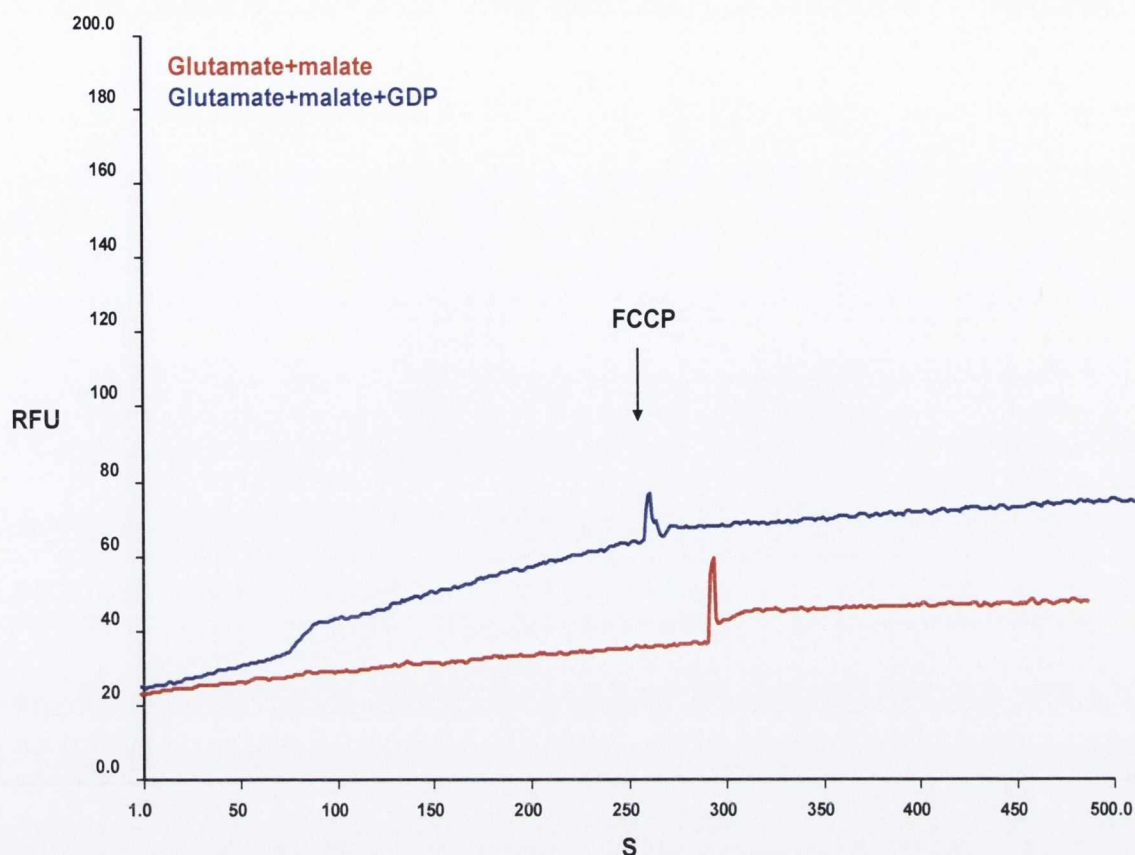


Figure 5.7 Representative trace of time course for the detection of H_2O_2 production by isolated thymus mitochondria respiring on **glutamate plus malate** and **glutamate plus malate plus GDP**.

Thymus mitochondria were isolated as described in section 2.4 from female Wistar rats. Subsequent H_2O_2 production rates by thymus mitochondria ($100\mu\text{g}/\text{ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g}/\text{ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA, 5 μM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glutamate + 6 mM malate or 10 mM glutamate + 6 mM malate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H_2O_2 production was measured for 250 seconds, whereupon 0.5 μM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H_2O_2 production was measured for a further 250 seconds.



Figure 5.8 H₂O₂ production in thymus mitochondria respiring on glutamate plus malate is GDP sensitive.

Thymus mitochondria were isolated as described in section 2.4 from female Wistar rats. Subsequent H₂O₂ production rates by thymus mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glutamate +6 mM malate or 10 mM glutamate + 6 mM malate plus 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05; ***=p<0.001).

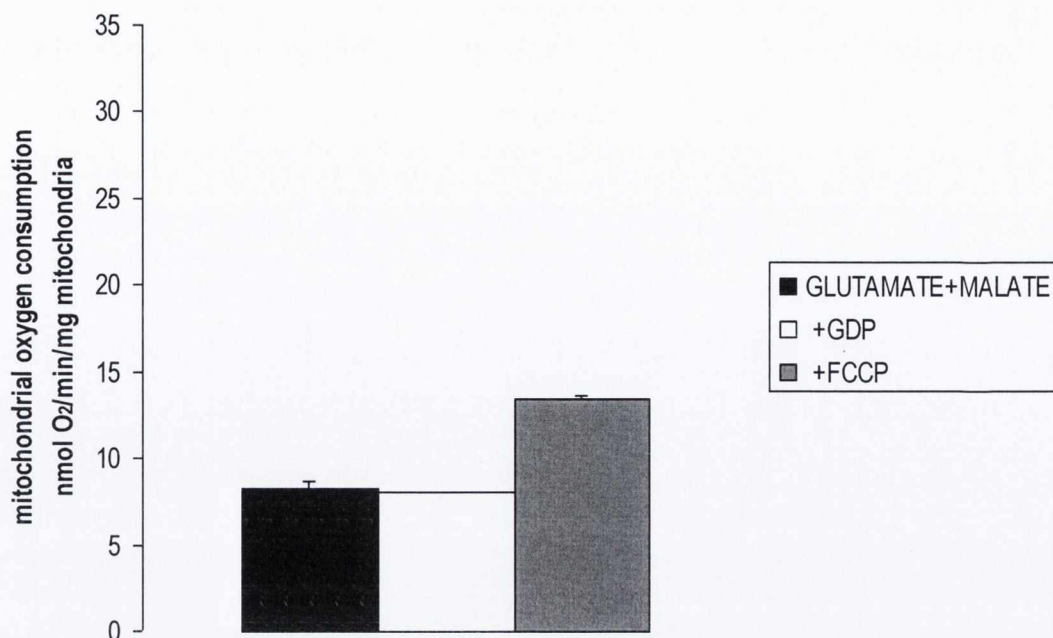


Figure 5.9 Non-phosphorylating oxygen consumption by isolated thymus mitochondria respiring on glutamate plus malate is GDP insensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wistar rats. Subsequent oxygen consumption rates by thymus mitochondria (100 μ g/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 μ g/ml oligomycin, 5 μ M atractyloside, 0.1% de-fatted BSA and 10 mM glutamate + 6 mM malate in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μ M FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate.

Figure 5.10 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by thymus mitochondria isolated from wild-type and UCP 1 knock out mice respiring on succinate, succinate plus GDP, succinate plus rotenone and succinate plus rotenone plus GDP. No significant differences in H_2O_2 production were observed by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate (WT=2.09 $\pm$ 0.22; KO=2.273 $\pm$ 0.38) or succinate plus rotenone (WT=0.75 $\pm$ 0.25; KO=1.15 $\pm$ 0.29). GDP addition did not affect H_2O_2 production by thymus mitochondria isolated from wild-type or UCP 1 knock out mice respiring on succinate (WT+GDP=1.92 $\pm$ 0.59; KO+GDP=2.2 $\pm$ 0.31) or succinate plus rotenone (WT+GDP=0.8 $\pm$ 0.27; KO+GDP=1.23 $\pm$ 0.34)

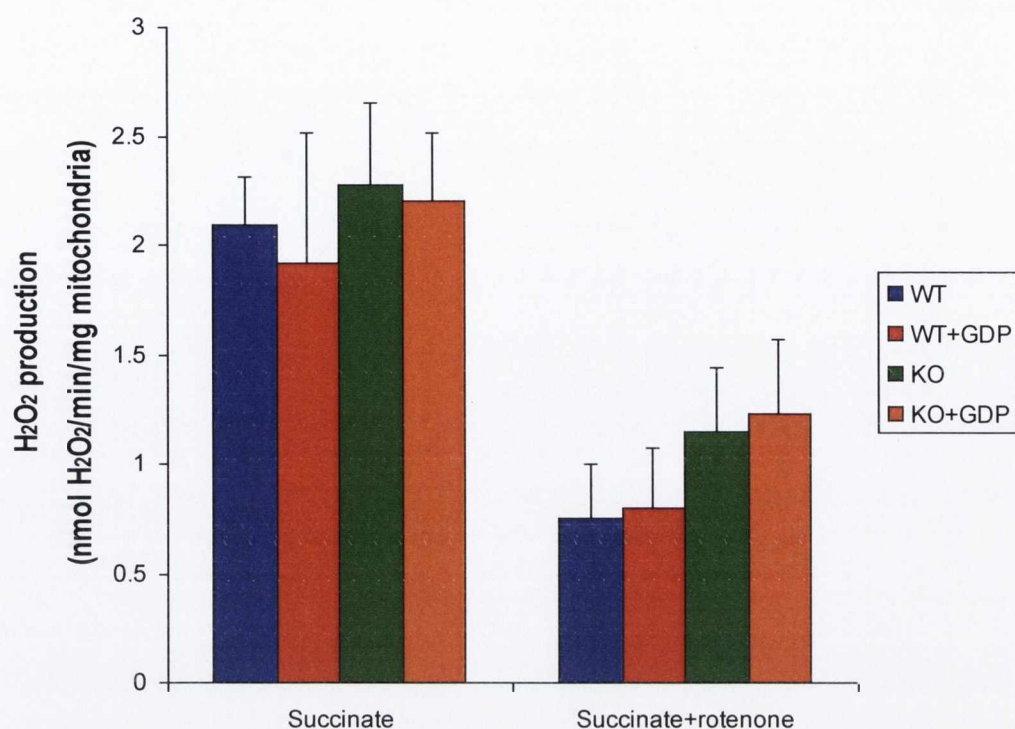


Figure 5.10 H₂O₂ production by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate is GDP insensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wild-type and UCP 1 knock-out mice. Subsequent H₂O₂ production rates by thymus mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM succinate, 10 mM succinate + 1 µM rotenone, 10 mM succinate + 1 mM GDP and 10 mM succinate + 1 µM rotenone + 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

Figure 5.11 compares non-phosphorylating O₂ consumption (nmol O₂/min/mg mitochondria) by thymus mitochondria isolated from wild-type and UCP 1 knock-out mice respiring on succinate and succinate plus rotenone. No difference in succinate supported state 2 oxygen consumption was observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out and wild-type mice (WT=24.106±3.28; KO=21.927±1.876). However significantly higher oxygen consumption was observed by thymus mitochondria isolated from wild-type mice respiring on succinate plus rotenone (WT=29.909±3.428; KO=15.629±2.43, p=0.0273). GDP addition did not affect oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on succinate (WT+GDP=27.147±4.12; KO+GDP=20.977±1.104) and succinate plus rotenone (WT=27.636±1.978; KO=14.518±2.35). No differences in state 4 uncoupled rates were observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate (WT=33.829±3.62; KO=31.183±2.748) or succinate plus rotenone (WT=28.485±3.236; KO=25.73±3.614).

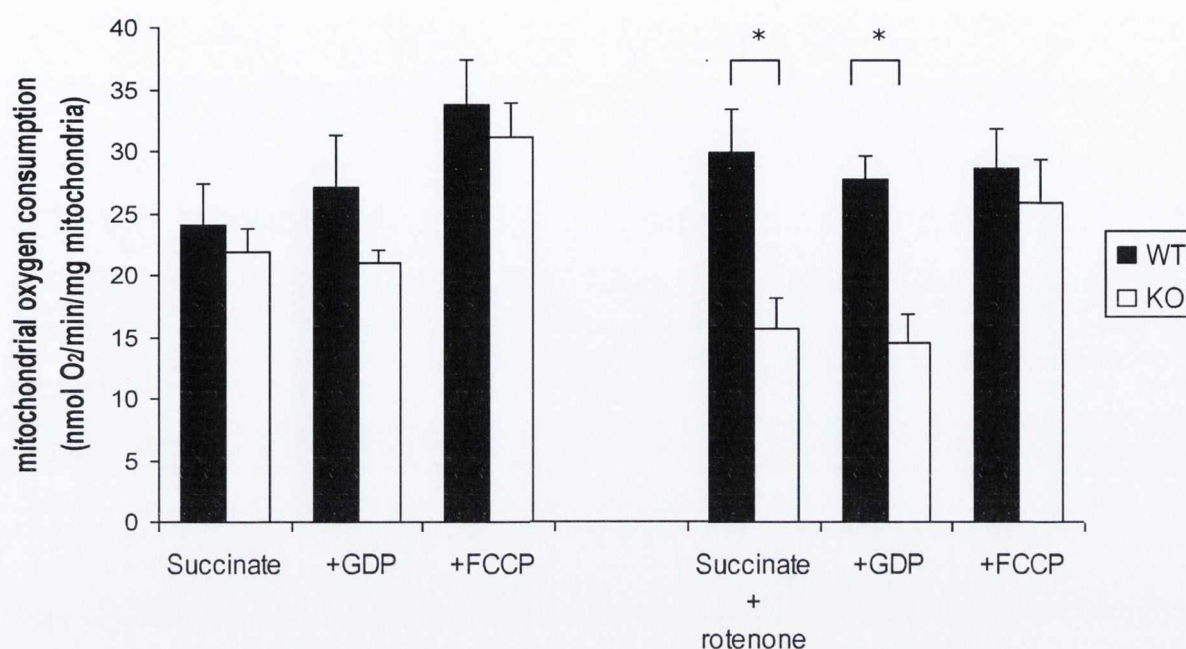


Figure 5.11 Non-phosphorylating oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate is GDP insensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wild-type and UCP 1 knock-out mice. Subsequent oxygen consumption rates by thymus mitochondria (100 µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 1µM rotenone, 0.1% de-fatted BSA and 10 mM succinate-KOH in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 µM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05).

Figure 5.12 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by thymus mitochondria isolated from wild-type and UCP 1 knock out mice respiring on glycerol-3-phosphate, glycerol-3-phosphate plus GDP, glycerol-3-phosphate plus rotenone and glycerol-3-phosphate plus rotenone plus GDP. H_2O_2 production by thymus mitochondria isolated from UCP 1 knock out mice was consistently higher compared to H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate, although not significantly higher (WT=1.54 $\pm$ 0.057; KO=1.844 $\pm$ 0.31). H_2O_2 production by thymus mitochondria isolated from UCP 1 knock out mice was significantly higher compared to H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate plus rotenone (WT=1.46 $\pm$ 0.12; KO=1.91 $\pm$ 0.033, $p=0.0224$). GDP consistently increased H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate (-GDP=1.54 $\pm$ 0.057; +GDP=2.17 $\pm$ 0.46) and glycerol-3-phosphate plus rotenone (-GDP=1.46 $\pm$ 0.12; +GDP=1.71 $\pm$ 0.46), although not significantly. GDP does not significantly increase H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out mice respiring on glycerol-3-phosphate (-GDP=1.844 $\pm$ 0.31; +GDP=1.647 $\pm$ 0.059) or glycerol-3-phosphate plus rotenone (-GDP=1.91 $\pm$ 0.033; +GDP=2.02 $\pm$ 0.13).

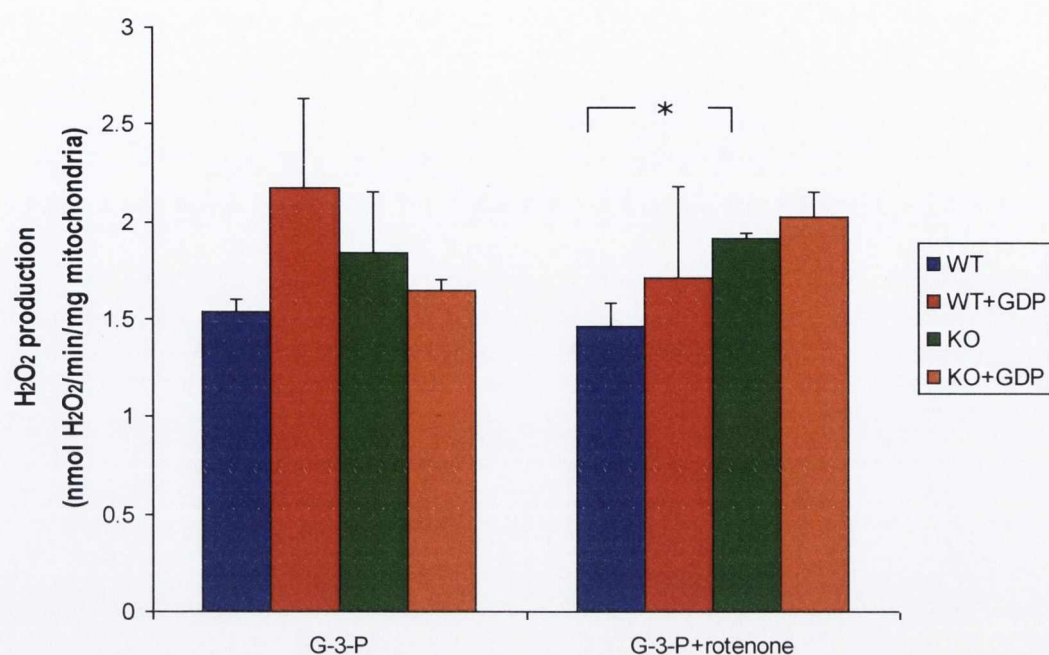


Figure 5.12 H₂O₂ production by thymus mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate and rotenone is GDP sensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wild-type and UCP 1 knock-out mice. Subsequent H₂O₂ production rates by thymus mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glycerol-3-phosphate, 10 mM glycerol-3-phosphate + 1 µM rotenone, 10mM glycerol-3-phosphate + 1 mM GDP and 10 mM glycerol-3-phosphate + 1 µM rotenone + 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate (*=p<0.05).

Figure 5.13 compares non-phosphorylating O₂ consumption (nmol O₂/min/mg mitochondria) by thymus mitochondria isolated from wild-type and UCP 1 knock-out mice respiring on glycerol-3-phosphate and glycerol-3-phosphate plus rotenone. No difference in glycerol-3-phosphate supported state 4 oxygen consumption was observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out and wild-type mice (WT=10.726±0.84; KO=13.324±1.6). No difference in glycerol-3-phosphate plus rotenone supported state 4 oxygen consumption was observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out and wild-type mice (WT=13.014±2.711; KO=10.949±1.83). GDP addition did not affect oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on glycerol-3-phosphate (WT+GDP=9.67±0.85; KO+GDP=12.65±0.47) or glycerol-3-phosphate plus rotenone (WT=17.353±2.184; KO=11.79±1.94). No differences in state 4 uncoupled rates were observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glycerol-3-phosphate (WT=16.03±1.368; KO=19.81±3.162) or glycerol-3-phosphate plus rotenone (WT=19.53±3.881; KO=18.997±3.401).

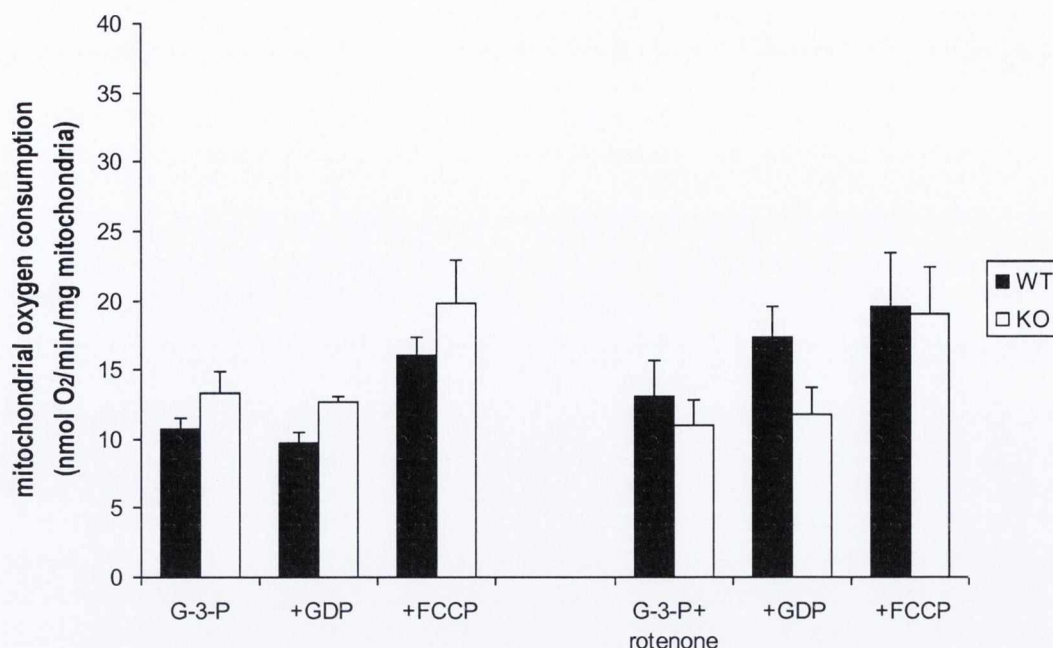


Figure 5.13 Non-phosphorylating oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glycerol-3-phosphate is GDP insensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wild-type and UCP 1 knock-out mice. Subsequent oxygen consumption rates by thymus mitochondria (100 $\mu\text{g/ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM atractyloside, 1 μM rotenone, 0.1% de-fatted BSA and 10 mM glycerol-3-phosphate in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate.

Figure 5.14 compares H_2O_2 production (nmol H_2O_2 /min/mg mitochondria) by thymus mitochondria isolated from wild-type and UCP 1 knock out mice respiring on glutamate plus malate and glutamate plus malate plus GDP. No differences in H_2O_2 production by thymus mitochondria isolated from UCP 1 knock out and wild-type mice was observed by mitochondria respiring on glutamate plus malate (WT=1.03 $\pm$ 0.12; KO=0.941 $\pm$ 0.1). GDP did not significantly affect H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on glutamate plus malate(WT+GDP=0.94 $\pm$ 0.03; KO+GDP=0.71 $\pm$ 0.12).

Figure 5.15 compares non-phosphorylating O_2 consumption (nmol O_2 /min/mg mitochondria) by thymus mitochondria isolated from wild-type and UCP 1 knock-out mice respiring on glutamate plus malate. No difference in state 4 oxygen consumption was observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out and wild-type mice (WT=12.393 $\pm$ 1.621; KO=15.492 $\pm$ 1.19). GDP addition did not affect oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice (WT+GDP=13.845 $\pm$ 2.69; KO+GDP=17.514 $\pm$ 3.06). No differences in state 4 uncoupled rates were observed (WT=16.68 $\pm$ 0.11; KO=20.64 $\pm$ 2.11)

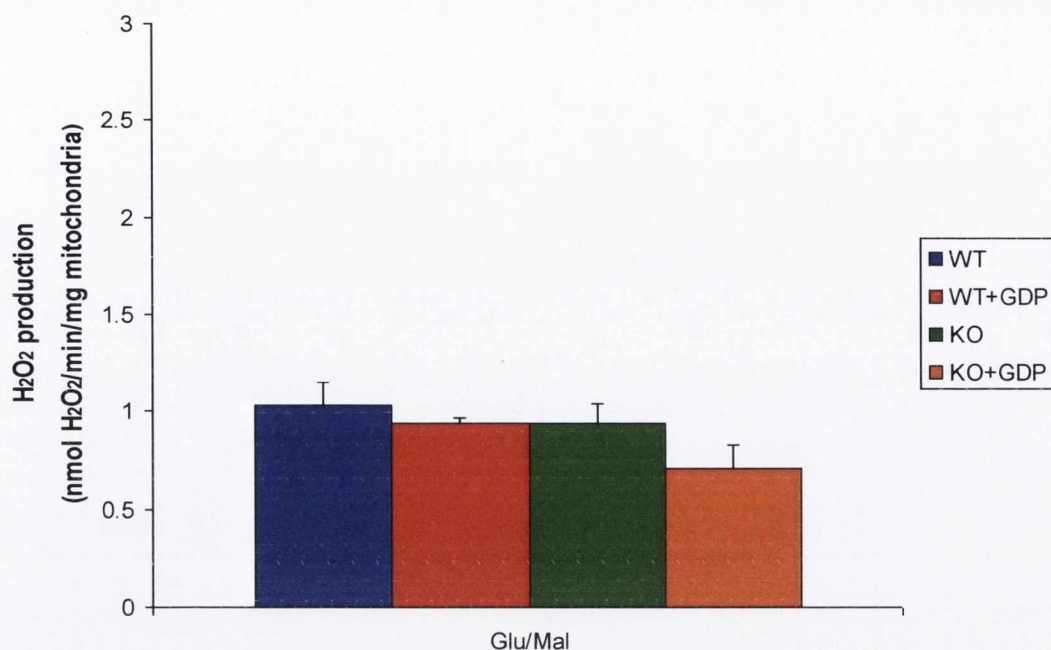


Figure 5.14 H₂O₂ production by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glutamate plus malate is UCP 1 independent.

Thymus mitochondria were isolated as described in section 2.4 from female wild-type and UCP 1 knock-out mice. Subsequent H₂O₂ production rates by thymus mitochondria (100µg/ml) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 µg/ml oligomycin, 5 µM atractyloside, 0.1% de-fatted BSA, 5 µM Amplex Red, 10 U/ml horseradish peroxidase and 30 U/ml superoxide dismutase. Substrates were 10 mM glutamate + 6 mM malate and 10 mM glutamate + 6 mM malate + 1 mM GDP. Fluorescence was detected by a Perkin Elmer LS 55 fluorometer with excitation set at 570 ± 8 nm and emission at 585 ± 4 nm. H₂O₂ production was measured for 250 seconds, whereupon 0.5 µM of the protonophore carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazine (FCCP) was added and H₂O₂ production was measured for a further 250 seconds. Fluorescence was calibrated using known amounts of H₂O₂ each experimental day. Data is expressed as mean ± S.E.M. of at least 3 experiments, each experiment performed in triplicate.

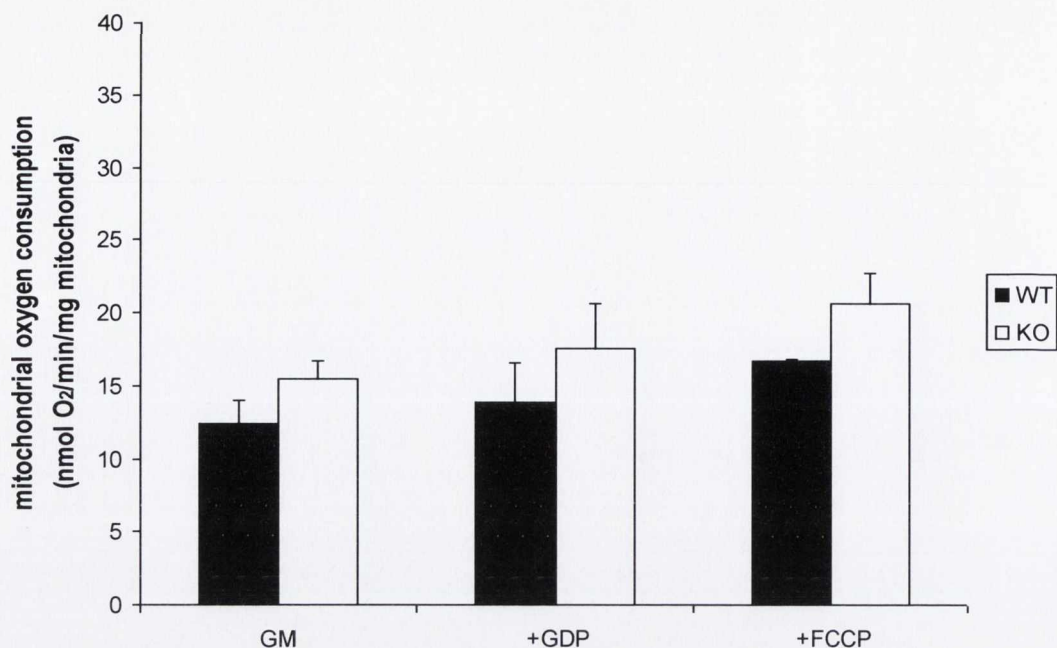


Figure 5.15 Non-phosphorylating oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glutamate plus malate is GDP insensitive.

Thymus mitochondria were isolated as described in section 2.4 from female wild-type and UCP 1 knock-out mice. Subsequent oxygen consumption rates by thymus mitochondria (100 $\mu\text{g/ml}$) were determined at 37°C in the presence of 120 mM KCl, 5 mM Hepes-KOH pH 7.4, 1 mM EGTA, 1 $\mu\text{g/ml}$ oligomycin, 5 μM atractyloside, 0.1% de-fatted BSA and 10 mM glutamate + 6 mM malate in a pre-calibrated Oroboros Oxygraph. Steady-state oxygen consumption rates were then obtained. 1 mM GDP was added to inhibit UCP 1. Finally 0.25 μM FCCP was titrated to achieve the state 4 uncoupled rate. Data is expressed as mean $\pm$ S.E.M. of at least 3 experiments, each experiment performed in triplicate.

[5.3] Discussion

In this chapter we set out to determine whether UCP 1 in thymus mitochondria could play a role in the attenuation of ROS production by thymus mitochondria. In chapter 3 we presented and discussed results which showed that UCP 1 in BAT mitochondria can attenuate ROS production by the respiratory chain. This is a new role for UCP 1 in BAT mitochondria, so therefore such a role for UCP 1 in thymus mitochondria warrants investigation.

Figures 5.1 (raw data) and 5.2 (collated data) illustrates H_2O_2 production by non-phosphorylating thymus mitochondria isolated from Wistar rats respiring on succinate alone (forward and reverse electron transport) and succinate plus rotenone (forward electron transport only). No significant difference in H_2O_2 production was observed upon addition of GDP to mitochondria respiring on succinate alone. There was consistently more H_2O_2 production by thymus mitochondria respiring on succinate plus rotenone in the presence of GDP, compared to H_2O_2 production in the absence of GDP, but this difference was not significant.

This contrasts with BAT mitochondria where GDP significantly increased H_2O_2 production by BAT mitochondria respiring on succinate alone and succinate plus rotenone, with the observed H_2O_2 increase in the absence of rotenone greater than the increase in the presence of rotenone. This indicates that although UCP 1 in succinate supported BAT mitochondrial respiration is able to attenuate H_2O_2 production due to reverse electron transport and to a lesser extent due to forward electron transport, UCP 1 in succinate supported thymus mitochondrial respiration is unable to attenuate H_2O_2 production due to reverse electron transport and can attenuate only a small amount of H_2O_2 production due to forward electron transport which is insignificant.

Addition of the protonophore FCCP significantly decreased H_2O_2 production by thymus mitochondria respiring on succinate

alone, succinate plus GDP, succinate plus rotenone and succinate plus rotenone plus GDP. This indicates that uncoupling of succinate supported thymus mitochondrial respiration can attenuate H_2O_2 production, and suggests that UCP 1 in thymus mitochondria does not uncouple the mitochondria in the presence of succinate.

The presence of rotenone reduced the rate of H_2O_2 production by thymus mitochondria respiring under all substrate combinations. This suggests that H_2O_2 production by thymus mitochondria due to reverse electron transport occurs both when UCP 1 is active and when it is inhibited. This is in contrast to H_2O_2 production by BAT mitochondria respiring on succinate, where rotenone addition only decreased H_2O_2 production in the presence of GDP indicating that H_2O_2 production due to reverse electron transport did not occur when UCP 1 was active.

Non-phosphorylating oxygen consumption rates were determined in parallel with the H_2O_2 production measurements presented in figure 5.2

Figure 5.3 illustrates non-phosphorylating oxygen consumption by thymus mitochondria respiring on succinate alone and succinate plus rotenone. GDP inhibited oxygen consumption only in the presence of rotenone. No GDP inhibition of oxygen consumption was observed in the absence of rotenone. This endorses our H_2O_2 production data presented in figure 5.2 where GDP addition increased H_2O_2 production only in the presence of rotenone.

It can be extrapolated from this data that inhibition of UCP 1 by GDP in isolated thymus mitochondria respiring on succinate can increase the membrane potential and thereby increase H_2O_2 production by forward electron transport but not by reverse electron transport.

However it is evident from our oxygen consumption measurements that UCP 1 in thymus mitochondria does not completely uncouple the mitochondria as occurs in BAT

mitochondria. The state 4 uncoupled rates are greater than the state 4 non-phosphorylating rates indicating that thymus mitochondria are not completely uncoupled in the presence of substrate.

We can conclude from our H_2O_2 and O_2 consumption measurements that UCP 1 in succinate supported thymus mitochondrial respiration isolated from rats cannot attenuate H_2O_2 production due to reverse electron transport, and may be able to attenuate H_2O_2 production due to forward electron transport, but this attenuation appears to be insignificant.

This may be due to the degree of uncoupling that UCP 1 in thymus mitochondria can achieve. UCP 1 protein levels in thymus mitochondria are 10-fold lower than UCP 1 protein levels in BAT mitochondria (unpublished data by Adams and Porter, 2011) so therefore the level of uncoupling in thymus mitochondria would be significantly less and by extrapolation so would its H_2O_2 attenuation potential.

Figures 5.4 (raw data) and 5.5 (collated data) illustrates H_2O_2 production by non-phosphorylating thymus mitochondria isolated from Wistar rats respiring on glycerol-3-phosphate alone (forward and reverse electron transport) and glycerol-3-phosphate plus rotenone (forward electron transport). GDP addition significantly increased H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate alone. The presence of rotenone all but abolished this GDP sensitive increase, with only a small non-significant increase observed. This contrasts with BAT mitochondria where rotenone had no significant effect on H_2O_2 production by BAT mitochondria respiring on glycerol-3-phosphate.

FCCP addition significantly decreased H_2O_2 production only in the presence of GDP. No significant decrease was observed by thymus mitochondria respiring on glycerol-3-phosphate alone or

glycerol-3-phosphate plus rotenone. The lack of an effect of FCCP on glycerol-3-phosphate supported H_2O_2 production was also observed in BAT mitochondria.

Non-phosphorylating oxygen consumption rates were determined in parallel with the H_2O_2 production measurements. Figure 5.6 illustrates non-phosphorylating oxygen consumption by thymus mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone. GDP significantly decreased oxygen consumption both in the presence and absence of rotenone. This correlates with our H_2O_2 measurements in figure 5.5 where GDP addition increased H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate.

From our H_2O_2 measurements, it would be expected that the decrease in oxygen consumption in the absence of rotenone would be greater than the decrease in the presence of rotenone. The apparent decrease in oxygen consumption due to increased coupling of the respiratory chain may be masked by the increase in H_2O_2 production which occurs in parallel.

This decrease in oxygen consumption is due to inhibition of UCP 1 and thus coupling of the mitochondria and correlates with a significant increase in H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate alone and a non-significant increase in thymus mitochondria respiring on glycerol-3-phosphate plus rotenone.

Addition of the protonophore FCCP increases oxygen consumption by BAT mitochondria respiring on glycerol-3-phosphate both in the absence and the presence of rotenone, indicating that the decrease in H_2O_2 production observed upon FCCP addition correlates with an uncoupled state.

The oxygen consumption data presented in figure 5.6 verifies that the observed increase in H_2O_2 production upon the addition of GDP correlates with coupling of the mitochondria, and the decrease in H_2O_2 production upon FCCP addition correlates with uncoupling of

the mitochondria.

We can conclude from our H_2O_2 and O_2 consumption measurements that UCP 1 in glycerol-3-phosphate supported thymus mitochondria isolated from rats is able to attenuate H_2O_2 production due to reverse electron transport, and may be able to attenuate H_2O_2 production due to forward electron transport, but this attenuation appears to be insignificant.

Greater attenuation of H_2O_2 production by UCP 1 in glycerol-3-phosphate supported thymus mitochondria compared to attenuation of H_2O_2 production in succinate supported thymus mitochondria contrasts with H_2O_2 attenuation by UCP 1 in BAT mitochondria, which is greater in succinate supported mitochondria compared to glycerol-3-phosphate supported mitochondria.

This difference may be due to the down regulation of glycerol-3-phosphate dependent ROS production in BAT mitochondria compared to glycerol-3-phosphate dependent ROS production in other tissues with much lower content of mitochondrial glycerol-3-phosphate dehydrogenase (Mracek et al., 2009).

mGPDH levels in thymus mitochondria were not quantified in this study but from examination of O_2 consumption by thymus mitochondria respiring on glycerol-3-phosphate (Fig 5.6), it can be estimated that mGPDH levels are ~ 10 times lower than mGPDH levels in BAT mitochondria.

If the observation by Mracek et al., (2009) holds true for mGDPH in thymus mitochondria, then ROS production relative to specific activity of mGPDH in thymus mitochondria would be greater than ROS production relative to specific activity of mGPDH in BAT mitochondria. This may explain why attenuation of glycerol-3-phosphate supported ROS production compared to succinate supported ROS production is greater in thymus mitochondria compared to BAT mitochondria.

The H_2O_2 production by isolated thymus mitochondria in the presence of glutamate plus malate, providers of complex I substrate was measured to determine whether UCP 1 can play a role in the attenuation of H_2O_2 production due to forward electron transfer from complex I in thymus mitochondria.

Figures 5.7(raw data) and 5.8 (collated data) illustrate that H_2O_2 production by thymus mitochondria respiring on glutamate plus malate is GDP sensitive. FCCP addition significantly decreased H_2O_2 production in the presence and absence of GDP. This contrasts with BAT mitochondria respiring on complex I substrates, where H_2O_2 production was GDP insensitive and where FCCP addition did not significantly decrease H_2O_2 production.

Non-phosphorylating oxygen consumption rates were determined in parallel with the H_2O_2 production measurements presented in figure 5.8.

Figure 5.9 illustrates that non-phosphorylating oxygen consumption by thymus mitochondria respiring on glutamate plus malate is not decreased by the addition of GDP. This does not correlate with the observed increase in H_2O_2 production by thymus mitochondria respiring under the same conditions. As has been suggested in this discussion before, the expected decrease in oxygen consumption upon GDP addition may be masked by the large increase in H_2O_2 production which was observed during GDP inhibition of UCP 1.

FCCP addition increases oxygen consumption which correlates with our observed decrease in H_2O_2 production.

We can conclude from our H_2O_2 and O_2 consumption measurements that UCP 1 is able to attenuate H_2O_2 production in thymus mitochondria respiring on complex I substrates glutamate plus malate. This is in contrast to UCP 1 in BAT mitochondria which has no significant effect on H_2O_2 production by complex I

substrates. H_2O_2 production by thymus mitochondria respiring on complex I substrates are significantly decreased by addition of the protonophore FCCP, which illustrates that H_2O_2 production by thymus mitochondria respiring on complex I substrates are sensitive to uncoupling.

H_2O_2 production by BAT mitochondria respiring on complex I substrates however are not significantly affected by the protonophore FCCP, which illustrates that H_2O_2 production by BAT mitochondria respiring on complex I substrates are insensitive to uncoupling. This differential in sensitivity to uncoupling may explain the difference in the ability of UCP 1 to attenuate H_2O_2 production in both tissues.

Determination of UCP 1 dependent H_2O_2 production by comparing H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice.

To corroborate our data so far presented in this chapter using isolated thymus mitochondria from rats, H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out mice was compared to H_2O_2 production by thymus mitochondria isolated from wild-type mice.

Like our study using thymus mitochondria isolated from rats, ROS production due to reverse and forward electron transport was examined using glutamate plus malate, succinate plus and minus rotenone and glycerol-3-phosphate plus and minus rotenone as substrates. Any UCP 1-sensitive ROS production was investigated by comparing the ROS production in thymus mitochondria isolated from UCP 1 knock-out and wild-type mice.

If the GDP-sensitive ROS production observed in thymus mitochondria isolated from rats is due to inhibition of UCP 1, then it would be expected that thymus mitochondria isolated from UCP 1 knock-out mice would have greater ROS production compared to

thymus mitochondria isolated from wild-type mice. Furthermore in order to determine that the GDP-sensitive ROS production observed in thymus mitochondria isolated from rats was in fact due to UCP 1 inhibition, GDP-sensitive ROS production was investigated in thymus mitochondria isolated from UCP 1 knock-out and wild-type mice.

Figure 5.10 illustrates H_2O_2 production by non-phosphorylating thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on succinate alone and succinate plus rotenone. The data presented here illustrates that H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out mice is greater than H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on succinate in the presence of rotenone. However this difference is not statistically significant. This difference was all but abolished in the absence of rotenone.

No GDP sensitive H_2O_2 production is observed in thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on succinate alone or succinate plus rotenone.

Non-phosphorylating oxygen consumption rates were determined in parallel with H_2O_2 production measurements presented in figure 5.10. Figure 5.11 shows that there is no difference in succinate supported non-phosphorylating, GDP sensitive or state 4 uncoupled oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice.

Significantly higher succinate plus rotenone supported non-phosphorylating oxygen consumption and GDP sensitive oxygen consumption was observed in thymus mitochondria isolated from wild-type mice compared to thymus mitochondria isolated from UCP 1 knock-out mice. No difference in the state 4 uncoupled oxygen

consumption rates were observed by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice. This indicates that there is no difference in the capacity of the respiratory chain in thymus mitochondria from UCP 1 knock-out or wild-type mice, and UCP 1 is responsible for the higher state 4 non-phosphorylating oxygen consumption in thymus wild-type mitochondria.

GDP did not decrease oxygen consumption by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on succinate alone or succinate plus rotenone. This correlates with our H_2O_2 production data in figure 5.10 where GDP had no effect on H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice.

The lack of a GDP effect in the thymus mitochondria from wild-type mice however does not correlate with our observation in thymus mitochondria from rats where GDP significantly decreased oxygen consumption and increased H_2O_2 production in thymus mitochondria respiring on succinate plus rotenone.

However the higher oxygen consumption and lower H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on succinate plus rotenone suggests that UCP 1 can attenuate H_2O_2 production and increase oxygen consumption by thymus mitochondria isolated from mice respiring on succinate plus rotenone.

Figure 5.12 illustrates H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone. It can be observed that H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out mice is greater compared to H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on glycerol-3-phosphate and significantly greater by mitochondria respiring on glycerol-3-phosphate plus rotenone.

GDP increased H_2O_2 production by thymus mitochondria isolated from wild-type mice but not from thymus mitochondria isolated from UCP 1 knock-out mice, although this increase was not statistically significant. This correlates with our H_2O_2 measurements using rat mitochondria and indicates that the GDP sensitive H_2O_2 production that we observed in thymus mitochondria isolated from rats respiring on glycerol-3-phosphate is due to inhibition of UCP 1. Similar to our rat H_2O_2 data, GDP had a greater effect in the absence of rotenone.

Non-phosphorylating oxygen consumption rates were determined in parallel with H_2O_2 production measurements presented in figure 5.12. No significant differences in non-phosphorylating state 4, GDP sensitive or uncoupled state 4 oxygen consumption was observed in a comparison of thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on glycerol-3-phosphate alone or glycerol-3-phosphate plus rotenone.

GDP did not decrease oxygen consumption in thymus mitochondria isolated from UCP 1 knock-out or wild-type mice. The absence of a GDP effect in thymus mitochondria from wild-type mice however does not correlate with our observation in thymus mitochondria from rats where GDP significantly decreased oxygen consumption and increased H_2O_2 production by thymus mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone.

The H_2O_2 production by isolated thymus mitochondria isolated from UCP 1 knock-out and wild-type mice in the presence of complex I substrates glutamate plus malate was also measured and the data is presented in figure 5.14. No difference in H_2O_2 production was observed in H_2O_2 production rates by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glutamate plus malate. GDP did not affect H_2O_2 production by thymus mitochondria isolated from UCP 1 knock-out

or wild-type mice. This contrasts with our H_2O_2 production data using thymus mitochondria isolated from rats where GDP significantly increased H_2O_2 production by thymus mitochondria respiring on glutamate plus malate.

Non-phosphorylating oxygen consumption was measured in parallel to the H_2O_2 production presented in figure 5.14. Figure 5.15 shows that no significant difference in non-phosphorylating state 4, GDP sensitive or state 4 uncoupled oxygen consumption was observed by thymus mitochondria isolated from UCP 1 knock-out and wild-type mice respiring on glutamate plus malate. However all oxygen consumption parameters measured were consistently higher by mitochondria isolated from UCP 1 knock-out mice. GDP did not decrease oxygen consumption in thymus mitochondria isolated from UCP 1 knock-out or wild-type mice. This is consistent with our oxygen consumption measurements by thymus mitochondria isolated from rats respiring on glutamate plus malate, where GDP had no effect on oxygen consumption.

Our H_2O_2 and O_2 consumption measurements in thymus mitochondria from UCP 1 knock-out and wild-type mice do not allow us to completely corroborate our data obtained from thymus mitochondria isolated from rats. H_2O_2 production is higher in thymus mitochondria isolated from UCP 1 knock-out mice compared to H_2O_2 production by thymus mitochondria isolated from wild-type mice respiring on succinate plus rotenone and glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone. This suggests that ablation of UCP 1 in thymus mitochondria increases H_2O_2 production in the presence of these substrates.

However UCP 1 in thymus mitochondria isolated from wild-type mice appears to be GDP insensitive. This contrasts with thymus mitochondria isolated from rats, where both H_2O_2 production and O_2 consumption were sensitive to UCP 1.

GDP had no observable effect on H_2O_2 production or O_2

consumption by thymus mitochondria isolated from wild-type mice respiring on succinate or succinate plus rotenone.

GDP increased H_2O_2 production in wild-type mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone, but this increase was not significant and had no effect on O_2 consumption by thymus mitochondria isolated from wild-type mitochondria respiring on glycerol-3-phosphate alone and glycerol-3-phosphate plus rotenone.

No difference in H_2O_2 production by thymus mitochondria respiring on glutamate plus malate, isolated from UCP 1 knock-out and wild-type mice was observed and GDP did not increase H_2O_2 production by thymus mitochondria isolated from wild-type mice. This suggests that UCP 1 cannot attenuate H_2O_2 production by thymus mitochondria respiring on glutamate plus malate.

This is supported by oxygen consumption measurements which show that there is no difference in O_2 consumption by thymus mitochondria isolated from UCP 1 knock-out or wild-type mice respiring on glutamate plus malate. It was also observed that GDP did not decrease O_2 consumption by thymus mitochondria isolated from wild-type mice.

This data suggests that ablation of UCP1 increases H_2O_2 production by thymus mitochondria isolated from mice respiring on succinate plus rotenone and glycerol-3-phosphate plus or minus rotenone. However inhibition of UCP 1 in wild-type mice by GDP does not allow us to corroborate the effect of ablation.

Purine nucleotide binding may be stronger in mice compared to rats, and our isolation procedure for thymus mitochondria may not unmask the binding sites.

Unlike UCP 1 in BAT mitochondria, a role for UCP 1 in thymus mitochondria in the attenuation of ROS production is not clear. The data obtained from rat mitochondria suggests a role for UCP 1 in thymus mitochondria in the attenuation of ROS. This is supported by the data obtained from the ablation of UCP 1 in mice which resulted in an increase in H_2O_2 production compared to the wild-type control. Corroboration of our GDP effect in rat mitochondria, by a comparison of the GDP effect in wild-type and UCP 1 knock-out thymus mitochondria however does not clarify that the GDP sensitive H_2O_2 in rat mitochondria was due to inhibition of UCP 1.

However the H_2O_2 production data from UCP 1 knock-out and wild-type mice do indicate that UCP 1 plays a role in the attenuation of H_2O_2 production in thymus mitochondria isolated from mice. The GDP sensitive H_2O_2 production in thymus mitochondria from rats indicate that UCP 1 plays a role in the attenuation of H_2O_2 production in thymus mitochondria isolated from rats.

The discrepancy in GDP sensitivity of thymus mitochondria from rats and mice may be due to different states of UCP 1 activity in thymus mitochondria from different species.

The level of H_2O_2 attenuation observed in rats and between UCP 1 knock-out and wild-type mice however is never as significant as the attenuation observed by UCP 1 in BAT mitochondria in chapter 3 and so a physiological role for UCP 1 in ROS attenuation in thymus mitochondria is not readily apparent.

To clarify whether UCP 1 in thymus mitochondria has a role in the attenuation of ROS production, the experiments presented in this chapter should be repeated in thymus mitochondria isolated from proliferating thymocytes.

Thymocyte activation results in a metabolic switch from a resting mode to a proliferative mode of energy metabolism (Fox *et al.* 2005). It has been shown by our group that stimulation of proliferation in thymocytes can increase the amount of UCP 1 protein in thymus mitochondria 2 fold (Adams and Porter, 2011

unpublished).

The thymus mitochondria used in the study presented in this chapter are isolated from unchallenged mice, and therefore the thymocytes are non-proliferating. The uncoupling effect of UCP 1 in proliferating cells may be increased by the increase in amount of UCP 1 protein, and therefore have a more significant effect on ROS production.

UCP 1 in thymus mitochondria may not be required to attenuate ROS production in non-proliferating cells due to the low oxidative capacity of the cells. However when stimulated to proliferate, thymocytes dramatically increase their glucose uptake, due to need for increased building blocks and energy required for proliferation. ROS production is reduced in proliferating thymocytes, and this is attributed mainly to a switch from oxidative phosphorylation to glycolysis. However, oxidative phosphorylation activity does increase in proliferating cells, mainly through allosteric activation of metabolic enzymes by Ca^{2+} (Brookes *et al.* 2004).

This activation of oxidative phosphorylation could increase the rate of ROS production. An increase in UCP 1 protein expression in thymus mitochondria of proliferating cells may be involved in the attenuation of any putative increase in ROS production by mitochondria during proliferation.

By investigating ROS production in thymus mitochondria isolated from proliferating cells, any putative role for UCP 1 in ROS attenuation may be clarified, and a physiological role for UCP 1 in thymus may be determined.

Chapter 6 A role for the ubiquitin-proteasome degradation system in the turnover of UCP 1

[6.1] Introduction

Post-translational modification of mitochondrial proteins is now emerging as an important means of controlling the diverse range of cellular processes which mitochondria are involved in (Boneh 2006; Pagliarini and Dixon 2006; Salvi *et al.* 2005). Our laboratory has recently shown that UCP 1 in BAT mitochondria is phosphorylated on serine 51 (Carroll *et al.* 2008) and that the proportion of phosphoserine on UCP1, purified from cold-acclimated rats, was significantly greater than that on UCP 1 from rats kept at room temperature.

Covalent modification by phosphorylation has been identified as a marker for ubiquitination (Myung *et al.* 2001). The purpose of serine phosphorylation of UCP 1 is unknown.

Until recently the mitochondrion was considered a ubiquitination-free organelle (Bota and Davies 2002). However recent reports published suggest that ubiquitination may play an important role in mitochondrial protein quality control (Margineantu *et al.* 2007; Radke *et al.* 2008).

Recently the cytosolic proteasome has been implicated in the turnover of uncoupling proteins 2 and 3 (UCP 2 and 3) (Azzu and Brand, 2010; Azzu *et al.*, 2010). In these reports by the Brand lab it was shown that inhibition of the cytosolic proteasome blocked UCP 2 and UCP 3 degradation. A reconstituted, cell-free system to study proteasome dependent degradation of UCP 2 and 3 was also used to show that the cytosolic proteasome is capable of degrading both

proteins with a half-life similar to the half-life observed in cyclohexamide treated cells. Furthermore a role for mitochondrial membrane potential was implicated in the unfolding and degradation of inner-membrane mitochondrial proteins in these studies.

With this in mind we decided to investigate whether UCP 1 could be ubiquitinated and if so, what the purpose of this covalent modification was.

[6.2] Results:

Figure 6.1 illustrates the detection of UCP 1 in BAT mitochondria following incubation at 37 °C in a ubiquitin conjugating system. The immunoblot shows that after 12 hours at 37 °C, UCP 1 (32kDa) can be detected at higher molecular weights (45-120 kDa) under denaturing conditions.

Figure 6.2 illustrates the immunoprecipitation of UCP 1 using an anti-ubiquitin antibody and the immunoprecipitation of ubiquitinated proteins using an anti-UCP 1 antibody from freshly isolated mitochondria from BAT and thymus tissue. The immunoblots illustrate that UCP 1 reactive bands can be detected in anti-ubiquitin immunoprecipitates from thymus mitochondria (lane 1) and BAT mitochondria (lane 2). It also illustrates that ubiquitin reactive bands can be detected in anti-UCP 1 immunoprecipitates from thymus mitochondria (lane 3) and BAT mitochondria (lane 5). An immunoprecipitation using an anti-ANT antibody in thymus and BAT mitochondria shows that ubiquitinated ANT is not detectable in BAT mitochondria (lane 6), but present in small amounts in thymus mitochondria (lane 4).

Figure 6.3 illustrates an immunoblot of cell lysates obtained from mature brown adipocytes which were treated with the proteasome inhibitor MG132 and as a control DMSO (a) proteasome inhibitor treatment of mature brown adipocytes increases the amount of ubiquitinated proteins present in cell lysates and (b) UCP 1 protein levels are sensitive to proteasome inhibition.

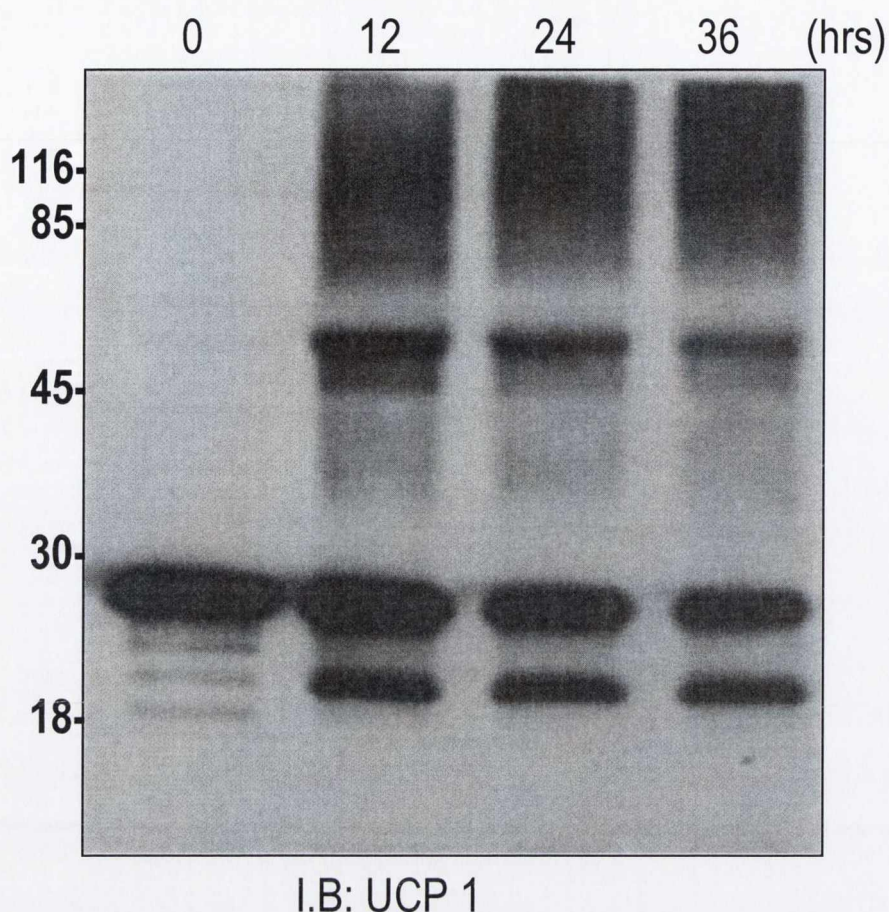


Figure 6.1 UCP 1 with a molecular weight >32 kDa can be detected in BAT mitochondria incubated with a ubiquitin conjugating system.

BAT mitochondria were isolated from Wistar rats as described in section 2.3 BAT mitochondria were resuspended to 500 µg/ml and incubated at 37° C in modified STE buffer containing an ATP regenerating system (1 mM ATP, 10 mM phosphocreatine and 20 U/ml creatine kinase), ubiquitin mix (100 µg ubiquitin, 1.4 µg fraction 1 and 1.4 µg fraction 2 from Calbiochem cat#662096) as described in Azzu and Brand, 2010. Samples (5µg) were taken at 0, 12, 24 and 36 hours and subjected to SDS-PAGE as described in section 2.6. Western blotting was performed as described in section 2.8 using an anti-UCP 1 antibody (1/500, Sigma, cat#U6382) for immunodetection.

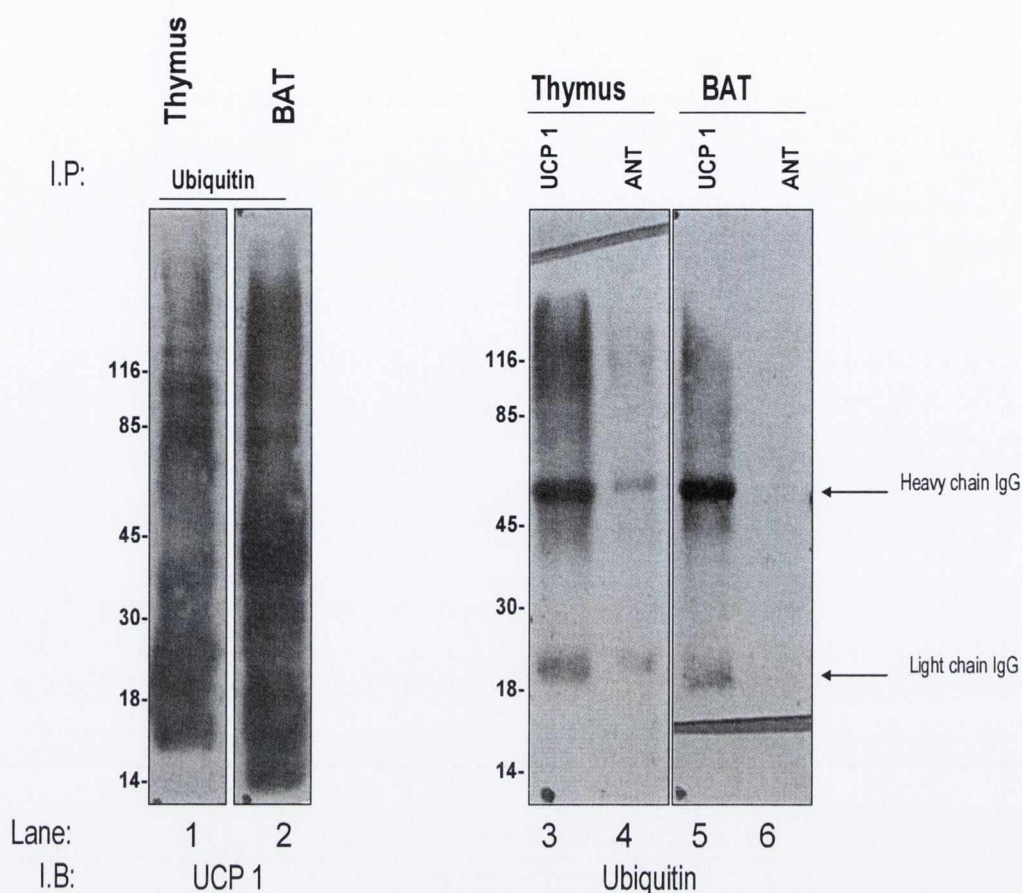


Figure 6.2 Immunoblots illustrating the immunoprecipitation of UCP 1 using an anti-ubiquitin antibody and the immunoprecipitaion of ubiquitinated proteins using an anti-UCP 1 antibody.

BAT and thymus mitochondria were isolated from Wistar rats as described in sections 2.3 and 2.4. Immunoprecipitations were performed as described in section 2.5 using anti-UCP 1 (5 μ g, Sigma, cat#U6382), anti-Ubiquitin (5 μ g, Santa Cruz, cat#sc-8017) and anti-ANT (5 μ g, Santa Cruz, cat#H188), as indicated. Immunoprecipitates were subjected to SDS-PAGE as described in section 2.6 and western blotting as described in section 2.8 using anti-UCP 1 (1/500, Sigma, cat#U6382) and anti-ubiquitin (1/500, Santa Cruz, cat#sc-8017) antibodies for immunodetection as indicated.

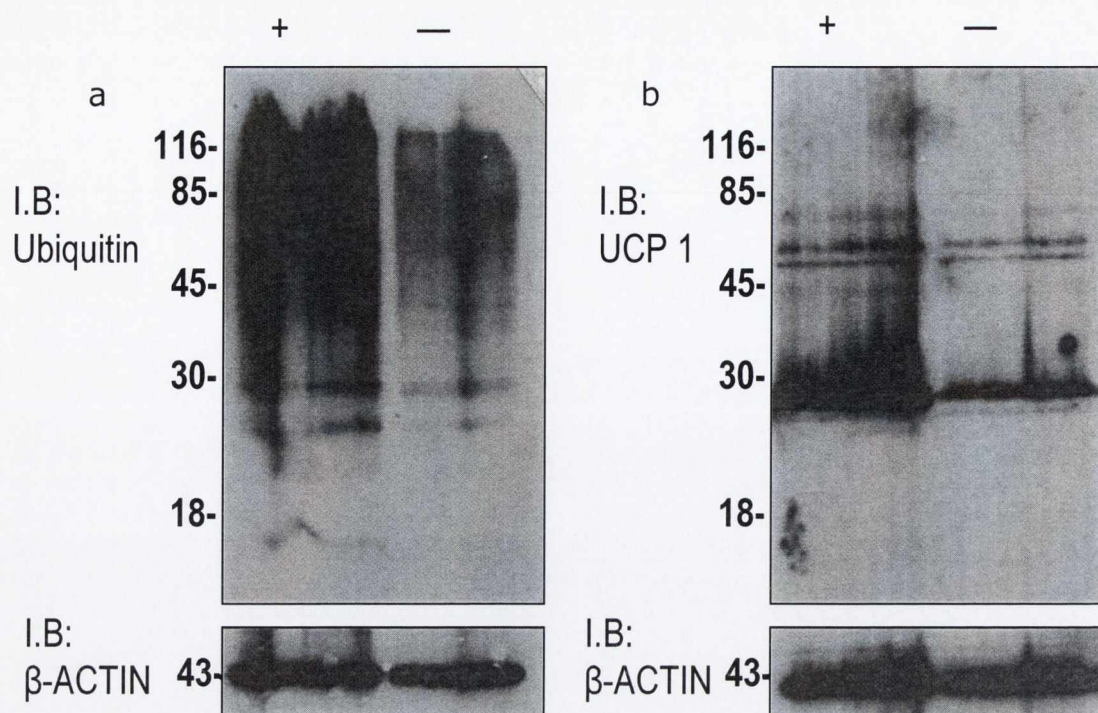


Figure 6.3 UCP 1 protein levels in mature brown adipocytes are sensitive to proteasome inhibition.

Brown adipocytes were isolated and differentiated as described in section 2.22 From C57BL6 mice. Mature brown adipocytes were treated with the proteasome inhibitor MG132 (30 μ M) (+) or the vehicle DMSO (-) for 12 hours. Cell lysates were collected and subjected to SDS-PAGE and western blotting using anti-UCP 1 (1/500, Sigma, cat#U6382), anti- β -Actin (1/1000, Millipore, cat#MAB1501) and anti-ubiquitin (1/500, Santa Cruz, cat#sc-8017) for immunodetection as indicated.

Figure 6.4 illustrates the detection of UCP 1 protein by immunoblotting in isolated BAT mitochondria incubated at 37 °C in STE buffer containing an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. The immunoblot illustrates that UCP 1 protein is stable in isolated BAT mitochondria incubated at 37 °C for 48 hours.

Figure 6.5 (a) illustrates the degradation of UCP 1 in BAT mitochondria in the presence of different components of an in vitro reconstituted ubiquitin-proteasome system between 0 and 48 hours. Where indicated 20 mM succinate, ubiquitin mix (35 µg ubiquitin, 1.4 µg fraction 1, 1.4 µg fraction 2 from Calbiochem cat. #662096) or 5 µg 26S proteasome fraction were added to the assay along with an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. The data indicates that greater degradation of UCP 1 at 48 hours occurs when succinate, ubiquitin mix and proteasome are all present together in the assay. The degradation of UCP 1 is decreased when any one of the three components are missing. (c) Illustrates representative immunoblots from the experiments described in (a). (b) illustrates a comparison of the degradation of UCP 1 in BAT mitochondria between 0 and 48 hours in an in vitro reconstituted ubiquitin-proteasome system consisting of the previously described ATP regenerating system plus succinate, ubiquitin mix and purified 26S proteasome plus or minus FCCP. The data illustrates that FCCP decreases the degradation of UCP 1 at 48 hours in this reconstituted in vitro system. (d) illustrates representative immunoblots from the experiments described in (b). All values are means ± S.E.M. (n=3), corrected for loading (ponceau stained membranes). All statistical significances were determined by repeated measures ANOVA (comparison of matching time points) with Dunnett's post-testing.

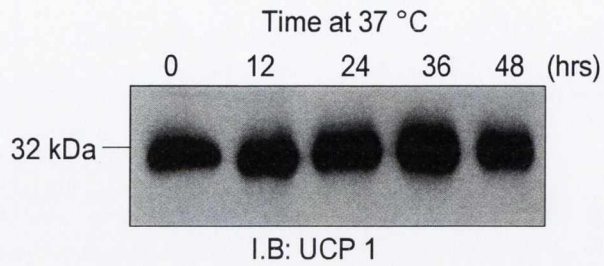


Figure 6.4 UCP 1 protein levels are stable in isolated BAT mitochondria incubated at 37 °C for 48 hours.

BAT mitochondria were isolated as described in section 2.3 Freshly isolated mitochondria were incubated at 37 °C in STE buffer plus an ATP regenerating system consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. At specified time points, samples were taken, boiled in 2X gel loading buffer and subjected to SDS-PAGE and western blotting using a UCP 1 antibody (1/500, Sigma) for immunodetection.

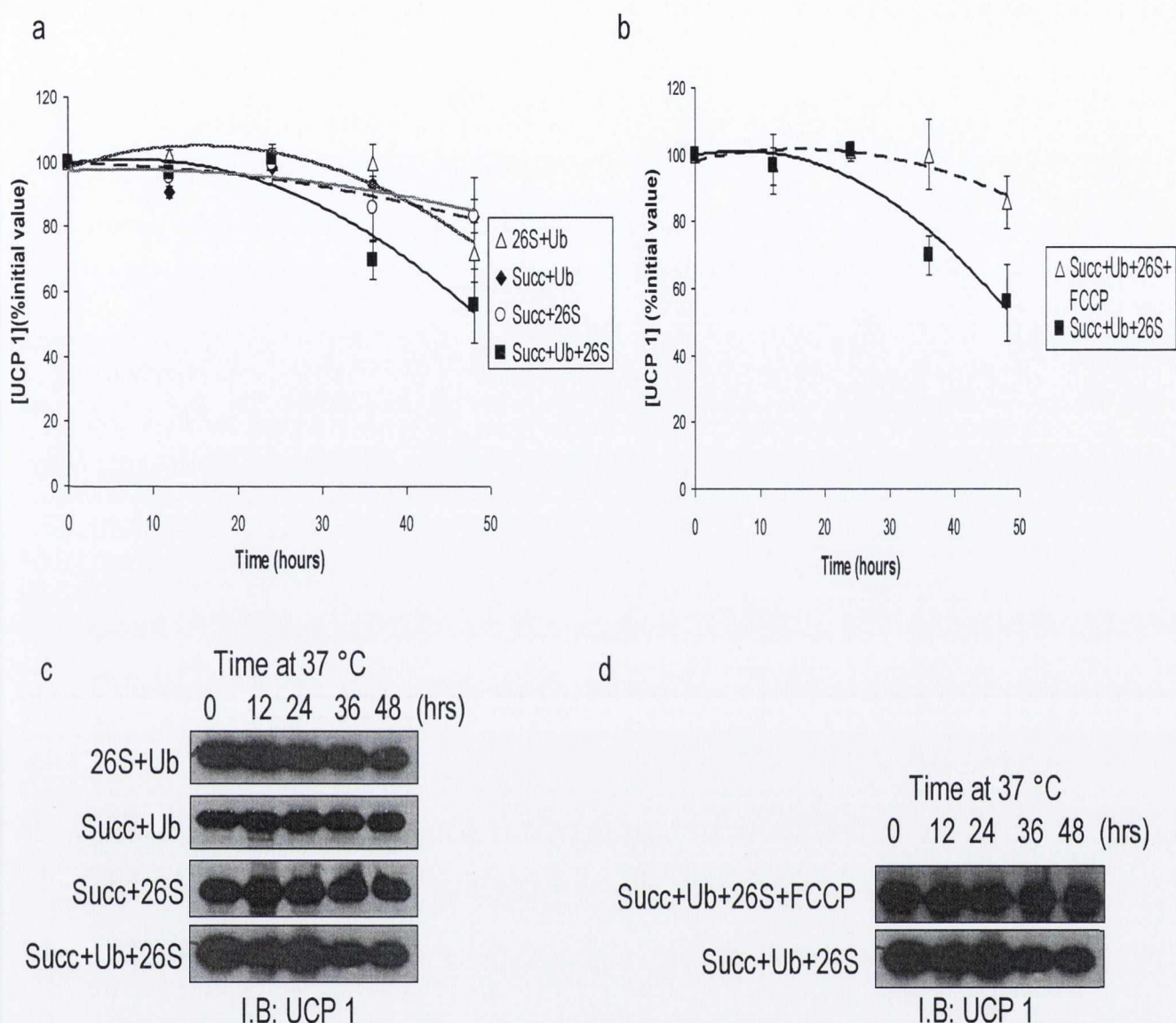


Figure 6.5 Reconstitution of UCP 1 degradation in brown adipose tissue mitochondria by a ubiquitin-proteasome system.

BAT mitochondria were isolated as described in section 2.3. An in vitro degradation assay for UCP1 was carried out essentially as described by Azzu and Brand, 2010 and in section 2.20. For each assay BAT mitochondria (500 µg/ml) were incubated with an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. Where indicated 20 mM succinate, 20 µM FCCP, ubiquitin mix (35 µg ubiquitin, 1.4 µg fraction 1, 1.4 µg fraction 2 from Calbiochem cat. #662096) and 5 µg 26S proteasome fraction were added to the assay. Mitochondria were incubated at 37 °C and samples (10 µg) were removed at the relevant time point, subjected to SDS-PAGE and western blotting using an anti-UCP 1 antibody (1/500, Sigma) for immunodetection. Densitometry was performed using scion imaging to determine relative intensity at each time point. (a) illustrates a comparison of UCP 1 degradation in the presence of the indicated components of a ubiquitin-proteasome degradation system and (c) illustrates typical immunoblots from each assay. (b) illustrates a comparison of UCP 1 degradation by a reconstituted ubiquitin-proteasome degradation system in the presence and absence of FCCP and (d) illustrates typical immunoblots in the presence and absence of FCCP.

Figure 6.6 illustrates the detection of UCP 1 protein by immunoblotting in isolated thymus mitochondria incubated at 37 °C in STE buffer containing an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. The immunoblot illustrates that UCP 1 protein is stable in isolated thymus mitochondria incubated at 37 °C for 8 hours.

Figure 6.7 (a) illustrates the degradation of UCP 1 in thymus mitochondria in the presence of different components of an in vitro reconstituted ubiquitin-proteasome system between 0 and 8 hours. Where indicated 20 mM succinate, ubiquitin mix (35 µg ubiquitin, 1.4 µg fraction 1, 1.4 µg fraction 2 from Calbiochem cat. #662096) or 5 µg 26S proteasome fraction were added to the assay along with an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. The data indicates that significantly greater degradation of UCP 1 at 8 hours occurs when succinate, ubiquitin mix and proteasome are all present together in the assay. The degradation of UCP 1 is decreased significantly when any one of the three components are missing. (c) Illustrates representative immunoblots from the experiments described in (a). (b) Illustrates a comparison of the degradation of UCP 1 in thymus mitochondria between 0 and 8 hours in an in vitro reconstituted ubiquitin-proteasome system consisting of the previously described ATP regenerating system plus succinate, ubiquitin mix and purified 26S proteasome plus or minus FCCP. The data illustrates that FCCP decreases significantly the degradation of UCP 1 at 8 hours in this reconstituted in vitro system. (d) Illustrates representative immunoblots from the experiments described in (b). All values are means ± S.E.M. (n=3), corrected for loading (ponceau stained membranes). All statistical significances were determined by repeated measures ANOVA (comparison of matching time points) with Dunnett's post- testing (**=p<0.01).

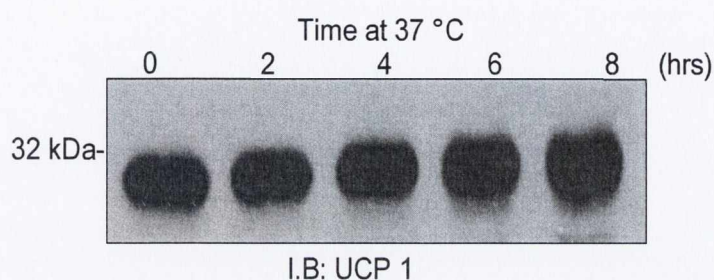


Figure 6.6 UCP 1 protein levels are stable in isolated thymus mitochondria incubated at 37 °C for 8 hours.

Thymus mitochondria were isolated as described in section 2.4. Freshly isolated mitochondria were incubated at 37 °C in STE buffer plus an ATP regenerating system consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. At specified time points, samples were taken, boiled in 2X gel loading buffer and subjected to SDS-PAGE and western blotting using a UCP 1 antibody (1/500, Sigma) for immunodetection.

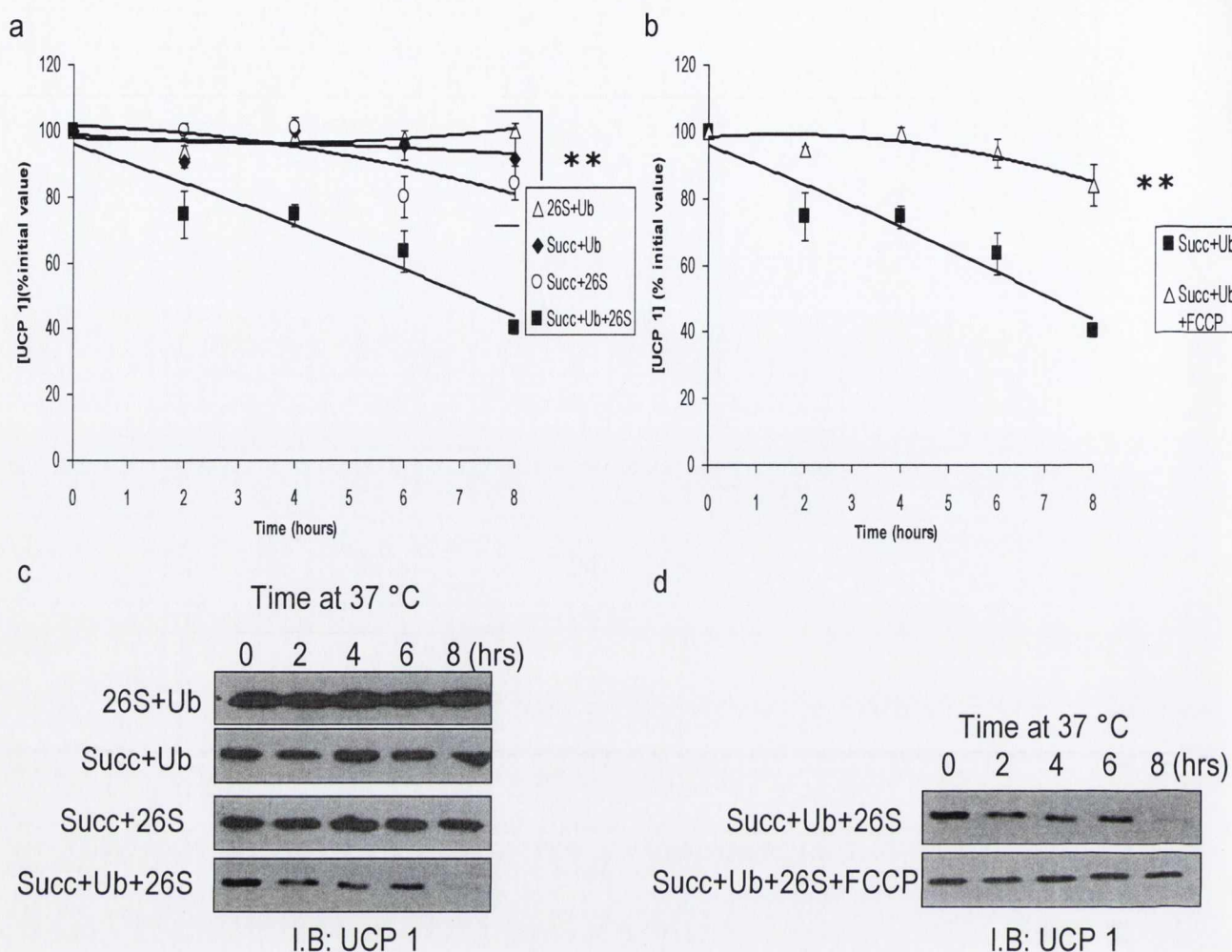


Figure 6.7 Reconstitution of UCP 1 degradation in thymus mitochondria by a ubiquitin-proteasome system.

Thymus mitochondria were isolated as described in section 2.4. An *in vitro* degradation assay for UCP1 was carried out essentially as described by Azzu and Brand, 2010 and in section 2.20. For each assay thymus mitochondria (1 mg/ml) were incubated with an ATP regenerating system, consisting of 1 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine kinase, 2 mM MgCl₂ and 0.1% defatted bovine serum albumin. Where indicated 20 mM succinate, 20 μM FCCP, ubiquitin mix (35 μg ubiquitin, 1.4 μg fraction 1, 1.4 μg fraction 2 from Calbiochem cat. #662096) and 5 μg 26S proteasome fraction were added to the assay. Mitochondria were incubated at 37 °C and samples (10 μg) were removed at the relevant time point, subjected to SDS-PAGE and western blotting using an anti-UCP 1 antibody (1/500, Sigma) for immunodetection. Densitometry was performed using scion imaging to determine relative intensity at each time point. (a) illustrates a comparison of UCP 1 degradation in the presence of the indicated components of a ubiquitin-proteasome degradation system and (c) illustrates typical immunoblots from each assay. (b) illustrates a comparison of UCP 1 degradation by a reconstituted ubiquitin-proteasome degradation system in the presence and absence of FCCP and (d) illustrates typical immunoblots in the presence and absence of FCCP.

Figure 6.8 compares the detection of UCP 1 in BAT mitochondria isolated from cold-acclimated rats and rats housed at room temperature by SDS-PAGE and western blotting. The immunoblot illustrated shows that UCP 1 with a molecular weight >32 kDa is detected in greater abundance in BAT mitochondria isolated from cold-acclimated rats compared to the abundance of UCP 1 with a molecular weight >32 kDa in BAT mitochondria isolated from rats housed at room temperature. As expected UCP 1 detected at 32 kDa is also in greater abundance in BAT mitochondria isolated from cold-acclimated rats. The loading control, pyruvate dehydrogenase- α -E1, shows that equal amounts of BAT mitochondria were used in the blot.

Figure 6.9 illustrates a colloidal Comassie Blue G-250 one dimensional stained SDS-PAGE gel of samples obtained from a UCP 1 immunoprecipitation performed with BAT mitochondria. Where indicated bands were excised and subjected to in-gel digestion with trypsin, prior to LC MS/MS analysis.

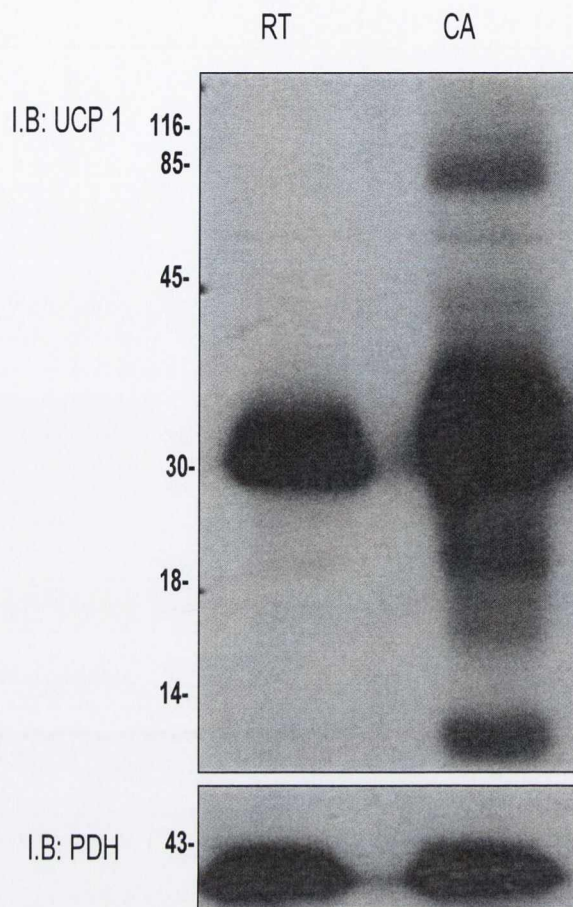


Figure 6.8 BAT mitochondria isolated from cold acclimated rats contain greater amounts of UCP 1 with a molecular weight >32 kDa compared to BAT mitochondria isolated from rats housed at room temperature.

BAT mitochondria were isolated from cold acclimated rats and rats housed at room temperature as described in section 2.3 Mitochondria were subjected to SDS-PAGE as described in section 2.6 followed by western blotting as described in section 2.8 Using anti-UCP 1 (1/500, Sigma) and anti-PDH-E1-alpha (1/500, Mitosciences, cat#MSPO3) for immunodetection as indicated.

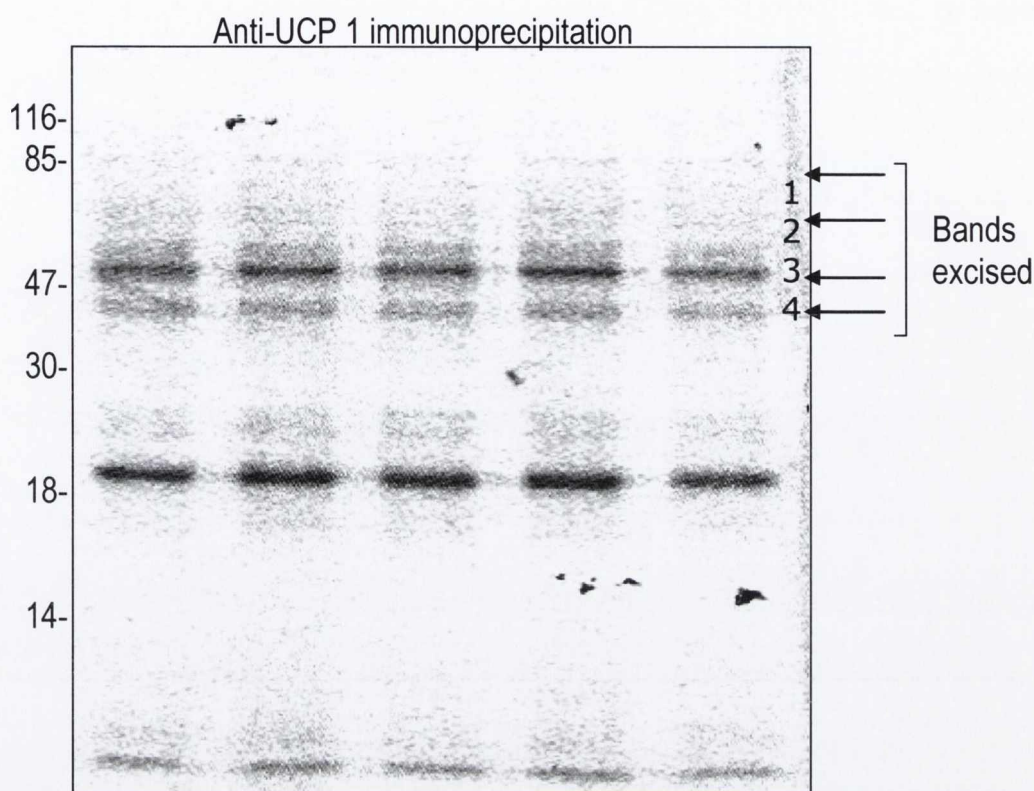


Figure 6.9 Colloidal Coomassie Blue R-250 stained SDS-PAGE gel of anti-UCP 1 immunoprecipitation

BAT mitochondria were isolated as described in section 2.3 An immunoprecipitation was performed with the resulting BAT mitochondria as described in section 2.5 using anti-UCP 1(5 μ g, Sigma). The resulting pull-down was subjected to SDS-PAGE. The gel was stained in Coomassie R-250. The indicated bands were excised and subjected to in-gel trypsinisation prior to mass spectrometric analysis.

Sample:	Sequence:	Residue No's:	
1	K.LYSGLPAGIQR.Q	74-84	12 % coverage of UCP 1
	R.VIATTESLSTLWK.G	163-175	
	R.IGLYDTVQEYFSSGR.E	93-107	
2	R.VIATTESLSTLWK.G	163-175	9% coverage of UCP 1
	R.IGLYDTVQEYFSSGR.E	93-107	
3	R.MQAQSHLHGKPR.Y	141-153	9% coverage of UCP 1
	R.IGLYDTVQEYFSSGR.E	93-107	
4	R.YKGVLTITTLAK.T	55-67	13% coverage of UCP 1
	R.VIATTESLSTLWK.G	163-175	
	R.IGLYDTVQEYFSSGR.E	93-107	

Table 6.10 Sequences of UCP 1 peptides identified in the excised bands as indicated in figure 6.2.9. Data was obtained from Dr. Terry W. Pearson at the University of Victoria, Canada.

[6.3] Discussion

The data presented in this chapter provide evidence that UCP1 is ubiquitinated *in vivo*. Our data presented in Fig. 6.1 of this chapter shows that UCP 1 in isolated BAT mitochondria has the potential to be ubiquitinated when incubated with purified ubiquitin and a ubiquitin conjugating system, as detected by immunoblotting.

The use of a western blotting technique which enhances the detection of ubiquitinated proteins (described in section 2.8) aided in the detection of any putative ubiquitinated UCP 1. The protocol for western blotting used here omits methanol from the western blotting process and uses a low voltage for a long period of time. This is followed by fixation of the membranes. By using this process small amounts of BAT mitochondria were used to detect putative ubiquitinated UCP 1, which would most likely be stoichiometrically lower when compared to unmodified UCP 1.

By using small amounts of protein in the SDS-PAGE and western blotting process we reduced the chance of unspecific reactivity which might occur if large amounts of sample were used in order to detect any putative ubiquitinated UCP 1. Our results demonstrate that UCP1 which has a molecular mass of 32kDa, was detectable at higher molecular masses (45-120kDa) on a denaturing gel, following incubation of brown adipose tissue mitochondria in the ubiquitination system *in vitro*, thus providing evidence of the potential of UCP1 to be (poly) ubiquitinated.

In light of the fact that UCP1 has the potential to be ubiquitinated *in vitro*, we set out to determine whether UCP1 was ubiquitinated *in vivo*. Figure 6.2 illustrates that UCP 1 can be immunoprecipitated by an anti-ubiquitin antibody from freshly isolated mitochondria from both BAT and thymus tissues (lanes 1 and 2). The reciprocal immunoprecipitation of UCP 1 followed by

immunodetection of ubiquitin, illustrates that ubiquitinated proteins can be immunoprecipitated by an anti-UCP 1 antibody in both BAT mitochondria and thymus mitochondria (lanes 3 and 5). The immunoreactive bands detected in the UCP 1 immunoprecipitation correspond to the bands detected in BAT mitochondria incubated with the ubiquitin conjugating system (figure 6.1). The detection of putative polyubiquitinated UCP 1 in freshly isolated BAT mitochondria suggests that the putative polyubiquitinated UCP 1 detected after incubating BAT mitochondria with a ubiquitin conjugating system is not due to an artefact of the system used in figure 6.1.

Immunoprecipitation using an adenine nucleotide translocator (ANT) antibody was also performed on BAT and thymus mitochondria followed by immunodetection of ubiquitinated proteins (lanes 4 and 6) due to the similarity in structure and location of ANT to UCP 1. It can be seen that only small amounts of ANT appear to be ubiquitinated in thymus mitochondria, and none can be detected in BAT mitochondria. This suggests that ubiquitination may not be a general feature of mitochondrial carriers.

One of the main functions of ubiquitination is to target the protein for degradation by the 26S proteasome (Ciechanover et al., 1980; Hershko and Ciechanover, 1998). To test whether polyubiquitination of UCP 1 could be linked to degradation by the proteasome, we pharmacologically inhibited the cytosolic 26S proteasome in mature brown adipocytes and measured, by immunodetection the amount of UCP 1 in the resulting cell lysates. Figure 6.3 shows that (a) inhibition of the 26S proteasome by MG132 increases the amount of ubiquitinated proteins present in cell lysates of mature brown adipocytes treated with MG132, compared to control lysates from mature brown adipocytes treated with just DMSO and (b) shows that the amount of UCP 1 present in cells treated with MG132 is greater compared to the amount of UCP 1 in cells treated with just DMSO. MG132 is a very potent

proteasome inhibitor but may also inhibit mitochondrial Lon protease (Granot *et al.* 2003). Due to this the more specific proteasome inhibitor epoxomicin (Meng *et al.* 1999) was also used to study the effect of proteasome inhibition on UCP 1 degradation. Mature brown adipocytes however were not viable after 12 hours of epoxomicin treatment and so the levels of UCP 1 protein were not possible to determine accurately. The half life of UCP 1 in BAT mitochondria is relatively long with a range of half-lives reported in the literature from 20 hours for acute adrenergically stimulated brown adipocytes and 72 hours for chronic adrenergically stimulated brown adipocytes (Puigserver *et al.* 1992) to 30 hours reported for UCP 1 expressed in CHO cells (Rousset *et al.* 2007). Due to this long half-life, sufficient incubation time with a proteasome inhibitor is needed to ensure that adequate turnover of UCP 1 has occurred, in order to determine whether proteasome inhibition has had any effect.

(Azzu *et al.* 2008) reported that UCP 2 was stable in isolated mitochondria, and from this implied that degradation of UCP 2 occurred outside the mitochondria, or at least the signal for degradation came from outside the mitochondria. In subsequent reports they provided evidence which suggested that the degradation of UCP 2 and UCP 3 may be proteasome dependent (Azzu and Brand, 2010; Azzu *et al.*, 2010). Based on these findings we decided to investigate whether UCP 1 in isolated BAT and thymus mitochondria are stable when incubated at 37 °C.

Figure 6.2.4 illustrates that UCP 1 protein in isolated BAT mitochondria is stable when incubated at 37 °C in STE buffer for 48 hours, suggesting that (a) UCP 1 degradation occurs outside the mitochondria, (b) the signal for its degradation comes from outside the mitochondria or (c) the protease involved in its degradation is located in the cytosol.

Following on from this we decided to investigate whether UCP 1 in isolated mitochondria can be degraded in the

presence of a reconstituted in-vitro ubiquitin-proteasome degradation system. The system used consisted of an ATP regenerating system (creatine kinase, phosphocreatine, ATP, $MgCl_2$ and defatted BSA), purified ubiquitin, ubiquitin conjugating enzymes, purified 26S proteasome and succinate for substrate supply to the respiratory chain (Azzu and Brand, 2010; Azzu et al., 2010).

Figure 6.5a illustrates that UCP 1 in isolated BAT mitochondria can be degraded in the presence of a reconstituted in-vitro proteasome degradation system and an ATP regenerating system. Omission of any of the components reduces the degradation of UCP 1, but not significantly. The presence of the protonophore FCCP also reduces the degradation of UCP 1 in the in-vitro proteasome degradation system (figure 6.5b).

Figure 6.6 illustrates that UCP 1 in isolated thymus mitochondria is stable when incubated at 37 °C for 8 hours. It has been shown by our laboratory that the half-life of UCP 1 in thymus mitochondria is between 3.5 and 5 hours (Adams et al., 2011, in preparation), a half-life much shorter than the 20-72 hour half-life of UCP 1 in BAT mitochondria (Rousset et al., 2007; Puigserver et al., 1992). For this reason thymus mitochondria were only incubated for 8 hours at 37 °C. The immunoblot in figure 6.6 shows that UCP 1 protein in isolated mitochondria is stable for up to 8 hours at 37 °C.

Figure 6.7a illustrates that UCP 1 in isolated thymus mitochondria can be degraded in the presence of a reconstituted in-vitro proteasome degradation system and an ATP regenerating system. Omission of any of the components of the in-vitro system significantly reduces the degradation of UCP 1 in isolated thymus mitochondria. Addition of the protonophore FCCP also significantly reduces UCP 1 degradation in the in-vitro proteasome degradation system (figure 6.7b).

The ability of a reconstituted in-vitro proteasome degradation system to degrade UCP 1, which we have shown here to be stable in isolated mitochondria (figures 6.4 and 6.6), suggests that the proteasome may be involved in the degradation of UCP 1 in both BAT and thymus mitochondria. This is supported by our finding that inhibition of the proteasome in brown adipocytes, increases the level of UCP 1 protein present in the cells (figure 6.3).

Differences however are evident between the degradation rate of UCP 1 in BAT and thymus mitochondria. The dramatic difference in the half-life of UCP 1 in both tissues is perhaps due to the function and physiology of the two different cells. The function of UCP 1 in both tissues may also contribute to the difference in half-life. The function of UCP 1 in the thymus, which is currently under investigation, may require a more dynamic turnover rate than the adaptive uncoupling activity function of UCP 1 in BAT mitochondria.

The degradation of UCP 1 in isolated mitochondria by the reconstituted in-vitro ubiquitin-proteasome degradation system used in this study mirrors the degradation of UCP 1 in cells; degradation of UCP 1 in BAT mitochondria was slow, with degradation only evident after 24 hours, but relatively fast in thymus, with degradation evident after 2 hours.

Apart from the degradation time other differences in the degradation of UCP 1 in the reconstituted in-vitro proteasome degradation system exist between BAT and thymus mitochondria. While the presence of a full proteasome degradation system resulted in a greater degradation rate of UCP 1 in BAT mitochondria compared to a system with any one of the components missing, this difference was not significant (figure 6.5a). In contrast a full proteasome degradation system was needed for degradation of UCP 1 in thymus mitochondria, and omission of any component significantly reduced degradation of UCP 1 (figure 6.7a). Also the presence of the protonophore FCCP inhibited the degradation of UCP 1 in both BAT and thymus mitochondria in the ubiquitin-proteasome

degradation system, but only significantly in thymus mitochondria (figures 6.5b and 6.7b).

The difference in the effect of FCCP on the degradation of UCP 1 in BAT and thymus mitochondria may be due to the length of time that the mitochondria were incubated for, i.e. 48 hours for BAT mitochondria and 8 hours for thymus mitochondria. Isolated mitochondria incubated at 37 °C for 48 hours would unlikely be completely coupled, and therefore the effect of uncoupling by FCCP may be understated, due to the mitochondria in the control system (minus FCCP) being partially/completely uncoupled also.

The incubation time may also explain the differences in the degradation of UCP 1 in the presence of a full ubiquitin-proteasome degradation system in BAT and thymus mitochondria. The ineffectiveness of a complete ubiquitin-proteasome degradation system in BAT mitochondria may be due to the presence of mitochondria which are uncoupled.

The inhibition of UCP 1 degradation in thymus mitochondria in the presence of FCCP suggests that the presence of a membrane potential may be necessary for the degradation of UCP1 by the proteasome, or possibly the extraction of the protein from the inner-membrane. The presence of FCCP also inhibited the degradation of UCP 1 in BAT mitochondria, but not significantly, and possible reasons have been discussed above for this.

Figure 6.8 illustrates that BAT mitochondria isolated from cold acclimated rats contain greater amounts of putative ubiquitinated UCP 1 with a molecular weight >32 kDa compared to BAT mitochondria isolated from rats housed at room temperature. During cold acclimation the thermogenic activity of BAT rises, as animals require heat production to maintain their body temperature (Nicholls and Locke 1984). An increase in the expression of UCP1 in BAT mitochondria contributes to this increased thermogenic activity of BAT (Nicholls and Locke, 1984). Figure 6.8 illustrates that the amount of putative ubiquitinated UCP 1 also increases following

chronic cold-acclimation. The level of putative ubiquitinated UCP 1 was not quantified, as the bands representing this form of UCP 1 are detected over a large range of molecular weights, possibly due to different numbers of ubiquitin monomers conjugated to UCP 1.

However figure 6.8 suggests that the level of putative ubiquitinated UCP 1 increases in cold-acclimated BAT as unmodified UCP 1 protein levels increase. This suggests that the ubiquitin-proteasome degradation system is involved in the general turnover of UCP 1 in BAT mitochondria.

Interestingly our laboratory has reported previously that UCP 1 is serine phosphorylated on serine 51 and that the proportion of phosphoserine on UCP1, purified from cold-acclimated rats, was significantly greater than that on UCP 1 from rats kept at room temperature (Carroll et al., 2008). Covalent modification by phosphorylation has been identified as a marker for ubiquitination (Myung *et al.* 2001). The purpose of serine phosphorylation of UCP 1 is unknown, but could possibly be a marker for our observed ubiquitination of UCP1.

Figure 6.9 illustrates a Colloidal Coomassie Blue G-250 stained SDS-PAGE gel following separation of the immunoprecipitate under denaturing conditions, of an anti-UCP 1 immunoprecipitation using isolated BAT mitochondria from 4 week cold-acclimated rats. Where indicated, bands which correspond to ubiquitin immunoreactive bands were excised and subjected to in-gel digestion by trypsin, prior to mass spectrometric analysis. Figure 6.10 shows the sequences of UCP 1 peptides identified by mass spectrometry in the excised bands indicated in figure 6.9. As can be seen from the table in figure 6.10, UCP 1 peptides were identified in all the excised bands. These samples are separated under denaturing conditions and all of these bands are >32 kDa, so therefore the UCP 1 present must be covalently modified resulting in an increased molecular weight. The presence of UCP 1 at these

molecular weights under denaturing conditions indicates that the UCP 1 immunoreactive bands previously identified in BAT and thymus mitochondria by immunoprecipitation and due to ubiquitin conjugation contain UCP 1 and not non-specific proteins cross-reacting with our antibodies.

The mechanism by which ubiquitinated mitochondrial proteins are exported to the cytosol where the bulk of the proteasome machinery is located is still under debate. The process whereby targeted proteins are retrotranslocated through one or two mitochondrial membranes is unknown. However a similar pathway through one membrane occurs in the endoplasmic reticulum (ER) called the ERAD pathway (ER-associated degradation pathway) (Meusser *et al.* 2005). This pathway involves ubiquitination of target proteins followed by retro translocation across the membrane and subsequent degradation by the proteasome in the cytosol.

However inner-mitochondrial and matrix proteins would require retrotranslocation through two membranes, so extra steps in any putative mitochondrial associated degradative pathway would be required. Neutzner *et al.* (2007) proposed such a pathway for mitochondrial outer membrane-associated proteins (OMMAD), and this proposal was extended for mitochondrial inner membrane proteins by Azzu and Brand (2010).

Azzu and Brand proposed a model whereby a mitochondrial inner membrane protein is ubiquitinated by an E3 ligase and unfolded from the mitochondrial inner membrane by a process which is possibly ATP or membrane potential dependent. At the outer membrane the ubiquitinated protein is recognized by the proteasome, whereby it extracts the protein and degrades it.

Our findings that the protonophore FCCP inhibits degradation of UCP 1 in an in vitro proteasome degradation system (Figures 6.5b and 6.7b), correlates with their findings that the process may be membrane potential dependent.

Future work:

One of the next steps in the investigation of ubiquitination of UCP 1 would be identification of the site of ubiquitination on UCP 1. During this study identification of this site was undertaken by purifying ubiquitinated UCP 1 by immunoaffinity. However sufficient pure ubiquitinated UCP 1 was not obtained for site identification by Q-trap mass spectrometry. A peptide tag like haemagglutinin or myc could be expressed attached to ubiquitin in BAT cells or thymocytes and used to purify and enrich ubiquitinated proteins from BAT cells and thymocytes (Ellison and Hochstrasser 1991). Tagged ubiquitin is correctly conjugated in vivo and in vitro and can be covalently extended into the multimeric ubiquitin chain that is normally required for the degradation.

Site directed mutagenesis could also be used to identify possible sites of ubiquitination on UCP 1 in BAT cells and thymocytes.

Identification of the site may also aid in the determination of the type of ubiquitin linkage involved in ubiquitination of UCP 1. Our results suggest that polyubiquitination of UCP 1 occurs, as multiple bands were identified by immunoblotting and mass spectrometry. However different types of polyubiquitination occur as identified by the lysine on ubiquitin used for linkage. The two most common types of linkages are K48 and K63 polyubiquitination. K48 ubiquitination is involved in proteasomal degradation, whereas K63 ubiquitination is involved in DNA repair (Woelk *et al.* 2007). The expression of mutant ubiquitin unable to form K48 or K63 linkages in BAT cells or thymocytes would aid in determining the type of linkage involved in UCP 1 ubiquitination and thus clarify that the polyubiquitination we have observed is linked to its degradation.

Chapter 7 General Discussion

The work presented in this thesis involved investigating a role for UCP 1 in the attenuation of ROS in BAT and thymus mitochondria. We also present for the first time evidence implicating the ubiquitin-proteasome system in the regulation of UCP 1 turnover.

In chapter 3 we showed that UCP 1 in isolated BAT mitochondria is capable of attenuating ROS production in the presence of a variety of substrates. We showed that ROS production due to reverse electron transport was particularly sensitive to attenuation by UCP 1. The attenuation of ROS production by UCP 1 was shown to be through dissipation of the membrane potential. These findings fit in with the proposed mechanism of 'mild uncoupling', for the attenuation of ROS production (Kurshunov *et al.*, 1997; Brand *et al.*, 2004).

Our laboratory has previously reported UCP 1 sensitive ROS production in isolated BAT mitochondria (Dlaskova *et al.*, 2010). However ROS production due to reverse electron transport was not investigated in this study. Furthermore in the course of that investigation, we noted that when BAT mitochondria were respiring on succinate alone (without rotenone) plus the UCP1 ligand GDP, the rate of reactive oxygen species production detected in mitochondria from wild-type mice was greater than in mitochondria from UCP1 knock-out mice, plus or minus GDP (A. Dlaskova, K.J. Clark and R.K. Porter, unpublished observation). In addition, we noted that the rate of oxygen consumption by BAT mitochondria respiring on complex I substrates ($\pm$ uncoupler) was greater in mitochondria from wild-type mice compared to those from UCP1 knock-out mice (Dlaskova *et al.*, 2010).

The results presented in chapter 3 explain the apparent anomalies in ROS production and oxygen consumption between BAT mitochondria from UCP 1 knock-out and wild-type mice. We have

shown for the first time that BAT mitochondria isolated from UCP 1 knock-out mice have significantly less complex I activity, and surprisingly have significantly more glycerol-3-phosphate dehydrogenase activity.

The reduced activity of complex I in BAT mitochondria isolated from UCP 1 knock-out mice may be due to ROS mediated inactivation of the iron-sulphur clusters of complex I (Rotig *et al.* 1997) , which would suggest that ablation of UCP 1 does increase ROS production in vivo.

The data in chapter 3 demonstrates the potential for UCP 1 to attenuate ROS production in vitro. Future studies will determine whether this happens in vivo. Our data show that ROS production due to reverse electron transport is particularly sensitive to attenuation by UCP 1 in vitro. Whether reverse electron transport occurs in vivo, has been disputed. However, there is strong evidence indicating that it does.

A number of studies have found an inverse correlation between the rate of ROS production and lifespan (Brunet-Rossinni, 2004; Ku and Sohal, 1993; Herrero and Barja, 1998; Csiszar *et al.*, 2007; Lambert *et al.*, 2010). Further investigation of this correlation has found low complex I content in mitochondria of long lived species, thereby reducing ROS production by reverse electron transport (Pierre *et al.*, 2002; Lambert *et al.*, 2010; Valle *et al.*, 2007). This suggests that reverse electron transport occurs in vivo.

However it has been suggested that the physiological conditions needed for reverse electron transport derived ROS production do not occur in vivo (Tretter and Adam-Vizi, 2007c). This was concluded from observations of an increase in ROS production upon rotenone addition to *in situ* synaptic mitochondria (Sipos *et al.*, 2003), which suggests that in situ electron supply is mainly through complex I and thus forward electron flow in synaptosomes.

Reverse electron transport however, has been shown to occur in intact hepatocytes during ketogenesis. During ketogenesis high-

energy electrons enter the respiratory chain at a greater rate than hepatocytes can generate ATP thereby generating a large membrane potential, conditions under which reverse electron transport can occur (McCarthy and Gustin, 1999).

It may be a case that reverse electron transport does not occur *in situ* in all cell types, or alternatively it may only occur under certain metabolic conditions. Reverse electron transport is still speculative, and further work into its physiological relevance is needed.

In chapter 4 we investigated the ability of UCP 1 in isolated BAT mitochondria to attenuate ROS production under conditions where increased oxidative capacity of BAT mitochondria could lead to an increase in ROS production.

We first of all investigated whether mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), which is a significant source of ROS, is activated by calcium in BAT mitochondria. Previous reports have shown that mGPDH in brain mitochondria is activated by calcium (Tretter *et al.*, 2007). We next investigated whether activation of mGPDH in BAT mitochondria resulted in an increase in ROS production. We have shown that an increase in ROS production only occurs when UCP 1 is inhibited thereby illustrating that UCP 1 is capable of attenuating an increase in ROS production due to calcium activation of mGPDH.

We have also shown that calcium activation of mGPDH increases non-phosphorylating oxygen consumption and UCP 1 dependent oxygen consumption. This is the first time that calcium activation of mGPDH has been shown to increase respiratory activity in isolated BAT mitochondria. The observation suggests that calcium activation of mGPDH could be of physiological relevance during initial adrenergic stimulation of BAT cells when mitochondrial calcium levels increase (Koivisto *et al.*, 2000). However, we predict

the increase in oxidative activity due to calcium activation of mGPDH in BAT tissue will not result in increased oxidative damage, due to the attenuation of ROS production by mitochondria by UCP 1.

To further investigate the ability of UCP 1 to attenuate ROS production we compared ROS production by BAT mitochondria isolated from cold-acclimated rats and rats housed at room temperature. BAT mitochondria isolated from cold-acclimated rats have greater oxidative capacity compared to BAT mitochondria isolated from rats housed at room temperature. With an increase in oxidative capacity there is the potential for greater oxidative damage due to a potential increase in ROS production.

Our results presented in chapter 4 show that no difference in ROS production is observed in BAT mitochondria isolated from cold-acclimated rats compared to rats housed at room temperature when UCP 1 is active.

However, significantly more ROS production due to succinate supported reverse electron transport is observed in BAT mitochondria isolated from cold-acclimated rats compared to BAT mitochondria isolated from rats housed at room temperature, when UCP 1 is inhibited by GDP.

This shows that BAT mitochondria have an increased capacity to produce ROS by reverse electron transport following cold-acclimation but this increased capacity to produce ROS may not manifest itself due to attenuation by UCP 1.

ROS production due to forward electron transport is actually lower in BAT mitochondria following cold-acclimation when UCP 1 is inhibited by GDP, but not significantly so. This probably reflects the increase in capacity of the mitochondrial respiratory chain following cold-acclimation which would increase the rate of electron flow and therefore decrease the length of time that transients of the electron transport chain are capable of reducing O_2 to $O_2^{\cdot-}$, such as CoQH-.

Until relatively recently BAT was only considered of physiological importance to infant humans. Recent studies however

have shown that active BAT can be found in adult humans (Cypess *et al.* 2009; Nedergaard *et al.* 2007).

This repositions BAT as an important physiological and pharmaceutical target for the treatment of obesity. The ability of UCP 1 to prevent oxidative damage in metabolically active BAT would allow any pharmaceutical or clinical intervention involving the activation of BAT to occur without increasing oxidative damage in BAT of the patient, and thereby preventing the development of any pathology associated with increased oxidative damage.

Our results also show that mGPDH may be involved in the initial response of BAT to adrenergic stimulation, via an increase in mitochondrial calcium levels. Calcium has been previously linked to the initial response of BAT to adrenergic stimulation, but it has not been linked to the activation of mGPDH in BAT mitochondria as a means of activating thermogenesis (Koivisto *et al.*, 2000).

Our results in chapter 3 show that BAT mitochondria isolated from UCP 1 knock-out mice have significantly greater mGPDH activity compared to BAT mitochondria isolated from wild-type mice. This may be a compensatory mechanism, and allow UCP 1 knock-out mice to increase the thermogenic capabilities of their brown adipocytes through glycerol-3-phosphate activity rather than through UCP1 activity.

In chapter 5 we showed that ROS production by thymus mitochondria isolated from UCP 1 knock-out mice was higher than ROS production by thymus mitochondria isolated from wild-type mice, but unlike BAT this difference was not significant. This is probably due to the low levels of UCP 1 protein in thymus mitochondria (10 fold lower than in BAT mitochondria) and suggests that UCP 1 may not attenuate ROS production in thymus mitochondria isolated from resting thymocytes.

It has been observed in our laboratory that UCP 1 protein

levels are two fold higher in thymus mitochondria isolated from mitogen challenged mice compared to UCP 1 protein levels in thymus mitochondria isolated from unchallenged mice (Adams and Porter, in preparation 2011). In the mitogen challenged mice thymocytes are stimulated to proliferate. In proliferating thymocytes, oxidative phosphorylation activity increases, mainly through allosteric activation of metabolic enzymes by Ca^{2+} (Brookes *et al.*, 2004). UCP 1 expression in mitochondria from proliferating thymocytes may increase in order to attenuate an increase in ROS production due to increased activity of the respiratory chain.

Future work into the investigation of the role of UCP 1 in the thymus should involve investigating ROS production by proliferating thymocytes in order to determine whether the increased expression of UCP 1 is involved in ROS attenuation during proliferation.

The 2 fold increase in UCP 1 protein levels in thymus mitochondria 3 days after mitogen injection of animals shows that the expression of UCP 1 in thymus mitochondria is quite dynamic. Proliferation of thymocytes is required to mount an immune response which is maximal around 72 hours after initial mitogenic stimulation in the system used in this study (Hick *et al.* 2006), so an increase in UCP 1 protein levels would need to occur in this time if it is involved in the response.

In comparison, UCP 1 in BAT is involved in non-shivering thermogenesis which is maximal after 3 weeks following initial cold-exposure. UCP 1 protein recruitment in BAT is maximal following this time also. (Cannon and Nedergaard, 2004).

The more dynamic nature of UCP 1 in thymus mitochondria compared to BAT mitochondria is also reflected in the results

presented in chapter 6. In this chapter we presented results which showed that UCP 1 in BAT and thymus mitochondria can be degraded by the cytosolic proteasome. Our in vitro proteasome

degradation studies of UCP 1 in isolated BAT and thymus mitochondria show that the cytosolic proteasome can degrade UCP 1, and the half-life of UCP 1 in such a system reflects the half-life of UCP 1 in intact cells (i.e 3.5-5 hours in thymocytes and 30-72 hours in brown adipocytes). This dramatic difference in the half-life of UCP 1 in two different tissues probably reflects the difference in function and regulation of UCP 1 in each tissue. UCP 1 in BAT is activated in response to cold exposure by noradrenaline and regulated by purine nucleotides and fatty acids (Cannon and Nedergaard, 2004). The signal for an increase in UCP 1 in the thymus following mitogenic stimulation is unknown. Observations in our lab have shown however thymocytes do not respond to noradrenaline directly, and UCP 1 levels in thymus mitochondria do not increase following cold-acclimation (Brennan *et al.*, 2006).

UCP 1 in the thymus may not be regulated by purine nucleotides *in vivo*. Our study using isolated thymus mitochondria from wild-type mice in chapter 5 suggests that this may be the case. Regulation of UCP 1 in thymus mitochondria may be through regulation of protein levels as opposed to direct regulation of activity.

In chapter 6 we also show for the first time evidence that UCP 1 in BAT and thymus mitochondria can be ubiquitinated, and that the cytosolic proteasome is capable of degrading UCP 1 in a reconstituted *in vitro* proteasome degradation system. We show for the first time that UCP 1 can be conjugated to ubiquitin in an *in vitro* conjugation system. We also show for the first time by immunoblotting and mass spectrometric analysis that UCP 1 can be found at molecular weights above 32 kDa which correspond to ubiquitinated forms of the protein.

Like the findings of Azzu and Brand, (2010) and Azzu *et al.*, (2010) we found that dissipation of the membrane potential by the protonophore FCCP inhibited degradation of UCP 1 in the reconstituted *in vitro* proteasome degradation system.

Ubiquitin-proteasome degradation of mitochondrial proteins and in particular mitochondrial inner-membrane proteins is a relatively new area of research, and the method by which inner membrane proteins are extracted from the membrane and degraded by the cytosolic proteasome is unknown. Our findings along with those of Azzu and Brand, (2010) and Azzu *et al.*, (2010) suggest that the membrane potential plays a role in this process. What this role is awaits elucidation.

Defects in proteasome dependent protein degradation of mitochondrial proteins has been implicated in neurodegenerative diseases including Parkinson's disease (Olanow and McNaught 2006). However proteasome dependent protein degradation of mitochondrial proteins is not very well understood. Research into proteasome dependent degradation of mitochondrial proteins could aid in the understanding neurodegenerative disorders such as Parkinson's.

The high levels of protein expression of UCP 1 in BAT mitochondria readily allow the study of its regulation, which may be used as a model for proteasome dependent protein degradation of mitochondrial proteins. The expression of UCP 1 in two different organs, where it has very different half-lives, would also allow the determination of factors which affect the ubiquitin-proteasome system in mitochondria.

References

- Acker, H. (1994). Mechanisms and meaning of cellular oxygen sensing in the organism. *Respir Physiol* **95**, 1-10.
- Adams, A. E., Hanrahan, O., Nolan, D. N., Voorheis, H. P., Fallon, P., and Porter, R. K. (2008). Images of mitochondrial UCP 1 in mouse thymocytes using confocal microscopy. *Biochim Biophys Acta* **1777**, 115-7.
- Adams, A. E., Kelly, O. M., and Porter, R. K. (2010). Absence of mitochondrial uncoupling protein 1 affects apoptosis in thymocytes, thymocyte/T-cell profile and peripheral T-cell number. *Biochim Biophys Acta* **1797**, 807-16.
- Akerman, K. E., and Wikstrom, M. K. (1976). Safranin as a probe of the mitochondrial membrane potential. *FEBS Lett* **68**, 191-7.
- Arrigo, A. P. (1999). Gene expression and the thiol redox state. *Free Radic Biol Med* **27**, 936-44.
- Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S., Miroux, B., Couplan, E., Alves-Guerra, M. C., Gubern, M., Surwit, R., Bouillaud, F., Richard, D., Collins, S., and Ricquier, D. (2000). Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat Genet* **26**, 435-9.
- Azad, N., Vallyathan, V., Wang, L., Tantishaiyakul, V., Stehlik, C., Leonard, S. S., and Rojanasakul, Y. (2006). S-nitrosylation of Bcl-2 inhibits its ubiquitin-proteasomal degradation. A novel antiapoptotic mechanism that suppresses apoptosis. *J Biol Chem* **281**, 34124-34.
- Azzu, V., Affourtit, C., Breen, E. P., Parker, N., and Brand, M. D. (2008). Dynamic regulation of uncoupling protein 2 content in INS-1E insulinoma cells. *Biochim Biophys Acta* **1777**, 1378-83.
- Azzu, V., and Brand, M. D. (2010). Degradation of an intramitochondrial protein by the cytosolic proteasome. *J Cell Sci* **123**, 578-85.
- Azzu, V., Mookerjee, S. A., and Brand, M. D. (2010). Rapid turnover of mitochondrial uncoupling protein 3. *Biochem J* **426**, 13-7.
- Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997). Epidermal growth factor (EGF)-

induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* **272**, 217-21.

Bai, Y., Onuma, H., Bai, X., Medvedev, A. V., Misukonis, M., Weinberg, J. B., Cao, W., Robidoux, J., Floering, L. M., Daniel, K. W., and Collins, S. (2005). Persistent nuclear factor-kappa B activation in Ucp2^{-/-} mice leads to enhanced nitric oxide and inflammatory cytokine production. *J Biol Chem* **280**, 19062-9.

Bar-Shalom, R., Gaitini, D., Keidar, Z., and Israel, O. (2004). Non-malignant FDG uptake in infradiaphragmatic adipose tissue: a new site of physiological tracer biodistribution characterised by PET/CT. *Eur J Nucl Med Mol Imaging* **31**, 1105-13.

Barrington, S. F., and Maisey, M. N. (1996). Skeletal muscle uptake of fluorine-18-FDG: effect of oral diazepam. *J Nucl Med* **37**, 1127-9.

Behrens, W. A., and Himms-Hagen, J. (1977). Alteration in skeletal muscle mitochondria of cold-acclimated rats: association with enhanced metabolic response to noradrenaline. *J Bioenerg Biomembr* **9**, 41-63.

Bienengraeber, M., Echtay, K. S., and Klingenberg, M. (1998). H⁺ transport by uncoupling protein (UCP-1) is dependent on a histidine pair, absent in UCP-2 and UCP-3. *Biochemistry* **37**, 3-8.

Boneh, A. (2006). Regulation of mitochondrial oxidative phosphorylation by second messenger-mediated signal transduction mechanisms. *Cell Mol Life Sci* **63**, 1236-48.

Boss, O., Samec, S., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1997a). Tissue-dependent upregulation of rat uncoupling protein-2 expression in response to fasting or cold. *FEBS Lett* **412**, 111-4.

Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1997b). Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett* **408**, 39-42.

Bota, D. A., and Davies, K. J. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat Cell Biol* **4**, 674-80.

Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* **134**, 707-16.

Brand, M. D. (1977). The stoichiometric relationships between electron transport, proton translocation and adenosine triphosphate synthesis and hydrolysis in mitochondria. *Biochem Soc Trans* **5**, 1615-20.

Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L., and Parker, N. (2004a). Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med* **37**, 755-67.

Brand, M. D., Buckingham, J. A., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Murphy, M. P., Pakay, J. L., Talbot, D. A., and Echtay, K. S. (2004b). Mitochondrial superoxide and aging: uncoupling-protein activity and superoxide production. *Biochem Soc Symp*, 203-13.

Brand, M. D., Chien, L. F., Ainscow, E. K., Rolfe, D. F., and Porter, R. K. (1994). The causes and functions of mitochondrial proton leak. *Biochim Biophys Acta* **1187**, 132-9.

Brand, M. D., Pamplona, R., Portero-Otin, M., Requena, J. R., Roebuck, S. J., Buckingham, J. A., Clapham, J. C., and Cadenas, S. (2002). Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3. *Biochem J* **368**, 597-603.

Braun, B., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P., Finlay, D., Schmidt, M. (1999). *Nat Cell Biol* **1**, 221-226

Brennan, C. M., Breen, E. P., and Porter, R. K. (2006). Cold acclimation and oxygen consumption in the thymus. *Biochim Biophys Acta* **1757**, 1463-8.

Bronnikov, G., Houstek, J., and Nedergaard, J. (1992). Beta-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via beta 1 but not via beta 3 adrenoceptors. *J Biol Chem* **267**, 2006-13.

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., and Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* **287**, C817-33.

Brown, G. C., and Brand, M. D. (1986). Changes in permeability to protons and other cations at high proton motive force in rat liver mitochondria. *Biochem J* **234**, 75-81.

Brown, L. J., Koza, R. A., Marshall, L., Kozak, L. P., and MacDonald, M. J. (2002). Lethal hypoglycemic ketosis and glyceroluria in mice lacking both the mitochondrial and the cytosolic glycerol phosphate dehydrogenases. *J Biol Chem* **277**, 32899-904.

Brown, L. J., MacDonald, M. J., Lehn, D. A., and Moran, S. M. (1994). Sequence of rat mitochondrial glycerol-3-phosphate dehydrogenase cDNA. Evidence for EF-hand calcium-binding domains. *J Biol Chem* **269**, 14363-6.

Brunet-Rossinni, (2004), Reduced free-radical production and extreme longevity in the little brown bat (*Myotis lucifugus*) versus two non-flying mammals, *Mech Ageing Dev*, **125**, 11-20.

Buetler, T. M., Krauskopf, A., and Ruegg, U. T. (2004). Role of superoxide as a signaling molecule. *News Physiol Sci* **19**, 120-3.

Bukowiecki, L., Collet, A. J., Follea, N., Guay, G., and Jahjah, L. (1982). Brown adipose tissue hyperplasia: a fundamental mechanism of adaptation to cold and hyperphagia. *Am J Physiol* **242**, E353-9.

Buttgereit, F., Grant, A., Muller, M., and Brand, M. D. (1994). The effects of methylprednisolone on oxidative phosphorylation in Concanavalin-A-stimulated thymocytes. Top-down elasticity analysis and control analysis. *Eur J Biochem* **223**, 513-9.

Cadenas, S., Echtay, K. S., Harper, J. A., Jekabsons, M. B., Buckingham, J. A., Grau, E., Abuin, A., Chapman, H., Clapham, J. C., and Brand, M. D. (2002). The basal proton conductance of skeletal muscle mitochondria from transgenic mice overexpressing or lacking uncoupling protein-3. *J Biol Chem* **277**, 2773-8.

Cannon, B., Hedin, A., and Nedergaard, J. (1982). Exclusive occurrence of thermogenin antigen in brown adipose tissue. *FEBS Lett* **150**, 129-32.

Cannon, B., and Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiol Rev* **84**, 277-359.

Carroll, A. M., Haines, L. R., Pearson, T. W., Brennan, C., Breen, E. P., and Porter, R. K. (2004). Immunodetection of UCP1 in rat thymocytes. *Biochem Soc Trans* **32**, 1066-7.

Carroll, A. M., and Porter, R. K. (2004). Starvation-sensitive UCP 3 protein expression in thymus and spleen mitochondria. *Biochim Biophys Acta* **1700**, 145-50.

- Carroll, A. M., Porter, R. K., and Morrice, N. A. (2008). Identification of serine phosphorylation in mitochondrial uncoupling protein 1. *Biochim Biophys Acta* **1777**, 1060-5.
- Casteilla, L., Rigoulet, M., and Penicaud, L. (2001). Mitochondrial ROS metabolism: modulation by uncoupling proteins. *IUBMB Life* **52**, 181-8.
- Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59**, 527-605.
- Chappell, J. B. (1972). *IN SUBCELLULAR COMPONENTS: PREPARATION AND FRACTIONATION*. Buttersworths, London.
- Cheng, J., Bawa, T., Lee, P., Gong, L., and Yeh, E. T. (2006). Role of desumoylation in the development of prostate cancer. *Neoplasia* **8**, 667-76.
- Chowdhury, S. K., Drahota, Z., Floryk, D., Calda, P., and Houstek, J. (2000). Activities of mitochondrial oxidative phosphorylation enzymes in cultured amniocytes. *Clin Chim Acta* **298**, 157-73.
- Chretien, D., Rustin, P., Bourgeron, T., Rotig, A., Saudubray, J. M., and Munnich, A. (1994). Reference charts for respiratory chain activities in human tissues. *Clin Chim Acta* **228**, 53-70.
- Ciechanover, A., Elias, S., Heller, H., Ferber, S., and Hershko, A. (1980). Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *J Biol Chem* **255**, 7525-8.
- Commins, S. P., Watson, P. M., Padgett, M. A., Dudley, A., Argyropoulos, G., and Gettys, T. W. (1999). Induction of uncoupling protein expression in brown and white adipose tissue by leptin. *Endocrinology* **140**, 292-300.
- Considine, M. J., Goodman, M., Echtay, K. S., Laloi, M., Whelan, J., Brand, M. D., and Sweetlove, L. J. (2003). Superoxide stimulates a proton leak in potato mitochondria that is related to the activity of uncoupling protein. *J Biol Chem* **278**, 22298-302.
- Cordoza, C. (1993). *Enzyme Protein* **47**, 296-305.
- Costa, A. D., Nantes, I. L., Jezek, P., Leite, A., Arruda, P., and Vercesi, A. E. (1999). Plant uncoupling mitochondrial protein activity in mitochondria isolated from tomatoes at different stages of ripening. *J Bioenerg Biomembr* **31**, 527-33.

Couplan, E., del Mar Gonzalez-Barroso, M., Alves-Guerra, M. C., Ricquier, D., Goubern, M., and Bouillaud, F. (2002). No evidence for a basal, retinoic, or superoxide-induced uncoupling activity of the uncoupling protein 2 present in spleen or lung mitochondria. *J Biol Chem* **277**, 26268-75.

Csiszar, A., Labinsky, N., Orosz, Z., Xiangmin, Z., Buffenstein, R., Ungvari, Z., (2007), Vascular aging in the longest-living rodent, the naked mole rat, *Am J Physiol Heart Circ Physiol*, **293**, H919-H927.

Cunningham, O., McElligott, A. M., Carroll, A. M., Breen, E., Reguenga, C., Oliveira, M. E., Azevedo, J. E., and Porter, R. K. (2003). Selective detection of UCP 3 expression in skeletal muscle: effect of thyroid status and temperature acclimation. *Biochim Biophys Acta* **1604**, 170-9.

Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., Kuo, F. C., Palmer, E. L., Tseng, Y. H., Doria, A., Kolodny, G. M., and Kahn, C. R. (2009). Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* **360**, 1509-17.

Deby, C., and Goutier, R. (1990). New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. *Biochem Pharmacol* **39**, 399-405.

Desautels, M., Zaror-Behrens, G., and Himms-Hagen, J. (1978). Increased purine nucleotide binding, altered polypeptide composition, and thermogenesis in brown adipose tissue mitochondria of cold-acclimated rats. *Can J Biochem* **56**, 378-83.

Dick, T., Nussbaum, A., Deeg, M., Heinemeyer, W., Groll, M., Schirle, M., Keiholz, W., Stevanovic, S., Wolf, D., Huber, R., Rammensee, H., Schild, H. (1998). *J Biol Chem* **273**, 25637-25646

Ding, Q., Dimayuga, E., and Keller, J. N. (2006). Proteasome regulation of oxidative stress in aging and age-related diseases of the CNS. *Antioxid Redox Signal* **8**, 163-72.

Blaskova, A., Clarke, K. J., and Porter, R. K. (2010). The role of UCP 1 in production of reactive oxygen species by mitochondria isolated from brown adipose tissue. *Biochim Biophys Acta* **1797**, 1470-6.

Blaskova, A., Spacek, T., Skobisova, E., Santorova, J., and Jezek, P. (2006). Certain aspects of uncoupling due to mitochondrial

uncoupling proteins in vitro and in vivo. *Biochim Biophys Acta* **1757**, 467-73.

Drahota, Z., Chowdhury, S. K., Floryk, D., Mracek, T., Wilhelm, J., Rauchova, H., Lenaz, G., and Houstek, J. (2002).

Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide. *J Bioenerg Biomembr* **34**, 105-13.

Dummler, K., Muller, S., and Seitz, H. J. (1996). Regulation of adenine nucleotide translocase and glycerol 3-phosphate dehydrogenase expression by thyroid hormones in different rat tissues. *Biochem J* **317** (Pt 3), 913-8.

Dumont, A., Hehner, S. P., Hofmann, T. G., Ueffing, M., Droge, W., and Schmitz, M. L. (1999). Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappaB. *Oncogene* **18**, 747-57.

Echtay, K. S., and Brand, M. D. (2001). Coenzyme Q induces GDP-sensitive proton conductance in kidney mitochondria. *Biochem Soc Trans* **29**, 763-8.

Echtay, K. S., Esteves, T. C., Pakay, J. L., Jekabsons, M. B., Lambert, A. J., Portero-Otin, M., Pamplona, R., Vidal-Puig, A. J., Wang, S., Roebuck, S. J., and Brand, M. D. (2003). A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *Embo J* **22**, 4103-10.

Echtay, K. S., Roussel, D., St-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roebuck, S. J., Morrison, A., Pickering, S., Clapham, J. C., and Brand, M. D. (2002). Superoxide activates mitochondrial uncoupling proteins. *Nature* **415**, 96-9.

Ellison, M. J., and Hochstrasser, M. (1991). Epitope-tagged ubiquitin. A new probe for analyzing ubiquitin function. *J Biol Chem* **266**, 21150-7.

Estabrook, R. W., and Sacktor, B. (1958). alpha-Glycerophosphate oxidase of flight muscle mitochondria. *J Biol Chem* **233**, 1014-9.

Fandrey, J., Frede, S., and Jelkmann, W. (1994). Role of hydrogen peroxide in hypoxia-induced erythropoietin production. *Biochem J* **303** (Pt 2), 507-10.

Finkel, T. (1998). Oxygen radicals and signaling. *Curr Opin Cell Biol* **10**, 248-53.

Finkel, T. (2003). Oxidant signals and oxidative stress. *Curr Opin Cell Biol* **15**, 247-54.

Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997). Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* **15**, 269-72.

Fox, C. J., Hammerman, P. S., and Thompson, C. B. (2005). Fuel feeds function: energy metabolism and the T-cell response. *Nat Rev Immunol* **5**, 844-52.

Garlid, K. D., Orosz, D. E., Modriansky, M., Vassanelli, S., and Jezek, P. (1996). On the mechanism of fatty acid-induced proton transport by mitochondrial uncoupling protein. *J Biol Chem* **271**, 2615-20.

Garrib, A., and McMurray, W. C. (1986). Purification and characterization of glycerol-3-phosphate dehydrogenase (flavin-linked) from rat liver mitochondria. *J Biol Chem* **261**, 8042-8.

Garruti, G., and Ricquier, D. (1992). Analysis of uncoupling protein and its mRNA in adipose tissue deposits of adult humans. *Int J Obes Relat Metab Disord* **16**, 383-90.

Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W., Gimeno, C. J., Iris, F., Ellis, S. J., Woolf, E. A., and Tartaglia, L. A. (1997). Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes* **46**, 900-6.

Gomez-Ambrosi, J., Fruhbeck, G., and Martinez, J. A. (2001). Rapid in vivo PGC-1 mRNA upregulation in brown adipose tissue of Wistar rats by a beta(3)-adrenergic agonist and lack of effect of leptin. *Mol Cell Endocrinol* **176**, 85-90.

Gong, D. W., He, Y., Karas, M., and Reitman, M. (1997). Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta3-adrenergic agonists, and leptin. *J Biol Chem* **272**, 24129-32.

Gong, D. W., Monemdjou, S., Gavrilova, O., Leon, L. R., Marcus-Samuels, B., Chou, C. J., Everett, C., Kozak, L. P., Li, C., Deng, C.,

Harper, M. E., and Reitman, M. L. (2000). Lack of obesity and normal response to fasting and thyroid hormone in mice lacking uncoupling protein-3. *J Biol Chem* **275**, 16251-7.

Granneman, J. G. (1988). Norepinephrine infusions increase adenylate cyclase responsiveness in brown adipose tissue. *J Pharmacol Exp Ther* **245**, 1075-80.

Granot, Z., Geiss-Friedlander, R., Melamed-Book, N., Eimerl, S., Timberg, R., Weiss, A. M., Hales, K. H., Hales, D. B., Stocco, D. M., and Orly, J. (2003). Proteolysis of normal and mutated steroidogenic acute regulatory proteins in the mitochondria: the fate of unwanted proteins. *Mol Endocrinol* **17**, 2461-76.

Hansford, R. G., Hogue, B. A., and Mildaziene, V. (1997). Dependence of H₂O₂ formation by rat heart mitochondria on substrate availability and donor age. *J Bioenerg Biomembr* **29**, 89-95.

Harder, Z., Zunino, R., and McBride, H. (2004). Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. *Curr Biol* **14**, 340-5.

Harper, J. A., Stuart, J. A., Jekabsons, M. B., Roussel, D., Brindle, K. M., Dickinson, K., Jones, R. B., and Brand, M. D. (2002). Artifactual uncoupling by uncoupling protein 3 in yeast mitochondria at the concentrations found in mouse and rat skeletal-muscle mitochondria. *Biochem J* **361**, 49-56.

Heaton, G. M., Wagenvoord, R. J., Kemp, A., Jr., and Nicholls, D. G. (1978). Brown-adipose-tissue mitochondria: photoaffinity labelling of the regulatory site of energy dissipation. *Eur J Biochem* **82**, 515-21.

Herrero, A., Barja, G., (1998), H₂O₂ production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved, *Mech Ageing Dev*, **103**, 133-146.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67**, 425-79.

Hesselink, M. K., Greenhaff, P. L., Constantin-Teodosiu, D., Hultman, E., Saris, W. H., Nieuwlaet, R., Schaart, G., Kornips, E., and Schrauwen, P. (2003). Increased uncoupling protein 3 content does not affect mitochondrial function in human skeletal muscle in vivo. *J Clin Invest* **111**, 479-86.

Hick, R. W., Gruver, A. L., Ventevogel, M. S., Haynes, B. F., and Sempowski, G. D. (2006). Leptin selectively augments thymopoiesis in leptin deficiency and lipopolysaccharide-induced thymic atrophy. *J Immunol* **177**, 169-76.

Himms-Hagen, J. (1990). Brown adipose tissue thermogenesis: interdisciplinary studies. *Faseb J* **4**, 2890-8.

Hinkle, P. C., Butow, R. A., Racker, E., and Chance, B. (1967). Partial resolution of the enzymes catalyzing oxidative phosphorylation. XV. Reverse electron transfer in the flavin-cytochrome beta region of the respiratory chain of beef heart submitochondrial particles. *J Biol Chem* **242**, 5169-73.

Hodny, Z., Kolarova, P., Rossmeisl, M., Horakova, M., Nibbelink, M., Penicaud, L., Casteilla, L., and Kopecky, J. (1998). High expression of uncoupling protein 2 in foetal liver. *FEBS Lett* **425**, 185-90.

Honzik, T., Drahota, Z., Bohm, M., Jesina, P., Mracek, T., Paul, J., Zeman, J., and Houstek, J. (2006). Specific properties of heavy fraction of mitochondria from human-term placenta - glycerophosphate-dependent hydrogen peroxide production. *Placenta* **27**, 348-56.

Horvath, T. L., Diano, S., Miyamoto, S., Barry, S., Gatti, S., Alberati, D., Livak, F., Lombardi, A., Moreno, M., Goglia, F., Mor, G., Hamilton, J., Kachinskas, D., Horwitz, B., and Warden, C. H. (2003). Uncoupling proteins-2 and 3 influence obesity and inflammation in transgenic mice. *Int J Obes Relat Metab Disord* **27**, 433-42.

Houstek, J., Cannon, B., and Lindberg, O. (1975). Glycerol-3-phosphate shuttle and its function in intermediary metabolism of hamster brown-adipose tissue. *Eur J Biochem* **54**, 11-8.

Huang, L. E., Arany, Z., Livingston, D. M., and Bunn, H. F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* **271**, 32253-9.

Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* **95**, 7987-92.

Huang, S. G., and Klingenberg, M. (1995). Nature of the masking of nucleotide-binding sites in brown adipose tissue mitochondria.

Involvement of endogenous adenosine triphosphate. *Eur J Biochem* **229**, 718-25.

Idahl, L. A., and Lember, N. (1995). Glycerol 3-phosphate-induced ATP production in intact mitochondria from pancreatic B-cells. *Biochem J* **312** (Pt 1), 287-92.

Jarmuszkiewicz, W., Sluse-Goffart, C. M., Hryniewiecka, L., and Sluse, F. E. (1999). Identification and characterization of a protozoan uncoupling protein in *Acanthamoeba castellanii*. *J Biol Chem* **274**, 23198-202.

Jezek, P., Zackova, M., Rehakova, Z., Ruzicka, M., Borecky, J., Skobisova, E., Brucknerova, J., Garlid, K. D., Gimeno, R. E., and Tartaglia, L. A. (1999). Existence of uncoupling protein-2 antigen in isolated mitochondria from various tissues. *FEBS Lett* **455**, 79-82.

Jung, C. H., and Wells, W. W. (1997). Ascorbic acid is a stimulatory cofactor for mitochondrial glycerol-3-phosphate dehydrogenase. *Biochem Biophys Res Commun* **239**, 457-62.

Karbowski, M., Neutzner, A., and Youle, R. J. (2007). The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. *J Cell Biol* **178**, 71-84.

Klaus, S., Casteilla, L., Bouillaud, F., and Ricquier, D. (1991). The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. *Int J Biochem* **23**, 791-801.

Klingenberg, M. (1970). Localization of the glycerol-phosphate dehydrogenase in the outer phase of the mitochondrial inner membrane. *Eur J Biochem* **13**, 247-52.

Klingenberg, M. (1984). Characteristics of the uncoupling protein from brown-fat mitochondria. *Biochem Soc Trans* **12**, 390-3.

Klingenberg, M. (1988). Nucleotide binding to uncoupling protein. Mechanism of control by protonation. *Biochemistry* **27**, 781-91.

Klingenberg, M. (1990). Mechanism and evolution of the uncoupling protein of brown adipose tissue. *Trends Biochem Sci* **15**, 108-12.

Klingenberg, M., and Buecher, T. (1961). [Glycerin-1-phosphate and flight muscle mitochondria.]. *Biochem Z* **334**, 1-17.

Klingenberg, M., and Huang, S. G. (1999). Structure and function of the uncoupling protein from brown adipose tissue. *Biochim Biophys Acta* **1415**, 271-96.

Koivisto, A., Siemen, D., and Nedergaard, J. (2000). Norepinephrine-induced sustained inward current in brown fat cells: alpha(1)-mediated by nonselective cation channels. *Am J Physiol Endocrinol Metab* **279**, E963-77.

Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* **416**, 15-8.

Kortelainen, M. L., Pelletier, G., Ricquier, D., and Bukowiecki, L. J. (1993). Immunohistochemical detection of human brown adipose tissue uncoupling protein in an autopsy series. *J Histochem Cytochem* **41**, 759-64.

Kourie, J. I. (1998). Interaction of reactive oxygen species with ion transport mechanisms. *Am J Physiol* **275**, C1-24.

Koza, R. A., Hohmann, S. M., Guerra, C., Rossmeisl, M., and Kozak, L. P. (2000). Synergistic gene interactions control the induction of the mitochondrial uncoupling protein (Ucp1) gene in white fat tissue. *J Biol Chem* **275**, 34486-92.

Koza, R. A., Kozak, U. C., Brown, L. J., Leiter, E. H., MacDonald, M. J., and Kozak, L. P. (1996). Sequence and tissue-dependent RNA expression of mouse FAD-linked glycerol-3-phosphate dehydrogenase. *Arch Biochem Biophys* **336**, 97-104.

Krauss, S., Zhang, C. Y., and Lowell, B. B. (2002). A significant portion of mitochondrial proton leak in intact thymocytes depends on expression of UCP2. *Proc Natl Acad Sci U S A* **99**, 118-22.

Krauss, S., Zhang, C. Y., Scorrano, L., Dalgaard, L. T., St-Pierre, J., Grey, S. T., and Lowell, B. B. (2003). Superoxide-mediated activation of uncoupling protein 2 causes pancreatic beta cell dysfunction. *J Clin Invest* **112**, 1831-42.

Krieger-Brauer, H. I., Medda, P. K., and Kather, H. (1997). Insulin-induced activation of NADPH-dependent H₂O₂ generation in human adipocyte plasma membranes is mediated by Galphai2. *J Biol Chem* **272**, 10135-43.

Ku, H. H., Sohal, R. S., (1993), Comparison of mitochondrial pro-oxidant generation and anti-oxidant defenses between rat and

pigeon: possible basis of variation in longevity and metabolic potential, *Mech Ageing Dev*, **72**, 67-76.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Lambert, A. J., and Brand, M. D. (2004). Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem J* **382**, 511-7.

Lambert, A. J., and Brand, M. D. (2009). Reactive oxygen species production by mitochondria. *Methods Mol Biol* **554**, 165-81.

Lambert, A.J., Buckingham, J.A., Boysen, H.M. and Brand, M.D., (2010), Low complex I content explains the low hydrogen peroxide production rate of heart mitochondria from the long-lived pigeon, *Columba Livia.*, *Aging Cell*, **9**, 78-91.

Larrouy, D., Laharrague, P., Carrera, G., Viguerie-Bascands, N., Levi-Meyrueis, C., Fleury, C., Pecqueur, C., Nibbelink, M., Andre, M., Casteilla, L., and Ricquier, D. (1997). Kupffer cells are a dominant site of uncoupling protein 2 expression in rat liver. *Biochem Biophys Res Commun* **235**, 760-4.

Lean, M. E., James, W. P., Jennings, G., and Trayhurn, P. (1986). Brown adipose tissue uncoupling protein content in human infants, children and adults. *Clin Sci (Lond)* **71**, 291-7.

Ledesma, A., de Lacoba, M. G., Arechaga, I., and Rial, E. (2002). Modeling the transmembrane arrangement of the uncoupling protein UCP1 and topological considerations of the nucleotide-binding site. *J Bioenerg Biomembr* **34**, 473-86.

Lee, S. C., Nuccitelli, R., and Pappone, P. A. (1993). Adrenergically activated Ca²⁺ increases in brown fat cells: effects of Ca²⁺, K⁺, and K channel block. *Am J Physiol* **264**, C217-28.

Li, B., Nolte, L. A., Ju, J. S., Han, D. H., Coleman, T., Holloszy, J. O., and Semenkovich, C. F. (2000). Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. *Nat Med* **6**, 1115-20.

Lin, C. S., and Klingenberg, M. (1980). Isolation of the uncoupling protein from brown adipose tissue mitochondria. *FEBS Lett* **113**, 299-303.

Lindquist, J. M., Fredriksson, J. M., Rehnmark, S., Cannon, B., and Nedergaard, J. (2000). Beta 3- and alpha1-adrenergic Erk1/2 activation is Src- but not Gi-mediated in Brown adipocytes. *J Biol Chem* **275**, 22670-7.

Liu, H., Colavitti, R., Rovira, II, and Finkel, T. (2005). Redox-dependent transcriptional regulation. *Circ Res* **97**, 967-74.

Liu, Y., Fiskum, G., and Schubert, D. (2002). Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* **80**, 780-7.

Los, M., Droge, W., Stricker, K., Baeuerle, P. A., and Schulze-Osthoff, K. (1995). Hydrogen peroxide as a potent activator of T lymphocyte functions. *Eur J Immunol* **25**, 159-65.

MacDonald, M. J. (1981). High content of mitochondrial glycerol-3-phosphate dehydrogenase in pancreatic islets and its inhibition by diazoxide. *J Biol Chem* **256**, 8287-90.

MacDonald, M. J., Tang, J., and Polonsky, K. S. (1996). Low mitochondrial glycerol phosphate dehydrogenase and pyruvate carboxylase in pancreatic islets of Zucker diabetic fatty rats. *Diabetes* **45**, 1626-30.

Margineantu, D. H., Emerson, C. B., Diaz, D., and Hockenbery, D. M. (2007). Hsp90 inhibition decreases mitochondrial protein turnover. *PLoS ONE* **2**, e1066.

Martinez-Botas, J., Anderson, J. B., Tessier, D., Lapillonne, A., Chang, B. H., Quast, M. J., Gorenstein, D., Chen, K. H., and Chan, L. (2000). Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. *Nat Genet* **26**, 474-9.

Mayinger, P., and Klingenberg, M. (1992). Labeling of two different regions of the nucleotide binding site of the uncoupling protein from brown adipose tissue mitochondria with two ATP analogs. *Biochemistry* **31**, 10536-43.

McCarthy, F.M. and Gustin, J.C., (1999), pyruvate and hydroxycitrate/carnitine may synergize to promote reverse electron transport in hepatocyte mitochondria, effectively 'uncoupling' the oxidation of fatty acids, *Medical Hypotheses* **52**, 407-416

McCormack, J. G., Halestrap, A. P., and Denton, R. M. (1990). Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* **70**, 391-425.

- McCormack, T., Cruikshank, A., Greiner, L., Melandri, F.D., Nunes, S., Plamondon, L., Stein, R., Dick, L. (1998). *Biochemistry* **37**, 7792-7800
- McLennan, H. R., and Degli Esposti, M. (2000). The contribution of mitochondrial respiratory complexes to the production of reactive oxygen species. *J Bioenerg Biomembr* **32**, 153-62.
- McLeod, C. J., Aziz, A., Hoyt, R. F., Jr., McCoy, J. P., Jr., and Sack, M. N. (2005). Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia. *J Biol Chem* **280**, 33470-6.
- Melov, S., Coskun, P., Patel, M., Tuinstra, R., Cottrell, B., Jun, A. S., Zastawny, T. H., Dizdaroglu, M., Goodman, S. I., Huang, T. T., Miziorko, H., Epstein, C. J., and Wallace, D. C. (1999). Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc Natl Acad Sci U S A* **96**, 846-51.
- Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N., and Crews, C. M. (1999). Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc Natl Acad Sci U S A* **96**, 10403-8.
- Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005). ERAD: the long road to destruction. *Nat Cell Biol* **7**, 766-72.
- Millard, S. M., and Wood, S. A. (2006). Riding the DUBway: regulation of protein trafficking by deubiquitylating enzymes. *J Cell Biol* **173**, 463-8.
- Miwa, S., and Brand, M. D. (2003). Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem Soc Trans* **31**, 1300-1.
- Miwa, S., and Brand, M. D. (2005). The topology of superoxide production by complex III and glycerol 3-phosphate dehydrogenase in *Drosophila* mitochondria. *Biochim Biophys Acta* **1709**, 214-9.
- Miwa, S., St-Pierre, J., Partridge, L., and Brand, M. D. (2003). Superoxide and hydrogen peroxide production by *Drosophila* mitochondria. *Free Radic Biol Med* **35**, 938-48.
- Mori, S., Yoshizuka, N., Takizawa, M., Takema, Y., Murase, T., Tokimitsu, I., and Saito, M. (2008). Expression of uncoupling proteins in human skin and skin-derived cells. *J Invest Dermatol* **128**, 1894-900.

Mracek, T., Pecinova, A., Vrbacky, M., Drahota, Z., and Houstek, J. (2009). High efficiency of ROS production by glycerophosphate dehydrogenase in mammalian mitochondria. *Arch Biochem Biophys* **481**, 30-6.

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13.

Myung, J., Kim, K. B., and Crews, C. M. (2001). The ubiquitin-proteasome pathway and proteasome inhibitors. *Med Res Rev* **21**, 245-73.

Nakamura, N., Kimura, Y., Tokuda, M., Honda, S., and Hirose, S. (2006). MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. *EMBO Rep* **7**, 1019-22.

Nechad, M. (1986). *In Brown Adipose Tissue*. Arnold, London.

Nedergaard, J., Bengtsson, T., and Cannon, B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* **293**, E444-52.

Nedergaard, J., and Cannon, B. (1987). Apparent unmasking of [3H]GDP binding in rat brown-fat mitochondria is due to mitochondrial swelling. *Eur J Biochem* **164**, 681-6.

Negre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Trolly, M., Salvayre, R., Penicaud, L., and Casteilla, L. (1997). A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *Faseb J* **11**, 809-15.

Neumcke, I., Schneider, B., Fandrey, J., and Pagel, H. (1999). Effects of pro- and antioxidative compounds on renal production of erythropoietin. *Endocrinology* **140**, 641-5.

Neutzner, A., Youle, R. J., and Karbowski, M. (2007). Outer mitochondrial membrane protein degradation by the proteasome. *Novartis Found Symp* **287**, 4-14; discussion 14-20.

Nicholls, D. G. (1974a). Hamster brown-adipose-tissue mitochondria. The control of respiration and the proton electrochemical potential gradient by possible physiological effectors of the proton conductance of the inner membrane. *Eur J Biochem* **49**, 573-83.

Nicholls, D. G. (1974b). The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner

membrane of rat-liver mitochondria as determined by ion distribution. *Eur J Biochem* **50**, 305-15.

Nicholls, D. G., and Locke, R. M. (1984). Thermogenic mechanisms in brown fat. *Physiol Rev* **64**, 1-64.

Nicholls, D. G., and Rial, E. (1989). Measurement of proton leakage across mitochondrial inner membranes and its relation to protonmotive force. *Methods Enzymol* **174**, 85-94.

Oelkrug, R., Kutschke, M., Meyer, C. W., Heldmaier, G., and Jastroch, M. (2010). Uncoupling protein 1 decreases superoxide production in brown adipose tissue mitochondria. *J Biol Chem* **285**, 21961-8.

Olanow, C. W., and McNaught, K. S. (2006). Ubiquitin-proteasome system and Parkinson's disease. *Mov Disord* **21**, 1806-23.

Orlowski, M., Cardoza, C., Michaud, C. (1993). *Biochemistry* **32**, 1563-1572.

Pagliarini, D. J., and Dixon, J. E. (2006). Mitochondrial modulation: reversible phosphorylation takes center stage? *Trends Biochem Sci* **31**, 26-34.

Palou, A., Pico, C., Bonet, M. L., and Oliver, P. (1998). The uncoupling protein, thermogenin. *Int J Biochem Cell Biol* **30**, 7-11.

Pecqueur, C., Alves-Guerra, M. C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F., and Miroux, B. (2001). Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation. *J Biol Chem* **276**, 8705-12.

Pedraza, N., Solanes, G., Iglesias, R., Vazquez, M., Giralt, M., and Villarroya, F. (2001). Differential regulation of expression of genes encoding uncoupling proteins 2 and 3 in brown adipose tissue during lactation in mice. *Biochem J* **355**, 105-11.

Petrovic, N., Shabalina, I. G., Timmons, J. A., Cannon, B., and Nedergaard, J. (2008). Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARgamma agonist. *Am J Physiol Endocrinol Metab* **295**, E287-96.

Pi, J., Zhang, Q., Fu, J., Woods, C. G., Hou, Y., Corkey, B. E., Collins, S., and Andersen, M. E. (2010). ROS signaling, oxidative

stress and Nrf2 in pancreatic beta-cell function. *Toxicol Appl Pharmacol* **244**, 77-83.

Porter, R. K., and Andrews, J. F. (1998). Effects of leptin on mitochondrial 'proton leak' and uncoupling proteins: implications for mammalian energy metabolism. *Proc Nutr Soc* **57**, 455-60.

Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B., and Nedergaard, J. (1992). Induction and degradation of the uncoupling protein thermogenin in brown adipocytes in vitro and in vivo. Evidence for a rapidly degradable pool. *Biochem J* **284 (Pt 2)**, 393-8.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829-39.

Radke, S., Chander, H., Schafer, P., Meiss, G., Kruger, R., Schulz, J. B., and Germain, D. (2008). Mitochondrial protein quality control by the proteasome involves ubiquitination and the protease Omi. *J Biol Chem* **283**, 12681-5.

Raimbault, S., Dridi, S., Denjean, F., Lachuer, J., Couplan, E., Bouillaud, F., Bordas, A., Duchamp, C., Taouis, M., and Ricquier, D. (2001). An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. *Biochem J* **353**, 441-4.

Rhee, S. G., Kang, S. W., Jeong, W., Chang, T. S., Yang, K. S., and Woo, H. A. (2005). Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Curr Opin Cell Biol* **17**, 183-9.

Ricquier, D., and Bouillaud, F. (2000). The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem J* **345 Pt 2**, 161-79.

Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J., and Penicaud, L. (1986). Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. Evidence for a rapid beta-adrenoreceptor-mediated and transcriptionally regulated step during activation of thermogenesis. *J Biol Chem* **261**, 13905-10.

Ricquier, D., Mory, G., Bouillaud, F., Thibault, J., and Weissenbach, J. (1984). Rapid increase of mitochondrial uncoupling protein and its mRNA in stimulated brown adipose tissue. Use of a cDNA probe. *FEBS Lett* **178**, 240-4.

Ritter (1992). *structure and development in: the thymus*. IRL press and oxford university press.

Roth, S., and Droge, W. (1987). Regulation of T-cell activation and T-cell growth factor (TCGF) production by hydrogen peroxide. *Cell Immunol* **108**, 417-24.

Rotig, A., de Lonlay, P., Chretien, D., Foury, F., Koenig, M., Sidi, D., Munnich, A., and Rustin, P. (1997). Aconitase and mitochondrial iron-sulphur protein deficiency in Friedreich ataxia. *Nat Genet* **17**, 215-7.

Rousset, S., Alves-Guerra, M. C., Ouadghiri-Bencherif, S., Kozak, L. P., Miroux, B., Richard, D., Bouillaud, F., Ricquier, D., and Cassard-Doulcier, A. M. (2003). Uncoupling protein 2, but not uncoupling protein 1, is expressed in the female mouse reproductive tract. *J Biol Chem* **278**, 45843-7.

Rousset, S., Mozo, J., Dujardin, G., Emre, Y., Masscheleyn, S., Ricquier, D., and Cassard-Doulcier, A. M. (2007). UCP2 is a mitochondrial transporter with an unusual very short half-life. *FEBS Lett* **581**, 479-82.

Rutter, G. A. (2001). Nutrient-secretion coupling in the pancreatic islet beta-cell: recent advances. *Mol Aspects Med* **22**, 247-84.

Rutter, G. A., Pralong, W. F., and Wollheim, C. B. (1992). Regulation of mitochondrial glycerol-phosphate dehydrogenase by Ca^{2+} within electroporated insulin-secreting cells (INS-1). *Biochim Biophys Acta* **1175**, 107-13.

Saitoh, H., Pu, R. T., and Dasso, M. (1997). SUMO-1: wrestling with a new ubiquitin-related modifier. *Trends Biochem Sci* **22**, 374-6.

Salvi, M., Brunati, A. M., and Toninello, A. (2005). Tyrosine phosphorylation in mitochondria: a new frontier in mitochondrial signaling. *Free Radic Biol Med* **38**, 1267-77.

Scarpace, P. J., Bender, B. S., and Borst, S. E. (1991). Escherichia coli peritonitis activates thermogenesis in brown adipose tissue: relationship to fever. *Can J Physiol Pharmacol* **69**, 761-6.

Schmid, E., Hotz-Wagenblatt, A., Hacj, V., and Droge, W. (1999). Phosphorylation of the insulin receptor kinase by phosphocreatine in combination with hydrogen peroxide: the structural basis of redox priming. *Faseb J* **13**, 1491-500.

Scholz, T. D., Koppenhafer, S. L., TenEyck, C. J., and Schutte, B. C. (1997). Developmental regulation of the alpha-glycerophosphate shuttle in porcine myocardium. *J Mol Cell Cardiol* **29**, 1605-13.

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scime, A., Devarakonda, S., Conroe, H. M., Erdjument-Bromage, H., Tempst, P., Rudnicki, M. A., Beier, D. R., and Spiegelman, B. M. (2008). PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**, 961-7.

Seemuller, E., Lupus, A., Zuhl, F., Zwickl, P., Baumeister, W. (1995). *FEBS Lett* **359**, 173-178.

Shabalina, I. G., Petrovic, N., Kramarova, T. V., Hoeks, J., Cannon, B. and Nedergaard, J. UCP1 and defense against oxidative stress. *Journal of Biological Chemistry*. 281, 13882-13893

Shi, Y., Chan, D. W., Jung, S. Y., Malovannaya, A., Wang, Y., and Qin, J. A dataset of human endogenous ubiquitination sites. *Mol Cell Proteomics*.

Shih, M. F., and Taberner, P. V. (1995). Selective activation of brown adipocyte hormone-sensitive lipase and cAMP production in the mouse by beta 3-adrenoceptor agonists. *Biochem Pharmacol* **50**, 601-8.

Silva, J. P., Shabalina, I. G., Dufour, E., Petrovic, N., Backlund, E. C., Hultenby, K., Wibom, R., Nedergaard, J., Cannon, B., and Larsson, N. G. (2005). SOD2 overexpression: enhanced mitochondrial tolerance but absence of effect on UCP activity. *Embo J* **24**, 4061-70.

Sipos, I., Tretter, L., Adam-Vizi, V., (2003), Quantitative relationship between inhibition of respiratory complexes and formation of reactive oxygen species in isolated nerve terminals, *J Neurochem*, **84**, 112-118.

Skulachev, V. P. (1996). Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. *Q Rev Biophys* **29**, 169-202.

Skulachev, V. P. (1997). Membrane-linked systems preventing superoxide formation. *Biosci Rep* **17**, 347-66.

Slater, A. F., Stefan, C., Nobel, I., van den Dobbelsteen, D. J., and Orrenius, S. (1995). Signalling mechanisms and oxidative stress in apoptosis. *Toxicol Lett* **82-83**, 149-53.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76-85.

St-Pierre, J., Buckingham, J. A., Roebuck, S. J., and Brand, M. D. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* **277**, 44784-90.

Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S., and Beal, M. F. (2004). Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. *J Neurosci* **24**, 7779-88.

Suzukawa, K., Miura, K., Mitsushita, J., Resau, J., Hirose, K., Crystal, R., and Kamata, T. (2000). Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. *J Biol Chem* **275**, 13175-8.

Suzuki, J., Gao, M., Ohinata, H., Kuroshima, A., and Koyama, T. (1997). Chronic cold exposure stimulates microvascular remodeling preferentially in oxidative muscles in rats. *Jpn J Physiol* **47**, 513-20.

Swick, A. G., and Swick, R. W. (1986). Rapid changes in number of GDP binding sites on brown adipose tissue mitochondria. *Am J Physiol* **251**, E192-5.

Tait, S. W., de Vries, E., Maas, C., Keller, A. M., D'Santos, C. S., and Borst, J. (2007). Apoptosis induction by Bid requires unconventional ubiquitination and degradation of its N-terminal fragment. *J Cell Biol* **179**, 1453-66.

Talbot, D. A., Hanuise, N., Rey, B., Rouanet, J. L., Duchamp, C., and Brand, M. D. (2003). Superoxide activates a GDP-sensitive proton conductance in skeletal muscle mitochondria from king penguin (*Aptenodytes patagonicus*). *Biochem Biophys Res Commun* **312**, 983-8.

Thompson, W. E., Ramalho-Santos, J., and Sutovsky, P. (2003). Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol Reprod* **69**, 254-60.

Thonberg, H., Fredriksson, J. M., Nedergaard, J., and Cannon, B. (2002). A novel pathway for adrenergic stimulation of cAMP-response-element-binding protein (CREB) phosphorylation:

mediation via alpha1-adrenoceptors and protein kinase C activation. *Biochem J* **364**, 73-9.

Timmons, J. A., Wennmalm, K., Larsson, O., Walden, T. B., Lassmann, T., Petrovic, N., Hamilton, D. L., Gimeno, R. E., Wahlestedt, C., Baar, K., Nedergaard, J., and Cannon, B. (2007). Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* **104**, 4401-6.

Toyomizu, M., Ueda, M., Sato, S., Seki, Y., Sato, K., and Akiba, Y. (2002). Cold-induced mitochondrial uncoupling and expression of chicken UCP and ANT mRNA in chicken skeletal muscle. *FEBS Lett* **529**, 313-8.

Tretter, L., Takacs, K., Hegedus, V., and Adam-Vizi, V. (2007a). Characteristics of alpha-glycerophosphate-evoked H₂O₂ generation in brain mitochondria. *J Neurochem* **100**, 650-63.

Tretter, L., Takacs, K., Kover, K., and Adam-Vizi, V. (2007b). Stimulation of H₂O₂ generation by calcium in brain mitochondria respiring on alpha-glycerophosphate. *J Neurosci Res* **85**, 3471-9.

Tretter, L. and Adam-Vizi, V., (2007c), Uncoupling is without an effect on the production of reactive oxygen species by in situ synaptic mitochondria, *J Neurochem*, **103**, 1864-1871.

Valle, A., Guevara, R., Garcia-Palmer, F.J., Roca, P. and Oliver, J., (2007), Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions, *Am. J. Physiol. Cell Physiol.* **293**, C1302-C1308

Vianna, C. R., Hagen, T., Zhang, C. Y., Bachman, E., Boss, O., Gereben, B., Moriscot, A. S., Lowell, B. B., Bicudo, J. E., and Bianco, A. C. (2001). Cloning and functional characterization of an uncoupling protein homolog in hummingbirds. *Physiol Genomics* **5**, 137-45.

Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J. S., and Lowell, B. B. (1997). UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem Biophys Res Commun* **235**, 79-82.

Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ido, Y., Szczepanik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D. M., and Lowell, B. B. (2000). Energy metabolism in uncoupling protein 3 gene knockout mice. *J Biol Chem* **275**, 16258-66.

- Vrbacky, M., Drahota, Z., Mracek, T., Vojtiskova, A., Jesina, P., Stopka, P., and Houstek, J. (2007). Respiratory chain components involved in the glycerophosphate dehydrogenase-dependent ROS production by brown adipose tissue mitochondria. *Biochim Biophys Acta* **1767**, 989-97.
- Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A* **92**, 5510-4.
- Warr, M. R., Acoca, S., Liu, Z., Germain, M., Watson, M., Blanchette, M., Wing, S. S., and Shore, G. C. (2005). BH3-ligand regulates access of MCL-1 to its E3 ligase. *FEBS Lett* **579**, 5603-8.
- Wasiak, S., Zunino, R., and McBride, H. M. (2007). Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. *J Cell Biol* **177**, 439-50.
- Watanabe, M., Yamamoto, T., Mori, C., Okada, N., Yamazaki, N., Kajimoto, K., Kataoka, M., and Shinohara, Y. (2008). Cold-induced changes in gene expression in brown adipose tissue: implications for the activation of thermogenesis. *Biol Pharm Bull* **31**, 775-84.
- Winkler, E., and Klingenberg, M. (1994). Effect of fatty acids on H⁺ transport activity of the reconstituted uncoupling protein. *J Biol Chem* **269**, 2508-15.
- Woelk, T., Sigismund, S., Penengo, L., and Polo, S. (2007). The ubiquitination code: a signalling problem. *Cell Div* **2**, 11.
- Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* **118**, 83-97.
- Yonashiro, R., Ishido, S., Kyo, S., Fukuda, T., Goto, E., Matsuki, Y., Ohmura-Hoshino, M., Sada, K., Hotta, H., Yamamura, H., Inatome, R., and Yanagi, S. (2006). A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. *Embo J* **25**, 3618-26.
- Yousefi, S., Green, D. R., Blaser, K., and Simon, H. U. (1994). Protein-tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils. *Proc Natl Acad Sci U S A* **91**, 10868-72.
- Yu, X. X., Mao, W., Zhong, A., Schow, P., Brush, J., Sherwood, S. W., Adams, S. H., and Pan, G. (2000). Characterization of novel UCP5/BMCP1 isoforms and differential regulation of UCP4 and UCP5

expression through dietary or temperature manipulation. *Faseb J* **14**, 1611-8.

Zacharchuk, C. M., Mercep, M., and Ashwell, J. D. (1991). Thymocyte activation and death: a mechanism for molding the T cell repertoire. *Ann N Y Acad Sci* **636**, 52-70.

Zhang, C. Y., Baffy, G., Perret, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A. J., Boss, O., Kim, Y. B., Zheng, X. X., Wheeler, M. B., Shulman, G. I., Chan, C. B., and Lowell, B. B. (2001). Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* **105**, 745-55.

Zhao, J. (2007). Sumoylation regulates diverse biological processes. *Cell Mol Life Sci* **64**, 3017-33.

Zhao, J., Cannon, B., and Nedergaard, J. (1997). alpha1-Adrenergic stimulation potentiates the thermogenic action of beta3-adrenoreceptor-generated cAMP in brown fat cells. *J Biol Chem* **272**, 32847-56.

Zhou, M., Lin, B. Z., Coughlin, S., Vallega, G., and Pilch, P. F. (2000). UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase. *Am J Physiol Endocrinol Metab* **279**, E622-9.

Zhou, Y. T., Shimabukuro, M., Koyama, K., Lee, Y., Wang, M. Y., Trieu, F., Newgard, C. B., and Unger, R. H. (1997). Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. *Proc Natl Acad Sci U S A* **94**, 6386-90.

Zunino, R., Schauss, A., Rippstein, P., Andrade-Navarro, M., and McBride, H. M. (2007). The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. *J Cell Sci* **120**, 1178-88.