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The Role of the Bile Acid Deoxycholate in Gastrointestinal Epithelial Cell Signalling

A Thesis Submitted for the Degree
of Doctor of Philosophy (Ph.D.)

By

Eileen Looby
B.Sc. (Biotech)

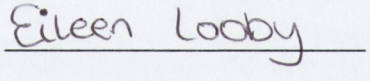
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Eileen Looby

SUMMARY

Environmental and dietary factors are implicated in the aetiology of human cancer. Bile acids, the amphiphilic derivatives of cholesterol, are strongly implicated in colonic and oesophageal carcinogenesis. While bile acids themselves cannot induce tumours, they are generally regarded as being tumour promoters. The exact mechanism of their tumour promoting activity is uncertain but it is likely to involve alterations in epithelial cell signalling cascades and gene expression systems. The ability of deoxycholic acid (DCA), a potent bile acid, to modify intracellular signalling cascades such as protein kinase C (PKC) and mitogen activated protein kinase (MAPK) was examined. As bile acids are also thought to mediate their tumour promoting effects through transcriptional deregulation, DCA-induced alterations in the transcription factor, activator protein-1 (AP-1), were investigated. The findings of this thesis provide a further understanding of the role of DCA in gastrointestinal tumour cell development through the persistent activation of MAPK and AP-1 contributing to deregulated expression of downstream targets such as COX-2.

Firstly DCA induces rapid translocation of PKC-β_1, PKC-ε but not PKC-δ from the cytosol to the plasma membrane. Interestingly sodium butyrate, the by-product of dietary fibre fermentation, is considered to be protective against colon cancer. It does not alter the translocation events induced by DCA in colonic cells. DCA-induced translocation of PKC-β_1 and PKC-ε to the plasma membrane is accompanied by rearrangements in the cytoskeletal elements actin and tubulin. As modulation of the cytoskeleton is known to affect the distribution of signalling molecules, reorganisation of the cytoskeletal elements in response to DCA is likely to be important in tumour cell progression.

Secondly the biological responses of oesophageal adenocarcinoma cells to DCA were examined. DCA does not alter PKC-β_1 subcellular localisation in oesophageal cells but induces morphological changes such as alteration in cell shape and vacuolisation. DCA induces a dose and time dependent decrease in cell proliferation. Low concentrations of DCA, below 300 μM, do not alter the proliferative responses of the cells. At 300 μM, DCA induces maximal COX-2 expression, which is concurrent with onset of DCA-induced PARP cleavage and DNA fragmentation that occurs in parallel. At higher concentrations of DCA, in particular 500 μM, PARP cleavage and DNA fragmentation is maximal while COX-2 expression is lost. Inhibition of DCA-induced COX-2 expression results in elevated PARP

cleavage and DNA fragmentation. Lower concentrations of DCA appear to mediate oesophageal tumour cell survival, which is lost at the higher concentrations of DCA due to the inherent cytotoxic effects of DCA. The survival mechanism mediated by DCA is occurring at concentrations known to occur in the oesophageal aspirates of Barrett's patients. As this mechanism can be blocked using pharmacological agents, this could represent a possible therapeutic strategy for patients on acid suppression, where bile acid concentrations in the refluxate are increased.

Thirdly DCA persistently activates the MAPKs, extracellular-signal-regulated-kinase (Erk), p38 but not c-Jun-N-terminal-kinase (JNK). As the MAPKs control the activation of the transcription factor AP-1, a key regulator of cellular processes such as proliferation, differentiation and apoptosis, the ability of DCA to activate AP-1 was analysed. DCA induces persistent activation of the AP-1. Analysis of the DCA-induced AP-1 complex, by EMSA and DNA affinity purification experiments, identified Fra-1 and JunB as the predominant components of the AP-1 complex. In addition to *de novo* protein synthesis, which occurred for Fra-1 and JunB, AP-1 binding activity is increased by the MAPKs. Both Erk and p38 mediate DCA-induced Fra-1 DNA binding activity, while Erk induces JunB activation. Additionally the downstream target of MAPK activation and AP-1 activity, COX-2 was examined. Erk and p38 specific inhibitors abolish DCA-induced COX-2 expression suggesting that DCA induces sustained activation of Erk and p38 resulting in persistent activation of AP-1 and COX-2 in oesophageal adenocarcinoma cells.

To conclude, DCA induces marked alterations in intracellular signalling and gene expression in gastrointestinal cells. In colonic cells, DCA differentially regulates PKC isoforms while in oesophageal cells, DCA activates the MAPKs, which mediate AP-1 and COX-2 expression. The regulation of these cascades and their significance in the abnormal morphogenesis of the gastrointestinal epithelium in response to bile acids is considered.

DEDICATION

To my Father Denis

"Every search begins with beginner's luck. And every search ends with the victor's being severely tested."

-Paulo Coehlo

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ABBREVIATIONS

ABTS	2,2'-azino-di[3-ethylbenz-thiazoline sulfonate-6-diammonium salt]
AIF	Apoptosis inducing factor
AO	Acridine Orange
AP-1	Activated Protein 1
APC	Adenomatous polyposis Coli
APS	Ammonium persulphate
BIS	Bisindolylmalemide
bZIP	Basic region-leucine zipper
BSA	Bovine serum Albumin
C/EBPβ	cAMP response element binding protein beta
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CMC	Critical micelle concentration
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CRE	Cyclic AMP response element
DAG	Diacylglycerol
DCA	Deoxycholic acid
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EB	Ethidium Bromide
ECACC	European Collection of Animal Cell Cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGTA	Ethyleneglycol bis-(aminoethlether)tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated kinase
FAP	Familial adenomatous polyposis
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FXR	Farnesoid X receptor
GADD153	Growth arrest and DNA damage-inducible gene 153
GFP	Green fluorescent protein
GORD	Gastro-oesophageal reflux disease
HBSS	Hanks buffered salts solution
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horse radish peroxidase
IAP	Inhibitors of apoptosis
IL-1β	Interleukin-1beta
IL-2	Interleukin-2
IL-8	Interleukin-8
IL-6	Interleukin-6

JNK	c-Jun NH_2-terminal kinase
KDa	Kilodaltons
LB	Luria-Bertani media
LOX	Lipoxygenases
LXR	Liver X receptor
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MEK	MAP/Erk Kinase
MEK	MAPK/Erk Kinase Kinase
MKK	MAP Kinase Kinase
MKP	MAP kinase phosphatase
MNK	MAP kinase interacting kinase
mPGES-1	Microsomal prostaglandin E synthase-1
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
NaB	Sodium butyrate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NOS2	Nitric oxide synthase
NP-40	Nonidet P40
NSAIDs	Non-steroidal anti-inflammatory drugs
p90RSK	90 kDa ribosomal S6 protein kinase
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PDVF	Polyvinylidene difluoride
PFA	Paraformaldehyde
PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PKC-ι	PKC-lambda
PKC-α	PKC-alpha
PKC-δ	PKC-delta
PKC-ε	PKC-epsilon
PKC-η	PKC-eta
PKC-γ	PKC-gamma
PKC-μ	PKC-mu
PKC-θ	PKC-theta
PKC-ζ	PKC-zeta
PKC-β_1	PKC-beta1
PKC-β_2	PKC-beta2
PKD	Protein kinase D
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenyl methyl sulphonyl fluoride
PP-2A	Protein phosphatase-2A
PPIs	Proton pump inhibitors
Rho	Ras homology

ROS	Reactive oxygen species
SAP-1a	Serum response factor accessory protein
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SEK	SAPK/Erk Kinase
SRE	Serum response element
SIE	Sis inducible enhancer
ST	Staurosporine
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate EDTA buffer
TBE	Tris-boric EDTA buffer
TBS	Tris buffered saline
TCFs	Ternary complex factors
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethyl ethylene diamine
TGFβ	Transforming growth factor beta
TRAIL	TNF-related apoptosis inducing ligand
TRE	TPA responsive element
Tris	Tris (hydroxymethyl) aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
UDCA	Ursodeoxycholate
UV	Ultraviolet

CHAPTER I

GENERAL INTRODUCTION

CHAPTER I

General Introduction

1.1 GASTROINTESTINAL CANCER

The accumulation of genetic abnormalities is important in the development of a malignant phenotype. Cancers of the upper and lower gastrointestinal tract such as colonic and oesophageal cancer, are characterised by the acquisition of genetic defects leading to invasive cancer. Despite recent advances, the exact mechanism responsible for the development of gastrointestinal cancer has yet to be elucidated. This thesis investigates the role of bile acids in perturbing colonic and oesophageal epithelial cell signalling, a prerequisite for malignant progression and sheds new light on the role of bile acids in the pathogenesis of gastrointestinal cancer.

1.1.1 Colon Cancer

Colon cancer is a leading cause of cancer related deaths in western countries and is characterised by a series of histopathologic and molecular changes that transform normal colonic epithelial cells into a colorectal carcinoma, with the adenomatous polyp as an intermediate step in the process **(Fig 1.1)**. A landmark study in 1988 revealed that several genetic abnormalities commonly occur during colon cancer. The cumulative total of these genetic abnormalities appears to be more important than the order of their appearance (Vogelstein et al. 1988).

Hereditary forms of colon cancer such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) exist where the presence of germline mutations in specific genes increase the potential for transformation in colonic cells (Fahy and Bold 1998). In patients with FAP, germline mutations in the adenomatous polyposis coli (*apc*) gene were identified as early causative events in this syndrome (Powell et al. 1992). The *apc* gene functions primarily as a regulator of epithelial cell proliferation. In addition, *apc* also functions as a negative regulator of β-catenin. Inactivation of *apc* through mutation leads to the nuclear accumulation of β-catenin, increased activation of the TCF transcription complex resulting in the transcription of growth related genes leading to hyperproliferation (Pennisi 1998), a hallmark of colon cancer. In HNPCC, the basic genetic defects underlying this syndrome occur in the DNA mismatch repair genes,

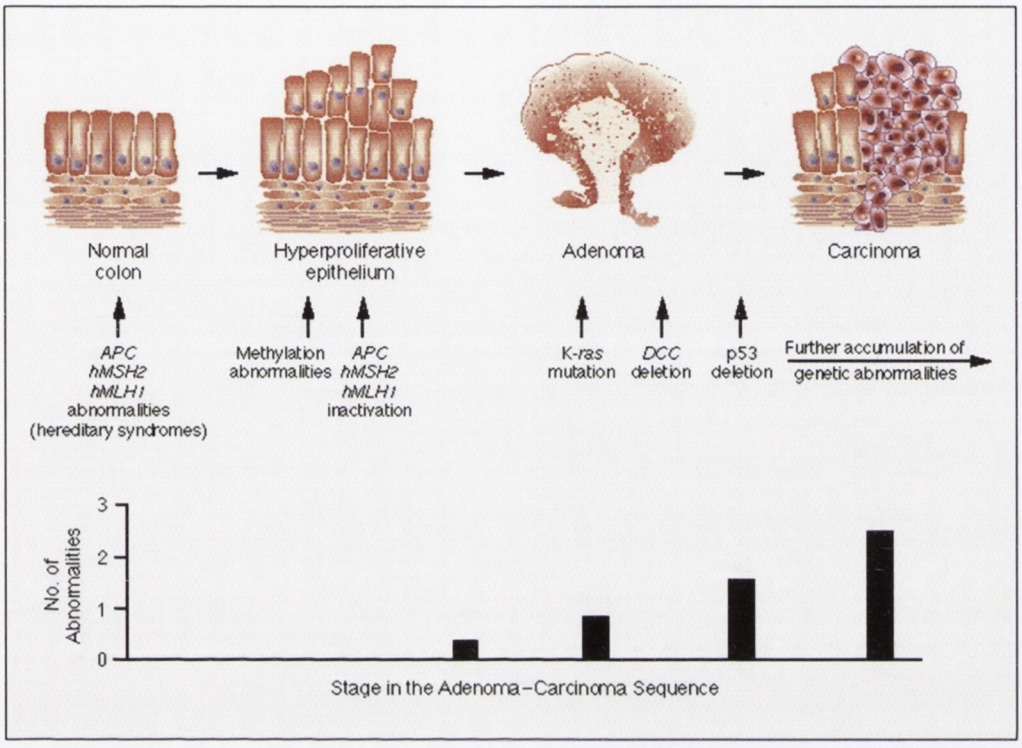


Fig 1.1. Events During Colorectal Carcinogenesis. Some of known chromosomal and genetic alterations that occur during the progression from the normal colonic epithelium to invasive cancer are shown. In the lower panel the average number of genetic abnormalities per tumour to the corresponding stage in the adenoma-carcinoma sequence is shown. Adapted from Toribara and Sleisenger 1995.

MLH1 and MSH2 (Bronner et al. 1994; Nicolaides et al. 1994). Loss of function of these genes is thought to allow for the accumulation of base-pair mismatches that result in a replication-error phenotype. Deletions and mutations in genes such as p53, DCC and k-ras are characteristic features of colonic neoplasms. While genetic defects are considered important for the onset of disease, dietary factors, in particular dietary fat, are thought to contribute to the development of the disease. A positive correlation between the incidence of colon cancer and the ingestion of high fat diets has been established (Lipkin et al. 1999). Against the backdrop of genetic instability, dietary factors are of major importance for the aetiology of colonic neoplasia (Lipkin et al. 1999).

1.1.2 Oesophageal Cancer

Oesophagitis, resulting from gastro-oesophageal reflux disease (GORD), is a common medical condition in western countries, with 10 % of patients progressing to Barrett's oesophagus (Cameron et al. 1990; Poynton et al. 1996). Barrett's oesophagus is best described as a premalignant lesion in which the normal squamous epithelium that lines the distal oesophagus is replaced by a metaplastic columnar epithelium (Jankowski et al. 2000). Three types of Barrett's oesophagus have been defined; however the specialised intestinal type has been consistently associated with increased risk of oesophageal cancer (Spechler et al. 1984; Drewitz et al. 1997). The genetic defects of oesophageal adenocarcinoma are less well characterised than those of colonic adenocarcinoma. Significant differences exist between the adenoma-carcinoma sequence in the colon and the proposed metaplasia-dysplasia-carcinoma sequence in the oesophagus, even though some of the same genes and regulatory systems are involved. Familial clusters of Barrett's oesophagus are known to exist (Chak et al. 2002) and it is likely follow an autosomal dominant model of inheritance (Drovdlic et al. 2003). Even though no germline mutations have been found in oesophageal adenocarcinoma, progression from metaplasia to carcinoma involves numerous genetic alterations including the allelic loss of chromosomes 17p, 18q and 5q resulting in aneuploid DNA content in dysplastic lesions. Their emergence is closely associated with inactivation of p53 (Wu et al. 1998). As depicted in **Fig 1.2**, deregulation of cell cycle is also characteristic of malignant progression in Barrett's oesophagus due in part to increased expression of cyclin D1 and hypermethylation or mutation of p16 with subsequent G2 phase accumulation (Tselepis et al. 2000). A well established and permanent alteration in Barrett's oesophagus is increased cell proliferation and decreased apoptosis (Gillen et al. 1988; Katada et al. 1997; Whittles et al. 1999; van

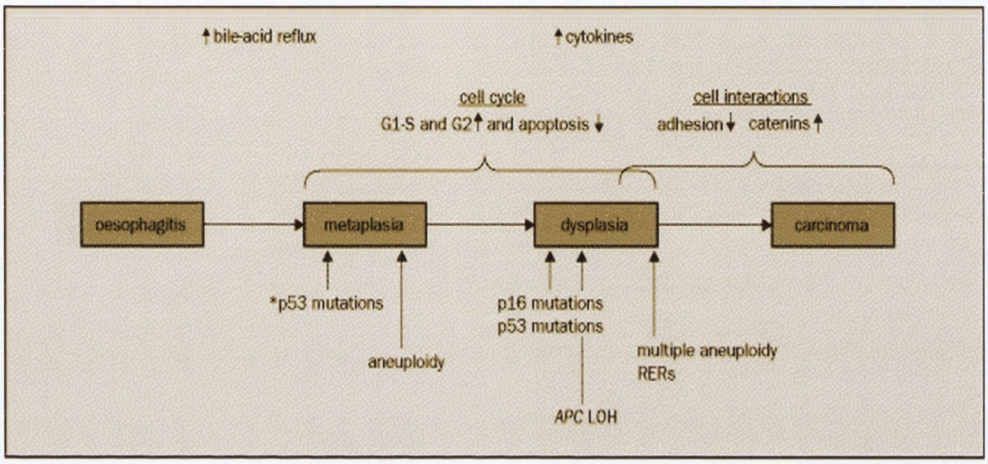


Fig 1.2. Molecular and Cellular Alterations in Progression of Barrett's Oesophagus. Progression of normal oesophageal cells to cancer cells along the metaplasia-dysplasia-carcinoma sequence. LOH= Loss of heterozygosity, RER= Random errors of replication, APC= adenomatous polyposis coli gene. *Early p53 mutations are unlikely to be important in the initial clonal expansion as inhibition of apoptosis (feature of p53 dysregulation) are not seen till late dysplasia. Reproduced from Jankowski *et al*. 2000.

der Woude et al. 2002) due in part to increased expression of anti-apoptotic proteins such as Bcl-2 and COX-2 early in the dysplasia-carcinoma sequence of Barrett's oesophagus (Goldblum and Rice 1995; Lauwers et al. 1997; Rioux-Leclercq et al. 1999). Furthermore, alterations in the apoptotic cascade such as reduced or loss of expression of pro-apoptotic proteins such as Fas has been noted in oesophageal adenocarcinoma (Hughes et al. 1997). The mechanism by which Barrett's oesophagus develops is not known, although it appears to result from chronic irritation of the oesophageal mucosa by bile acids and gastric acid.

1.2 BILE ACIDS AND GASTROINTESTINAL CANCER

An association between Barrett's oesophagus and colon cancer has been reported. Sontag *et al.* demonstrated that patients with Barrett's oesophagus and other GORDs had colonic tumours as well, which was later confirmed by Roberston *et al.* (Sontag et al. 1985; Robertson et al. 1989). It was speculated that a common aetiological agent, such as a dietary factor, might be involved. Diet-related factors are thought to account for almost 30 % of cancers in Western countries in particular cancers of the gastrointestinal tract (Key et al. 2002; Bingham and Riboli 2004).

1.2.1 Role of Bile Acids in Colon Cancer

Initially, a casual relationship between the bile acids and colon cancer was demonstrated when intrarectal instillation of bile acids in rats promoted the development of colonic adenomas and carcinomas (Narisawa et al. 1974; Reddy and Maeura 1984). Subsequent epidemiological studies have demonstrated that a typical Western diet, high in fat and low in fibre, contributes to the development of colon cancer (Woutersen et al. 1999). It is now suspected that almost 50% of colon cancers are attributed to dietary factors. Animal studies have demonstrated that not only is the total fat content but the type of fat is extremely important for tumour promotion in the colon (Reddy et al. 1996). Diets, high in beef tallow or lard increase the concentration of colonic faecal secondary bile acids such as deoxycholic acid, which are credited for the colon tumour promoting effects of dietary fat (Reddy 1994; Reddy et al. 1996). Elevated levels of bile acids such as deoxycholic acid (DCA) have been consistently associated with increased risk of developing colon cancer. Patients with colonic adenomas and carcinomas have higher levels of bile acids in their serum and stool (Bayerdorffer et al. 1995; Kishida et al. 1997) and thereby supporting the role of bile acids in contributing to the development of colon cancer. The most compelling data for the involvement of bile acids in the pathogenesis of colon cancer comes from

animal models. Azoxymethane (AOM) treated rodents maintained on a diet of secondary bile acids had significantly higher numbers of tumours than their control counterparts (Sutherland and Bird 1994). Based on the mounting evidence from epidemiological and animals studies, it is reasonably to conclude that bile acids play a prominent role in malignant development in the colonic epithelium.

1.2.2 Bile Acid Reflux in Oesophageal Cancer

Initial reports that oesophagitis was caused by gastric reflux were first made by Quincke in the last decade of the 19th century and were expanded by Winkelstein (Winkelstein 1935). However, evidence later suggested that gastric acid was not the sole agent responsible for oesophageal erosion as oesophagitis was frequently seen in patients after total and subtotal gastrectomy where the acid-producing part of the stomach was removed (Helsingen 1960). Gillison *et al.* demonstrated the importance of bile acids in reflux in primates, when rhesus monkeys underwent surgery to establish the role of reflux of acid alone and reflux of acid with bile on the development of oesophagitis. The severity of oesophagitis was increased in monkeys that had bile reflux (Gillison et al. 1972).

It is now believed that reflux of gastric and duodenal contents into the oesophagus are important factors in the development of oesophagitis. Gastro-oesophageal reflux occurs when the anatomical and physiological nature of the oesophagogastric junction fails. Individuals, with a high fat diet, have increased bile acid secretions and thereby increasing the bile acid content of the refluxate (Hill 1990). Evidence now suggests that bile acids alone are extremely important in Barrett's oesophagus as studies have demonstrated that bile acids are active in the refluxate (Sharma and Sampliner 1997). Increased bile acid exposure has been associated with increased oesophageal mucosal damage and severity of disease (Gillen et al. 1988). Bile acids have been associated with the promotion of goblet-cell containing metaplasia in other gastrointestinal epithelia including the stomach, duodenum, intestine and bile ducts (Jankowski et al. 2000).

1.3 BILE ACIDS

1.3.1 Bile Acid Structure and Metabolism

Bile acids are naturally occurring trophic factors of the gastrointestinal tract and are steroid metabolites of cholesterol. Primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA), derived from cholesterol in the liver, are secreted into the

cannaliculi as taurine and glycine conjugates in hepatic bile, which flows into the bile ducts (Vlahcevic et al. 1999). Hepatic bile, which is composed of large amounts of bile acids, cholesterol and phospholipids, is modified in the bile ducts by the addition of a bicarbonate rich aqueous secretion from the ductal epithelial cells. During fasting, the gallbladder stores and concentrates the bile by the removal of inorganic electrolytes and water by absorption. Micelle formation further concentrates the bile in the gall bladder **(Fig 1.3)**. When chime from an ingested meal enters the small intestine, acid and partial digested fats and proteins prompt the release of cholescystokinin and secretin, which stimulates gall bladder contraction and relaxation of the sphincter of Oddi culminating in the discharge of bile into the duodenum (Hofmann 1992).

Upon entry into the duodenum each of the bile constituents undergoes a different fate. Phospholipids, predominantly phosphatidylcholine, are hydrolysed to lysolecithin, which can solubilise free cholesterol. Conjugated bile acids solubilise partially digested fat such as fatty acids and monoglycerides in mixed micelles and assist in their diffusion to the enterocytes. Approximately 85% of bile acids are absorbed in the small intestine. In the duodenum, passive diffusion of mixed micelles of bile acids, fatty acids and monoglycerides is higher for hydrophobic bile acids. In the ileum, active transport favours conjugated over unconjugated bile acids. The remaining unabsorbed bile acids (15 %) pass into the colon **(Fig 1.3)**. Secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid (LCA) are formed from conjugated forms of CDCA and CA respectively through bacterial deconjugation and 7α-dehydroxylation by enteric anaerobic bacteria **(Fig 1.4)**. Tertiary bile acids such as ursodeoxycholic acid (UDCA) and sulpho-LCA (SLCA) are produced through epimerisation of CDCA or sulponation of LCA **(Fig 1.4)**. About 10 % of the bile acids, predominantly unconjugated dihydroxy bile acids, are reabsorbed through passive diffusion in the colon and merge in the portal venous blood with the flux of other conjugated and unconjugated bile acids from the distal small intestine (Nagengast et al. 1995; Debruyne et al. 2001). Reabsorbed bile acids are returned to the liver by binding to specific proteins that facilitate their transport across intestinal enterocytes (Lu et al. 2001). Bile acids are extracted from the portal venous blood by hepatocytes in the liver where they are reconjugated and so completing the enterohepatic circulation **(Fig 1.3)** (Small et al. 1972; Stellaard et al. 1984; Hofmann et al. 1987). Bile acids that pass the liver without being taken up by hepatocytes enter the peripheral blood into systemic circulation. Any remaining unabsorbed bile acids in the colon are secreted in the stool **(Fig 1.3)**.

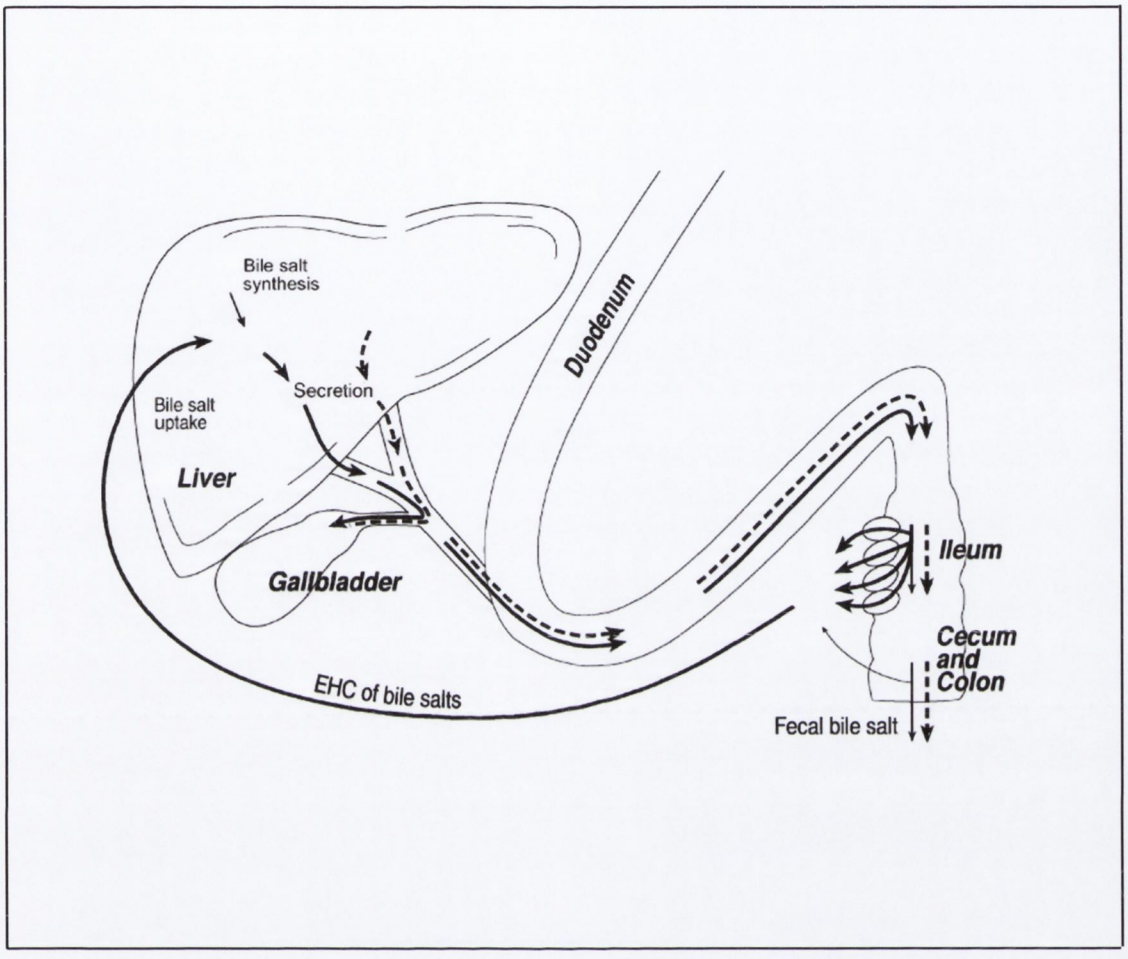


Fig 1.3. Dynamics of Enterohepatic Circulation. Large amounts of bile acids are secreted from the gall bladder into the duodenum. Most of the bile acids are reabsorbed from the duodenum in the venous blood in the ileum, which enters into the portal vein and from there to the sinusoids of the liver. Hepatocytes extract bile acids from sinusoidal blood preventing the bile acids entering into systemic circulation. Bile acids are subsequently transported across the hepatocytes to be resecreted into canaliculi and for reuse. Reproduced from Vítek and Carey 2003.

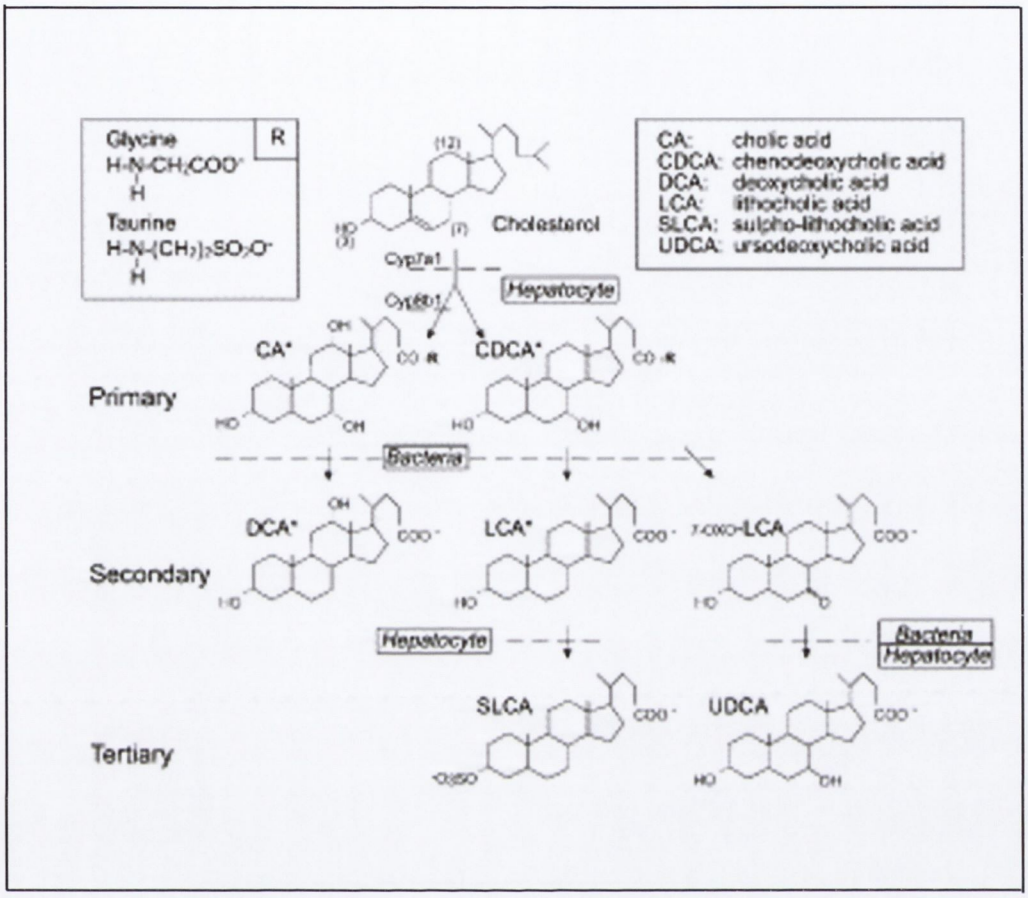


Fig 1.4. Structures of the Primary, Secondary and Tertiary Bile acids and their Amino Acid (R) Conjugates. The sites of metabolism either by the hepatocytes or by intestinal bacteria is indicated by the dashed lines. * This bile acid stimulates invasion *in vitro*. 7-oxo-LCA is unstable and is immediately converted to UDCA. The rate limiting enzymes in the bile acid synthesis, cholesterol 7α-hydroxylase (CYP7a1) and sterol 12α-hydroxylase (CYP8b1), are indicated. Reproduced from Debruyne *et al* 2001.

1.3.2 Regulation of Bile Acid Biosynthesis

Bile acid biosynthesis represents the main mechanism of cholesterol elimination from the body and plays an important role in cholesterol homeostasis. Accretion of dietary cholesterol in the liver stimulates the synthesis of oxysterols that bind to the orphan nuclear receptor, liver X receptor (LXR), which in turn amplifies the catabolic clearance of cholesterol by increasing bile acid synthesis and excretion as depicted in **Fig 1.5** (Repa and Mangelsdorf 1999; Lu et al. 2001). Bile acid homeostasis is regulated through feedback repression of its synthesis and transport. As the bile acid pool increases in size, transcription of cholesterol 7-α-hydroxylase (CYP7A1), the rate limiting enzyme in bile acid synthesis, is repressed through the orphan nuclear receptor, farnesoid X receptor (FXR) (Parks et al. 1999; Xu et al. 2002). FXR not only regulates bile acid synthesis but bile acid transport as well thereby preventing over accumulation of bile acid in hepatocytes **(Fig 1.5)**. Binding of bile acids to FXR regulates two principal transporters, the sodium taurocholate cotransporter polypeptide (NTCP) and the bile salt export pump (BESP). Activation of FXR by bile acids represses NTCP, which prevents bile acid uptake into the hepatocytes while stimulating BESP, a protein responsible for promoting bile acid efflux into bile. In the small intestine, bile acids upregulate the ileal bile acid binding protein via FXR, which assists in reducing the free concentrations of bile acids and thereby limiting their toxicity and circulation (Repa and Mangelsdorf 1999; Lu et al. 2001). Therefore FXR serves as a bile acid sensor and plays a critical role in bile acid homeostasis.

1.3.3 Function of Bile Acids as Normal Constituents of the Gastrointestinal Tract

Bile acids are amphipathic as they contain both hydrophobic and hydrophilic moieties. Their amphipathic nature allows bile acids to perform two important functions. Due to their inherent detergent properties, bile acids can emulsify fat globules to microscopic droplets. This greatly increases the surface area of the lipid making it available for digestion by lipases that cannot otherwise access the interior of the lipid globules (Carlier et al. 1986). Secondly bile acids function as lipid carriers in an aqueous environment as they are able to solubilise lipids by forming micelles, which are aggregates of lipids such as fatty acids, cholesterol and monoglycerides that remain suspended in water (Matsuoka and Moroi 2002; Stamp 2002). The critical micelle concentration (CMC) is the concentration at which aggregates or micelles form. Bile acids with low CMCs can overcome their pKa's effect, which can sometimes prevent them crossing the mucosal

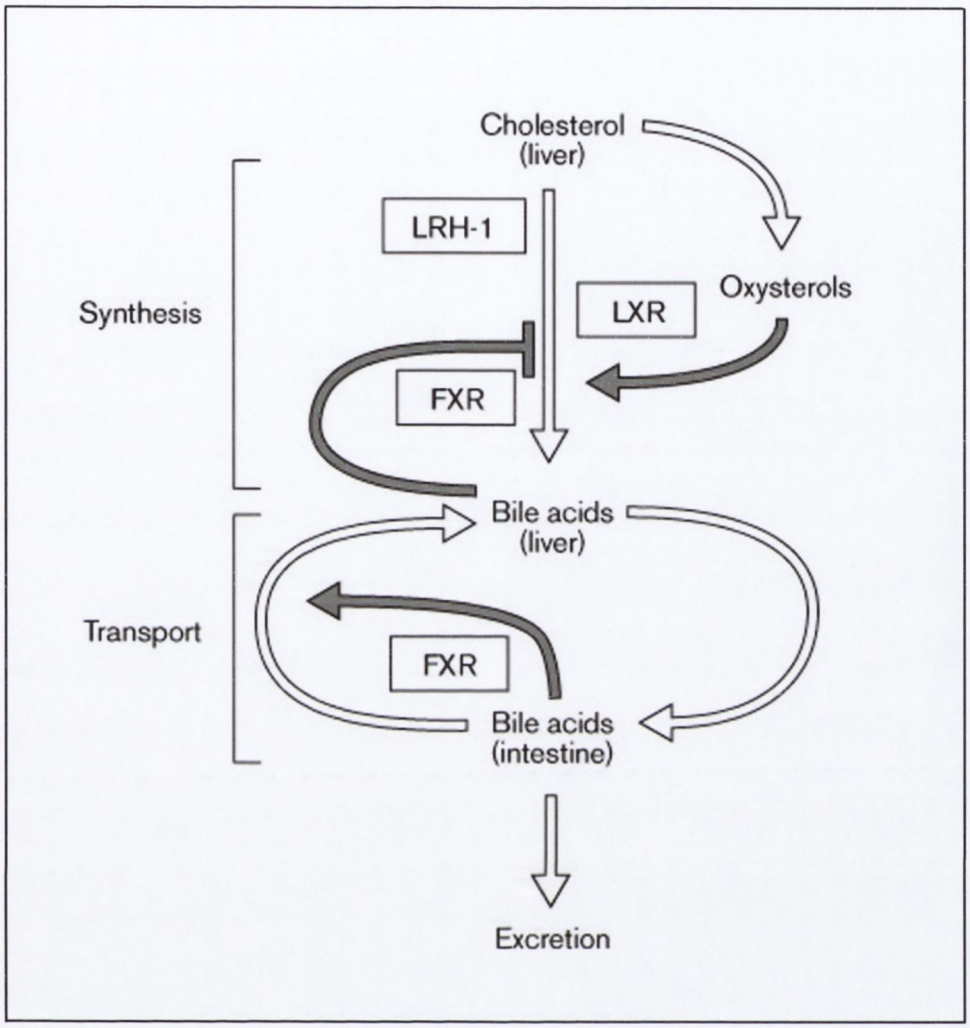


Fig 1.5. Transcriptional Control of Cholesterol and Bile Acid Homeostasis by Nuclear Receptors. White arrows are indicative of enzymatic reactions or transport steps, whereas grey arrows indicate positive or negative regulation by nuclear receptors. Reproduced from Repa and Mangelsdorf 1999.

barrier (Schweitzer et al. 1986). The CMC values for bile acids range from 3 mM for DCA to 10 mM for glycocholic acid (Matsuoka and Moroi 2002). In addition, bile acids are critical for the adsorption of fat soluble vitamins such as vitamin D (Holt 1972).

1.4 BILE ACID INDUCED ALTERATIONS IN GASTROINTESTINAL PATHOPHYSIOLOGY

Bile acids have been implicated in alterations in colonic and oesophageal physiology and thereby contributing to the development of a malignant phenotype. In the colon, increased concentrations of bile acids modify colonic microflora, which results in increased counts of bacteroides, a higher ratio of anaerobes over aerobes and more strains of non-spore forming anaerobes that are able to 7α-dehydroxylate bile acids (Hill et al. 1971). Alterations in colonic microflora increases the concentration of toxic secondary bile acids (Hill 1990), which serves as an amplification loop.

Secondary bile acids, including DCA, are produced by intestinal microflora along the gastrointestinal tract (Domellof et al. 1980). Intestinal microflora, capable of 7α-dehydroxylating bile acids to produce toxic secondary bile acids, have been detected in oesophageal aspirates from Barrett's patients (Nehra et al. 1998; Nehra et al. 1999). These bile acids are implicated in oesophageal mucosal injury and inflammation in Barrett's oesophagus and in animal models of reflux (Kivilaakso et al. 1980; Zhang et al. 2001a). Barrett's oesophagus is associated with inflammatory and neoplastic complications and is characterised by increased expression of pro-inflammatory cytokines such as interleukin - 1beta (IL-1β) and interleukin-8 (IL-8) as well as a T-cell infiltration (Biddlestone et al. 1998; Fitzgerald et al. 2002). Studies have demonstrated that bile acids induce the expression of pro-inflammatory cytokines such as IL-8, which are known to recruit and activate lymphocytic cells (Proost et al. 1996). The chronic inflammatory cell infiltrate, which exists in Barrett's metaplasia in response to acid and bile damage, is thought to maintain and promote Barrett's metaplasia even after reflux has been reduced through acid suppression therapy or ablation therapy (Biddlestone et al. 1998).

One of the defining features of Barrett's oesophagus, at a histological level, is the presence of mucin-producing goblet cells resembling those of the small intestine and colon (Jankowski et al. 2000). Bile acids have been associated with the promotion of goblet cell

containing metaplasia in gastrointestinal epithelia and are known to induce mucin secretion (Jankowski et al. 2000; Mariette et al. 2004). Recently it has been demonstrated that the MUC4 mucin gene, which is overexpressed in Barrett's adenocarcinoma (Arul et al. 2000; Corfield et al. 2000), is regulated by bile acids at both transcriptional and translation levels in oesophageal cells (Mariette et al. 2004). Therefore the extent of bile acid-induced alterations in gene expression is an important parameter in the determining the quantity of changes induced in gastrointestinal physiology.

1.5 GASTROINTESTINAL EPITHELIAL RESPONSES TO BILE ACIDS

Deregulation of cellular processes such as proliferation and cell death, which are essential for normal epithelial homeostasis, is a common defect in the pathogenesis of gastrointestinal cancer. Decreased propensity to undergo apoptosis or increased proliferation will not only lead to clonal expansion but will increase the likelihood of developing mutations.

1.5.1 Bile Acid-Induced Alterations in Epithelial Cell Proliferation

In a normal population of proliferating cells, if a cell acquires a growth advantage either through a mutation or an epimutation, it will expand clonally at the expense of neighbouring cells. In colon cancer, aberrant cellular proliferation has been noted. In normal colonic mucosa, the proliferative compartment is located in the lower two-thirds of the crypts. In patients with increased risk of colon cancer, the proliferative zone is expanded and extends towards the top of the crypt (Scalmati and Lipkin 1993). *In vivo,* DCA induces the proliferation of the colonic epithelium as judged by increased thymidine labelling after administration of DCA to rats (Deschner et al. 1981). Similarly in human colonic biopsies, higher bromodeoxyuridine labelling was observed after incubation with DCA *in vitro* (Bartram et al. 1993). It has also been suggested that bile acids cause compensatory hyperproliferation of colonic epithelial cells in response to bile acid-induced apoptosis (Martinez et al. 1998; Qiao et al. 2001a).

In the oesophagus, aberrant cell proliferation has been noted along the dysplasia-carcinoma sequence. Increased proliferation, as judged by Ki-67 staining, was observed along the dysplasia-carcinoma sequence in comparison to metaplastic tissue (Rioux-Leclercq et al. 1999; Whittles et al. 1999). Expression of c-myc is increased in 50 % of Barrett's metaplasia and 90 % of oesophageal adenocarcinoma patients. Furthermore, acidified bile

acids induce marked upregulation of c-myc at the mRNA and protein level, which is associated with alterations in cellular proliferation (Abdelatif et al. 1991; Tselepis et al. 2003). Studies with Barrett's explants have demonstrated that pulses of bile acids induce hyperproliferation as evidenced by PCNA staining that is COX-2 dependent (Kaur and Triadafilopoulos 2002). In animal models of duodenal reflux, increased Ki-67 and cyclin D1 expression in the proliferative zone suggested that increased cellular proliferation contributed to the observed mucosal thickening (Zhang et al. 2001a). It thus appears that the field in which oesophageal cancer arises experiences marked alterations in cellular proliferation.

1.5.2 Apoptosis Resistance

Cells, defective in the ability to undergo apoptosis in response to DNA damage, not only have a growth advantage but also have increased potential to undergo excessive DNA damage and mutations. Bile acids, at concentrations similar to those in faecal water after high fat meals, can induce apoptosis in colonic goblet cells (Payne et al. 1995). It is speculated that bile acid-induced apoptosis selects for apoptosis resistant cells as indicated in **Fig 1.6**. Bile acids induce DNA damage in colonic cells (Kandell and Bernstein 1991; Venturi et al. 1997), most of which undergo apoptosis, while at the same time selecting for the survival of cells with apoptosis resistant mutations or apoptosis resistant epigenetic changes as indicated at level 3 in **Fig 1.6**. This is also observed *in vivo* as goblet cells, from the "normal" appearing colonic epithelium in colon cancer patients, are more resistant to bile acid-induced apoptosis than goblet cells from colons of healthy individuals (Garewal et al. 1996; Bernstein et al. 1999a). Apoptosis resistant subpopulations may arise through aberrant overexpression of anti-apoptotic proteins such as Bcl-2, nuclear factor-kappa B (NF-κB) and nitric oxide synthase (NOS2) with simultaneous reduction in expression of pro-apoptotic proteins such as p53 and APC. Overexpression of Bcl-2 is frequently detected in colonic adenomas and carcinomas (Bosari et al. 1994; Bronner et al. 1994; Hague et al. 1994). Colonic cells lines selected for resistance to bile acid-induced apoptosis have marked upregulation of Bcl-2 and NF-κB (Crowley-Weber et al. 2002; Bernstein et al. 2004). Payne *et al.* found that inhibition of DCA-induced NF-κB sensitises colonic cells to bile acid-induced apoptosis (Payne et al. 1998). *In vivo*, abnormal expression of NF-κB and NOS2 is detected in the "normal" appearing colonic epithelium of colon cancer patients, which is associated with loss of apoptosis competence (Bernstein et al. 2002).

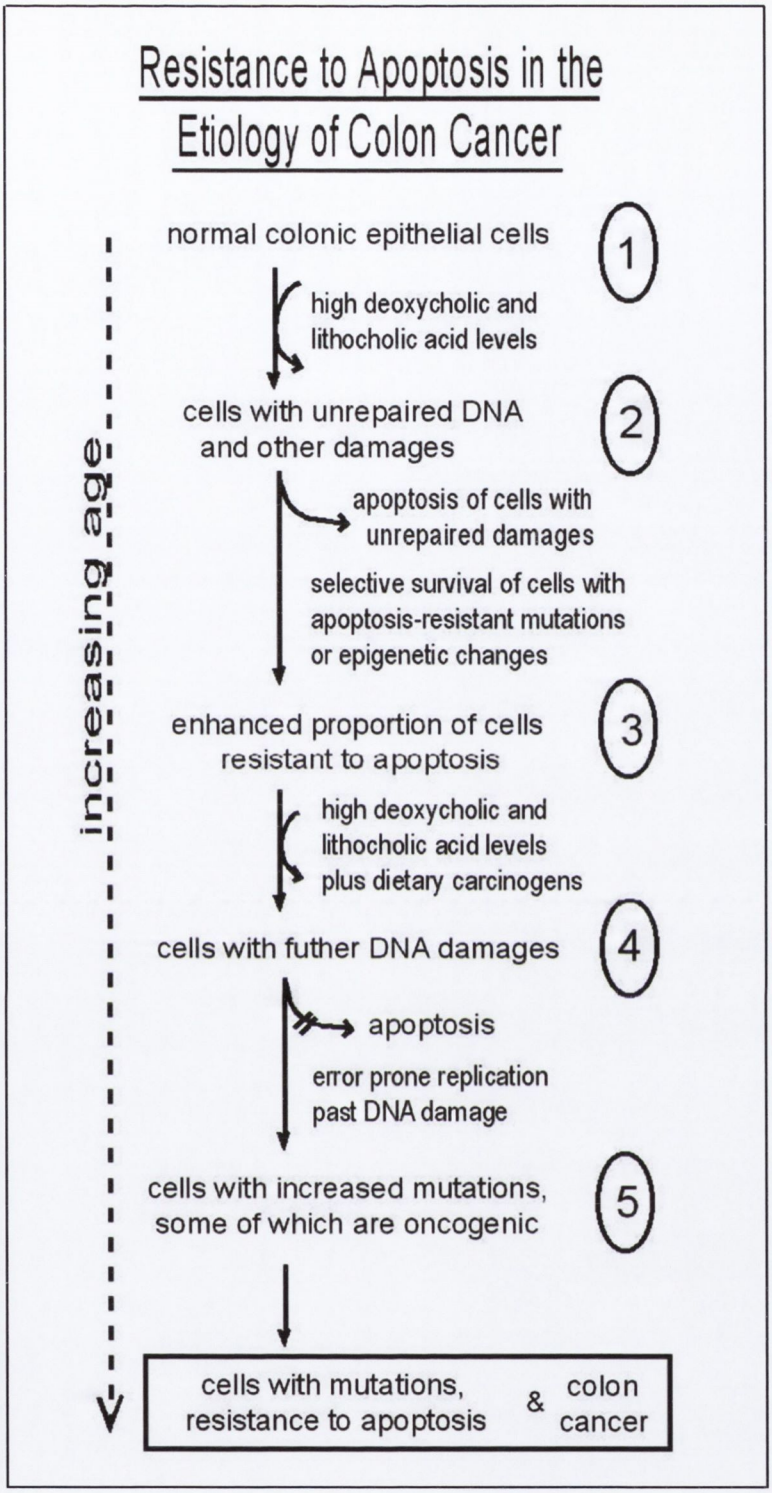


Fig 1.6. High Levels of Bile Acids Select for Apoptosis Resistant Cells. Level 1 indicates normal colonic cells encountering elevated bile acid concentrations following a high fat diet. Bile acids induce DNA damage in cells (level 2) after which most undergo apoptosis, facilitating the selection of apoptosis resistant cells as indicated in level 3. Additional DNA damage (level 4) results in a subpopulation of apoptosis resistant cells that cannot undergo apoptosis. Apoptosis resistant cells permit error prone replication resulting in increased mutations (level 5). Reproduced from Bernstein *et al* 2000.

Similar studies have shown that malignant transformation of the colonic epithelium involves progressive inhibition of apoptosis (Bedi et al. 1995).

Similar to colon cancer, inhibition of apoptosis along the metaplasia-dysplasia-carcinoma sequence of Barrett's oesophagus has been observed in several studies (Katada et al. 1997; Wetscher et al. 1998; Whittles et al. 1999). Early events in the malignant progression to oesophageal carcinoma are characterised by large amounts of oxidative damage increasing the likelihood of epimutations (Olyaee et al. 1995; Wetscher et al. 1995b; Wetscher et al. 1995a; Wetscher et al. 1997). The transcription factor NF-κB, which is known to inhibit apoptosis (LaCasse et al. 1998), is overexpressed in Barrett's oesophagus and adenocarcinoma and is induced by DCA in oesophageal adenocarcinoma cells (Abdel-Latif et al. 2004). Overexpression of anti-apoptotic proteins such as NOS2 and cyclooxygenase-2 (COX-2) has been demonstrated in both Barrett's oesophagus and associated adenocarcinoma. Bile acids can induce COX-2 expression both *in vivo* and *in vitro* (Zhang et al. 1998; Shirvani et al. 2000; Zhang et al. 2001a). It appears that a reduced apoptosis capacity is a feature of Barrett's oesophagus in which bile acids are implicated. A field of defective cells, such as those in Barrett's metaplasia, is likely to contain mutations and/or epimutations that can promote carcinogenesis through either resistance to apoptosis or more frequent cell division.

1.6 PROTEIN KINASE CASCADES

Once bile acids cross the mucosal barrier and interact with epithelial surface receptors, intracellular signalling cascades such as the protein kinase C (PKC) and the mitogen activated protein kinase (MAPK) cascades are initiated. Alterations to signal transduction pathways such as PKC and MAPK represent one of the primary means by which bile acids alter gene expression to elicit abnormal morphogenesis of the gastrointestinal epithelium. In this thesis, DCA-induced alterations in PKC and MAPK signalling are examined.

1.6.1 Protein Kinase C (PKC)

PKC, initially discovered 27 years ago as a calcium activated enzyme (Inoue et al. 1977; Kishimoto et al. 1980), is now known to consist of a family of serine/threonine kinases that play a fundamental role in a variety of cellular processes such as signal transduction leading to cell growth and differentiation (Nishizuka 1986; Blobe et al. 1996). Initial studies demonstrated that PKC is single polypeptide with an N-terminal regulatory domain

and a C-terminal catalytic domain. Four conserved (C1-C4) and five variable (V1-V5) regions have been identified **(Fig 1.7)**. The C1 and C2 regions are present in the N-terminal regulatory domain. The C1 region, consisting of two cysteine-rich zinc fingers, is responsible for binding of DAG and phorbol esters whereas the C2 region is involved in Ca^{2+} binding (Hug and Sarre 1993). The C3 site represents the catalytic site that is involved in binding ATP and is present the catalytic domain. The C4 site, containing the protein kinase domain, is responsible for recognition of target substrates and resides in the catalytic domain **(Fig 1.7)** (Toker 1998).

The PKC family consists of 12 members, which are categorised based on their structure and their requirements for DAG and calcium (Ca^{2+}) (Nishizuka 1986; Toker 1998). The conventional PKCs i.e. PKC-alpha (PKC-α), -beta1 (PKC-β_1), -beta2 (PKC-β_2) and –gamma (PKC-γ), have four conserved regions and require DAG and Ca^{2+} for activation as illustrated in **Fig 1.7**. The novel PKCs, of which PKC-epsilon (PKC-ε), -delta (PKC-δ), -eta (PKC-η) and -theta (PKC-θ) are members, contain a C2 like domain and require DAG for activation but are unresponsive to Ca^{2+} **(Fig 1.7)**. The atypical PKCs, PKC-zeta (PKC-ζ) and PKC-lambda (PKC-ι) possess a C2 like domain and only one zinc finger. They are Ca^{2+} independent and do not bind phorbol esters **(Fig 1.7)** (Hug and Sarre 1993). Another family member, discovered independently by two laboratories, called PKC-mu (PKC-μ) or protein kinase D (PKD) has been identified (Johannes et al. 1994; Valverde et al. 1994). The PKC isoforms have a very specific tissue distribution. PKC-α, PKC-δ and PKC-ζ are the most widely expressed of the PKC isoforms (Wetsel et al. 1992; McGarrity et al. 1996), whereas PKC-γ is expressed predominantly in the central nervous system (Nishizuka 1988).

1.6.1.1 Activation of PKC

The PKC isoenzymes are responsive to multiple lipid second messengers both *in vitro* and *in vivo*. In all PKCs, the N-terminal regulatory domain is responsible for autoinhibition of the native enzyme. Activation of phospholipase type C enzymes, in response to agonists, results in the hydrolysis of membrane phospholipids with the subsequent generation of DAG and soluble inositol phosphates **(Fig 1.8)**. Soluble inositol phosphates stimulate the release of Ca^{2+} from intracellular stores (Deacon et al. 1997; Toker 1998). Production of DAG or the presence of phorbol esters such as phorbol 12-myristate 13-acetate (PMA),

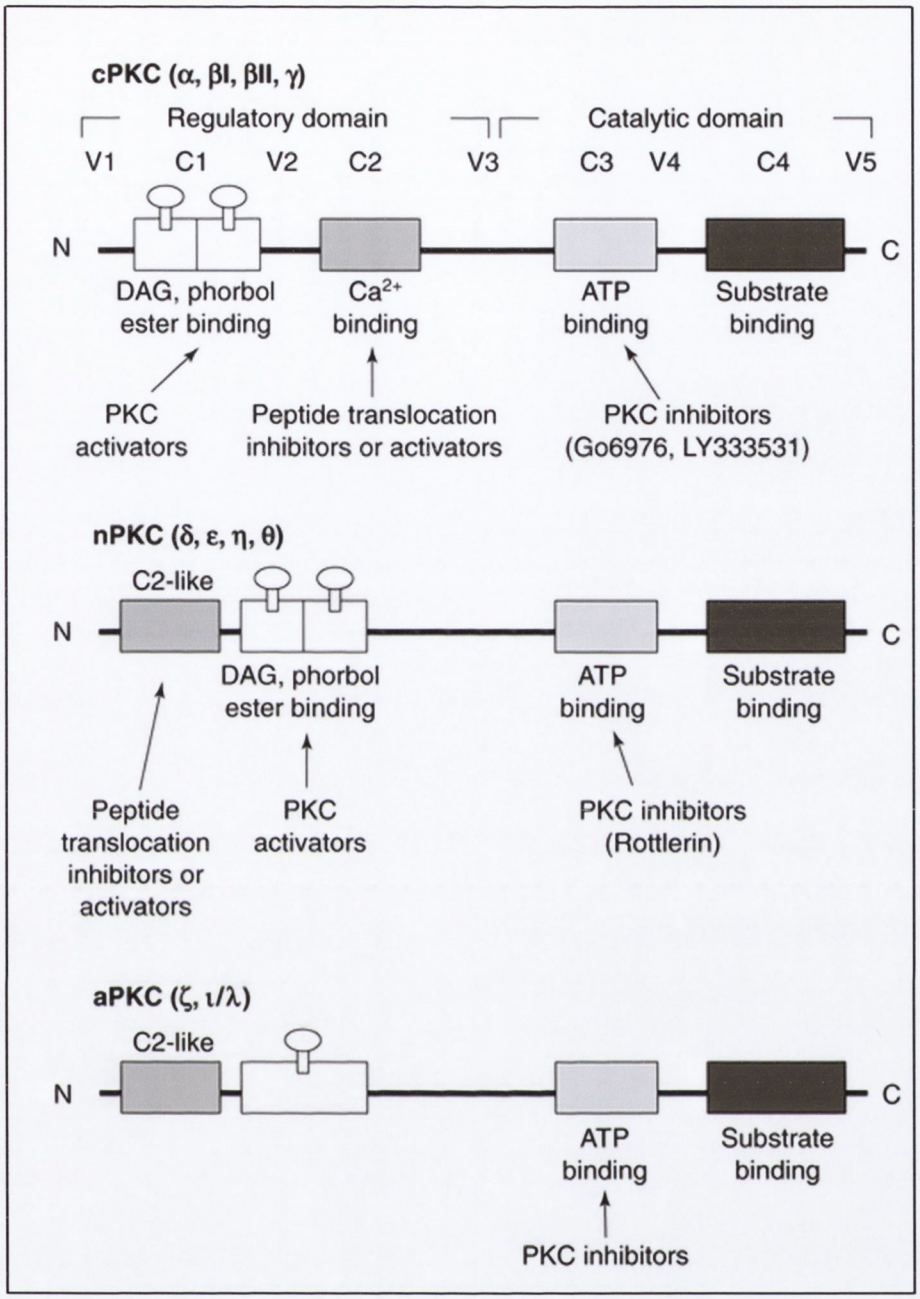


Fig 1.7. Sequence Homology Between The Primary Structures of The PKC Isoforms. The structure of PKC isoforms can be separated into the N-terminal (regulatory) domain and the C-terminal catalytic domain. (**A**) The conventional PKCs are comprised of four conserved regions (C1-C4) and five variable regions (V1-V5). The C1 region is involved in binding to DAG and phorbol ester activating compounds, whereas the C2 region is involved in Ca^{2+} binding. C3 and C4 regions are involved in binding to ATP and PKC substrates respectively. (**B**) The novel PKCs (nPKC) possess a C2 like region in V1 and are regulated by DAG and phosphatidylserine (PS) but are Ca^{2+} independent. (**C**) The atypical PKCs contain a C2 like region but are Ca^{2+} independent. They do not require DAG for activation although PS regulates their activity. Reproduced from Way *et al* 2000.

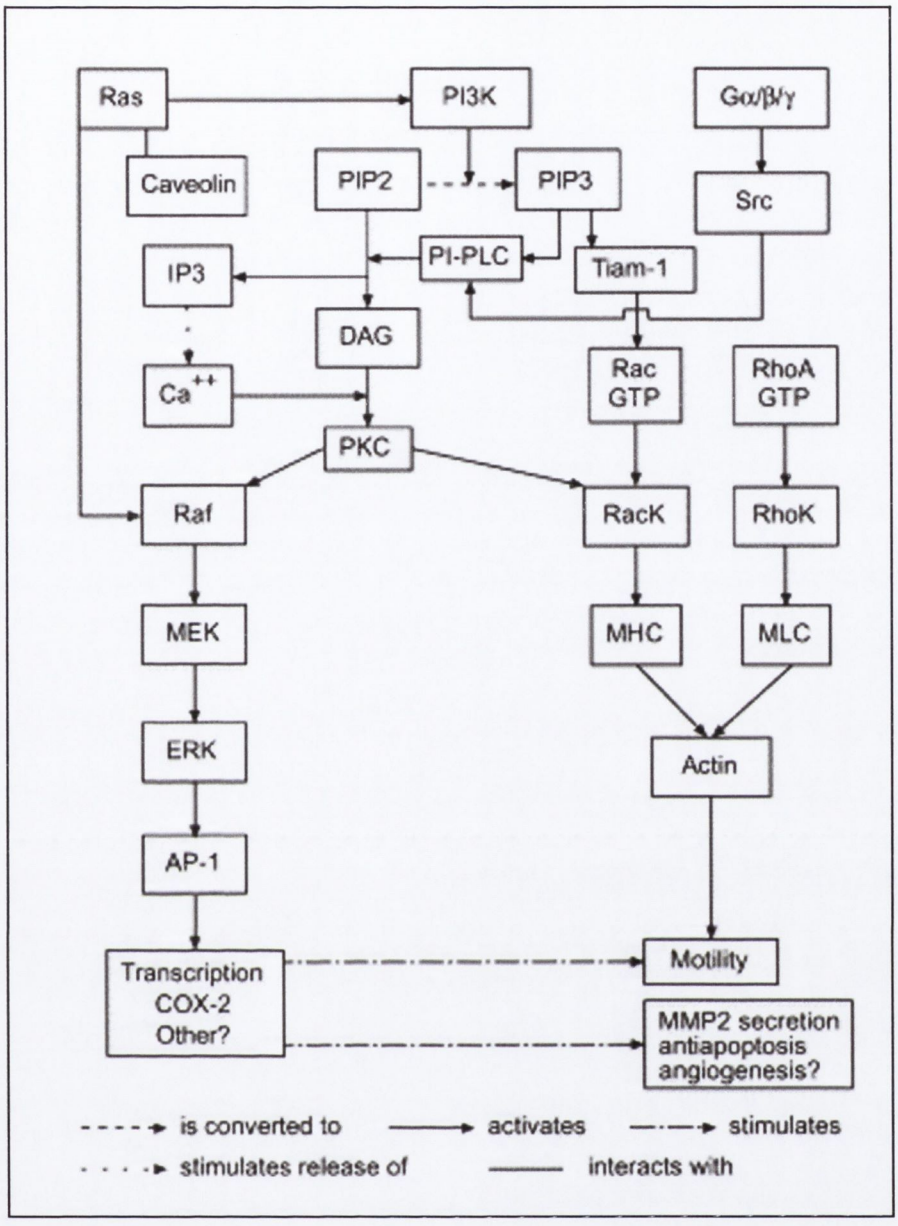


Fig 1.8. Potential Mechanisms Leading to PKC Activation. Ras and caveolins are upstream regulators of PI3-K and Raf. Activation of PI3-K is implicated upstream of phosphoinositide-specfic phospholipase C (PI-PLC). PI3-K can also signal through Tiam-1 towards the Rho GTPase family, which in turn regulates all aspects of cell motility and proliferation. Heterotrimetric G proteins (Gα/β/γ) activate PKC via Src tyrosine kinase. Raf can signal through to Mek leading to activation of Erk, which in turn activates activator protein-1 (AP-1). Cyclooxygenase-2 (COX-2) is a downstream target of AP-1 and is possibly responsible for apoptosis inhibition, motility and invasion. Reproduced from Debruyne *et al.* 2001.

results in activation of PKC through binding to the C1 domain and thereby relieving autoinhibition. Binding of the cofactor PS induces a conformational change that results in the removal of the regulatory domain from the catalytic site. The C2 like domain, present in the novel and atypical PKCs, is thought to reflect the C2/ Ca^{2+} conformation in the conventional PKCs.

The fact that lipids are required for PKC activation stipulates the recruitment of PKC isoforms from the inactive cytosolic pool to intracellular membranes where activation occurs, a process known as PKC translocation. Several lipid second messengers such as phosphoinositide 3-kinase (PI3-K) products are known to induce translocation and activation of both novel (PKC-ε, PKC-δ, PKC-η) **(Fig 1.8)** and atypical PKCs (PKC-ζ) *in vitro* (Singh et al. 1993; Toker et al. 1994). Fatty acids such as arachidonic acid can induce translocation and activation of multiple members of the PKC family (Shirai et al. 1998b; O'Flaherty et al. 2001). Furthermore secondary bile acids can activate phospholipases, especially phospholipase PLA_2, resulting in elevated membrane phospholipid turnover (Takenawa and Nagai 1981; DeRubertis et al. 1984). Increased phospholipid turnover and the release of arachidonic acid can activate PKCs. In primary rat hepatocytes, bile acids have been shown to induce PKC translocation to the membrane (Stravitz et al. 1996). In the same cells, bile acids increase the concentration of DAG resulting in activation of PKC-α and PKC-δ (Rao et al. 1997). Clearly, the lipid species generated as second messengers following receptor ligation, will stipulate the PKC isoenzymes activated and will thereby confer specificity. Furthermore chronic activation of the PKC isoforms has been shown to lead to downregulation and even loss of expression of individual isoforms in numerous cell types (Hug and Sarre 1993). Transcriptional and post-transcriptional downregulation of the PKC isoforms can occur in addition to post-translational regulation (Assert et al. 1996), which is both isoform and cell type specific (Freire-Moar et al. 1991; Tsutsumi et al. 1993).

1.6.1.2 Cellular Function of PKC

In vitro the PKC isoforms exhibit only subtle differences in regards to enzymatic properties and substrate specificity. However, *in vivo,* they have specific tissue and cellular expression patterns that suggests distinct functions for the individual PKC isoforms (Hug and Sarre 1993). The PKC family plays a fundamental role in a variety of cellular processes such as signal transduction leading to cell growth and differentiation (Masur et

al. 2001), secretion (Song et al. 1999), cytoskeleton function (Keenan and Kelleher 1998; Zhang et al. 2004) and gene expression (Orford et al. 1997; Murray et al. 1999). Given that multiple members of the PKC family are expressed in intestinal epithelial cells, this suggests that the PKCs are extensively involved in the homeostasis of the gastrointestinal epithelium (Jiang et al. 1995; Verstovsek et al. 1998). For example, in colonic epithelial cells PKC-ε regulates basolateral endocytosis, which is critical for mucosal surface hydration, through actin reorganisation involving the redistribution of the F-actin cross linking protein and PKC substrate, MARCKS (Song et al. 1999). In the normal colonic mucosa, PKC isoform expression increases from the base of the crypt to the mucosal surface suggesting a role for the PKC isoforms in mitotic and post-mitotic events (Saxon et al. 1994). A critical role for the PKC isoforms in proliferative responses of gastrointestinal epithelial cells has been demonstrated in overexpression studies (Livneh and Fishman 1997). Overexpression of certain PKC isoforms, PKC-β_1 and PKC-δ in particular, has been associated with negative effects on intestinal cell growth. PKC-β_1 overexpression in colonic adenocarcinoma cells, inhibited growth and markedly decreased tumourigenicity in nude mice (Choi et al. 1990; Goldstein et al. 1995). In malignant colonocytes, overexpression of PKC-δ resulted in a G0/G1 cell cycle arrest with a decrease in the number of cells in the S phase (Cerda et al. 2001). Conversely, overexpression of PKCβ_2 is associated with hyperproliferation and invasion both *in vivo* and *in vitro* (Murray et al. 1999; Zhang et al. 2004). Overexpression of PKC-ε stimulates proliferation and induces transformation in colonic epithelial cells (Perletti et al. 1998; Weller et al. 1999; Marras et al. 2001). It appears that the PKC isoforms can function as tumour suppressors (PKC-β_1 and PKC-δ) as well as protooncogenes (PKCβ_2 and PKC-ε) in the intestinal epithelium.

1.6.1.3 Role of PKC in Gastrointestinal Cancer

PKCs are implicated in malignant transformation in several cell types including the intestinal epithelium, through alterations in isoform activity and subcellular localisation (Dlugosz et al. 1992; Koren et al. 2004). Initial studies demonstrated that total PKC activity was reduced in colonic neoplasms relative to the normal colonic mucosa (Guillem et al. 1987; Kopp et al. 1991; Wali et al. 1991). Subsequently, decreased protein expression and/or mRNA abundance of various PKC isoforms, in particular PKC-β_1, has been reported in human colonic tumours and in carcinogen treated rodents (Doi et al. 1994; Gokmen-Polar et al. 2001). Aberrant PKC expression occurs early in the adenoma-

carcinoma sequence. Gokmen-Polar *et al.* demonstrated that decreased expression of PKC-β_1 and increased expression of PKC-β_2 is an early event occurring in the aberrant crypt foci, a premalignant lesion of the colon. In addition the expression of other members of the PKC family, such as PKC-δ, is lost at a later stage, usually in malignant colonocytes (Craven and DeRubertis 1994).

While the expression and activity of various PKC isoforms has been well characterised in colonic epithelial cells, little is known about their expression in normal or malignant oesophageal cells. PKC-α and PKC-η are known to be abundantly expressed in oesophageal epithelial cells (Osada et al. 1993; McGarrity et al. 1996). Hashimoto *et al.* measured PKC activity in oesophageal tumours and adjacent normal mucosa. Although they found considerable variability, the PKC activities in oesophageal tumours were reported to be the same as in adjacent normal mucosa (Hashimoto et al. 1989). However several *in vitro* studies have demonstrated the importance of PKCs in oesophageal carcinogenesis. In oesophageal explants, bile acid-induced upregulation of COX-2 and hyperproliferation has been shown to be PKC dependent (Kaur and Triadafilopoulos 2002). Alterations in PKC signalling in gastrointestinal cells are likely to deregulate downstream targets **(Fig 1.8)** such as Raf kinase, an upstream regulator of the MAPK pathway, resulting in abnormal morphogenesis of the gastrointestinal epithelium.

1.6.2 Mitogen Activated Protein Kinase (MAP kinase)

The MAPK signalling cascade plays a key role in transducing extracellular signals from membrane receptors to the nucleus thereby altering the transcriptional activity of cells and their subsequent biological responses (Hipskind and Bilbe 1998). The MAPKs are regulated by phosphorylation and participate in a phospho-relay system **(Fig 1.9)** that is composed of three sequentially activated kinases (Johnson and Lapadat 2002). The MAPK/Erk kinase kinases (Mekk or MKKK) are responsible for the phosphorylation and activation of specific MAPK/Erk kinases (Meks or MKKs), which in turn catalyse the phosphorylation of MAPKs, the third component of the phospho-relay system **(Fig 1.9)**.

1.6.2.1 Mekks

Mekk1, 2, 3 and 4, originally cloned based on their homology to Mekks from yeast (Lange-Carter et al. 1993), are serine/threonine kinases with an N-terminal regulatory

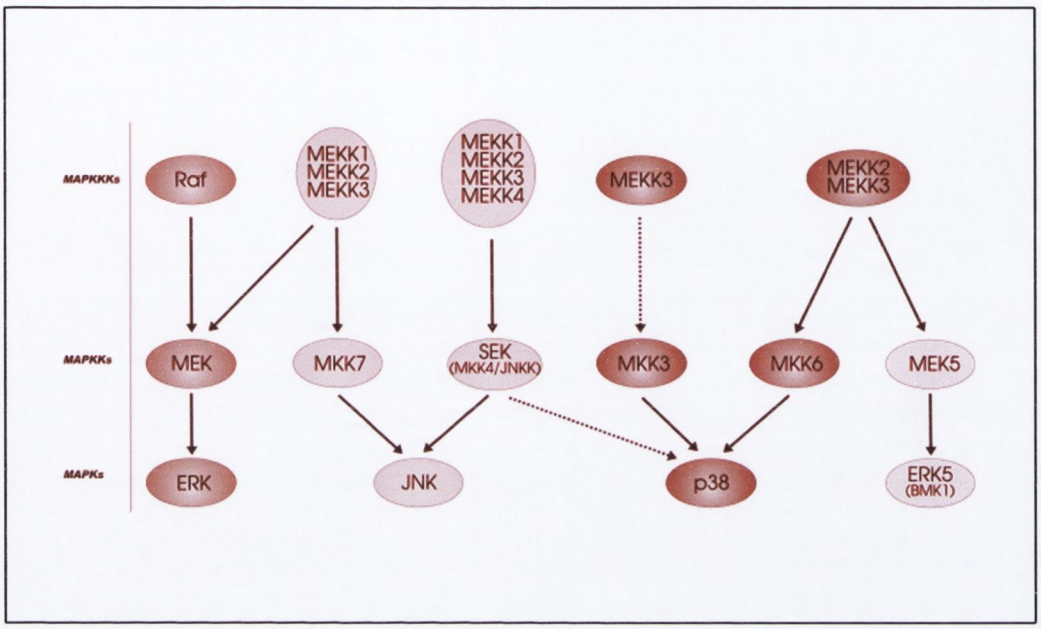


Fig 1.9. MAPK Signalling Cascade in Mammalian Cells. MAPK are regulated by phosphorylation and form part of a phospho-relay system that is composed of three sequentially activated kinases, which propagate extracellular signals to the nucleus thereby altering the transcriptional activity of the cell and its subsequent biological response. For abbreviations see text. Broken arrows indicates coupling that cannot be demonstrated directly. Reproduced from Hagemann and Blank 2001.

domain and a C-terminal catalytic domain. The first known Mekks are the members of the Raf family, which were originally studied as oncogene products (Wu et al. 1996). The Raf family members are characterised by their singular ability to phosphorylate Mek1/2 with A-Raf, unlike Raf-1 and B-Raf, being selective for Mek1 over Mek2 (Wu et al. 1996). The Mekks are distinct from the Raf kinases in their ability to activate Meks involved in p38 and JNK activation **(Fig 1.9)**.

The regulation of Mekk1, a large protein of 195 kDa, is poorly understood but it is known to phosphorylate Mek on serine 218 and 222, respectively, the same sites phosphorylated by Raf (Xu et al. 1995; Hagemann and Blank 2001). Studies have suggested that Mekks preferentially activate the stress activated protein kinase/Erk (SAPK/Erk or Sek) over Mek. Mekk1 phosphorylates and activates Sek by binding to the N-terminal extension of Sek (Xu and Cobb 1997; Xia et al. 2000; Gallagher et al. 2002). Activated Sek has lower affinity for Mekk1 and binds to c-Jun NH_2-terminal kinase (JNK) through the same N-terminal extension. It is thought that the sequential binding and phosphorylation confers specificity to the MAPK module. Mekks regulate the activity of a diverse group of substrates. In regards to the JNK pathway, Mekk1, 2 and 3 phosphorylate and activate MKK7 while Mekk2 and 3 regulate p38 activation via MKK3/6 **(Fig 1.9)** (Pearson et al. 2001). Studies have also shown that Mekk2 and 3 are involved in the regulation of Mek5, which activates the recently discovered extracellular signal regulated kinase 5 (Erk5) or big MAPK (BMK) (Chao et al. 1999). Initially, Mekk4 was thought to solely regulate JNK activation; however it has also been shown to regulate p38 activation as well (Chan-Hui and Weaver 1998).

In addition to the regulation of MKKs, Mekks have a broader spectrum of functions of which the roles of Mekk1 has been characterised the most. A role for Mekk1, in the regulation of cell motility and migration, has been demonstrated. In epithelial cells, expression of Mekk1 is associated with lamellipodia formation, which is necessary for cell migration (Yujiri et al. 2000). Mekk1 is known to have both pro- and anti-apoptotic functions depending on the nature of the initiating signal. Mekk1 represses apoptosis in response to microtubule interfering drugs (Yujiri et al. 1998; Yujiri et al. 1999), while stimulation with tumour necrosis factor alpha (TNF-α) activates Mekk1 and stimulates apoptosis (Avdi et al. 2001). Mekk1 is also a caspase substrate. Cleavage of Mekk1 to yield a 91 kDa fragment, results in Mekk1 translocating from the membrane to the cytosol

where it amplifies caspase activation and induces apoptosis (Widmann et al. 1998). The activation of Mekk1 by caspases serves as an important switch between Mekk1 functioning as a cell survival mediator and a pro-apoptotic effector. In addition Mekk1 has been shown to regulate transcription factors such as activator protein-1 (AP-1), NF-κB and signal transducer and activator of transcription 3 (STAT3) (Hagemann and Blank 2001).

A limited number of studies have addressed the specific functions of Mekk2, 3 and 4. A role for Mekk2 and 3 in activating IKKα and IKKβ resulting in NF-κB activation has been demonstrated (Ellinger-Ziegelbauer et al. 1997; Zhao and Lee 1999), whereas Mekk4 appears to be incapable of activating NF-κB (Zhao and Lee 1999). Ligation of the T-cell receptor has been shown to result in the redistribution of Mekk2 from the cytoplasm to the T-cell/antigen presenting cell contact sites (Schaefer et al. 1999). Mekk2 appears to be activated in response to T-cell receptor stimulation and has been implicated in T-cell adhesion (Schaefer et al. 1999). On the other hand, Mekk3 is involved in cell cycle regulation as Mekk3 activation is associated with cell cycle arrest by preventing entry into the S-phase through cyclin D1 inhibition (Ellinger-Ziegelbauer et al. 1999). Mekk3 mediated cell cycle arrest is dependent on p38 activity, which has previously been shown to repress cyclin D1 activation (Lavoie et al. 1996). Murine knockouts of Mekk3 display an embryonic lethal phenotype (Yang et al. 2000) unlike Mekk1 knockouts whose activity appears to be compensated by the presence of other Mekks (Yujiri et al. 2000). Mekk4 activation is associated with early mouse development. Constitutively active Mekk4 mediated retinoic acid-induced JNK activation and differentiation (Kanungo et al. 2000). Collectively these studies suggest diverse roles for Mekk2, 3 and 4 in T-cell activation, development and differentiation, respectively. However their mechanism of regulation is still poorly understood and further studies in different cell types are required to precisely define the roles of the Mekk isoforms.

1.6.2.2 Meks

The Mek family, of which they are seven members, are dual specific kinases that can phosphorylate serine/threonine and tyrosine residues in their MAPKs substrates. The substrate specificity of Mek is narrow, usually confined to one or two MAPKs. In the case of Erk1/2, which is activated by Mek1 and 2, they are first phosphorylated on the tyrosine residue before the threonine residue. This facilitates the establishment of a threshold. As

the tyrosine-phosphorylated proteins are not active, they must first accumulate before phosphorylation of threonine. Once the threshold is reached, they are rapidly phosphorylated on the threonine residue and converted to the active state (Pearson et al. 2001). Meks also act as intermediates involved in the detection of signals from other signaling pathways resulting either in the enhancement or suppression of the signal to the MAPKs (Pearson et al. 2001). Furthermore Meks are involved in signal amplification. The MAPK cascade is characterised by a successively more abundant protein at each tier of the kinase cascade so that the signal is amplified within the module (Hagemann and Blank 2001). Subsequently the Mek proteins are more abundant than Mekks.

Mek1 and 2 relay Ras and Raf signals through phosphorylation of Erk1/2 and are known to be localised in the cytoplasm but can traffic in and out of the nucleus to phosphorylate Erk **(Fig 1.10)** (Robinson and Cobb 1997). Murine knockouts have demonstrated unique roles for Mek1 and 2. Mice lacking Mek1 display an embryonic lethal phenotype and exhibit defective placental vascularisation (Wada and Penninger 2004). On the other hand, Mek2 knockout mice are viable and do present morphological alterations. Mek1 has been implicated in thymocyte development while no such involvement has been demonstrated for Mek2 (Wada and Penninger 2004). A role for Mek1, in addition to Erk activation, has been suggested in cell migration assays. Fibroblasts lacking Mek1 cannot be induced to migrate in response to fibronectin even though the levels of Mek2 protein and Erk activation are normal. These finding suggest that besides Erk, Mek1 has other specific substrates, which are possible involved in cell migration (Giroux et al. 1999).

The MKK3 and 6 (also known as Mek3 and 6) are dual specific kinases that phosphorylate p38 at threonine and tyrosine residues. MKK3 preferentially phosphorylates p38-α and -β isoforms whereas MKK6 phosphorylates all the members of the p38 family. In addition, MKK3 and 6 have been shown to phosphorylate JNK (Herlaar and Brown 1999). MKK3 gene disruption studies have demonstrated that MKK3 is intrinsically involved in p38 activation and pro-inflammatory cytokine production. Mice lacking MKK3 are viable and fertile. However, macrophages and dendritic cells from these mice display a marked reduction in p38 activation and impaired transcription and secretion of pro-inflammatory cytokines such as interleukin-12 (IL-12), IL-1α and IL-1β in response to lipopolysaccharide (Pearson et al. 2001). Alternatively, murine knockouts of MKK6 were viable, fertile and have no detectable developmental abnormalities. Unlike MKK3

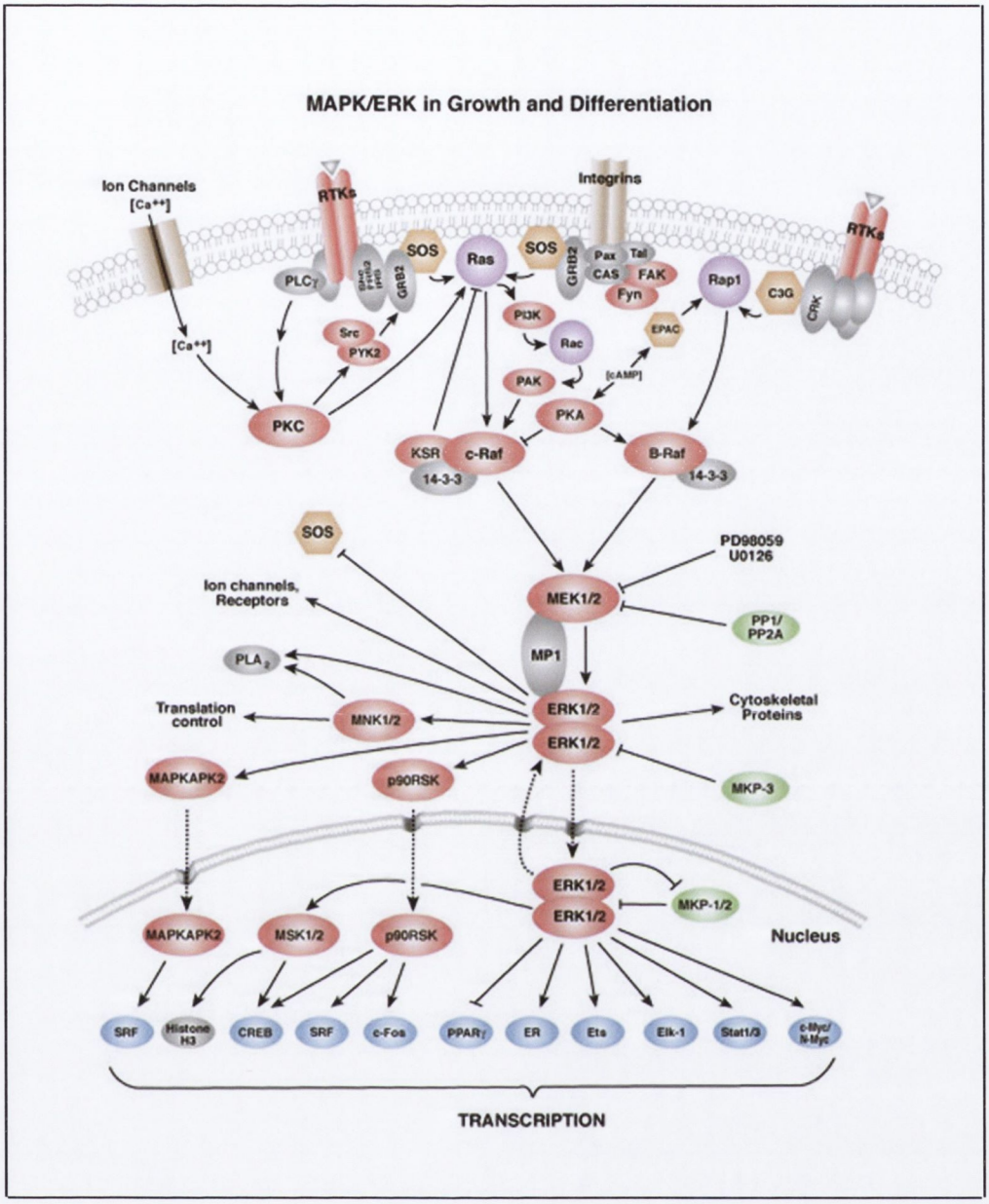


Fig 1.10. Extracellular Signal Regulated Kinase (Erk) Pathway. Activation of Erk is triggered by a broad range of receptors involved in growth and differentiation such as tyrosine kinase receptors, integrins and ion channels. The architecture of the pathway varies with stimuli but usually involves a subset of adapters (Shc, GRB2, Crk, etc.) linking the receptor to a guanine nucleotide exchange factor (Sos, C3G, etc.) thereby transducing the signal to small GTP binding proteins (Ras, Rap1). These subsequently turn activate the core Erk module consisting of a Mekk (Raf), Mek1/2 and Erk. Activated Erk can phosphorylate cytoplasmic proteins as well as a wide variety of transcription factors upon translocation to the nucleus. Reproduced from http://www.cellsignal.com/reference/pathway/MAPKERK.asp

knockout, T-cells from MKK6 knockouts are sensitive to activation-induced cell death and cytokine withdrawal. Furthermore in activated macrophages, the lethal factor from the bacterium *Bacillus anthracis* induces apoptosis by cleaving the N-terminal domains of MKK3 and 6 (Wada and Penninger 2004). While these studies demonstrate an important role for MKK3 and 6 in T-cell development, gene disruption studies in other cell types are required to define the role of MKK3 and 6 in other tissue.

MKK4 (Mek4 or Sek1) and MKK7 (Mek7) lie upstream of JNK and were initially identified using cDNA cloning strategies. MKK4 and 7 preferentially activate JNK but can also phosphorylate the p38 family *in vitro* and when overexpressed (Robinson and Cobb 1997). Studies have suggested that while both MKK4 and 7 cooperate to activate JNK, MKK4 preferentially phosphorylates the tyrosine residue in JNK whereas MKK7 preferentially phosphorylates the threonine residue (Pearson et al. 2001). A number of studies have shown a role of MKK4/7 in T-cell activation and proliferation (Nishina et al. 1997; Dong et al. 2000). Mice lacking MKK4 display an embryonic lethal phenotype with liver defects. Inactivation of MKK7 results in embryonic lethality with defects in liver formation and hepatocytes proliferation (Wada et al. 2004). Clearly, both MKK4 and 7 are important for mouse embryogenesis and hepatocytes proliferation, as they cannot substitute for one another.

Mek5, initially identified using cDNA-cloning strategies, is characterised by its singular ability to activate Erk5 and shares high sequence homology to Mek1 and 2 (Zhou et al. 1995). Splice variants of Mek5 exist such as Mek5-α and -β. Mek5-α is predominantly expressed in the liver and the brain whereas Mek5-β is ubiquitously expressed (English et al. 1995). These splice variants also have different subcellular localisation patterns suggesting unique roles for each of the isoforms. Mek5 has been shown to be intimately involved in Raf-1 signalling even though Raf-1 does phosphorylate or activate Mek5. Fibroblasts, harbouring kinase defective Mek5, cannot form foci in response to Raf stimulation, whereas constitutively active Mek5 synergises with Raf to form foci (English et al. 1999b). Studies with Mek5 are limited and as of yet its only known substrate is Erk5.

1.6.2.3 MAPKs

The MAPKs share a similar phosphorylation motif, threonine-Xxx-tyrosine, where X differs between the MAPK isoforms. For Erk, p38 and JNK the amino acid X is glutamic

acid, glycine and proline, respectively. This phosphorylation sequence resides near the ATP and substrate binding sites in the activation loop, the length of which varies amongst the individual MAPKs. The sequence of phosphorylation events is conserved between the MAPK members occurring with the sequential addition of a phosphate to the tyrosine and then to the threonine residue. Upon activation, the MAPKs phosphorylate either cytoplasmic or nuclear localised transcription factors leading to alterations in gene expression and subsequent biological responses (Hipskind and Bilbe 1998).

1.6.2.3.1 Erk

The Erk signalling cascade was the first of the MAPK to be characterised and it is now known to consist of at least six members. Erks are activated in response to broad range of mitogenic factors such as serum, growth factors, cytokines, ligands for the G-coupled protein receptors and transforming agents such as phorbol esters (Hommes et al. 2003). Upon activation by Mek1 and 2, Erk translocates to the nucleus, where it can phosphorylate transcription factors such as ternary complex factors (TCFs) namely Elk-1 and its homologue serum response factor accessory protein (SAP-1a) (Marais et al. 1993). These Ets domain proteins are responsible for mediating transcription from serum response elements (SRE) in the promoters of genes for Fos and other serum-induced genes. Erk is known to phosphorylate other transcription factors such as STATs (Chung et al. 1997), Myc (Davis 1995) and cAMP response element binding protein beta (C/EBPβ) **(Fig 1.10)** (Tan et al. 1996). In addition to transcription factors, Erk can phosphorylate cytoplasmic proteins such as 90 kDa ribosomal S6 protein kinase (RSK or MAPKAP-K1), MAPKAP-K2, MAP kinase interacting kinase (MNK) 1, MNK2, cytosolic phospholipase A_2 and several microtubule-associated proteins such as MAP-1 and Tau **(Fig 1.10)** (Zhang and Liu 2002). Two classes of phosphatases regulate the activity of Erk by dephosphorylation rendering it inactive. The MAP kinase phosphatase (MKP) class regulates Erk through its dual specificity for phospho-threonine and –tyrosine (Hunter 1995). In certain circumstances, induction of MKPs is too slow to account for the inactivation of Erk. Under these conditions, inactivation of Erk is likely to occur via threonine dephosphorylation mediated by protein phosphatase-2A (PP-2A) (Alessi et al. 1995). The co-ordinate phosphorylation and dephosphorylation of Erk ensures the appropriate transient activation of Erk.

The ubiquitously expressed Erk1/2 appear to be the major transducers of mitogenic signals to the nucleus and plays a crucial role in proliferation, cell cycle progression and differentiation. Studies in fibroblasts have demonstrated that Erk1/2 are required for cyclin D1 expression, a key regulator of the G_1/S phase transition during the cell cycle (Lavoie et al. 1996). Erk1/2 are known to phosphorylate $p27^{kip1}$, the inhibitor of cyclin E-cyclin dependent kinase 2 complexes, which are required for G_1/S phase transition. Phosphorylation of $p27^{kip1}$ marks the protein for ubiquitination and proteasomal degradation (Zhang and Liu 2002). In most instances, Erk1/2 functions as a survival factor. In PC12 cells, sustained activation and nuclear retention of Erk by nerve growth factor is associated with differentiation whereas nerve growth factor withdrawal results in Erk1/2 inhibition with subsequent induction of apoptosis (Xia et al. 1995). However, there are some reports of Erk1/2 functioning as a pro-apoptotic factor. In HeLa cells, Erk 1/2 activation is required for cisplatin-induced apoptosis (Wang et al. 2000b). Therefore Erk1/2 can have pro- and anti-apoptotic effects depending on the nature and the duration of the inducing signal. Gene disruption studies have being conducted for Erk1 only. Murine knockouts of Erk1 are viable, fertile and normal but thymocytes from these animals display defective proliferation in response to activation via a T-cell receptor antibody. Activation-induced apoptosis is not impaired in these cells, which would suggest that Erk2 in part compensates for Erk1 (Pages et al. 1999).

Erk3 was isolated from a cDNA library probed with Erk1. Two splice variants of Erk3 have been identified, Erk3-α and -β. Even though Erk3-α and -β display high sequence homology (50%) to Erk1/2, marked differences exist. Erk3 isoforms are characterised by a singular phosphoacceptor site, serine 189, in the phosphorylation motif of the activation loop. Erk3-α is able to autophosphorylate and is constitutively expressed in the nucleus unlike the other MAPKs (Pearson et al. 2001). At present, data identifying other substrates for Erk3 is weak (Cheng et al. 1996) and its regulation is still poorly understood.

Erk5, known to be homologous to Erk2, has a predicted molecular mass of 90 kDa and as such is also known as Big MAPK, BMK. Unlike Erk1/2, it contains ten consensus phosphorylation sites and can autophosphorylate (Pearson et al. 2001). Erk5 is ubiquitously expressed and has been shown to phosphorylate the transcription factors, myocyte enhancer factor 2A and C (MEF2A and C) and SAP1 (Kato et al. 1997; Kamakura et al. 1999). A role for Erk5 in cell survival has been suggested. In PC12 cells

deprived of growth factors, Erk5 is activated and mediates a survival advantage (Suzaki et al. 2002), which has also been demonstrated in cortical neurons (Liu et al. 2003). Murine knockouts for Erk5 display embryonic lethality with defective cardiovascular systems (Regan et al. 2002). Erk5 appears to be important for the proliferative responses of the cells and its function cannot be compensated by the presence of other MAPKs.

Erk7, a 61 kDa MAPK, has been identified and is constitutively activated by autophosphorylation through the interaction with its C-terminal domain (Abe et al. 2001). A role for Erk7 in growth inhibition has been proposed (Abe et al. 1999). Another MAPK, Erk8, has been cloned and shares 69% sequence homology with Erk7 (Abe et al. 2001). Unlike Erk7, Erk8 is not constitutively active. Erk8 contains two SH3 binding domains in the C-terminal, which allow it to associate with the SH3 domain in c-Src. Activation of Erk8 via Src has been demonstrated in response to serum stimulation (Wada and Penninger 2004). Murine knockouts of Erk7 and 8 have not been conducted; therefore their biological function *in vivo* has yet to be established.

1.6.2.3.2 p38

The p38 MAPK was identified independently in three different contexts; firstly as a tyrosine phosphoprotein present in cells treated with pro-inflammatory cytokines, secondly as a direct target of pyridinyl imidazole drugs and thirdly as kinase capable of activating MAPKAP-K2. The p38 family is now known to be activated in response to cytokines, hormones, G-coupled protein receptor ligation, genotoxic stress, protein synthesis inhibitors (anisomycin) and lipopolysaccharide **(Fig 1.11)** (Ichijo 1999). At least five isoforms of p38 have been identified, p38-α, p38-β, p38-β2, p38-δ and p38γ. Expression profiles have demonstrated the ubiquitous expression of p38-α, -β and -δ whereas p38-γ is expressed only in skeletal muscle (Herlaar and Brown 1999). The isoforms are also characterised by their sensitivity to pyridinyl imidazole drugs such as the SB203580 compound. The alpha and beta isoforms are inhibited whereas the delta and gamma isoforms are not (Davies et al. 2000; English and Cobb 2002). A sixth member, Mxi, has been identified and is known to be a splice variant of p38-α. Unlike p38-α, Mxi is insensitive to pyridinyl imidazole drugs and is activated by growth factors as well as stress. Together Mxi and p38-α bind Myc (Zervos et al. 1995; Sanz et al. 2000).

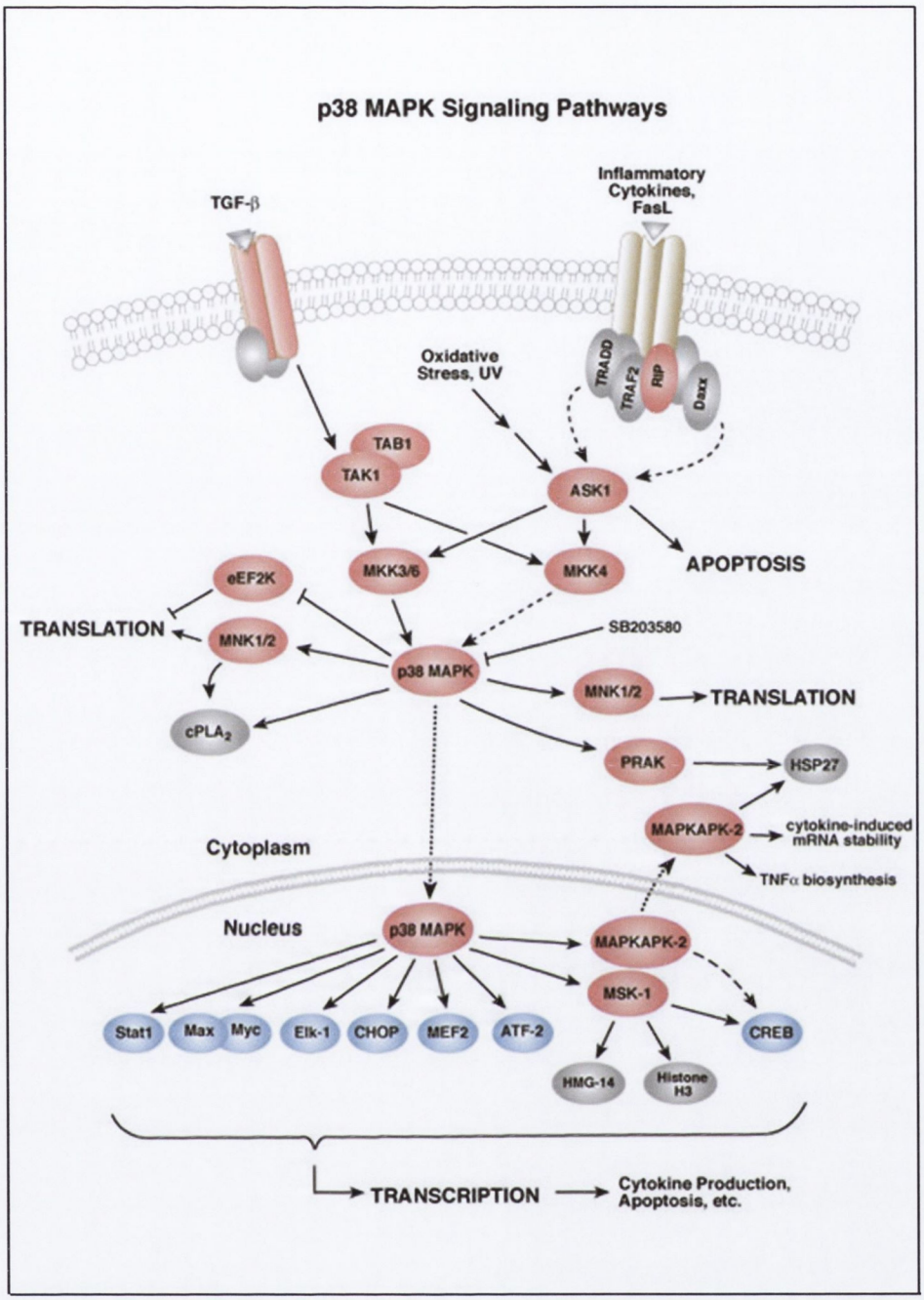


Fig 1.11 p38 Signal Transduction Pathway. The p38 signalling cascade is activated by a variety of environmental stresses and pro-inflammatory cytokines. A membrane proximal kinase usually Mekk2/3 or a member of the mixed lineage kinases (MLK) phosphorylate and activate MKK3 or MKK6. Additionally ASK1, which is stimulated by apoptotic stimuli, can directly activate MKK3/6 leading to activation of p38. Upon activation, p38 regulates the cytosolic proteins HSP27 and MAPKAP-2 as well as numerous transcription factors such as ATF-2, Stat1, MEF-2 and Elk-1. Reproduced from http://www.cellsignal.com/reference/pathway/p38MAPK.asp

The p38 family can phosphorylate and thereby activate the transcription factors TCFs Elk-1 and SAP-1a, growth arrest-and DNA damage-inducible gene 153 (GADD153), STAT1 and activating transcription factor 2 (ATF-2) **(Fig 1.11)**. Upon translocation to the nucleus, p38 can also regulate NF-κB dependent transcription. Similar to Erk, p38 can activate substrates other than transcription factors such as MAPKAP-K2, MAPKAP-K3, MAPKAP-K5 and the related protein MNK1 **(Fig 1.11)**. A major role for p38 in apoptosis, differentiation, inflammation and stress responses has been implicated. Unphosphorylated p38 has been shown to associate with the anti-apoptotic protein, cellular FLICE inhibitory protein (cFLIP). Phosphorylation of p38 results in its disassociation from cFLIP and induction of apoptosis in hepatocytes (Grambihler et al. 2003). In adipocytes, p38 phosphorylates GADD153 and thereby inhibits adipocyte differentiation (Wang and Ron 1996). A negative role for p38 in cell cycle progression has been suggested in studies using fibroblasts where activation of p38 is associated with inhibition of cyclin D1 expression (Lavoie et al. 1996). In some instances, activation of p38 is been associated with cell survival and growth (Juretic et al. 2001; Park et al. 2002). In fetal thymocytes expressing a constitutively active form of p38, no increase in cell death is observed upon activation of the p38 pathway (Diehl et al. 2000).

A prominent role for p38 in inflammatory processes has been suggested with p38-α being the major isoform activated in inflammatory cells (Herlaar and Brown 1999). The infiltration of leukocytes to a site of injury involves the coordinate expression and activation of adhesion molecules as well as the generation of leukocyte specific chemotactic gradients, which are regulated by p38 (Herlaar and Brown 1999). In conclusion, p38 plays a central role in mammalian cell differentiation, inflammation, proliferation and cell death.

1.6.2.3.3 JNK

The JNK MAPK family was identified by two different means, Firstly by their ability to phosphorylate the N-terminal transactivation domain of *c-jun* on Ser63 and Ser73 and secondly by the fact that they are activated by environmental stresses and hence are also known as stress activated protein kinases (SAPKs) (Hipskind and Bilbe 1998). JNK is activated in response to a broad spectrum of cellular stress such as UV irradiation, protein synthesis inhibitors (anisomycin), heat shock, anti-cancer drugs (cisplatin or etoposide), ceramide and oxidative stress **(Fig 1.12)** (Wada and Penninger 2004). At least three

isoforms of JNK are known to exist namely p46, p54α and p55β. JNK has been shown to phosphorylate the transcription factors c-Jun, ATF-2, Elk-1, c-Myc and p53 as well as non-transcriptional targets such as Bcl-2 and Bcl-X_L **(Fig 1.12)** (Davis 2000). JNK activation is also negatively regulated by the MKP family **(Fig 1.12)** (Chang and Karin 2001).

The role of JNK as a pro-apoptotic factor has been extensively demonstrated. *In vivo,* murine JNK1 and 2 double knockouts display an embryonic lethal phenotype with defective neural tube morphogenesis and impaired apoptosis in hindbrain regions (Kuan et al. 1999). However single deletions are viable but exhibit defective apoptosis in lymphocytes suggesting that JNK1 and 2 compensate for each other and function as pro-apoptotic factors in lymphocytes (Sabapathy et al. 1999). In cell free systems, JNK induces the release of pro-apoptotic factors such as cytochrome C and Diablo from isolated mitochondria suggesting that JNK activation regulates mitochondrial-dependent apoptosis (Aoki et al. 2002). In certain circumstances, JNK can promote cell survival (Sasaki et al. 2001) and has been shown to protect cardiomyocytes from oxidative stress (Dougherty et al. 2002). JNK activity can also be induced in response to growth factors, phorbol esters and heterotrimetric G proteins and as such has been implicated in cellular transformation in many of these pathways through its ability to transactivate c-Jun (Karin 1995; Davis 2000). These data suggest that JNK is not solely involved in the induction of apoptosis but rather regulates apoptosis in a signal specific and perhaps even cell type dependent manner.

Erk, p38 and JNK target an overlapping but not identical set of genes suggesting that the specific interaction and cross talk between these pathways determines the final biological response of the cell to extracellular stimuli and will varies depending on cell type and stimuli.

1.6.2.4 Role of MAPK in Cancer

Preneoplastic or neoplastic cells arise when the delicate balance between cell cycle, proliferation, differentiation and apoptosis is disrupted resulting in cells with a growth advantage due either to decreased propensity to undergo apoptosis or increased proliferation. Deregulation of the MAPK expression is a frequent occurrence (Hoshino et al. 1999). Constitutive Mek1 and Erk activation has been detected in renal carcinomas (Oka et al. 1995), breast carcinomas (Sivaraman et al. 1997) and oesophagogastric cancer (Barry et al. 2001). Colonic tumours have been shown to have elevated MAPK activation

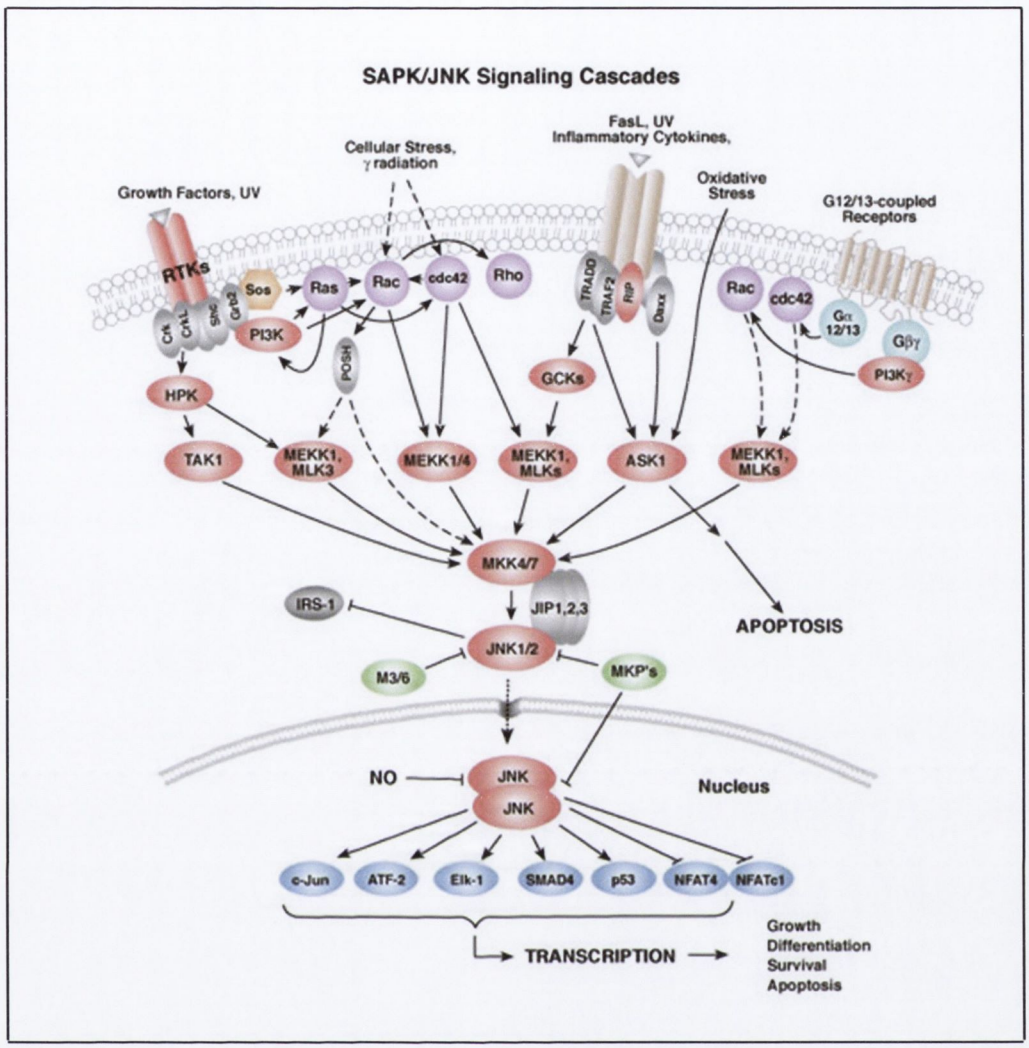


Fig 1.12. c-Jun N-Terminal Kinase (JNK) Signal Transduction Pathway. The JNK signalling cascade is activated by environmental stresses, pro-inflammatory cytokines, growth factors and G-coupled protein receptors. The members of small GTPases of the Rho family (Rac, Rho, cdc42) transduce signals to the JNK core unit. A membrane proximal kinase usually MEKK1/4 or a member of the mixed lineage kinases (MLK) phosphorylate and activate MKK4 (SEK) or MKK7. Additionally, members of the germinal center kinase (GCK) family can activate MKK4/7 in a GTPase-independent manner. Active JNK translocates to the nucleus where it phosphorylates and activates a broad spectrum of transcription factors such as c-Jun, ATF-2 and p53. Reproduced from http://www.cellsignal.com/reference/pathway/SAPKJNK.asp

particularly in the later stages of carcinoma (Licato et al. 1997; Hoshino et al. 1999). Approximately half of all colonic tumours have mutations in *ras* (Bos 1989), which convert *ras* to an activated oncogene. Oncogenic Ras induces persistent activation of the Mek-Erk cascade (Cook et al. 1999; Treinies et al. 1999), which is essential for increased invasion (Vial et al. 2003), angiogenesis (Eliceiri et al. 1998) and anchorage independent growth (Amundadottir and Leder 1998; Vial and Marshall 2003). *In vivo*, Mek inhibitors suppress the growth of colonic tumours in mice (Sebolt-Leopold et al. 1999). For these reasons, Mek inhibitors are being considered in the management of colon cancer.

The role of the p38 and JNK MAPKs in cancer is more complex due to the fact that both can have oncogenic and pro-apoptotic functions. For example, the p38 MAPKs can promote and suppress proliferation of leukaemia cells (Platanias 2003), while JNK activates c-Jun, a protein highly expressed in Hodgkin lymphoma cells and which promotes cell proliferation (Mathas et al. 2002). JNK is constitutively active in leukocytes from adult leukaemia patients (Xu et al. 1996). On the other hand, JNK is often a target of chemotherapeutic agents. Well known anti-cancer agents such as etoposide, adriamycin and camptothecin activate JNK to induce apoptosis (Seimiya et al. 1997). It is likely that cell type dependent inhibition or activation of p38 and JNK is required in cancer therapies modulating these pathways.

1.6.2.5 Bile Acids Activate MAPKs

Bile acids have been implicated in MAPK deregulation in several cell types. As previously discussed, Erk can be activated by ligation of G-coupled protein receptors and tyrosine kinase receptors such as epidermal growth factor receptor (EGFR) **(Fig 1.10)** (Pierce et al. 2001). Bile acids are known to activate G-coupled protein receptors such as TGF5, which subsequently activates Erk (Kawamata et al. 2003). Bile acids can also activate Erk via EGFR activation in hepatocytes, cholangiocarcinoma and colonic adenocarcinoma cells (Qiao et al. 2000; Qiao et al. 2001b; Yoon et al. 2002a; Im and Martinez 2004). Activatio of Erk by bile acids is associated with cell survival (Qiao et al. 2001a; Qiao et al. 2001b). JNK activation, in response to bile acids, is cell type dependent. In hepatocytes and cholangiocarcinoma cells, DCA activates JNK but not in colonic adenocarcinoma cells (Qiao et al. 2000; Yoon et al. 2002a; Qiao et al. 2003). In hepatocytes, JNK activation was cytoprotective (Qiao et al. 2003). Bile acids have been shown to activate p38 in multiple cell types (Qiao et al. 2000; Yoon et al. 2002a). In colonic adenocarcinoma cells, DCA-

induced p38 functions both as a pro- and anti-apoptotic factor. In HCT116 cells, DCA induces p38 and its transcriptional target GADD153, which mediates DCA-induced apoptosis (Qiao et al. 2002). In the same cells, DCA induces the anti-apoptotic protein COX-2 via p38 activation (Glinghammar et al. 2002), which has also been reported in cholangiocarcinoma cells (Yoon et al. 2002a).

Bile acids appear to induce apoptotic programs in tandem with survival cascades through MAPK deregulation. Bile acids are thought to mediate their tumour promoting activity via induction of apoptosis, which results in the selection and compensatory proliferation of apoptotic resistant cells (Bernstein et al. 2000). In colonic cells, prolonged exposure to DCA results in resistance to DCA-induced apoptosis. In these cells, several constituents of the MAPK cascade are deregulated. EGFR, Mek2 and the ras family members are upregulated while pro-apoptotic members of the MAPK cascade such as JNK1 are downregulated (Crowley-Weber et al. 2002; Bernstein et al. 2004). Collectively these data suggest an important role for the MAPKs in bile acid-induced alterations in gene expression and biological responses of cells from the gastrointestinal tract.

1.7 TRANSCRIPTION FACTORS

Transcription factors such as activator protein-1 (AP-1) are important regulators of cellular proliferation, differentiation and apoptosis and as such are essential for the maintenance of a proliferative steady state (Shaulian and Karin 2002). DCA has been shown to activate AP-1 and its upstream regulators, the MAPKs in other cell types (Qiao et al. 2000; Qiao et al. 2003). Therefore it was proposed that DCA activates AP-1 in oesophageal adenocarcinoma cells. AP-1 activation and the signalling pathways that mediate AP-1 activation were examined in this thesis. It is likely that the prolonged deregulated expression of AP-1 by DCA will contribute to oesophageal tumourigenesis as AP-1 is implicated in cellular transformation as well as tumour promotion and metastasis (Jochum et al. 2001; Eferl and Wagner 2003).

1.7.1 Activator Protein-1 (AP-1)

AP-1 was initially described as a transcription factor, responsive to the phorbol ester 12-0-tetradecanoylphorbol-12-acetate (TPA) that bound to the TPA-response element (TRE) in the *c-jun* promoter (Angel et al. 1987; Angel and Karin 1991). A plethora of physiological and environmental insults are now known to activate AP-1. The ability of AP-1, to respond

to diverse stimuli, stems from its structural and regulatory complexity. AP-1 exist as a dimer of basic region-leucine zipper (bZIP) proteins that belong to the Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD), MAF (c-Maf, v-Maf, MafB, MafA, MafG/F/K, Nrl) and ATF (ATF-2, ATF-3/LRF, B-ATF, JDP1, JDP2) subfamilies that bind to either the TRE (5'-TGAG/CTCA-3') site or the cyclic AMP response element (CRE, 5'-TGACGTCA-3') (Chinenov and Kerppola 2001). The Fos proteins do not form stable homodimers but dimerise with Jun to form heterodimers that are more stable than Jun homodimers **(Fig 1.13)** (Halazonetis et al. 1988; Kouzarides and Ziff 1988). Jun homodimers and Jun-Fos heterodimers bind preferentially to the TRE site. Conversely, the ATF proteins bind preferential to the CRE site and can form homodimers and heterodimeres with the Jun proteins **(Fig 1.13)** (Hai and Curran 1991). The MAF proteins (c-Maf, v-Maf and Nrl) can heterodimerise with Fos or Jun but the MAF related proteins (MafB, MafG/F/K) can only heterodimerise with Fos (Fujiwara et al. 1993; Kataoka et al. 1994; Kataoka et al. 1995). This thesis focused on the Fos and Jun family members.

1.7.1.1 Members of the Fos Family

1.7.1.1.1 c-Fos

Transcription of *c-fos* can be induced rapidly and transiently in response to a variety of stimuli (Treisman 1992; Piechaczyk and Blanchard 1994) and is regulated by a number of *cis* elements. The first *cis* element is the CRE located close to the TATA box. This is most likely occupied by either CREB or ATF proteins, all of which induce *c-fos* expression via cAMP and Ca^{2+} dependent signalling cascades in response to neurotransmitters and hormones **(Fig 1.14)** (Sheng et al. 1991). A second *cis* element, Sis inducible enhancer (SIE), is recognised by the STAT family of transcription factors (Darnell et al. 1994). The SRE represents the third *cis* element that is recognised by a dimer of serum-response factor (SRF), which recruits TCF. The SRE mediates *c-fos* transcription in response to growth factors and mitogens via phosphorylation of Elk-1 by Erk **(Fig 1.14)** (Gille et al. 1992; Marais et al. 1993). Persistent activation of Erk1/2 can also expose an Erk1/2 docking domain in c-Fos leading to phosphorylation of threonine 325 and 331, thereby altering the transcriptional activity of *c-fos* (Murphy et al. 2002). Transcription of *c-fos* is also mediated by SRE in response to stimuli such as IL-1 and UV irradiation through p38 and JNK-stimulated phosphorylation of Elk-1 **(Fig 1.14)**. The marked induction of c-Fos in response to growth factors and mitogens suggests an important role for c-Fos in cell proliferation. However murine knockouts of *c-fos* are viable, exhibiting only defective

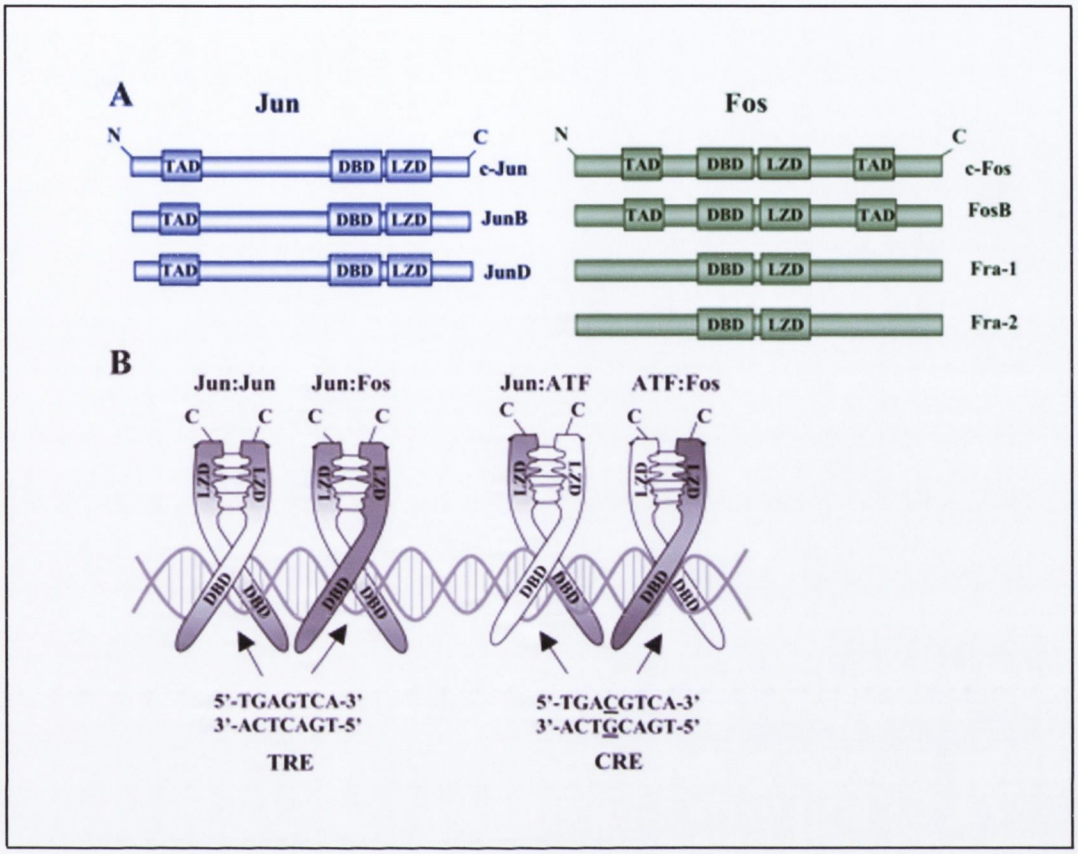


Fig 1.13. Schematic Representation of the Molecular Structures of the Jun and Fos Family Members. (*A*) The molecular structures of the Jun and Fos family members are shown and the positioning of the various domains is indicated. TAD, transcription-activating domain; LZD, leucine-zipper domain; DBD, DNA binding domain; N, amino terminus; C, carboxyl terminus. *B*: The LZD facilitates dimerisation between the DBD of Jun/Jun proteins and Jun/Fos proteins, which enables the interaction of protein dimers with DNA. The CRE binding site has an extra base (underlined) compared with the TRE site. Adapted from Reddy and Mossman 2002.

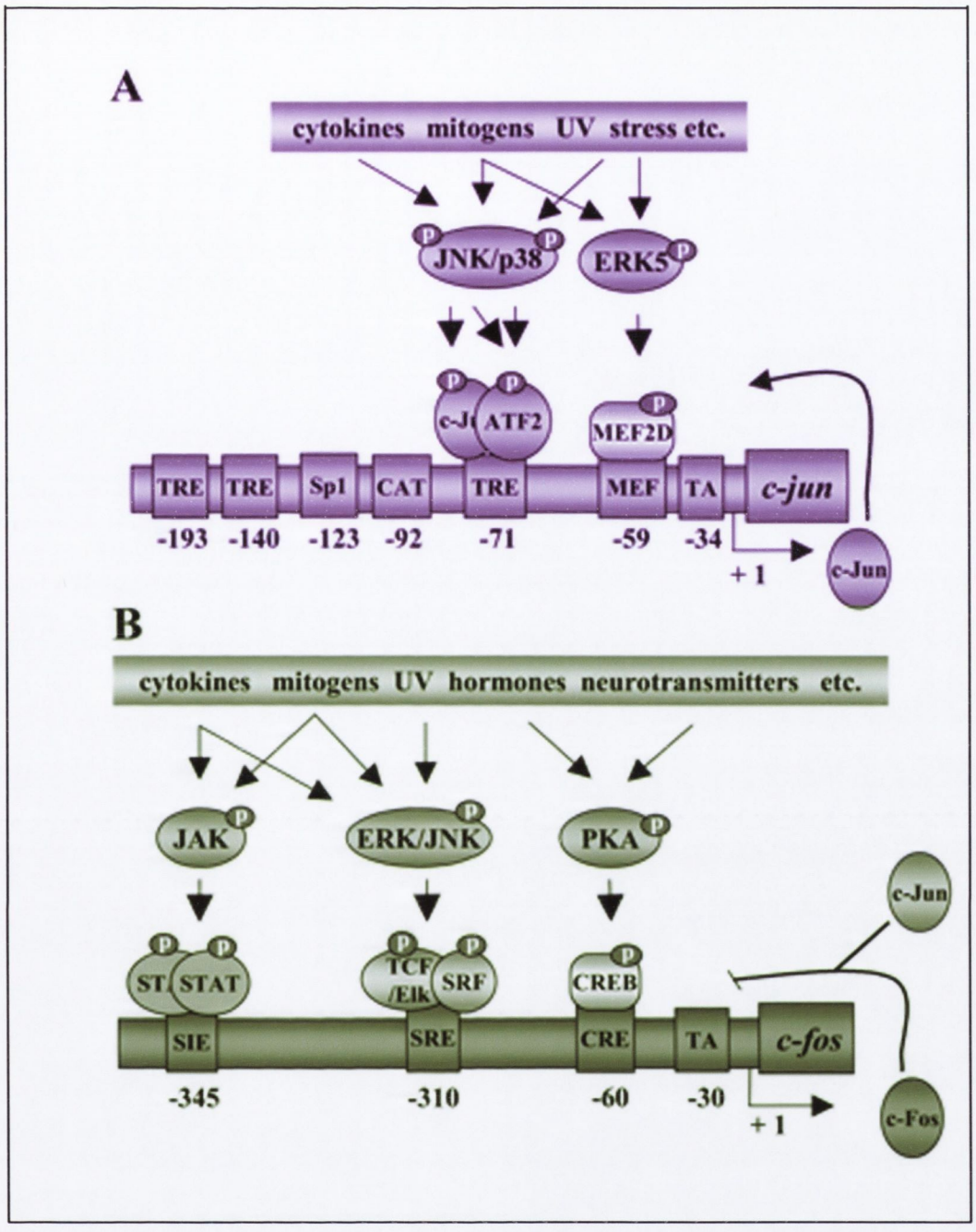


Fig 1.14. Regulation of *c-jun* and *c-fos* Transcription. A broad range of environmental and genetic stresses intracellular signalling cascades such as MAPKs, Janus kinase (JAK) and protein kinase A (PKA), which then translocate into the nuclei and activate both c-*jun* (*A*) and c-*fos* (*B*) transcription. The major regulatory *cis*-elements are indicated relative to the transcriptional start site (+1), which include TATA box; CCAAT protein binding site; CRE, MEF, SIE, SRE, Sp1, TRE, CREB, SRF and STAT. See text for abbreviations. P represents phosphorylation site while arrows show positive activation and blunt lines show an inhibition of the response. Adapted from Reddy and Mossman 2002.

bone remodelling (Johnson et al. 1992). T-cells, from mice deficient in *c-fos*, display no defects in differentiation or activity of peripheral T-cells (Jain et al. 1994). Therefore, its is speculated that *c-fos* is dispensable during embryonic development and in the adult organism as other members of the Fos family can compensate for the loss of *c-fos* (Jochum et al. 2001).

1.7.1.1.2 FosB

The *c-fos* homologue, *fosB*, is induced with similar kinetics to *c-fos*. The promoter of the *fosB* gene contains a SRE site enabling *fosB* to be induced by serum, growth factors and phorbol esters (Zerial et al. 1989). FosB activation is regulated by phosphorylation of a cluster of serine residues located within the C-terminal transactivation domain **(Fig 1.13)** (Skinner et al. 1997), which is Erk independent (Treinies et al. 1999). FosB, like c-Fos, is a potent activator of *fra-1* transcription. At least three splice variants of FosB have been identified, FosB, FosB2 and DFosB. FosB differs from FosB2 and DFosB by the presence of the C-terminal transactivation domain but all can efficiently bind Jun proteins (Mumberg et al. 1991; Wisdom and Verma 1993). It is speculated that one of the functions of the FosB2 and DFosB isoforms is to sequester the Jun proteins in less active AP-1 complexes (Yen et al. 1991; Bergers et al. 1995). During oncogenic transformation, the levels of FosB are generally unaffected (Mechta et al. 1997). Targeted inactivation of *fosB in vivo* established that FosB is dispensable during embryo development. However it is critical for behaviour as mice lacking *fosB* display a profound nurturing defect (Brown et al. 1996; Jochum et al. 2001).

1.7.1.1.3 Fos Related Antigen-1 (Fra-1)

Fra-1 was initially identified as an immediate early gene encoding a Fos-related antigen that cross reacts with c-Fos antibodies (Cohen and Curran 1988). It can be activated in response to serum and growth factors with delayed and protracted kinetics in comparison to c-Fos and FosB (Cohen and Curran 1988). Fra-1 shares strong sequence homology to c-Fos and FosB within its bZIP domain but unlike c-Fos and FosB, it lacks a C- and N-terminal transactivation domain **(Fig 1.13)**. Until recently Fra-1 reportedly had no transactivation domain (Bergers et al. 1995). Young *et al.* demonstrated that Fra-1 has a transactivation domain, which is dependent upon Erk-stimulated phosphorylation of threonine 231 (Young et al. 2002). Even though the regulation of *fra-1* is still poorly understood, it is known that the expression of *fra-1* can be regulated by AP-1 itself,

possibly via AP-1 sites located within intron-1 of the *fra-1* gene (Bergers et al. 1995). Consistent with this, overexpression of c-Jun, c-Fos, FosB or Fra-1 itself results in elevated transcription of the *fra-1* gene (Bergers et al. 1995). Fra-1 can also repress *c-fos* transcription (Bergers et al. 1995). Fra-1 is expressed at high levels in proliferating cells relative to c-Fos and FosB and its activity is necessary for DNA synthesis in cycling cells suggesting an essential role for Fra-1 in cell cycle progression (Kovary and Bravo 1992). Fra-1 expression is critical for embryonic development as demonstrated by targeted inactivation of the murine *fra-1* gene resulting in embryonic lethality and vascular defects (Schreiber et al. 2000). Unlike c-Fos and FosB, the presence of other Fos proteins cannot compensate for the loss of *fra-1* expression *in vivo*.

1.7.1.1.4 Fra-2

The Fra-1 homologue, Fra-2, is induced in response to mitogenic stimulation. The regulation of Fra-2 has been extensively studied in v-src transformed chicken embryo fibroblasts (Murakami et al. 1997). Similar to *fra-1*, the *fra-2* gene, which contains a CRE site, requires sustained Mek-Erk activation (Gruda et al. 1994). The presence of a positive autoregulatory loop, which is responsive to MAPK phosphorylation, allows for high levels of Fra-2 expression (Murakami et al. 1999). The expression patterns of Fra-1 and Fra-2 are very similar accounting for the speculation that they are functionally equivalent. However specific functions have been ascribed to Fra-2. For example, c-Jun/Fra-2 heterodimers rescued fibroblasts from cell growth arrest via a cyclin A dependent mechanism unlike c-Jun/Fra-1 and c-Jun/c-Fos heterodimers (Bakiri et al. 2000). Fra-2 is associated with terminal differentiation of ovarian granulosa cells into luteal cells (Sharma and Richards 2000). In *ras* transformed cells, Fra-1 is distinctly upregulated while no changes in the levels of Fra-2 are detected suggesting that Fra-2 is dispensable during *ras* transformation (Vallone et al. 1997; Vial and Marshall 2003). While Fra-2 may resemble Fra-1 in terms of structure and regulation, the two proteins appear to be functionally distinct.

1.7.1.2 Members of the Jun Family

1.7.1.2.1 c-Jun

The *c-jun* gene is constitutively expressed at a low level in most cell types and can be activated by a broad spectrum of stimuli such as growth factors, cytokines and UV irradiation (Mechta-Grigoriou et al. 2001). The *c-jun* promoter, which is highly conserved amongst species, contains several potential transcription factor binding sites **(Fig 1.14)**. A

TRE-like site (jun1) mediates induction of *c-jun* expression in response to serum, phorbol esters and TGFβ and is preferentially recognised by c-Jun/ATF-2 heterodimers (van Dam et al. 1998). A second AP-1 site (jun2) mediates the responsiveness of *c-jun* to PMA, growth factors and insulin treatment (Stein et al. 1992; Hagmeyer et al. 1993) and binds c-Jun/ATF-2 heterodimers. Since ATF2 alone cannot induce *c-jun*, the *c-jun* gene is upregulated by its own product **(Fig 1.14)**. Therefore in most cells, the TRE site in the *c-jun* promoter is constitutively occupied and a basal level of the c-Jun protein exists, even though its expression can be induced **(Fig 1.14)** (Angel et al. 1988; Rozek and Pfeifer 1993). Activation of c-Jun is regulated by the MAPKs in particular JNK, which is known to activate pre-existing c-Jun proteins (Devary et al. 1992; Gupta et al. 1995). JNK stimulates transcriptional activity of *c-jun* by phosphorylating Ser63 and Ser73 in the N-terminal transactivation domain (Kyriakis et al. 1994). Therefore JNK mediated *c-jun* activation in response to stimuli such as genotoxic stress and pro-inflammatory cytokines occurs through two distinct steps: Firstly endogenous c-Jun is activated by JNK dependent phosphorylation and secondly the phosphorylated c-Jun potentiates its own transcription via a positive auto-regulatory loop. The Erk1/2 MAPKs are thought to contribute *c-jun* activation by phosphorylating inhibitor residues residing next to the basic domain (Mechta-Grigoriou et al. 2001). Inactivation of *c-jun* in mice demonstrated that c-Jun is essential for embryonic development. Mice lacking *c-jun* exhibit an embryonic lethal phenotype (Hilberg et al. 1993; Johnson et al. 1993) with abnormalities in the liver (Hilberg et al. 1993; Eferl et al. 1999) and cardiac outflow tract (Eferl et al. 1999). Knock in mice having *junB* replacing *c-jun* do not die and have normal livers but defective cardiac outflow tract, indicating that JunB cannot fully compensate for c-Jun (Passegue et al. 2002).

1.7.1.2.2 JunB

The *junB* promoter, which is highly conserved amongst species, contains several potential regulatory elements such as SRE, CRE (Kitabayashi et al. 1993) and STAT3 (Coffer et al. 1995). Two SRE sites have been identified in the *junB* promoter; SRE1 is induced in response to growth factors and recruits TCFs while SRE2 is located distantly and does not respond to growth factors indicating that *junB* is regulated via the complex interaction between proximal and distal regulatory regions (Phinney et al. 1994). The STAT3 and a CRE like binding site mediate *junB* induction in response to interleukin-6 (IL-6) in HepG2 liver cells (Nakajima et al. 1993). Phorbol esters and protein kinase A can induce *junB* expression via an IL-6 regulatory domain containing an inverted region (de Groot et al.

1991b). Smad binding elements (CAGACA), present in the promoter region, allow *junB* to respond to members of the TGFβ family. Knockout studies with *junB* deficient mice demonstrated that JunB is essential for embryonic development and vascularisation (Schorpp-Kistner et al. 1999). Mice deficient in *junB* have an identical phenotype to *fra-1* deficient mice suggesting that *fra-1* and *junB* might have a common function (Schorpp-Kistner et al. 1999; Schreiber et al. 2000). JunB varies considerably from its homologue c-Jun as the transactivation potential of JunB is less than that of c-Jun. JunB does not contain a proline residue flanking its phosphoacceptor site and therefore cannot be phosphorylated by JNK, decreasing its dimerisation potential (Kallunki et al. 1996). Unlike *c-jun*, *junB* does not have a positive autoregulatory loop and therefore the basal level of JunB is lower than that of c-Jun. However *junB* is known to be the most inducible member of the Jun family (Mechta-Grigoriou et al. 2001).

1.7.1.2.3 JunD

The *junD* promoter contains several potential binding sites including CRE, an SP1 site, an octamer motif, an AP-1 motif and a GC rich region recognised by early growth response-1 (Egr-1). A high basal expression of JunD is present in most cell types owing to the presence of the octamer motif. As a major regulator of *junD* expression, the octamer motif is recognised by the widely expressed Oct1 protein, which mediates high basal levels of *junD* and accounts for its ubiquitous expression pattern (de Groot et al. 1991a). Serum stimulation of *junD*, unlike *c-jun* and *junB*, is relatively unpredictable (Hirai et al. 1989). The AP-1 motif is unresponsive to PMA, in the presence of the octamer motif, suggesting a role for the octamer motif as a repressor. This is likely to account for the low induction of the *junD* gene in response to certain stimuli (Mechta-Grigoriou et al. 2001). JunD expression is also regulated by the MAPKs. *In vivo*, JunD is phosphorylated by JNK in response to platelet-derived growth factor and anisomycin (Gupta et al. 1996; Kallunki et al. 1996; Wang et al. 1996). Since JunD lacks a proficient docking site, it is phosphorylated less efficiently than c-Jun by JNK (Kallunki et al. 1996). However, the MAPKs Erk1/2 can directly phosphorylate the N-terminal domain of JunD at Ser100 (Gallo et al. 2002). Unlike its homologues, inactivation of *junD* does not cause death *in utero* indicating that *junD* is not essential for embryonic development. Mice deficient in *junD* are healthy with males displaying age related defects in reproduction (Thepot et al. 2000).

1.7.1.3 Regulation of AP-1 Activity

The regulation of AP-1 activity is complex and occurs at many different levels through: Firstly changes in *fos* or *jun* gene transcription **(Fig 1.14)**; the effects on Fos and Jun protein turnover; post-translational modifications of Fos or Jun proteins that modify their transactivation potential and finally their interactions with ancillary proteins and other transcriptional factors that can either synergise or interfere with AP-1 activity (Karin et al. 1997; Chinenov and Kerppola 2001). The regulation of *c-fos* and *c-jun* transcription has been extensively studied and are the best understood.

The *fos* and *jun* genes are known as immediate early genes as their transcription can be rapidly induced in response to activating signals. AP-1 components, such as *c-fos,* are regulated at a transcriptional level through binding to the SRE, CRE and TRE sites **(Fig 1.14)**. In the case of *c-fos*, the SRE site mediates c-Fos induction following binding of SPF and TCFs. In response to stimulation, activated Erk phosphorylates Elk-1. Phosphorylation of Elk-1 facilitates the formation of a ternary complex consisting of Elk-1 itself, SRF and the SRE, which stimulates transcription of *c-fos* without altering its DNA binding activities (Marais et al. 1993). As the *c-fos* SRE site, as well as the *c-jun* TRE site, is constitutively occupied *in vivo* (Rozek and Pfeifer 1993), the phosphorylation of Elk-1 and other proteins occurs while bound to the DNA. The increased synthesis of c-Fos results in c-Fos combining with pre-existing Jun proteins to form heterodimers, which are more stable than Jun homodimers (Smeal et al. 1989). This leads to increased AP-1 DNA binding activity as the balance is moved towards stable dimer formation, which is critical for increased AP-1 DNA binding activity (Karin 1995). Accordingly, AP-1 DNA binding activity is also regulated by the composition of the AP-1 dimers. Dimers of c-Jun/JunB are known to be antagonistic and sometimes inactive (Deng and Karin 1993). Furthermore, dimer composition is a key determinant of the target genes induced and thereby the biological responses of the cells. Dimers of c-Jun/Fra-2 prevent growth arrest of serum deprived fibroblasts (Bakiri et al. 2002), whereas dimers of c-Jun/ATF-2 trigger growth factor independence. Variations in dimer composition will confer specificity to the actions of AP-1 and the subsequent target genes induced.

AP-1 activity is not only regulated at a transcriptional level but also by post-translation modifications of the Fos and Jun proteins. For example, post-translational modifications of c-Fos are critical in determining its activity. Differential Fos phosphorylation accounts for

the ability of c-Fos to function both as an activator and repressor of transcription. The capacity of c-Fos to repress transcription is regulated both by the rapid turnover of c-Fos and the phosphorylation of the C-terminal of c-Fos mediated by Erk and RSK **(Fig 1.14)** (Wilson and Treisman 1988; Ofir et al. 1990; Chen et al. 1993). N-terminal phosphorylation of c-Jun by JNK strongly augments c-Jun activity. Therefore the post-translation modifications of the Fos and Jun proteins can either positively or negatively regulate their transactivation potential.

The activity of the Fos and Jun proteins can additionally be regulated by their interaction with oncoproteins and ancillary proteins. The Ras oncoprotein is a major partner of AP-1 in mediating cellular transformation. Fra-1, JunB and c-Jun can co-operate with oncogenic Ras to mediate transformation (Mechta et al. 1997; Vallone et al. 1997). In addition to oncoproteins, Fos and Jun proteins can co-operate with ancillary proteins such as the glucocorticoid (Herrlich 2001) and retinoic-acid receptors. The glucocorticoid receptor can synergise with AP-1 and activate common target genes (Herrlich 2001). Conversely, it can inhibit AP-1 target gene expression by transrepression (Schule et al. 1990; Yang-Yen et al. 1990). The composition of the AP-1 dimers determines whether the glucocorticoid receptor enhances or represses AP-1 activity. The retinoic-acid receptor inhibits AP-1 activation by transrepresssion and therefore acts as tumour suppressor gene (Altucci and Gronemeyer 2001). Therefore some of the biological effects of AP-1 occur from the specific interaction of AP-1 with transcriptional co-repressors. For example, AP-1 mediated inhibition of Smad2 activity occurs when c-Jun interacts with the co-repressor, TG-interacting factor (Pessah et al. 2001).

The regulation of AP-1 is extremely complex and varies between the Fos and Jun proteins in accordance with the existence of distinct regulator regions within the individual proteins. However all of the AP-1 components are regulated by transcriptional and post-translational modifications and by their specific interactions with transcriptional co-activators and repressors.

1.7.1.4 AP-1 in Cellular Proliferation

AP-1 is intrinsically involved in the regulation of cell proliferation with the Jun proteins acting as the major determinant of cell fate while the Fos proteins appearing to have lesser effect (Shaulian and Karin 2001). Studies using neutralising antibodies, against individual

members of the Jun family, demonstrated that inactivation of any of the Jun proteins is sufficient to prevent serum-stimulated cell re-entry of serum starved cells. Microinjection of antibodies against any of the Fos proteins only partially inhibits cell re-entry suggesting some redundancy of function within the Fos family members (Kovary and Bravo 1991).

Direct evidence for the role of AP-1 proteins in regulating the cell cycle machinery was provided by the cyclin D1 gene, which contains AP-1 binding sites in its promoter. Fibroblasts, from *c-fos/fosB* double mutants, undergo cell cycle arrest and have reduced cyclin D1 expression (Brown et al. 1998). A positive role for c-Jun in cell cycle progression and proliferation is demonstrated in mouse embryo fibroblasts (MEFs) lacking *c-jun*, which display severe defects in proliferation as a result of *c-jun* inactivation (Schreiber et al. 1999). MEFs expressing a variant c-Jun, in which the JNK phosphoacceptor sites have been replaced with alanines, exhibit a less severe proliferation defect (Behrens et al. 1999). Inhibition of proliferation due to *c-jun* inactivation was attributed in part to inhibition of cyclin D1 expression (Wisdom et al. 1999). It is now known that c-Jun stimulates cell cycle progression and proliferation through two distinct mechanisms involving the stimulation of cyclin D1 expression with the simultaneous repression of $p21^{cip}$ (Schreiber et al. 1999; Shaulian et al. 2000).

JunB, unlike c-Jun, is regarded as being a negative regulator of cell proliferation depending on the cellular context and the presence of *c-jun*. MEFs overexpressing *junB* have retarded growth owing to a longer G_1 phase, which is credited to inhibition of cyclin D1 transcription by *junB* (Bakiri et al. 2000). JunB mediates its negative effect on cell cycle progression through the upregulation of the cell cycle inhibitor $p16^{ink4a}$, which prevents S phase entry (Passegue and Wagner 2000). JunB can negatively regulate cell proliferation by antagonising c-Jun. This is dependent on the cell type and the relative expression levels of both proteins. In the absence of c-Jun, JunB is thought to positively regulate cell growth. In knock in mice, replacement of *c-jun* with *junB* prevents the developmental and cellular defects related to the inactivation of *c-jun* (Passegue et al. 2002). However in the presence of c-Jun, JunB functions as a negative regulator of cell proliferation possibly through the formation of anti-proliferative c-Jun/JunB heterodimers (Schutte et al. 1989b; Deng and Karin 1993).

The effects of JunD on cell proliferation are paradoxical. In some instances such as in MEFs, inactivation of *junD* is credited for premature senescence and proliferation defects. This is associated with increased expression of the $p19^{ARF}$ protein that potentiates p53 activity (Weitzman et al. 2000). On the other hand, immortalised fibroblasts lacking *junD* have increased proliferation and cyclin D1 transcription (Weitzman et al. 2000). Furthermore, the tumour suppressor menin inhibits JunD phosphorylation and therefore activation (Gallo et al. 2002) whilst menin derived from tumours lack this ability suggesting a role for JunD in neoplastic growth (Agarwal et al. 1999). While the role of JunD in cell proliferation is still nebulous, it is certain that JunD is not as potent a regulatory of cell proliferation as c-Jun.

1.7.1.5 AP-1 in Cellular Transformation

Initially c-Fos and c-Jun were identified as homologues of the viral oncoproteins v-Fos and v-Jun (van Straaten et al. 1983; Maki et al. 1987), which suggested the involvement of AP-1 in neoplasia. This "guilt by association" was confirmed by the responsiveness of AP-1 components to tumour promoters such as phorbol esters (Angel and Karin 1991). AP-1 components can mediate oncogenic transformation by co-operating with activated oncogenes such as *ras*. The AP-1 components most frequently upregulated during oncogenic transformation include Fra-1, Fra-2, c-Jun and JunB while the levels of c-Fos and FosB are generally unaffected. In some instances, c-Fos can mediate cellular transformation by enhancing the transforming activity of c-Jun, which transforms immortalised fibroblasts (Miller et al. 1984; Schutte et al. 1989a). c-Fos is often required by malignant tumours for angiogenesis and tumour metastasis. In addition to Fra-1, c-fos regulates the expression of matrix metalloproteinases (MMP), MMP1 and MMP3, and the urokinase-type plasminogen activator system, which are essential for angiogenesis and invasive growth **(Fig 1.15)** (Hu et al. 1994; Kustikova et al. 1998).

The predominant Fos protein upregulated in *ras* and *mek1* transformed fibroblasts is Fra-1 (Mechta et al. 1997; Treinies et al. 1999). Upregulation of Fra-1 in cells containing oncogenic *ras* is associated with protection from anoïkis (Vial and Marshall 2003), the process whereby cells that detach from the extracellular matrix undergo cell death (Frisch and Francis 1994). Many tumour cells evade anoïkis and can survive from the extracellular matrix in a Ras dependent manner (Khwaja et al. 1997). Upregulation of Fra-1, in colonic and epithelioid adenocarcinoma cells, causes increased cell motility and elevated

Gene product	Activity	Main regulator
DNMT1	DNA methylation	c-FOS (upregulates)
EGFR	Stimulates proliferation	c-JUN (upregulates) JUNB (upregulates)
HB-EGF	Stimulates proliferation	c-JUN (upregulates)
GM-CSF	Stimulates proliferation	c-JUN (upregulates) JUNB (downregulates)
KGF	Stimulates proliferation	c-JUN (upregulates) JUNB (downregulates)
Cyclin D1	Stimulates proliferation	c-JUN (upregulates) JUNB (downregulates)
WAF1	Inhibits proliferation	c-JUN (downregulates)
p53	Inhibits proliferation Stimulates apoptosis	c-JUN (downregulates)
ARF	Inhibits proliferation Stimulates apoptosis	JUND (downregulates)
INK4A	Inhibits proliferation Stimulates apoptosis	c-JUN (downregulates) JUNB (upregulates)
FASL	Stimulates apoptosis	c-JUN (upregulates) c-FOS (upregulates)
FAS	Stimulates apoptosis	c-JUN (downregulates)
BIM	Stimulates apoptosis	c-JUN (upregulates)
BCL2	Inhibits apoptosis	JUNB (downregulates)
BCL-XL	Inhibits apoptosis	JUNB (downregulates)
BCL3	Inhibits apoptosis	c-JUN (upregulates)
VEGFD	Angiogenesis	c-FOS (upregulates)
uPA	Angiogenesis	FRA1 (upregulates)
uPAR	Angiogenesis	FRA1 (upregulates)
Proliferin	Angiogenesis	c-JUN (upregulates) JUNB (upregulates)
MMP1	Invasiveness	c-FOS (upregulates) FRA1 (upregulates)
MMP3	Invasiveness	c-FOS (upregulates) FRA1 (upregulates)
CD44	Invasiveness	c-FOS (upregulates) c-JUN (upregulates)
Cathepsin L	Invasiveness	c-FOS (upregulates)
MTS1	Invasiveness	c-FOS (upregulates)
KRP1	Invasiveness	c-FOS (upregulates)
TSC36/FRP	Invasiveness	c-FOS (upregulates)
Ezrin	Invasiveness	c-FOS (upregulates)
Tropomyosin 3	Invasiveness	c-FOS (upregulates)
Tropomyosin 5b	Invasiveness	c-FOS (upregulates)

Fig 1.15. AP-1 Target Genes in Tumour Development and Suppression. The Fos proteins mediate angiogenesis and tumour invasion in malignant tumours whereas the Jun proteins regulates genes involved in cell proliferation and apoptosis. DNMT. DNA methyltransferase; EGFR, epidermal growth factor receptor; FASL, Fas ligand; GM-CSF, granulocyte-macrophage colony stimulating factor; HB-EGF, heparin binding EGF; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VEGFD, vascular endothelial growth factor D. Reproduced from Eferl and Wagner 2003.

expression of the receptor for urokinase-type plasminogen activator (uPAR) **(Fig 1.15)** (Kustikova et al. 1998; Vial et al. 2003). In general, the Fos proteins function as enhancers of cellular transformation in conjunction with other oncoproteins and are frequently required by tumours to mediate angiogenesis and tumour invasion **(Fig 1.15)** (Eferl and Wagner 2003).

The ability of c-Jun to mediate oncogenic transformation is well documented (Schutte et al. 1989b; Mechta-Grigoriou et al. 2001; Shaulian and Karin 2001, 2002). Studies suggest that the dimerisation partner of c-Jun not only regulates c-Jun transformation activity but also influences the target genes induced **(Fig 1.15)** and therefore the biological response of the cells. Heterodimers of c-Jun/ATF-2 induce autocrine growth whereas heterodimers of c-Jun/c-Fos stimulate anchorage independent growth (van Dam et al. 1998). Unlike c-Jun, its homologue JunB can have opposing roles in transformation due primarily to the c-Jun/JunB antagonism, which is undoubtedly context dependent. In rat embryo fibroblasts, cotransfecting of JunB with c-Jun, reduces the capacity of c-Jun to participate in transformation (Schutte et al. 1989a). Similarly in mouse fibroblasts, overexpression of JunB reduces v-ras or v-src mediated transformation (Schreiber et al. 1999). Conversely, both c-Jun and JunB activate the angiogenic factor proliferin and mediate angiogenesis in the culture model of fibrosarcoma (Toft et al. 2001). Both Jun proteins are upregulated during fibrosarcoma tumour progression (Bossy-Wetzel et al. 1992). In colorectal carcinoma cells, c-Jun and JunB are upregulated while the levels of JunD remain high both in normal and malignant colonic cells (Wang et al. 2000a). A contrasting role for JunD in oncogenesis has been reported. JunD is known to be a negative regulatory of cell growth (Pfarr et al. 1994). However mice lacking JunD do not form spontaneous tumours (Thepot et al. 2000). The tumour suppressor menin inhibits JunD, which is associated with inhibition of tumour cell growth suggesting that JunD can function as an oncoprotein (Agarwal et al. 1999). Collectively these data suggest that the Fos family members and c-Jun function as positive tumour promoters whereas the functions of JunB and JunD are complex and dependent on the stimulus and cellular context.

1.7.1.6 AP-1 in Apoptosis and Survival

AP-1 transcription factors have been implicated in the co-ordinate regulation of cell death and survival, which is important for cellular homeostasis. Initially observations linking AP-1 to cell death came from studies in which c-Fos and c-Jun were upregulated by known

apoptotic agents. It is now known that upregulation of AP-1 components in neuronal and lymphoid cultures precedes induction of apoptosis triggered by growth factor withdrawal. Antisense oligonucleotides against *c-fos* and *c-jun* promote the survival of growth factor deprived cells (Colotta et al. 1992; Smeyne et al. 1993; Ham et al. 1995). In neuronal tissue from mice treated with kainic acid, a strong inducer of apoptosis in hippocamal neurons, c-Fos is persistently upregulated (Smeyne et al. 1993). Genotoxic stresses, such as alkylating agents and UV irradiation, persistently induce c-Jun prior to induction of cell death (Shaulian et al. 2000).

The pro-apoptotic activity of the AP-1 components has been attributed to the induction of target genes involved in apoptosis such as Fas ligand (FasL) and BIM **(Fig 1.15)**. The *fasL* promoter can be directly activated by c-Jun and c-Fos (Kasibhatla et al. 1998). Fibroblasts lacking c-Jun are resistant to UV irradiation and alkylating agent-induced apoptosis, which is associated with reduced FasL expression (Kolbus et al. 2000). However anti-apoptotic functions have also been attributed to AP-1 components such as c-Jun. In some cells, c-Jun can prevent UV-induced apoptosis by co-operating with STAT3 to suppress *fas* (Wisdom et al. 1999; Ivanov et al. 2001). Fibroblasts with inactive *c-jun* exhibit increased spontaneous apoptosis. In growth factor deprived T-cells, c-Jun mediates survival by upregulating the survival factor Bcl-3 **(Fig 1.15)** (Rebollo et al. 2000). Its is proposed that the AP-1 components function only as pro-apoptotic factors in response to extreme conditions such as high levels of genotoxic stress, UV irradiation and hippocampal neuronal stimulation (Shaulian and Karin 2001).

Two mechanisms have been suggested for the role of AP-1 as mediator of both cell death and survival. The first model proposes that the sum of the pro- and anti-apoptotic target genes induced in response to increased AP-1 activity will determine the life/death decision of the cells (Shaulian and Karin 2002). The second model suggests that AP-1 acts as a homeostatic factor. Under normal conditions, AP-1 maintains the cells in a steady state of proliferation. In response to environmental changes, AP-1 activity is increased resulting in the proliferation and survival of cells that still have the capacity to proliferate. However, AP-1 activation in cells with DNA damage leads to defective proliferation and possibly even apoptosis (Shaulian and Karin 2002). While it is unclear which model is occurring *in vivo,* it is probably that the functions of AP-1 will depend on the cell type and the nature

and duration of the stimulus activating AP-1 (Karin et al. 1997; Shaulian and Karin 2001, 2002).

1.7.1.7 Bile Acids Activate AP-1

Carcinogenesis is thought to proceed according to a two step model beginning with an irreversible "initiation" step such as the activation of *ras* through mutations, which frequently occurs in colon cancer followed by a "promotion" step ensuring sustained tumour development possibly involving AP-1 activation (Hanahan and Weinberg 2000). *In vivo*, *k-ras* mutations have been observed in aberrant crypts of AOM-treated mice. However the frequency and number of *k-ras* mutations increases when AOM-treated mice are fed high fat diets (Bird et al. 1989). Furthermore, the expression of AP-1 components such as c-Fos is increased in aberrant crypt foci as compared to normal crypts in the colon (Stopera et al. 1992). It was therefore speculated that prolonged AP-1 activity caused by increased concentrations of secondary bile acids resulting from high fat diets co-operates with activated *ras* to contribute to tumour development (Bird et al. 1989; Matheson et al. 1996). It is now known that oncogenic *ras* can induce persistent activation of Fra-1 in colonic adenocarcinoma cells, which is essential for avoiding anoïkis and tumour motility (Vial and Marshall 2003; Vial et al. 2003). Bile acids have been shown to induce AP-1 in several cell types including colonic cells (Matheson et al. 1996; Qiao et al. 2000; Glinghammar and Rafter 2001; Qiao et al. 2003). In colonic cells, the DCA-induced AP-1 complex consisted of c-Fos and JunD, known to be expressed at a high levels in premalignant and malignant colonic cells (Stopera et al. 1992; Wang et al. 2000a). Fra-1 is also a member of the DCA-induced AP-1 complex and can co-operate with oncogenic *ras* (Vial and Marshall 2003). Therefore it is likely that DCA induces AP-1 to facilitate tumour promotion in colonic adenocarcinoma cells.

In oesophageal carcinoma cells, overexpression of c-Fos has been reported (Wu et al. 2004). Fra-1 is known to be upregulated in oesophageal squamous cell carcinoma (Hu et al. 2001), but studies in oesophageal adenocarcinoma have not been conducted. The bile acid CDCA has been shown to induce AP-1 binding activity in oesophageal cells (Zhang et al. 1998). It is possible that the deregulated expression of AP-1 components such as c-Fos observed *in vivo* may be due to bile acid reflux.

A characteristic effect of bile acids is their ability to induce concurrent pro- and anti-apoptotic cascades. In colonic cells DCA-induced AP-1 activation stimulated COX-2 expression (Glinghammar et al. 2002), which is important for tumour promotion (Tsujii et al. 1997). In the same cells, DCA is in part responsible for apoptosis by activating GADD153 (Qiao et al. 2002). As discussed earlier, bile acids are thought to facilitate tumour promotion by inducing apoptosis to select for apoptotic resistant cells (Bernstein et al. 2000). As AP-1 target genes include pro-and anti-apoptotic factors **(Fig 1.15)**, bile acids might utilise AP-1 to induce apoptosis while at the same time promoting the survival and development of apoptotic resistant cells.

1.8 CYCLOOXYGENASE (COX)

Cyclooxygenase (COX), also known as prostaglandin H_2 synthase, is the key enzyme in the biosynthesis of prostaglandins. Due to its inherent cyclooxygenase and peroxidase activities, COX catalyses the conversion of arachidonic acid or other 20-carbon fatty acids to prostaglandin G_2 (PGG_2) and PGH_2. PGH_2 is subsequently converted to other eicosanoids including PGE_2, thromboxane A_2 (TXA_2) and prostacylins **(Fig 1.16)** (Dubois et al. 1998). The array of prostanoids produced, which is cell dependent, facilitates normal physiological functions such as homeostasis, gastric and renal function, reproduction, pain and fever (Dubois et al. 1998). Two cyclooxygenase isoforms, COX-1 and COX-2, have been identified. COX-1 is referred to as the housekeeping enzyme due to its constitutive expression in nearly all tissue whereas COX-2 is inducible usually in response to inflammatory mediators, growth factors and tumour promoters (Fosslien 2000). The expression of COX-2, due to its well-known association with gastrointestinal cancer (Eberhart et al. 1994; Wilson et al. 1998; Lagorce et al. 2003), was examined in oesophageal adenocarcinoma cells in response to DCA. The signalling pathways mediating COX-2 expression were also defined as they could possible facilitate the identification of therapeutic targets.

1.8.1 COX-1

COX-1 is constitutively expressed in almost all tissue under basal conditions (Crofford 1997) and its primary function is to provide maintenance levels of prostaglandins in tissues such as the kidneys and stomach for homeostatic regulation. In the kidney, COX-1 regulates renal plasma flow and glomerular infiltration rate by producing vasodilating prostaglandins in response to angiotensin, which is secreted during times of lowered renal

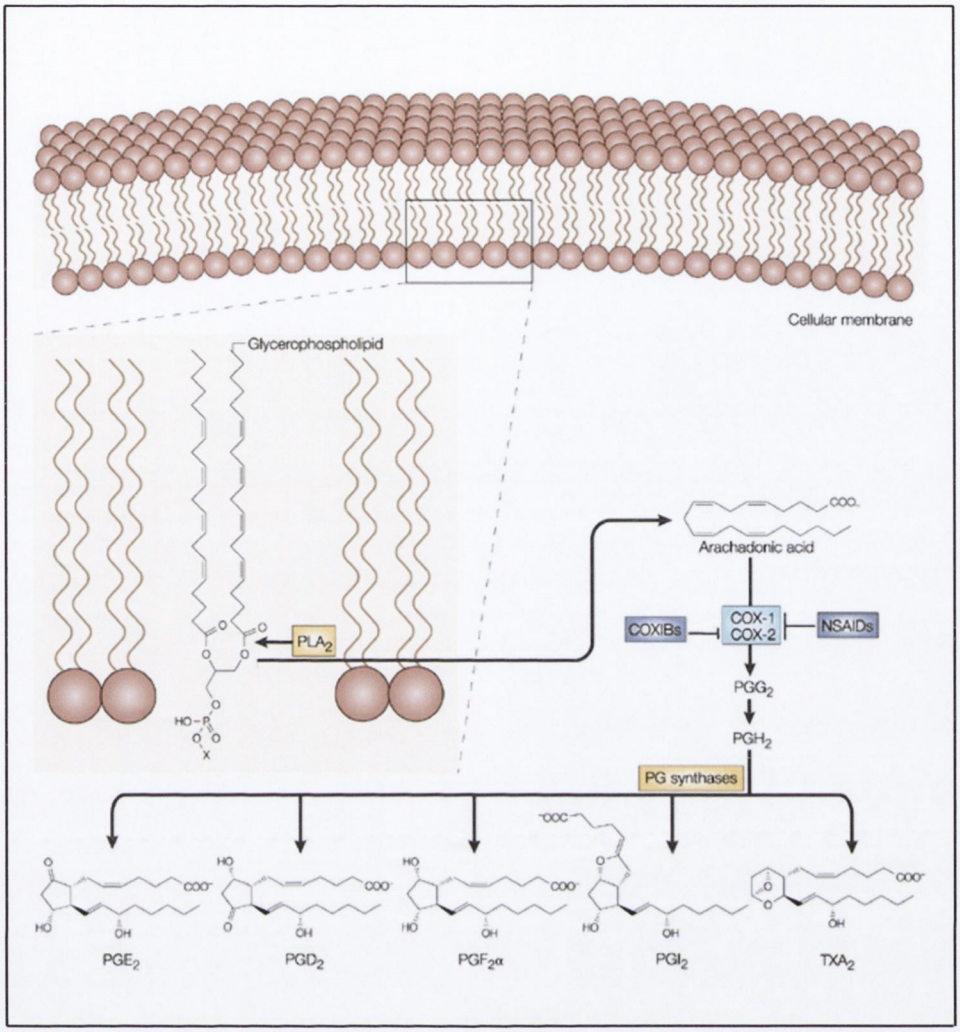


Fig. 1.16. Cyclooxygenases Catalyse Arachidonic Acid Metabolism. Arachidonic acid is released from the membrane by the action of phospholipase A_2 family of enzymes. The cyclooxygenase (COX) enzymes catalyse the biosynthesis of the intermediate PGG_2, which is reduced to PGH_2. Subsequently PGH_2 can be converted to several structurally related prostaglandins (PGs), including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TxA_2). COX-1 and COX-2 activity can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs). COXIBs selectively inhibit COX-2 activity. Reproduced from Gupta and Dubois 2001.

blood flow (Palmer and Henrich 1995). In the stomach, COX-1 is important for crypt cell survival by producing prostaglandins that regulate blood flow around the gastric mucosa (Trevethick et al. 1995). In platelets, COX-1 supplies the precursors for thromboxane synthesis necessary for platelet aggregation and wound healing (Schafer 1995). In these settings, COX-1 acts a homeostatic regulator due in part to its requirement for exogenous supplies of substrate. Therefore its activity is modulated by the physiological requirements of neighbouring cells (Dubois et al. 1998). There have been only a few reports implicating COX-1 in cancer. Elevated expression of COX-1 was detected in stromal cells adjacent to malignant breast cells overexpressing COX-2 (Hwang et al. 1998). It was speculated that the expression of COX-1 was elevated in neighbouring tissue in response to hormones, prostaglandins and other substances released by the cancer cells overexpressing COX-2 (Hwang et al. 1998; Fosslien 2000). Deregulation of COX-1 expression has only been demonstrated to occur via a paracrine pathway as result of COX-2 overexpression.

1.8.2 COX-2

The COX-2 protein, despite having similar enzymatic properties to COX-1, is functionally distinct and has unique expression patterns. The crystal structure of COX-2 demonstrated that it contains a larger substrate-binding site than COX-1 and can therefore oxygenate a wide array of fatty acid substrates. COX-2 contains a membrane binding motif and an epidermal growth factor (EGF) binding domain, which explains its responsiveness to EGF (Taketo 1998). Tumour promoters, growth factors and cytokines can stimulate the transcription of *cox-2*. Numerous potential transcription factor binding sites such as CRE, NF-κB, nuclear factor-IL6 (NF-IL6), PEA3, SP1, and an Ets binding site as well as regulatory regions such a TATA box are located in the *cox-2* promoter. The peroxisome proliferator-activated receptors (PPARs), transcription factors involved in fatty acid catabolism and storage, also regulate COX-2 expression. But PPAR regulation of COX-2 is complex as the PPARs can function as both positive and negative regulators of COX-2 depending on the cell type (Subbaramaiah et al. 2001; Glinghammar et al. 2003).

Regulation of *cox-2* occurs through both transcriptional and post-transcriptional modifications (Chun and Surh 2004). At a transcriptional level, transactivation of the *cox-2* promoter is stimulated by binding of NF-κB, TGF-β, Fos, Jun and other proteins to their respective binding sites (Guo et al. 2001). However the tumour suppressor gene, p53, can suppress *cox-2* transcription by competing with the TATA-binding protein for binding to

the TATA box of the *cox-2* promoter. Only wild type p53 can suppress *cox-2* transcription (Subbaramaiah et al. 1999). At a post-transcriptional level, the MAPKs have been shown to contribute to *cox-2* mRNA stability (Lasa et al. 2000; Guo et al. 2001). In human monocytes, lipopolysaccharide-induced p38 enhances COX-2 protein expression by stabilising *cox-2* mRNA (Dean et al. 1999). Similarly TGF-β augmented Ras-induced COX-2 protein expression by stabilised *cox-2* mRNA at the 3'-untranslated region (Sheng et al. 2000). Stabilisation of *cox-2* mRNA is regulated by its 3'-untranslated region, which contains an AU-rich sequence element (ARE) that can negatively regulate *cox-2* transcript stability and thereby modulate translation (Dixon et al. 2000). A role for COX-2 in maintaining and protecting the gastrointestinal epithelium has been demonstrated. *In vivo*, COX-2 is induced in response to infectious organisms, which results in enhanced prostaglandin production. Subsequently, the prostaglandins stimulate chloride and fluid secretions, which cleanse the intestine of infectious organisms and parasites (Eckmann et al. 1997). Therefore COX-2, under normal circumstances, is cytoprotective in the gastrointestinal epithelium.

1.8.3 Role of COX-2 in Cancer

COX-2 has been implicated in several human malignancies. Initial evidence, suggesting a role for COX-2 in neoplasia, emerged when transformed and tumour cells were found to have increased prostaglandin synthesis in comparison to normal cells (Bennett et al. 1980; Bennett et al. 1982). The most compelling evidence linking COX-2 with tumourigenesis came from animal studies where mice, overexpressing COX-2 in mammary glands, developed mammary gland dysplasia and metastatic cancer (Liu et al. 2001). Similarly *cox-2* null mice carrying the *apc* mutation, which genetically predisposes to colon cancer, exhibited a reduction in the frequency and multiplicity of tumours in comparison to wild type mice carrying the mutation (Oshima et al. 1996). Consistent with these reports, overexpression of COX-2 has since been observed in numerous human malignancies including Barrett's oesophagus, oesophageal cancer (Wilson et al. 1998), gastric cancer (Ristimaki et al. 1997), and colorectal cancer (Eberhart et al. 1994).

The mechanisms contributing to overexpression of COX-2 in malignant cells is still nebulous but it is likely to be a consequence of multiple cellular effects since no mutations for *cox-2* have been identified (Battu et al. 1998; Spirio et al. 1998). COX-2 expression can be upregulated by several oncogenes including Ras. PDGF-induced COX-2 expression

requires Ras-Mekk1-JNK activation (Xie and Herschman 1996). Similarly erbB-2 and EGFR, both of which are overexpressed in oesophageal cancer (al-Kasspooles et al. 1993), can upregulate COX-2 expression via the Ras-MAPK-AP-1 signalling module (Subbaramaiah et al. 2002; Yoon et al. 2002a). Furthermore, constitutive expression of several MAPKs and AP-1 components has been demonstrated in colonic and oesophageal cancer cells (Stopera et al. 1992; Barry et al. 2001; Wu et al. 2004). Deregulated expression of these molecules is likely to upregulate COX-2 expression via binding to the CRE site in the *cox-2* promoter. Moreover, aberrant expression of TGF-β is repeatedly observed in human neoplasia of the colon and oesophagus and can increase COX-2 expression either directly by increasing the stability of the *cox-2* transcript (Sheng et al. 2000) or indirectly by activating AP-1. Increased transcriptional activity of *cox-2* may be consequence of defective regulation of the *cox-2* gene itself. It is speculated that the ARE binding proteins that normally negatively regulate *cox-2* transcript stability by interacting with the 3'-untranslated region, are defective in tumour cells contributing to the deregulated expression of COX-2 (Dixon et al. 2000). Similarly loss of function of p53, which negatively regulates *cox-2* transcription, is a frequent occurrence in human malignancies. In Barrett's adenocarcinoma, loss of heterozygosity of the chromosomal region 17p13.1, where the p53 gene is located, frequently occurs (Dunn et al. 1999) and is likely to result in increased COX-2 expression. The constitutive expression of COX-2 in neoplasia is likely to be the result of a combination of transcriptional and posttranscriptional effects.

COX-2 is frequently upregulated in neoplasia due to its ability to increase prostaglandin synthesis, mediate the production of mutagens, confer resistance to apoptosis, and induce angiogenic markers (Howe et al. 2001). COX-2 directly regulates the biosynthesis of prostaglandins, which are upregulated in malignant cells. Increased production of prostaglandins contributes to tumourigenesis by inducing DNA damage in part through the effects of malondialdehyde (MDA) (Marnett 1992), stimulating cell growth (Nolan et al. 1988) and preventing immune surveillance (Huang et al. 1996). PGE_2 can inhibit TNF-α production while stimulating IL-10, which has potent immunosuppressive effects (Huang et al. 1996). Prostaglandins can also prevent antigen processing of dendritic cells (Saito et al. 2002). Consequently, prostaglandins are likely to contribute to carcinogenesis by allowing damaged cells evade detection. This process is possibly aided by the ability of COX-2 to suppress apoptosis. In rat intestinal epithelial cells, overexpression of COX-2

increased resistance to apoptosis (Tsujii and DuBois 1995). In malignant tissue overexpressing COX-2, reduced levels of the pro-apoptotic proteins Bax and Bcl-x_L have been observed (Liu et al. 2001). It is proposed that COX-2 confers resistance to apoptosis by increased the expression of anti-apoptotic proteins such as Bcl-2 (Sheng et al. 1998), which is thought to favour tumourigenesis. A crucial facet of tumourigenesis is neovascularisation. In colonic cells, COX-2 positively regulates angiogenesis by upregulating angiogenic factors including VEGF (Tsujii et al. 1998; Cianchi et al. 2001) whose expression is correlated with increased metastatic potential (Mandriota et al. 1995; Nakata et al. 1998). COX-2 also increases the metastatic potential of tumour cells by activating the MMPs, which can degrade extracellular matrices crucial for invasion (Tsujii et al. 1997). All of these effects constitute the mechanisms by which overexpression of COX-2 possibly mediates tumourigenesis.

1.8.4 Inhibition of COX-2 as a Therapeutic Tool

COX activity can be inhibited by nonsteroidal anti-inflammatory drugs (NSAIDS) such as aspirin and sulindac, which are commonly used for their analgesic, anti-pyretic and anti-inflammatory effects (Flower 2003; Tarnawski and Caves 2004). Since COX-2 is highly expressed in inflammatory conditions such as rheumatoid arthritis, COX-2 inhibitors have been used in the management of this disease. Celecoxib (commercially known as celebrex ™) was one of the first COX-2 specific inhibitors to be approved for the treatment of rheumatoid arthritis and osteoarthritis (Chun and Surh 2004). Given that COX-2 is known to be upregulated in neoplasia and that the use of NSAIDs is correlated with a reduced incidence of colon cancer and other malignancies (Thun et al. 1991; Greenberg et al. 1993; Farrow et al. 1998), the use of NSAIDS in the management of malignancies of the gastrointestinal tract is being considered. A wealth of pharmacological, genetic and expression data indicates that COX-2 selective NSAIDS inhibit the growth of malignant cells, including colonic and oesophageal carcinoma cells, by inducing apoptosis (Elder et al. 1996; Li et al. 2000; Souza et al. 2000). In the US, COX-2 selective inhibitors have been approved for use as an adjunct to existing treatment. For example, celecoxib has been approved for the management of FAP patients in conjunction with existing therapies (Steinbach et al. 2000; Chun and Surh 2004). However, the long-term use of some NSAIDS is limited by their gastrointestinal side effects. Adverse effects such as peptic ulcers and gastrointestinal haemorrhaging have been reported due to their non selective inhibition of COX-1 and COX-2 (Cryer and Feldman 1999; Derry and Loke 2000).

Modified derivatives of aspirin and other COX-2 inhibitors, which appear to have improved therapeutic efficacy, have being developed in an effort to minimise the gastrointestinal side effects associated with some NSAIDS (Lichtenberger et al. 2001; Fiorucci et al. 2003). However, the therapeutic benefits of NSAIDs and their derivatives outweigh the relatively small risk of gastrointestinal complications (Flower 2003; Tarnawski and Caves 2004). Clearly, pharmacological inhibition of COX-2 represents a viable approach for the treatment and even prevention of gastrointestinal malignancies.

1.8.5 Bile Acids Activate COX-2

Bile acids have been shown to activate COX-2 both *in vitro* and *in vivo* in multiple cell types (Zhang et al. 1998; Shirvani et al. 2000; Zhang et al. 2000; Zhang et al. 2001a; Yoon et al. 2002a). In colonic neoplastic cells, bile acids augment COX-2 expression by increasing *cox-2* transcription (Glinghammar and Rafter 2001) as well as ensuring *cox-2* transcript stability (Zhang et al. 2000). Similarly in oesophageal adenocarcinoma cells, bile acids stimulated *cox-2* promoter activity, which results in increased prostaglandin synthesis (Zhang et al. 1998). Due to its well-known anti-apoptotic activity, it is speculated that COX-2 expression is upregulated by bile acids to mediate the survival of pre-malignant cells. In colonic adenocarcinoma cells, inhibition of DCA-induced COX-2 expression sensitises the cells to the inherent cytotoxic effects of DCA (Washo-Stultz et al. 2002). In addition to its anti-apoptotic activity, COX-2 is known to stimulate invasion as cells overexpressing COX-2 have increased metastatic and invasive potential (Tsujii et al. 1997). In colonic adenocarcinoma cells, COX-2 inhibition suppressed lithocholic acid-induced haptotaxis and invasion (Debruyne et al. 2002). Moreover, COX-2 can stimulate angiogenic factors and subsequently mediate angiogenesis. As bile acids are regarded as tumour promoters and neovascularisation is crucial for malignant progression, bile acids have been shown to induce angiogenic factors via COX-2 dependent pathways. It is probably that bile acids induce COX-2 expression to mediate angiogenesis and thereby ensure sustained growth of malignant cells (Wilson 2002). The bile acid UDCA, which is thought to be cytoprotective and lacks the tumour promoting activity of DCA and CDCA, has potent anti-angiogenic effects suggesting a role for bile acids in angiogenesis (Suh et al. 1997). Upregulation of COX-2 expression is likely to assist the tumour promoting activities of bile acids by reducing the apoptosis capacity and increasing the invasive and metastatic potential of cells along the gastrointestinal tract.

1.9 SUMMARY

Malignancies of the gastrointestinal tract have been the focus of much research in the last number of years, with certain neoplasms such as colon cancer being quite extensively characterised (Toribara and Sleisenger 1995). A wealth of *in vivo* and *in vitro* data has correlated the presence of bile acids with increased severity of disease and tumour formation (Narisawa et al. 1974; Debruyne et al. 2001; Zhang et al. 2001a). Bile acids have been implicated in several gastrointestinal malignancies, in particular colon cancer and oesophageal adenocarcinoma. Even though some of the earliest steps of bile acid promotion have yet to be defined, an improved understanding exists of some of the signal transduction pathways involved (Zhang et al. 1998; Qiao et al. 2000), which potentially affect the cells' survival and proliferation. Abnormal intracellular signalling and altered transcriptional activity underlies the ability of bile acids to mediate aberrant morphogenesis of the gastrointestinal epithelium. Despite intensive investigation, the molecular events by which DCA controls the functions of intracellular signalling pathways remains poorly described, particularly in oesophageal cells. Therefore this thesis examined the responses of the PKC and MAPK signalling cascades as well as the transcription factor AP-1 to DCA stimulation. The regulatory mechanisms controlling the signaling pathways utilized by DCA were defined and may represent possible therapeutic targets and thereby providing a new approach to the treatment of gastrointestinal disease.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

Materials and Methods

2.1 CHEMICALS

Poly (dI-dC), T4 polynucleotide kinase and 10X Kinase buffer was purchased from Pharmacia (Biosystems, Milton Keynes, UK). [γ-^{32}P]dATP (35pmol, 3000Ci/mmol) and ECL detection kit were obtained from Amersham International (Aylesbury, UK). The MTT assay system used was from Promega Inc. (Madison, WI). Bovine Serum Albumin, ammonium persulphate, Acrylamide: Bisacrylamide (29:1), Nonidet P40 (NP-40), PMA, DCA, UDCA, CDCA, leupeptin, 2-mercaptoethanol, dimethylsulphoxide, ampicillin, kanamycin, EDTA, PMSF, Sodium dodecyl sulphate, TEMED, Tris (hydroxymethyl) aminomethane (Tris), dithiothreitol, Tween-20, streptavidin agarose beads and sodium orthovanadate were obtained from Sigma (Poole, Dorset, UK and St. Louis, MO., US). Tris buffer Saline (TBS) was from Pierce (Medical Supply Company, Dublin Ireland). Protease inhibitor cocktail was purchased from Roche Diagnostics (East Sussex, UK). Agarose was purchased from GIBCO BRL (Life Technologies, Paisley, Scotland). Sodium chloride, magnesium chloride, potassium chloride, ethanol, methanol, acetone, butanol, glycerol, acetic acid and boric acid were obtained from BDH Ltd. (Poole, UK). PD98059 (2'-amino-3'-methoxyflavone), SB203580 (4-[4'-fluorophenyl]-2[4'-methylsulfinylphenyl]-5-[4'-pyridyl] imidazole), U0126 (1,4-diamino-2, 3-dicyano-1, 4-bis [2-aminophenylthio] butadiene), Bisindoylmaleimide-4, GÖ6976 (12H-indolo(2,3-a)pyrrolo(3,4-c)carbazole-12-propanenitrile,5,6,7,13-tetrahydro-13-methyl-5-oxo[MESH]), sodium butyrate, phorbol 12,13-dibutyrate (PdBu), Z-VAD-FMK (Z-Val-Ala-Asp-CH_2F), Z-DEVD-FMK (Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK), and anisomycin were purchased from Calbiochem novabiochem corp. (LA Jolla, CA). Cell death detection plus Kit was obtained from Pierce (Perbio Science UK Ltd., Cheshire, UK). All buffer reagents for SDS-PAGE were prepared in deionised water (Elga Prima reverse osmosis).

2.2 ANTIBODIES AND OLIGONUCLEOTIDES

PKC Beta antibody was purchased from Seikagaku America (Rockville, MD). PKC-Delta and PKC-Epsilon were purchased from Transduction labs (Lexington, KY). Phospho-Erk1/2 (sc-7383), total-Erk1/2 (sc-4024), total-p-38 (sc-535), phospho-JNK (sc-6254), total-JNK

(sc-7345), Fra-1 (sc-605X), JunD (sc-74X), JUNB (sc-73X) and c-Jun (sc-45) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-p-38 (9211) was from Cell Signal (New England Biolabs, Hertfordshire, UK). An antibody to c-Fos (PC05L) was purchased from Oncogene research products (San Diego, CA). COX-2 antibody (160112) was from Cayman chemicals (Alexis Corp, UK). PARP antibody (44-698Z) was purchased from Biosource international (UK). AP-1 consensus oligonucleotide was purchased from Promega Inc. (Madison, WI). Biotinyalted AP-1 probe for affinity precipitation was synthesised by Sigma-Genosys (UK).

2.3 CELL CULTURE

2.3.1 Source of Cell Lines and Cell Culture Reagents

HCT116, a colonic epithelial cell line, derived from a colonic adenocarcinoma was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, UK). SKGT4 cells were established from a well-differentiated adenocarcinoma of Barrett's epithelium and were kindly provided by Dr. David Schrump. The oesophageal adenocarcinoma cell line, OE-33, and the gastric cancer cell line AGS were obtained from the ECACC (Porton Down, Salisbury, UK). RPMI 1640 medium, McCoy's 5A medium, foetal calf serum (FCS), Hank's balanced salt solution (HBSS) and trypsin-EDTA were obtained from GIBCOBRL (Life Technologies, Renfrewshire, Paisley, Scotland). Penicillin/Streptomycin/L-glutamine prepared aliquots were purchased from Sigma (Poole, Dorset, UK).

2.3.2 Maintenance of Cell Cultures

Cell lines were maintained in complete media at 37°C in a humidified atmosphere containing 5% CO_2. The media was supplemented with 10% FCS, 4mM L-Glutamine, 50 units/ml Penicillin and 50μg/ml streptomycin. Supplemented medium was stored at 4°C. A stock solution of the antibiotics was prepared [Appendix 1] and stored in aliquots at –20°C until use. FCS was heat inactivated (56°C, 1 hr) to inactivate complement and then aliquoted for storage at –20°C. Cells were routinely maintained in 75 cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) at 37°C in 5% CO_2 in a humidified incubator. Cells were passaged upon reaching 80% confluence. For passage, the spent medium was aspirated and the cells were washed twice with HBSS medium containing hepes buffer [Appendix A]. Cells were then detached from the culture flasks by trypsinisation and

centrifuged (1500 rpm, 5 min). Cells were resuspended in complete media for enumeration or seeding.

2.3.3 Cell Freezing and Revival

A cryopreservation solution was prepared containing 90% FCS and 10% DMSO. Cells were harvested as in section 2.3.2. The supernatant was decanted and cells at a cell density of 2 x 10^6 cells/ml were resuspended in ice-cold cryopreservative medium. 1 ml of cell suspension was aliquoted into sterile cryovials (Nunclon, Roskilde, Denmark). The cryovials were then transferred to liquid nitrogen for long-term storage. For cell revival, cryovials removed from storage were quickly thawed in a 37°C water bath. Cells were washed with pre-warmed RPMI media. Gentle centrifugation (1000 rpm, 3 min) was used to pellet the cells which were subsequently resuspended in 10 ml of RPMI supplemented with 20% FCS and transferred to a 25 cm^2 tissue culture flask and placed at 37°C, 5% CO_2 in a humidified incubator.

2.3.4 Cell Enumeration and Viability

To assess cell yield and viability, Ethidium bromide/acridine orange (0.1% w/v) (EB/AO) (Lee et al. 1975) was used. Cells were counted on a Neubauer haemocytometer under ultraviolet (UV) light (Hudson and Hay 1976). Under UV excitation, live cells appear green and non-viable cells stain orange. Enumerations were performed in duplicate and cells were routinely resuspended at a density of 2 x 10^5 cells/ml and used when less than 90% confluent.

2.3.5 Coculture of Cells with Bile Acids and Other Stimuli

HCT116 cells were removed from the cell culture flasks by trypsinisation, counted (Section 2.3.4) and seeded in 8-well chamber slides (Nunclon, Roskilde, Denmark) at a density of 1 x 10^5 cells/ml. 24 hr post transfection (Section 2.6.4) spent media was replaced with fresh media. Cells were incubated with 1 μM PMA and 300 μM DCA (diluted in pre warmed media) at different time points. For sodium butyrate experiments, cells were pretreated with 4 mM sodium butyrate for 50 min and then DCA was added for 1 hour in the presence of sodium butyrate.

SKGT4 cells and OE-33 cells were detached from the tissue flasks by trypsinisation (Section 2.3.2) and seeded in 6-well plates (Nunclon, Roskilde, Denmark) at a density of 2

x 10^5 cells/ml and 5 x 10^5 cells/ml respectively. Spent media was replaced with 1.5ml of prewarmed RPMI media and incubated for 30 min at 37°C. For inhibitor studies, PD98059, SB203580, BIS and GÖ6976 were added to the culture media and incubated for 30 min prior to addition of DCA. SKGT4 and OE-33 cells were also treated with CDCA and UDCA. SKGT4 cells were treated with PdBu and anisomycin. For every experiment, cells were incubated with culture media alone and experiments were preformed in triplicate. Representative results are shown.

2.3.6 MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Promega Inc., Madison, WI.) was employed to assess the effects of DCA on cell numbers. SKGT4 cells (2.5 x 10^4) were plated in a flat-bottomed micro-titre plate and incubated for 24 hr at 37°C and 5% CO_2. Cells were incubated either with increasing concentrations of DCA (0 - 500 μM) or over a period of 1-24 hr with 300 μM DCA. Control wells containing medium alone were also included. 20 μl of MTT solution was then added to the wells and further incubated for between 1 - 3 hr. Following incubation, absorbance was measured at 490 nm using an ELISA plate reader (Labsystems Multiskanplus, Type 314, Finland). The optical density of the control well was subtracted from the average of the test results (n = 3), and viability was calculated for the dose response and time course of DCA.

2.3.7 Apoptosis Assay

DCA induced toxicity was quantified by cell death detection kit from Roche diagnostics (Penzberg, Germany) according to the manufacturer's standard protocols. Briefly SKGT4 cells were seeded at 2.5 x 10^5 cells/ml and stimulated either with increasing concentrations of DCA (0 - 500 μM) or over a period of 0 - 6hr with 300 μM DCA. Following stimulations, ice-cold PBS is added to the cells and the plate is centrifuged at 2000 rpm for 10mins. The spent medium was removed and 1 ml of lysis buffer was added to each well and incubated for 1 hr with mild agitation at room temperature. The plate was centrifuged and 20 μl of supernatant was added to the pre-coated wells. Control wells contained 20 μl of incubation buffer. 80 μl of immunoreagent was added to each well and incubated for 2 hr with gentle shaking (approx 200 rpm) at room temperature. Wells were washed 3 times with incubation buffer and then 100 μl of ABTS (2,2'-azino-di[3-ethylbenz-thiazoline sulfonate-6-diammonium salt]) substrate was added to each well and the colour was

allowed to develop (2 - 20 min). Absorbance was measured at 405 nm using an ELISA plate reader.

2.4 CELL PROTEIN PREPARATION

2.4.1 Total Cell Lysates

HCT116 cells were lysed in 500 µl of buffer C [Appendix A] and sonicated for 30 sec. The cells were then boiled for 5 min and centrifuged at 12,000 rpm for 10 min to remove unlysed cells and nuclei. The supernatant was designated the total cell lysate.

To obtain oesophageal cell lysates; cells were lysed 200 µl of lysis buffer [Appendix A] for 20 min on ice. Cell nuclei and debris were eliminated by centrifugation for 5 min at 14,000 rpm.

2.4.2 Cell Fractionation

For subcellular fractionation HCT116 cells were lysed in ice-cold hypotonic buffer [Appendix A] and sonicated for 1 min on ice and centrifuged at 600 X g for 5 min at 4°C. Supernatant was centrifuged at 100,000 X g for 10 min and the supernatant was designated the cytosolic fraction. The pellet was resuspended in buffer B [Appendix A] and centrifuged at 100,000 X g for 30 min at 4°C and the supernatant was the membrane fraction.

2.4.3 Determination of Protein Concentration

Where indicated, the protein content in the cell lysates was standardised using the BioRad protein assay according to manufacturer's instructions. Briefly, 5-10 µl of cell lysate were mixed with 1 ml of BioRad staining solution (500-0006, diluted 1:5) and incubated for 5 min at room temperature before its OD_{595} was measured. Protein content was calculated against a bovine serum albumin standard curve. Volumes were adjusted with lysis buffer and equal protein content per sample was then used for affinity precipitation with biotinylated oligonucleotides, EMSA or acetone precipitation.

2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

2.5.1 Sample and Molecular Weight Standard Preparation

Following protein estimation, samples were generally aliquoted to yield 50 µg of protein. For protein concentration, this volume was diluted 1:5 with ice-cold acetone and incubated

at –20°C for at least 30 min. The samples were then centrifuged at 12,000 rpm for 5 min and the supernatant was aspirated. Any remaining acetone was allowed to evaporate at room temperature for approximately 15 min. Precipitated protein (50 μg) was resuspended in 5 to 20 μl 1X reducing sample buffer as required [Appendix A]. Protein samples and appropriate molecular weight standards were boiled at 100°C for 5 min and centrifuged briefly (30 s) to remove any insoluble solids.

2.5.2 SDS-PAGE

An ATTO system was used for all SDS-PAGE gels (ATTO Corporation, Japan) and protein samples were electrophoresed using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels prepared using a discontinuous buffer system as described by (Laemmli 1970) and adapted by (Sambrook and Gething 1989). Resolving and stacking acrylamide gels were prepared to the required percentage acrylamide in the order indicated in **Tables 1 and 2**. APS and TEMED were added last with gentle swirling of the mixture. Electrophoresis was carried out at 25 mA per gel for approximately 1.5 h until the dye front had reached just above the gel base, at which stage electrophoresis was discontinued.

Component	10%	12.5%	15%	20%
Acryl/Bis 30%	6.66 ml	8.32 ml	10 ml	13.32 ml
Tris 1.5M	5.0 ml	5.0 ml	5.0 ml	5.0 ml
H_2O	8.32 ml	6.56 ml	4.89 ml	2.78 ml
APS 10%	100 μl	100 μl	100 μl	100 μl
TEMED	10 μl	10 μl	10 μl	10 μl

TABLE 1. Composition of resolving gels for SDS-PAGE.

Component	Amount
Acryl/Bis 30%	1.33 ml
Tris 1.0M	3.05 ml
H_2O	5.55 ml
APS 10%	50 μl
TEMED	10 μl

TABLE 2. Composition of stacking gel for SDS-PAGE.

2.5.3 Western Immunoblotting

Western immunoblotting was carried out using the semi-dry method for the transfer of electrophoresed proteins to immobilising membranes as described by (Towbin et al. 1979) and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies, Japan). During SDS-PAGE, polyvinylidene fluoride (PVDF) transfer membrane (0.45 μm, Pall Life Sciences) was briefly saturated with methanol for 10 – 15 s and then equilibrated in transfer buffer for approximately 20 – 30 min prior to semi-dry transfer. Whatmann 3mm filter paper of similar dimensions was also saturated in transfer buffer prior to semi-dry blot sandwich construction, which was assembled in the order of cathode; filter paper, PVDF, acrylamide gel, filter paper and finally anode. Electrophoretic transfer was performed at 100 mA per gel for 60 min. Following semi-dry transfer; PVDF membrane was removed and processed for immunoblotting. The lane containing the molecular weight marker was stained with Coomassie Blue R-250 and destained in 50% methanol

2.5.4 Immunodetection and Development

Following transfer, non-specific sites on the membrane were blocked with freshly prepared 5% non-fat skimmed milk (Blocking solution) [Appendix A] for 1 h with gentle agitation at room temperature. Blots were then washed twice with PBS and incubated with appropriate primary antibodies diluted (range 1:200 to 1:1000) in primary and secondary antibody diluent solution [Appendix A] for 1 h shaking in a sealed plastic bag. Following incubation with primary antibody, blots were washed several times over 30 min with 0.1% PBS-Tween washing solution [Appendix A]. Blots were then incubated with the relevant horseradish peroxidase-conjugated secondary antibody in Primary and Secondary Antibody Diluent Solution for 1 h with shaking in a sealed plastic bag. Following incubation with secondary antibody, blots were finally washed 4 to 5 times with 0.1% PBS-Tween Washing Solution over 30 min. prior to development, blots were placed in PBS. Blots were stored in PBS at 4°C if development could not be undertaken immediately. Detection of immunoblots was performed using the enhanced chemiluminescence (ECL) method. Membranes were incubated for 1 min in a solution of iodophenol (400 μM), luminol (1.25 mM), and hydrogen peroxide (0.1% (v/v)) in 0.1 M Tris-HCl (pH 8.8) [Appendix 1]. The membrane was removed after 1 min and placed between acetate sheets, which were then exposed to Kodak X-OMAT S film for the appropriate time period (range 30 s to 30 min). Exposed films were developed using an

automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.6 TRANSIENT TRANSFECTION ASSAY

2.6.1 DNA Constructs

The plasmids PKC-ε-GFP, PKC-δ-GFP and pEGFP-N1, encoding rat PKC-ε cDNA, rat PKC-δ cDNA and humanised GFP cDNA, were kindly provided by Dr. Naoaki Saito. The PKC-β_1-GFP plasmid, containing 6.7 Kb PKC-β cDNA, was purchased from Clontech (Palo Alto, CA).

2.6.2 Transformation of Bacterial Cells

Competent *E. Coli* DH5α cells were transformed with plasmid DNA by incubating 100 µl of competent cells with between 5 – 100 ng of plasmid DNA on ice for 30 min. The cells were heat shocked for 1 - 2 min at 42°C. Cells were cooled on ice for 1 - 2 min and 1 ml of warm broth (LB) medium [Appendix A] was added. Cells were allowed to recover for 2 hr at 37°C with gentle agitation (50 rpm). Transformed cells were plated onto selective LB agar plates and left for 12 - 24 hours at 37°C. Single colony purification was performed where a single colony was selected and grown on selective LB agar plates for 12 - 24 hours at 37°C. This was performed three times.

2.6.3 Preparation and Purification of Plasmid DNA from *E. Coli* DH5α

A starter culture of 10 ml of LB Broth was inoculated with pure colonies and incubated for 12 - 16 hr at 37°C under ampicillin or kanamycin antibiotic selection. Stock solutions were made by adding 930 µl of growing culture to 70 µl of DMSO and stored in sterile cryovials at -70°C. 500 µl of starter culture was added to 40 ml of LB media with antibiotics and incubated for 12 - 16hr at 37°C. Plasmid DNA was purified from *E. Coli* DH5α using the Sigma GeneElute kit (Poole, Dorset, UK). Plasmid DNA concentration was calculated based on the OD_{280}/OD_{260} ratio and verified from a 0.8% agarose gel.

2.6.4 Transient Transfection

HCT116 cells (1 x 10^5 cells/well) were cultured on 8 well permanox glass chamber slides (Nunc, Naperville, IL) for 24hr until grown to 50-70% confluence. Cells were transfected with 1 µg of DNA using the Gene Porter transfection reagent (Gene Therapy Systems, San

Diego, CA) according to the manufacturer's standard protocol. Briefly 1 μg of DNA was diluted in serum free media using half the transfection volume (100μl). The Gene Porter reagent was diluted in serum free media in the other half of the transfection volume. The volume of Gene Porter required for transfection of the different GFP plasmids is outlined in **Table 3**. An equal volume of diluted DNA was added to an equal volume of diluted Gene Porter reagent and mixed by pipetting up and down 5 times. The DNA/Gene Porter mixture was incubated for 15 - 45 min at room temperature. The culture media was aspirated from the cells and the DNA/Gene Porter mixture was added and incubated for 3-5 hr at 37°C. 200 μl of cell culture media with 20% FCS was added to each well and the cells were allowed to recover overnight at 37°C in 5% CO_2. All experiments were performed 24 hr after transfection with transfection efficiencies of greater than 75%.

pEGFP	PKC-β_1-GFP	PKC-ε-GFP	PKC-δ-GFP
1 μg DNA	1 μg DNA	1 μg DNA	1 μg DNA
4 μl Geneporter	4 μl Geneporter	3 μl Geneporter	4 μl Geneporter

TABLE 3. Optimised Volumes of DNA and Gene Porter for Transfection of GFP Plasmids.

2.6.5 Visualisation of PKC-GFP Translocation by Digital Microscopy

HCT116 cells expressing pEGFP, PKC-β_1, -ε and -δ-GFP were washed with CO_2 independent medium (GIBCO BRL Grand Island, NY) pre-warmed to 37°C. Translocation was observed following the addition of either 1 μM PMA or 300 μM DCA. To analyse the effect of sodium butyrate on PKC translocation, HCT116 cells transfected with either EGFP (Empty Vector), PKC-β_1-GFP, PKC-ε-GFP or PKC-δ-GFP were pretreated with 4 mM sodium butyrate for 50 min. Following stimulation the cells were washed and then stimulated with 300 μM DCA in the presence of 4 mM sodium butyrate. All experiments were performed at 37°C in CO_2 independent media. For the real-time imaging of the PKC translocation, a Nikon Diaphot TE300 (Nikon Europe, Badhoevedorp, The Netherlands) inverted microscope equipped with a cooled CCD camera (Photometrics, Tucson, AZ) and fluorescence attachment with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) optimised filter cubes was used initially. Sequential images were

obtained under the Qwin-standard acquisition program. 800 millisecond exposure intervals were routinely used in all experiments. Dynamic PKC intracellular localisation was subsequently verified using the Live Cell Imager confocal microscopy work station (Perkin Elmer).

2.7 IMMUNOFLUORESCENCE ANALYSIS

HCT116 transfected cells were treated with 1 μM PMA and 300 μM DCA for appropriate periods of time as determined by real time translocation experiments. Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were washed twice with PBS and stained for 20 min at room temperature with a TRITC conjugate of phalloidin (Sigma) to observe the filamentous actin (F-Actin). After washing twice with PBS, the cells were permeabilised with 0.1% Triton X-100 at 4°C for 24 hr and stained with a mAb to α-tubulin (Sigma) and a 7-amino-4-methyl coumarin-3-acetic acid (AMCA) labelled secondary affinity purified Ab (Dako, Bucks, UK). Analysis of cell immunofluorescence on the slides and microphotography were performed on the previously described microscope using a 100x oil immersion lens and equipped with Leica DC-100 colour digital camera.

2.8 ELECTROPHORETIC MOBILITY SHIFT ASSAY

2.8.1 Preparation of Nuclear Fractions

Nuclear extracts were prepared as described by Osborn *et al.* (1989). Cells seeded in 6-well cell culture plates (1.5 ml volume) were treated as described earlier (section 2.3.5). Experiments were terminated by removal of media and washing the cells twice with ice-cold PBS [Appendix A]. All subsequent steps were performed on ice with ice-cold buffers, which were prepared fresh. All centrifugation steps were preformed at 4°C. Cells were scraped and centrifuged at 1400 rpm for 5 min and resuspended in 1 ml hypotonic Buffer A [Appendix A]. Following centrifugation (10,000 rpm, 10 min), the pellet was resuspended in 20 μl Buffer A containing 0.1% (v/v) NP-40 and incubated on ice for 10 min for cellular lysis. Samples were centrifuged at 10,000 rpm for 10 min. The pellets were resuspended in 15 μl Buffer C [Appendix A] and incubated on ice for 20 min prior to centrifugation (10,000 rpm, 10 min). The supernatant was retained and diluted with 75 μl of Buffer D [Appendix A]. Extracts were stored for up to six months at –70°C until required.

2.8.2 Consensus Oligonucleotide Labelling

5'-end labelling of the 21 base pair oligonucleotide containing AP-1 (*c-jun*) consensus sequence (5'- CGC TTG ATG AGT CAG CCG GAA-3') (Promega Corp., WI, U.S.) was performed according to the protocol of (Sambrook and Gething 1989). The reaction mixture was prepared in a sterile eppendorf tube in a final volume of 50 µl (**Table 4**).

Component	Amount	Final Volume
5'-ends of DNA probe (1-50 pmol)	5.7 µl	
T4 polynucleotide kinase (10-20 U)	2.5 µl	
10X Kinase Buffer	5.0 µl	50 µl
[γ-^{32}P] ATP (3000 Ci/mmol, 10 mCi/ml, 50 pmol)	15.0 µl	
Sterile water	21.8 µl	

TABLE 4. Consensus oligonucleotide labelling reaction components.

The reaction mixture was incubated in a pre-warmed Perspex box for 10 min at 37°C and the reaction was ended by the addition of 2 µl of 0.5 M EDTA. To the reaction mixture, 50 µl of phenol: chloroform solution (1 part TE-saturated phenol and 1 part chloroform: isoamyl alcohol, (24:1 ratio) was added to extract the DNA. The reaction mixture was vortexed for 1 min and centrifuged at 13,000 g for 2 min. The upper aqueous layer was transferred to a fresh tube and 2 µl of 5 M NaCl was added. The tube was vortexed and 100 µl of ethanol was added followed by incubation at –70°C for 30 min to precipitate the DNA. Following centrifugation at 13,000 g for 5 min, the supernatant was removed and the pellet dried in a vacuum dryer. Finally, the pellet was resuspended in 50 µl TE buffer [Appendix A]. To calculate the activity of the labelled oligonucleotide, 1 µl of this solution was diluted in 5 ml Ecosint and counted using a Wallac 1409 DSA Liquid Scintillation Counter. 10,000 cpm of labelled oligonucleotide was used per EMSA.

2.8.3 EMSA DNA-Protein Binding Reaction

The binding reaction was prepared for detection of AP-1 activity by electrophoresis as described by Sen and Baltimore (1986). Nuclear extracts (2 - 4 µg protein) were incubated with 10,000 cpm of ^{32}P-labelled AP-1 consensus oligonucleotide. The assay was performed in a 20 µl binding reaction volume in the presence of binding buffer [Appendix

A] and 2 μg poly (dI-dC) as non-specific competitor as per **Table 5**. The reaction mixture was incubated for 30 min at room temperature. The binding reaction was terminated by the addition of one-tenth volume of gel loading dye [Appendix A]. The samples were then ready for electrophoresis.

Component	Amount
Nuclear Extract	2 - 4 μg
10 X Binding Reaction Buffer	2 μl
[γ-^{32}P] ATP labelled probe	1 μl
Poly (dI-dC)	2 μl
Sterile water (to a final volume of 20 μl)	X μl

TABLE 5. DNA-Protein Binding Reaction.

2.8.4 Gel/Buffer Preparation and DNA-Protein Complex Electrophoresis

Nuclear proteins were separated on 5% polyacrylamide non-reducing 1-mm thick gels using the ATTO gel system (ATTO, Medical Supply Corporation, Japan). **Table 6** indicates the composition of the gel. Distilled water, Accugel (29:1) (National Diagnostics, Kimberly Research, GA, US) and 0.5 X TBE running buffer [Appendix A] were mixed carefully. Nuclease free ammonium persulphate and TEMED were added last and the gels were allowed to polymerise for 30 - 45 min at room temperature.

Component	Amount
Acrylamide Mix (40%)	3.125 ml
10X TBE Buffer	2.5 ml
Ammonium persulphate	50 mg
Distilled water	19.4 ml
TEMED	15 μl
DTT	5 μl

TABLE 6. EMSA Gel Solution Components.

The polymerised gels were pre-electrophoresed in 0.5 X TBE running buffer [Appendix A] at 80 V for 30 min. After sample loading, the gels were electrophoresed (Consort EUSS Unit) in the same buffer at 150 V for 2 - 3 hr or until the dye front had migrated a sufficient distance. At this stage gels were transferred to filter paper, covered with cling-film and dried on an automatic gel-drying unit. The filter paper was subsequently exposed to Kodak X-OMAT S film for 24 - 48 h in a film cassette with intensifying screens at –70°C. Exposed Kodak X-OMAT S film was developed using a Curix 60 developer (AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.8.5 Positive Controls For AP-1 Activation

Positive controls for AP-1 activation were included in all EMSA experiments performed and were obtained as follows. Serum starved AGS cells were exposed to PMA (50 ng/ml) (Sigma, Dorset, U.K.) for a period of 3 h. This compound is known to induce AP-1 activation at a concentration of 50 ng/ml (Naumann et al. 1999). Nuclear extracts were prepared as described in Section 2.8.1 and the Bradford assay was used to estimate the protein concentration of the extract. 4 μg of this nuclear extract was run in parallel with experimental samples in which the DNA-binding activity of AP-1 was being examined by EMSA.

2.8.6 Supershift Assay

Supershift assay was performed in order to identify the components of the AP-1 complexes induced and secondly to ensure their specificity. 2.0 μl of rabbit polyclonal antibodies to specific AP-1 subunits (anti-c-Fos, anti-Fra-1, anti-c-Jun and anti-JunD) were incubated with nuclear extracts for 30 min at room prior to the binding reaction.

2.9 AFFINITY PRECIPITATION OF DNA BINDING PROTEINS

2.9.1 Oligonucleotide Annealing

Affinity precipitation of DNA binding proteins was performed with the following oligonucleotide sequence: the AP-1 consenus sequence (5'- CGC TTG ATG AGT CAG CCG GAA-3'). Oligonucleotides of the 3' and 5' complementary strands where synthesised by sigma genosys UK. 5' strand contained a biotin moiety at the 5' end. To obtain double stranded oligonucleotides, the 3' and 5'-biotin strands were resuspended to 1 μg/ μl with TE and equal volumes were mixed. The mixture was incubated at 95°C in a

water bath for 10 min and left to slowly cool to room temperature. Double stranded oligonucleotides were diluted to 0.1 μg/ μl with TE/NaCl and stored at 4°C.

2.9.2 Affinity Purification with Biotinylated Oligonucleotides

Cells were stimulated and lysed in high salt lysis buffer [Appendix A]. Centrifuging for 5 min at 14,000 rpm eliminated cell nuclei and debris. Lysates were diluted with salt free lysis buffer [Appendix A] to 15 mM NaCl and pre-cleared with 30 μl streptavidin agarose beads, while rotating, for 15 min at 4°C. Pre-cleared lysates were incubated with 1 μg of corresponding double stranded oligonucleotide and 30 μl streptavidin agarose beads for 2 hours at 4°C with continuous rotation. Beads containing the oligonucleotide bound proteins were washed three times with cold lysis buffer and re-suspended in reducing sample buffer. All spins were at 6,500 rpm for 5 minutes.

2.10 DENSITOMETRY

The intensity of the bands obtained by Western blotting was electronically determined by densitometry using the Kodak 1D software on scanned Western blotting films. The band corresponding to the negative control or inactivated state was given the arbitrary value of 1 and relative fold inhibition or induction was calculated thereafter.

2.11 DATA ANALYSIS

Experiment were conducted a minimum of three times and the data was analysed after calculation of the mean and the standard deviation (SD) of each experiment, using a student unpaired T-test and the results are expressed as mean ± SD.

CHAPTER III

EFFECTS OF THE BILE ACID DEOXYCHOLATE ON THE PKC ISOFORMS β_1, ε AND δ

CHAPTER III

3.1 INTRODUCTION

3.1.1 Deoxycholate and Sodium Butyrate in Colon Cancer

Colorectal cancer is the third most common cause of cancer deaths in developed countries (Key et al. 2002). Bile acids, in particular deoxycholic acid (DCA), have been consistently associated with colorectal cancer risk. These bile acids are normal constituents of the gastrointestinal tract. They are synthesised in the liver as primary bile acids and are converted in the colon by enteric bacteria to secondary bile acids, of which DCA is the most potent (Armstrong and Doll 1975; Debruyne et al. 2001). DCA is thought to act as a tumour promoter by altering intracellular signalling and gene expression as demonstrated by Martinez and co-workers. Bile acid induction of AP-1 and COX-2 was shown to be mediated by activation of PKC (Qiao et al. 2000; Glinghammar and Rafter 2001), which is a family of serine/threonine kinases that play a fundamental role in a variety of cellular processes such as signal transduction leading to cell growth and differentiation (Nishizuka 1986; Blobe et al. 1996). Given that DCA levels in the large intestine may be modulated by dietary factors, it is conceivable that high fat/low fibre diets associated with colorectal cancer might impact on PKC activation over a sustainable period of time. Other dietary factors such as sodium butyrate, generated in the intestine from anaerobic fermentation of dietary fibre, may also be important in this context. Sodium butyrate has been shown to suppress proliferation (Kim et al. 1980; Gamet et al. 1992; McBain et al. 1996) and induce differentiation (Gamet et al. 1992) in many colonic cell lines. Treatment of colonic cancer cells with sodium butyrate results in increased alkaline phosphatase activity as well as the induction of several markers of differentiation such as lactate dehydrogenase (Rickard et al. 1999). *In vitro* experimental studies revealed that alterations in the PKC status in colonic cells can augment a range of butyrate dependent cellular processes (Rickard et al. 1999).

3.1.2 PKC and Colon Cancer

PKC is a major target for diacylglycerol (DAG), which is produced following agonist stimulated phospholipid metabolism (Hug and Sarre 1993; Toker 1998) and serves as a receptor to the tumour promoting phorbol esters such as PMA. At least 12 isoforms of PKC have been identified to date and are classified into three categories based on their

structure and their requirements for Ca^{2+} and DAG for activation. PKC isoforms have been implicated in the homeostasis of the colonic epithelium including β-catenin stability (Orford et al. 1997) as well as in malignant transformation through alterations in isoform activity (Levy et al. 1993; Doi et al. 1994). Decreased protein expression and/or mRNA abundance of various PKC isoforms, in particular PKC-β_I, has widely been reported in human colorectal tumours (Levy et al. 1993; Doi et al. 1994). Furthermore decreased PKC levels are also observed following prolonged activation with PMA (Darbon et al. 1986b; Freire-Moar et al. 1991). Reduced PKC-β_I expression appears to be an early event in the pathogenesis of colon cancer as it is seen in human colonic adenomas (Doi et al. 1994). PKC-β_I seems to be preferentially lost in comparison to the novel isoforms such as PKC-ε and PKC-δ, which were used for comparison purposes. Another classical PKC isoform, PKC-α, appears to play an important role in colon cancer progression and has been linked with integrin activation, tumour cell migration and loss of E-cadherin expression (Chakrabarty and Huang 1996; Masur et al. 2001). Colonic cells resistant to DCA-induced apoptosis have reduced PKC-β_I expression (Crowley-Weber et al. 2002), which therefore suggests a functional consequence for reduction in PKC-β_I expression. In addition, cells resistant to DCA-induced apoptosis constitutively overexpress PI3-K (Bernstein et al. 2004). PI3-K and the Ras signal transduction pathways has been shown to be involved in PKC-ε mediated colonic cell transformation (Perletti et al. 1996; Perletti et al. 1998; Marras et al. 2001). Bile acid stimulated invasion of colonic cells was dependent on Rac1 and RhoA GTPases (Debruyne et al. 2002). On the other hand, overexpression of PKC-δ in a Src transformed cells results in growth suppression and reversed the transformed phenotype (Perletti et al. 1999). Therefore, PKC-ε and PKC-δ were included in this study to examine the effects of DCA on two functionally different PKC isoforms, which have important consequences in colon morphogenesis.

Given that the colonic epithelium is physiologically exposed to luminal contents such as DCA and sodium butyrate and that PKC signalling elements regulate many of their biological effects, we explored the hypothesis that DCA might induce PKC-β_I translocation, an isoenzyme that is preferentially lost at the adenocarcinoma stage. A hallmark of PKC activation has been the translocation of its isoforms from the cytosol to the plasma membrane (Mochly-Rosen et al. 1990) with persistent activation resulting in down regulation and loss of expression (Darbon et al. 1986b; Freire-Moar et al. 1991;

Strulovici et al. 1991). In this chapter, we examine the effects of DCA on PKC isoenzyme localisation and whether sodium butyrate can modulate those responses in a colonic cell line, HCT116. To date the involvement of the PKC isoforms in bile acid mediated events in colon cancer cells has been implied by the use of inhibitors specific for individual PKC isoforms (Glinghammar and Rafter 2001) or from PKC alterations detected in cancer tissue (Pongracz et al. 1995; Reddy et al. 1996). In this chapter, we use PKC-GFP constructs to directly monitor the effects of DCA and sodium butyrate on the PKC isoforms in real time.

3.2 OBJECTIVES

The aims of this chapter were to examine:

1. The expression of endogenous PKC isoforms in HCT116 cells.
2. The effects of PMA and DCA stimulation on subcellular localisation of the endogenously and exogenously expressed PKC isoforms.
3. The effects of sodium butyrate on PKC-GFP subcellular localisation.
4. The cytoskeletal element actin and tubulin in association with the PKC-GFP isoforms after PMA and DCA stimulation

3.3 RESULTS

3.3.1 HCT116 Express Endogenous PKC-ε And -δ But Not PKC-β_I

In order to elucidate whether PKC-β_I, -ε and -δ are endogenously expressed in HCT116 cells, Western blot analysis was performed on total cell lysates from untransfected cells and cells transfected with either PKC-β_I-GFP, PKC-ε-GFP or PKC-δ-GFP. As a control, HCT116 cells were also transfected with the pEGFP plasmid, which encodes only the green fluorescent protein. A monoclonal anti-PKC-β_I antibody recognises PKC-β_I-GFP as a band at 105kDa **(Fig 3.1A)**. HCT116 cells do not express endogenous PKC-β_I as no band of 78 kDa is seen **(Fig 3.1A)**. This was verified when lysates from HUT78 cell line and HCT116 cell lysate were probed for PKC-β_I expression. HUT78 cells endogenously express PKC-β_I (Volkov et al. 2001). Endogenous expression of PKC-β_I is only detected in HUT78 cells **(Fig 3.1B)**. Monoclonal antibodies against PKC-ε and PKC-δ detect endogenous expression of these isoforms in HCT116 cells **(Fig 3.1A)**. In addition, HCT116 cells transfected with either PKC-ε-GFP or PKC-δ-GFP express endogenous and fluorescently tagged proteins that are observed on Western blots as bands of the predicted higher molecular weight, 117 kDa and 105 kDa respectively **(Fig 3.1A)**. The appropriate

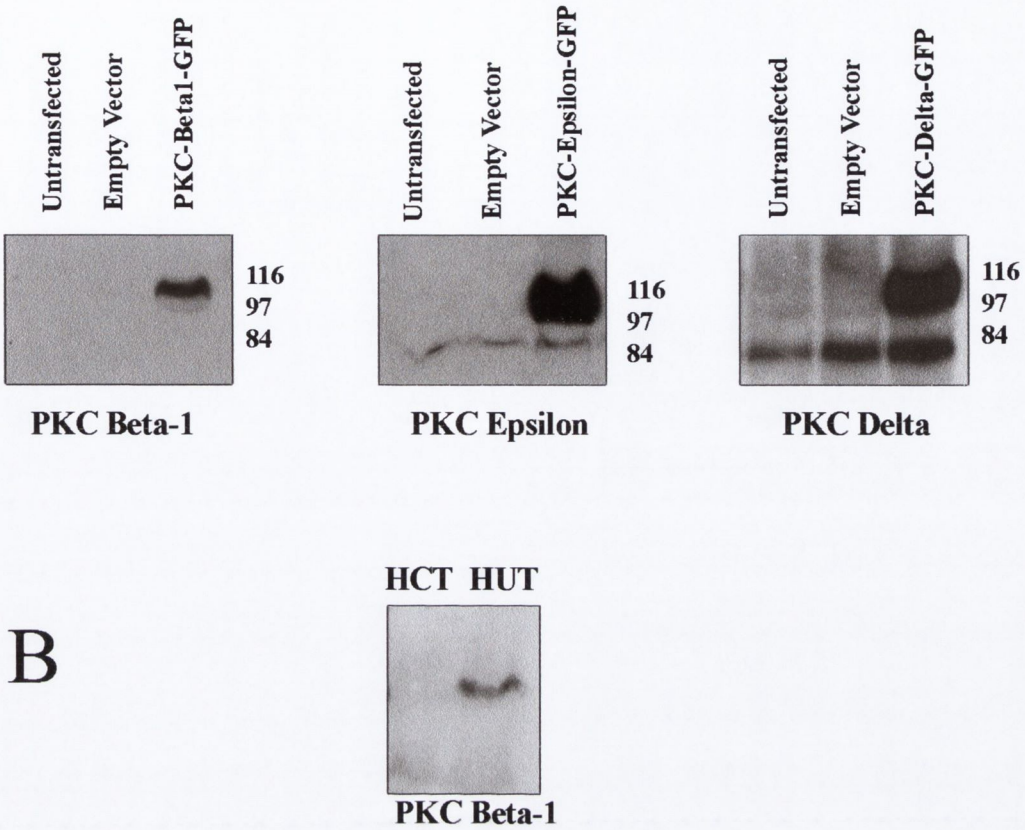


Fig 3.1. HCT116 Express Endogenous PKC-ε And -δ But Not PKC-β_1. (**A**) Antibodies specific to PKC-β_1,PKC-ε and PKC-δ were used to examine the PKC status of the HCT116 untransfected cells and cells transfected with pEGFP (empty vector), PKC-β_1-GFP, PKC-ε-GFP or PKC-δ-GFP. Total cell lysates were standardised to 50μg as described in chapter two and assessed by Western blotting using anti-PKC-β_1, anti-PKC-ε and ant-PKC-δ antibodies. Cells transfected with PKC-β_1-GFP express only the exogenous PKC-β_1 as a band of the predicted higher molecular weight, 105 kDa. HCT116 cells transfected with either PKC-ε-GFP or PKC-δ-GFP express endogenous protein, as bands of approximately 90 kDa and 78 kDa respectively, and fluorescently tagged proteins that are observed on Western blots as bands of the predicted higher molecular weight, 117 kDa and 105 kDa respectively (**B**) Endogenous PKC-β expression was assessed in HCT116 cells (HCT) and HUT78 cells (HUT), which served as a positive control. Total cell lysates were standardised to 50μg as described in chapter two and assessed by Western blotting using anti-PKC-β_1 antibody. Endogenous expression of PKC-β_1 is observed only in HUT78 cells. Blots are representative of more than three experiments.

expression of GFP and PKC-GFP is confirmed using an anti-GFP antibody. This detects a 27 kDa band corresponding to GFP in cells transfected with the pEGFP plasmid alone, a 105 kDa band in cells transfected either with PKC-β_I-GFP or PKC-δ-GFP and a 117 kDa band in cells transfected with PKC-ε-GFP **(Fig 3.2A)**. Likewise no catalytic fragments of native PKC or PKC-GFP were observed by Western blotting using antibodies specific to each isoform and GFP.

3.3.2 DCA Induces Endogenous PKC-ε But Not PKC-δ Translocation

The potential of the bile acid DCA to induce endogenous PKC translocation in HCT116 cells was investigated. As a control for PKC activation, these cells were stimulated with 1 μM PMA, which has previously been shown to induce translocation of PKC-ε and -δ in other cell types (Lin and Chen 1998; Wang et al. 1999; Weller et al. 1999). Subcellular fractionation clearly illustrates that treatment with PMA, for 1 hr, induces translocation of endogenous PKC-ε from the cytosol to the membrane **(Fig 3.2B)**. PMA treatment results in loss of cytosolic PKC-δ. Downregulation of PKC by PMA has been reported in other cell types (Tsutsumi et al. 1993; Rivero and Adunyah 1998). Treatment of HCT116 cells with 300 μM DCA induces endogenous PKC-ε but not PKC-δ membrane translocation demonstrating that the PKC isoforms are differentially activated by DCA **(Fig 3.2B)**. In addition, HCT116 cells express endogenous PKC-alpha (data not shown). Parallel studies conducted in our laboratory demonstrate that treatment of HCT116 cells with either 1μM PMA or 300 μM DCA induces endogenous PKC-α membrane translocation **(Fig 3.3)** (Shah Thesis in preparation). As PKC-ε and -δ are endogenously expressed and functionally distinct PKC isoforms, the response of these isoforms as well as PKC-$\beta_I$was assessed in all subsequent experiments.

3.3.3 DCA-Induced PKC-β_I-GFP Translocation is Dose Dependent

Since PKC-β_I expression is lost early in the adenoma-carcinoma sequence, PKC-β_I is exogenously expressed in HCT116 cells to elucidate the effects of DCA on PKC-β_I expression. PKC-β_I-GFP fluorescence is observed predominantly in the cytosol. Initially, PKC-β_I-GFP transfected cells were treated with varying concentrations of DCA ranging from 0 – 500 μM. Low concentrations of DCA such as 100 - 200 μM does not induce PKC-β_I-GFP translocation. DCA, at concentrations at and above 300 μM, induces PKC-β_I-GFP plasma membrane directed translocation **(Fig 3.4A)**. However high concentrations

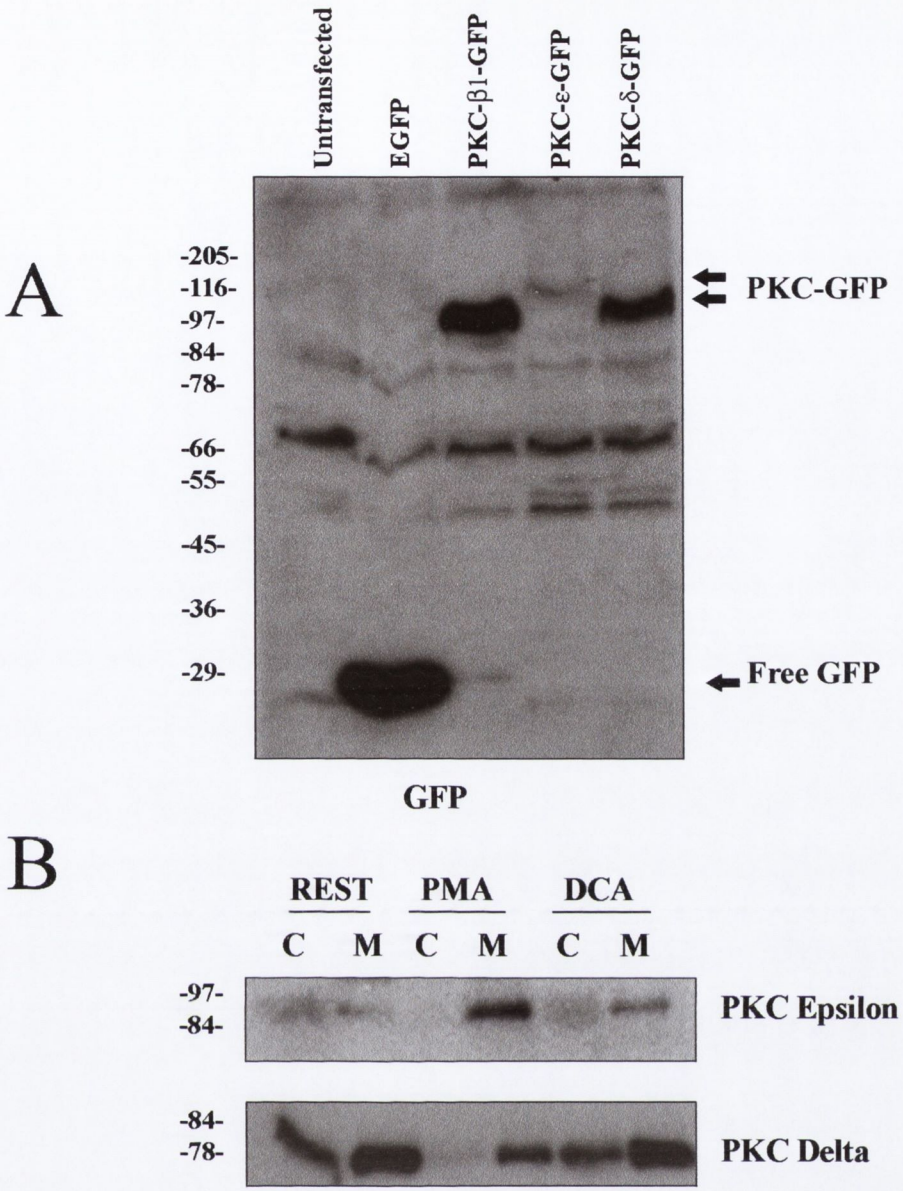


Fig 3.2. DCA Induces Endogenous PKC-ε Translocation but Not PKC-δ Translocation (**A**) HCT116 cells untransfected or cells transfected with pEGFP, PKC-β_1-GFP, PKC-ε-GFP and PKC-δ-GFP were lysed and the correct expression of PKC-GFP was verified by Western blotting using a polyclonal antibody specific for GFP. PKC-ε-GFP band is comparatively weaker to that of PKC-β_1-GFP and PKC-δ-GFP due to fractionally lower transfection efficiencies for PKC-ε-GFP. (**B**) Untransfected HCT116 cells were treated with 1 μM PMA and 300 μM for 1 hr. Cytosolic (c) and membrane (m) fractions were obtained as described in chapter two and were resolved by SDS-PAGE. Immunoblot analysis was performed using antibodies specific to PKC-ε and PKC-δ. Blots are representative of more than three experiments.

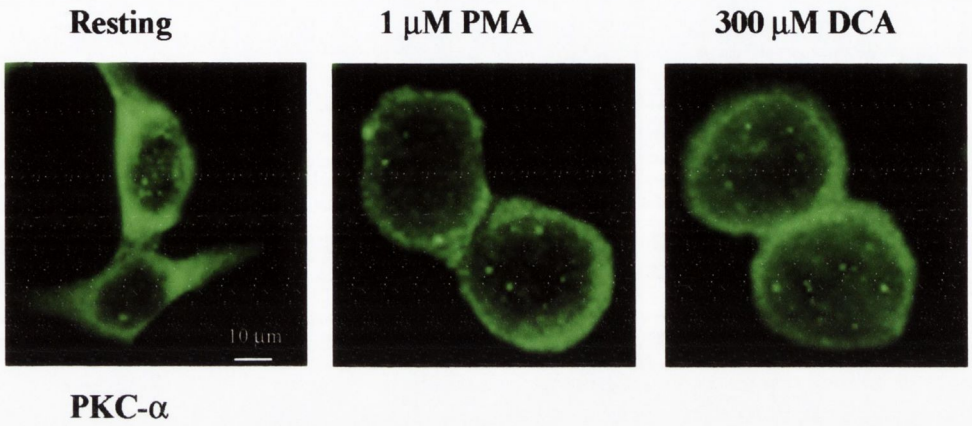


Fig 3.3. DCA Induces Endogenous PKC-α Translocation. The effect of DCA on other endogenously expressed PKC isoforms such as PKC-α was examined to determine whether DCA-induced PKC-ε translocation was specific event. Untransfected HCT116 cells were treated with 1 μM PMA or 300 μM for 1 hr. Cells were fixed and stained for PKC-α using an antibody specific for PKC-α. Scale bar represents 10 μm. Results are representative of more than three experiments. Adapted from Shah 2004 (thesis in preparation).

of DCA in particular 500 μM are associated with dramatic morphological changes indicative of apoptosis such as membrane blebbing and were deemed unsuitable for these studies **(Fig 3.4B)**. From this point on, 300 μM DCA was used for all subsequent studies unless otherwise stated. PMA (1 μM) induces rapid translocation of PKC-β_I-GFP from the cytosol to the plasma membrane in 10 min **(Fig 3.5)**. Similarly, treatment with 300 μM DCA, induces plasma membrane directed translocation in 15 min **(Fig 3.5)**. The fluorescence remains at the membrane even 3 hr after treatment with both PMA and DCA **(Fig 3.4B)**.

3.3.4 DCA and PMA Induce PKC-ε-GFP Translocation

PKC-ε-GFP is observed predominantly in the cytoplasm and in association with membrane ruffles. Stimulation with PMA induces rapid translocation of PKC-ε-GFP to the plasma membrane within 10 min **(Fig 3.5)**. DCA elicits a similar response and induces plasma membrane translocation in 15 min **(Fig 3.5)**. Translocation induced by the bile acid DCA is somewhat slower than that of PMA. Following prolonged stimulation, 30 min, the cells acquire a rounded phenotype (data not shown).

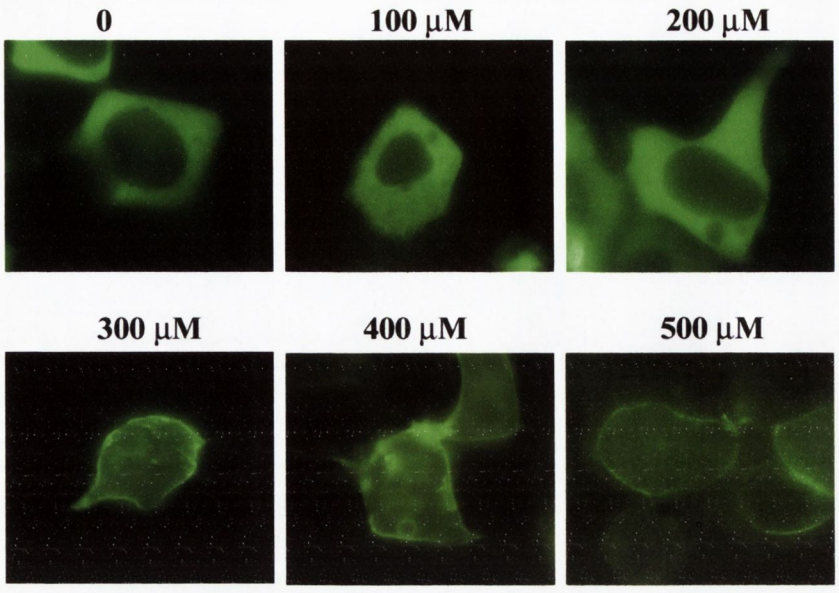
3.3.5 DCA Does Not Induce PKC-δ-GFP Translocation

PMA-induced translocation of PKC-δ-GFP requires a longer time frame. Translocation of PKC-δ-GFP to the plasma membrane and perinuclear region after PMA treatment is complete after 1 hr **(Fig 3.5)**. DCA does not induce translocation of PKC-δ-GFP within a 1 hr time frame as with PKC-β_I-GFP and PKC-ε-GFP. Following prolonged treatment with DCA (18 hr), PKC-δ-GFP resides predominantly at the plasma membrane with some localised at the cytoplasm. This is also associated with apoptotic changes and therefore cannot be attributed to a DCA specific effect (data not shown).

3.3.6 DCA and PMA Do Not Induce EGFP Translocation

Control HCT116 cells, transfected with pEGFP were treated either with 1 μM PMA or 300 μM DCA for identical periods of time. GFP localisation does not change upon treatment and retains a uniform fluorescence distribution pattern throughout the nucleus and cytoplasm **(Fig 3.5)**. The translocation patterns observed are not restricted to a subset of the population but occur in the majority of the cells **(Fig 3.6)** and are therefore not an artefact of transfection.

A



B

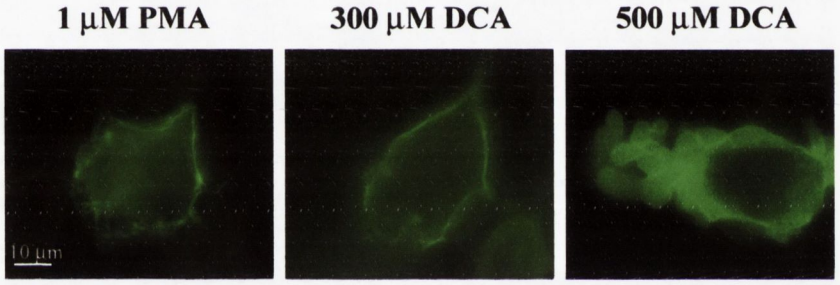


Fig 3.4. DCA-Induced PKC-β_1-GFP Translocation is Dose Dependent. (A) HCT116 cells transfected with PKC-β_1-GFP were treated with different concentrations of DCA ranging from 0 – 500 μM for 30 min and the PKC-GFP translocation patterns were monitored by time lapse microscopy (**B**) PKC-β_1-GFP transfected cells were treated with 1 μM PMA, 300 μM DCA and 500 μM DCA for 3 hr and PKC-GFP distribution was monitored by a Nikon Diaphot TE300 (Nikon Europe, Netherlands) inverted microscope equipped with a cooled CCD camera (Photometrics, Tucson, AZ) and fluorescence attachment.

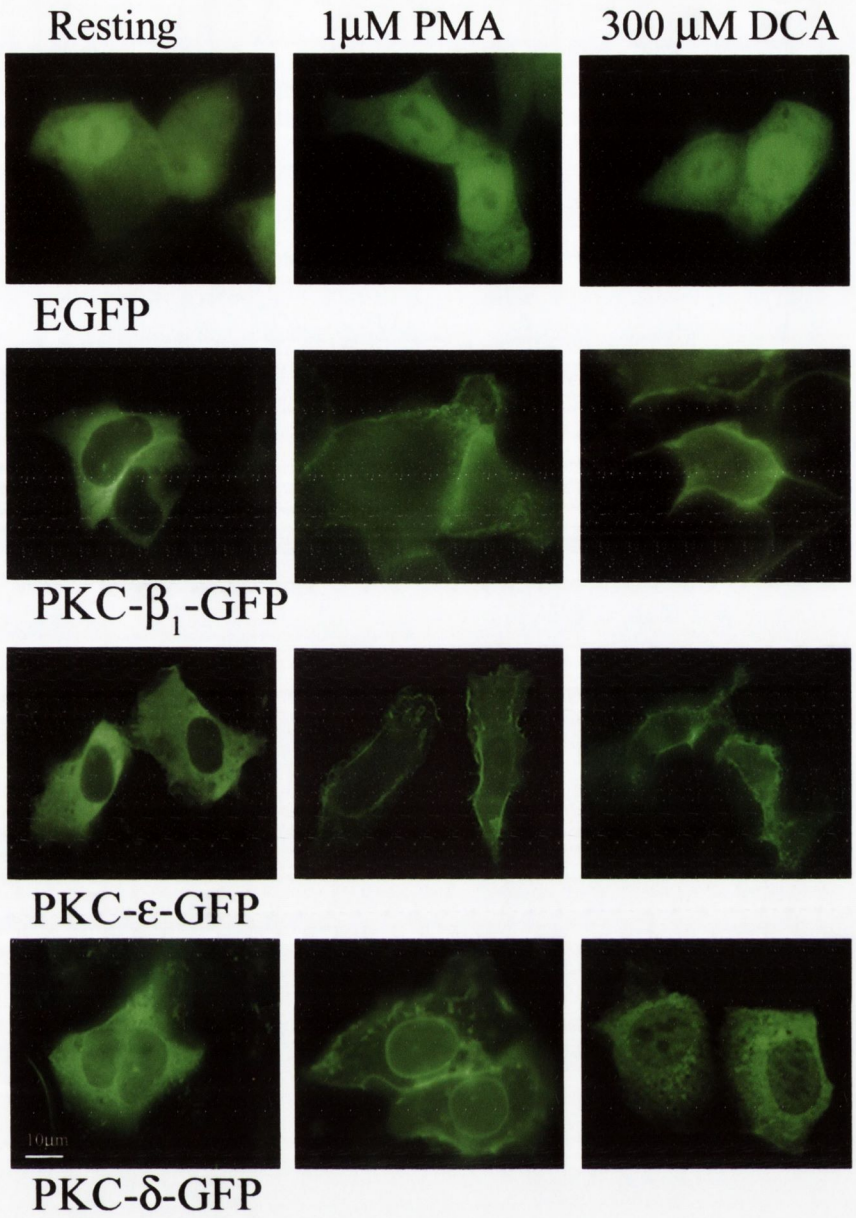


Fig 3.5. DCA Induces PKC-β_1-GFP, PKC-ε-GFP but PKC-δ-GFP Translocation. Left column: resting (non-activated cells), middle column: PMA treated cells, right column: DCA treated cells. Individual PKC isoforms are indicated under each row. Cells transfected with pEGFP, PKC-β_1-GFP, PKC-ε-GFP and PKC-δ-GFP were treated with 1 μM PMA and 300 μM DCA for 15 min. The results shown here and on the subsequent figures are representative for >5 independent experiments, all of which gave consistent results. Scale bar represents 10 μm on all figures unless otherwise stated.

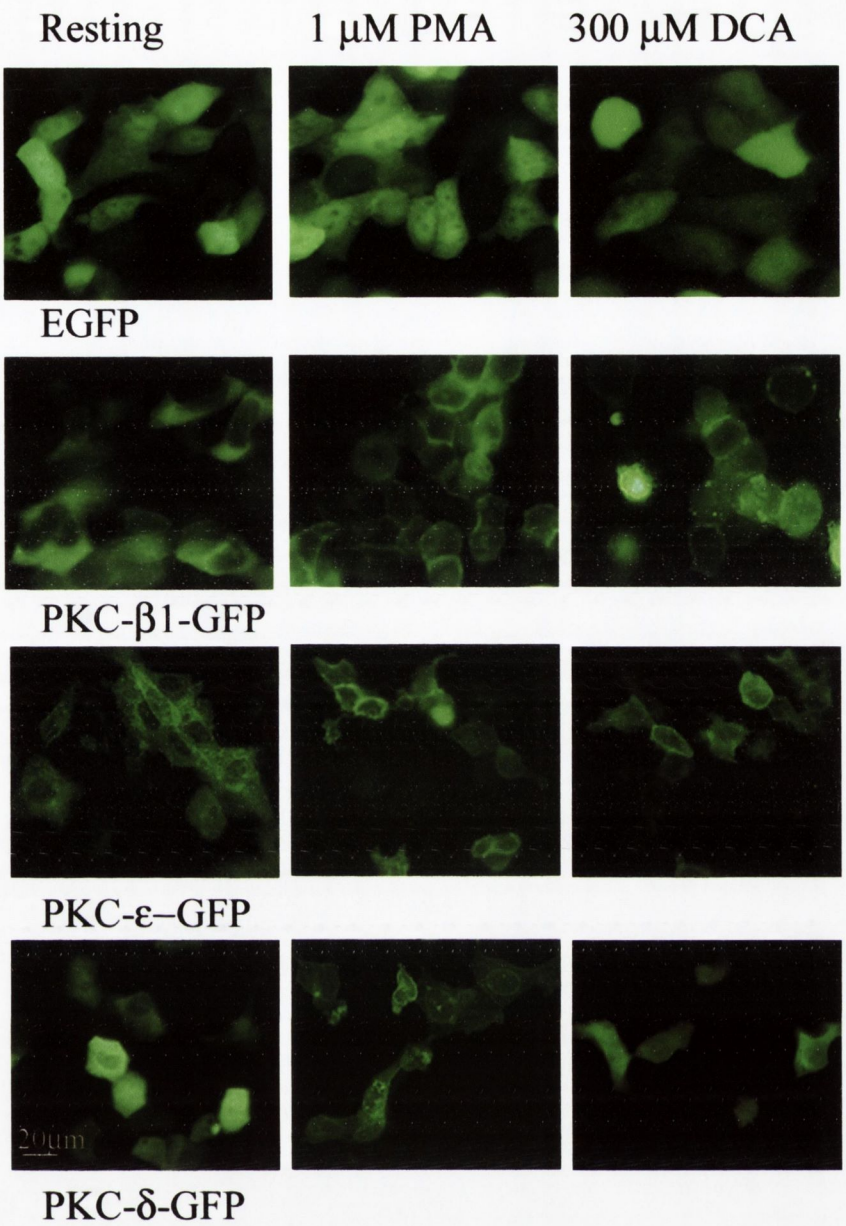


Fig 3.6. PMA and DCA induce PKC Isoform Translocation in large cell populations. Left column: resting (non-activated cells), middle column: PMA treated cells, right column: DCA treated cells. Cells transfected with pEGFP, PKC-β_1-GFP, PKC-ε-GFP and PKC-δ-GFP were treated with 1 μM PMA and 300 μM DCA for 15 min Individual PKC isoforms are indicated under each panel. Micrographs were taken at 40x magnification. Scale bar represents 20μm.

3.3.7 Sodium Butyrate Does Not Modulate PKC Translocation in Response to DCA

Sodium butyrate, which is present in the gastrointestinal tract at millimolar concentrations (Cummings 1981; Hill 1995), can modulate the colonic cell proliferation and differentiation (Hague et al. 1995; Glinghammar et al. 1999; Coradini et al. 2000). Therefore we examined whether sodium butyrate could modulate the responses of the PKC isoforms to DCA activation. Cells were pretreated for 50 min with 4 mM sodium butyrate. Sodium butyrate alone does not induce activation or translocation of the PKC isoforms to the plasma membrane as observed with DCA treatment **(Fig 3.7 left column, 0 min)**. However DCA, in the presence of sodium butyrate, induced plasma membrane translocation of PKC-β_1-GFP and PKC-ε-GFP but does not induce PKC-δ-GFP translocation in 15 min **(Fig 3.7 middle column)**. PKC-δ-GFP fluorescence remains unaltered even 1 hour after treatment with DCA in the presence of sodium butyrate. In cells transfected with EGFP, the pattern of GFP localisation remains unaltered and is expressed homogeneously throughout the cell **(Fig 3.7 upper panel)**.

3.3.8 PKC-β_1, -ε and -δ Association with the Cytoskeletal Element Actin

In A7v5 vascular smooth muscle cells, PMA induced PKC activation resulting in actin reorganisation (Brandt et al. 2002). Following stimulation with PMA and DCA, rounding of cells is observed which has been reported in other cell types (Romanova et al. 1999). In HCT116 cells in the resting state, actin is distributed at the plasma membrane and in the stress fibres bundles. **(Fig 3.8, 3.9, 3.10, 3.11 upper panels)**. DCA treatment induces some reorganisation of the cytoskeletal element, F-actin, in cells transfected with the empty vector, EGFP. In addition, PMA induces F-Actin and microtubule reorganisation **(Fig 3.8)**. HCT116 cells transfected with either PKC-β_1-GFP or PKC-ε-GFP were treated with 1 μM PMA (as a control) and 300 μM DCA. Upon treatment with DCA, remodelling of the actin cytoskeleton is observed in HCT116 cells transfected with either PKC-β_1-GFP or PKC-ε-GFP **(Fig 3.9)**. Similar results are observed with the phorbol ester PMA **(Fig 3.9)**. In cells transfected with PKC-ε-GFP, stimulation with DCA or PMA results in attenuation of the actin stress fibres bundles and staining is condensed around the periphery of the cell **(Fig 3.10)**. In resting PKC-δ-GFP transfected cells, PKC-δ-GFP is associated with the actin cytoskeleton at the cell periphery. Nevertheless PMA-induced PKC-δ-GFP translocation results in a specific and consistent pattern of association of this PKC isoform with the actin

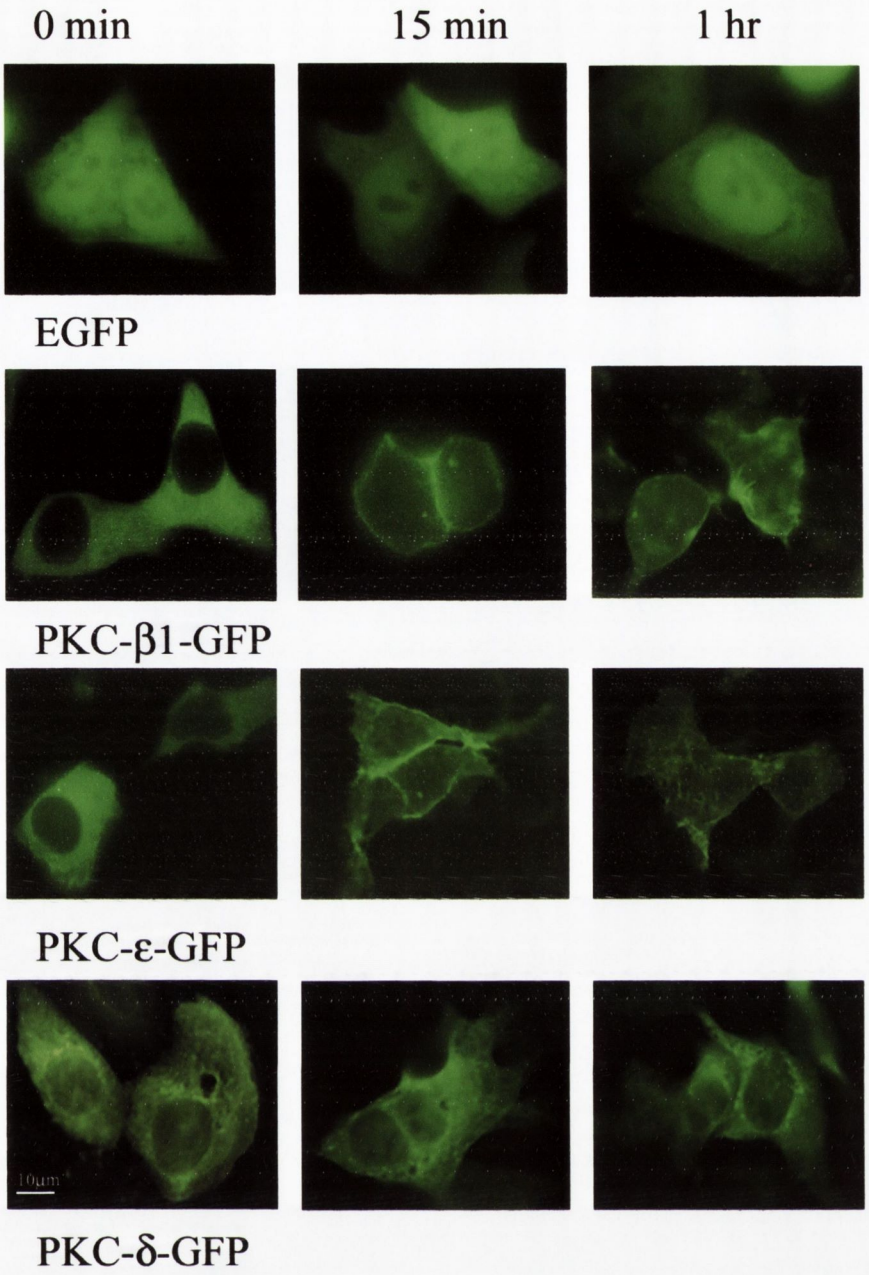


Fig 3.7. Sodium Butyrate Does Not Modulate PKC Translocation in Response to DCA. Left column: cells pretreated with sodium butyrate (no DCA), middle column: sodium butyrate pretreated cells with DCA after 15 min, right column: sodium butyrate pretreated cells with DCA after 1 hour. The PKC isoforms are indicated under each row. Following pre-treatment with 4mM sodium butyrate, HCT116 transfectants were stimulated with 300 μM DCA for up to one hour. All images are representative of >5 experiments.

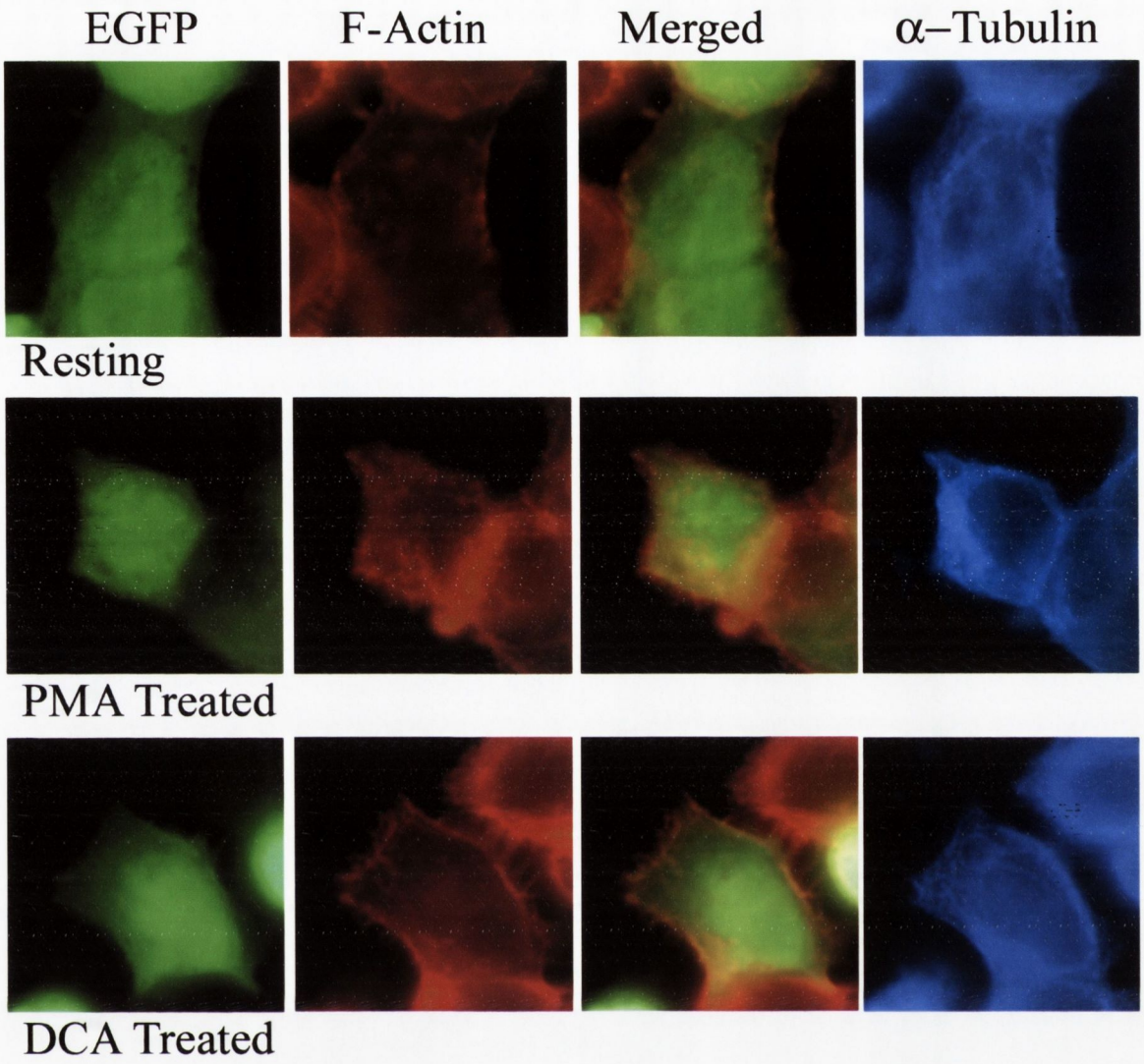


Fig 3.8. DCA and PMA Treated Free GFP Transfectants are Associated with Some Microfilament and Microtubule Cytoskeletal Rearrangement. F-actin (red) and α-tubulin (blue) were visualised in resting HCT116 cells and cells treated with either 1μM PMA or 300 μM DCA for 30 minutes. GFP expression pattern was merged with actin only. The microtubules are shown in a separate column due to over complications with the triple colour composite.

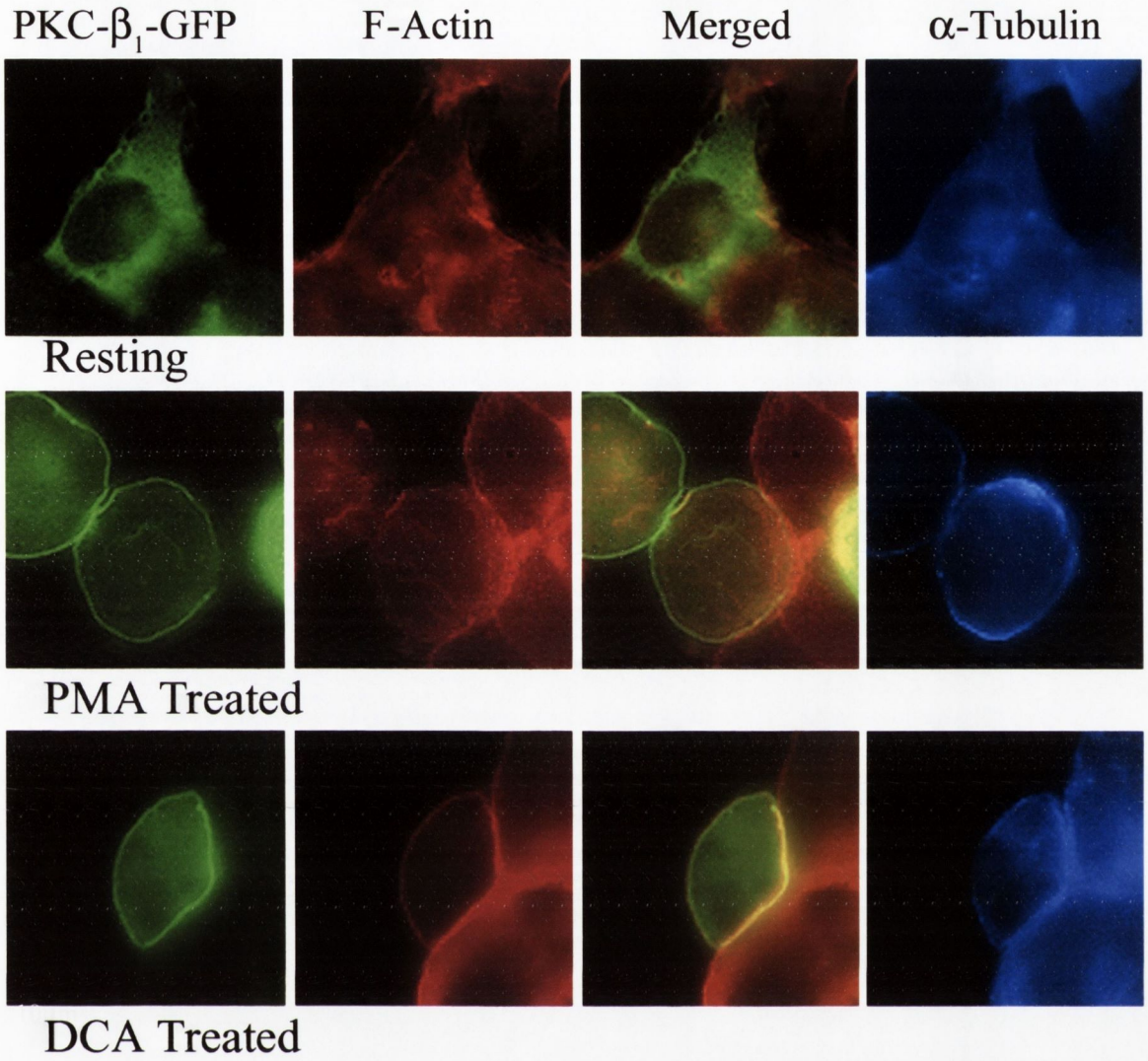


Fig 3.9. PKC-β_1-GFP is Associated with Cytoskeletal Systems in HCT116 Transfectants. Cells fixed after 30 min exposure to PMA and DCA, were stained for F-actin (red) and α-tubulin (blue) as outlined in chapter two. Yellow overlay regions in merged images reflect an association between PKC-β_1-GFP and actin microfilaments. Corresponding microscopic fields illustrating the reorganisation pattern of the microtubule cytoskeleton are given in a separate column (left column).

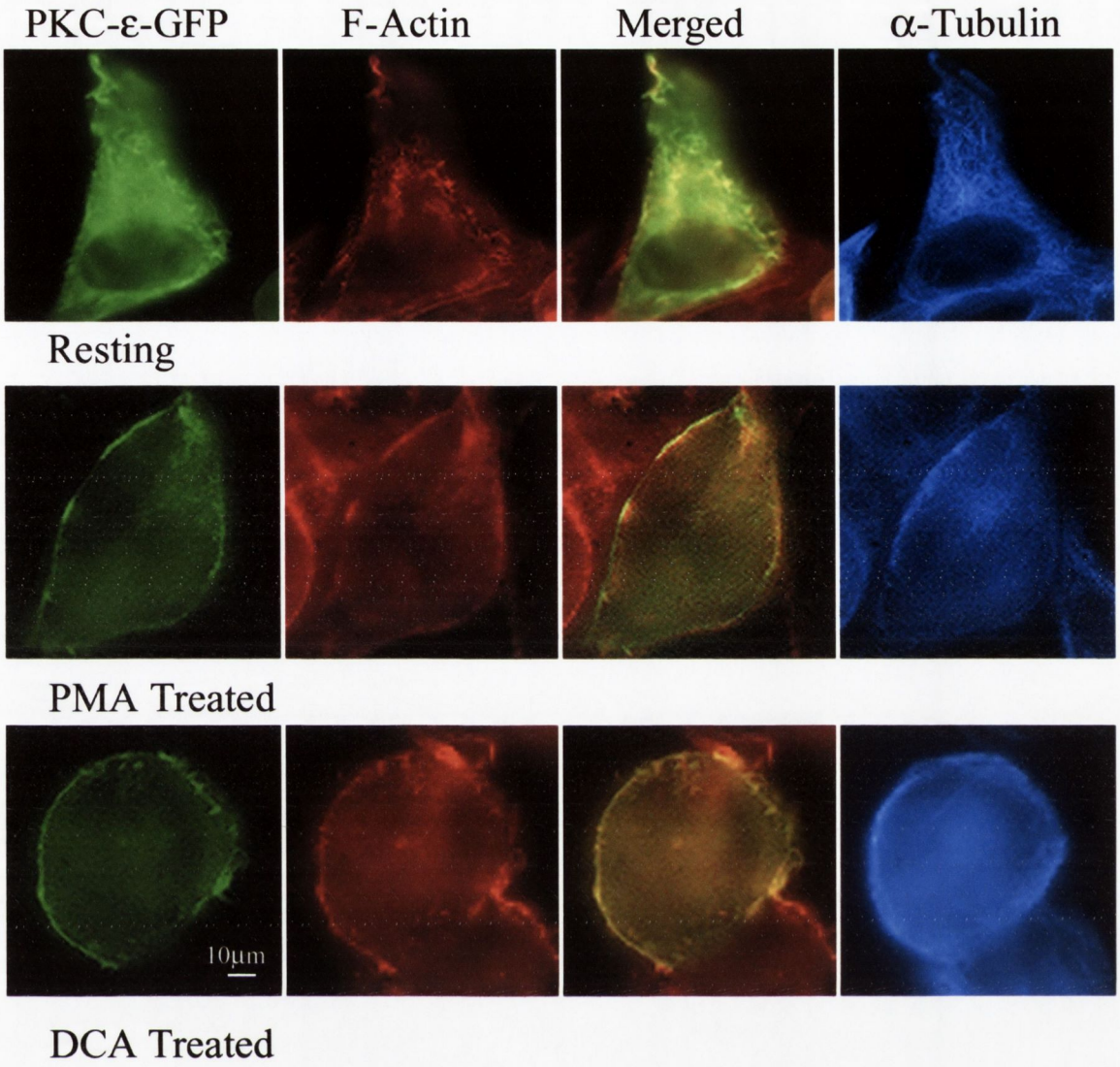


Fig 3.10. DCA and PMA-induced PKC-ε-GFP Translocation is Associated with Cytoskeletal Reorganisation. HCT116 cells transfected with PKC-ε-GFP were treated for 30 min with either 1 μM PMA or 300 μM DCA and stained for actin (red) and tubulin (blue) as outlined in chapter two. Equivalent experiments gave consistent results.

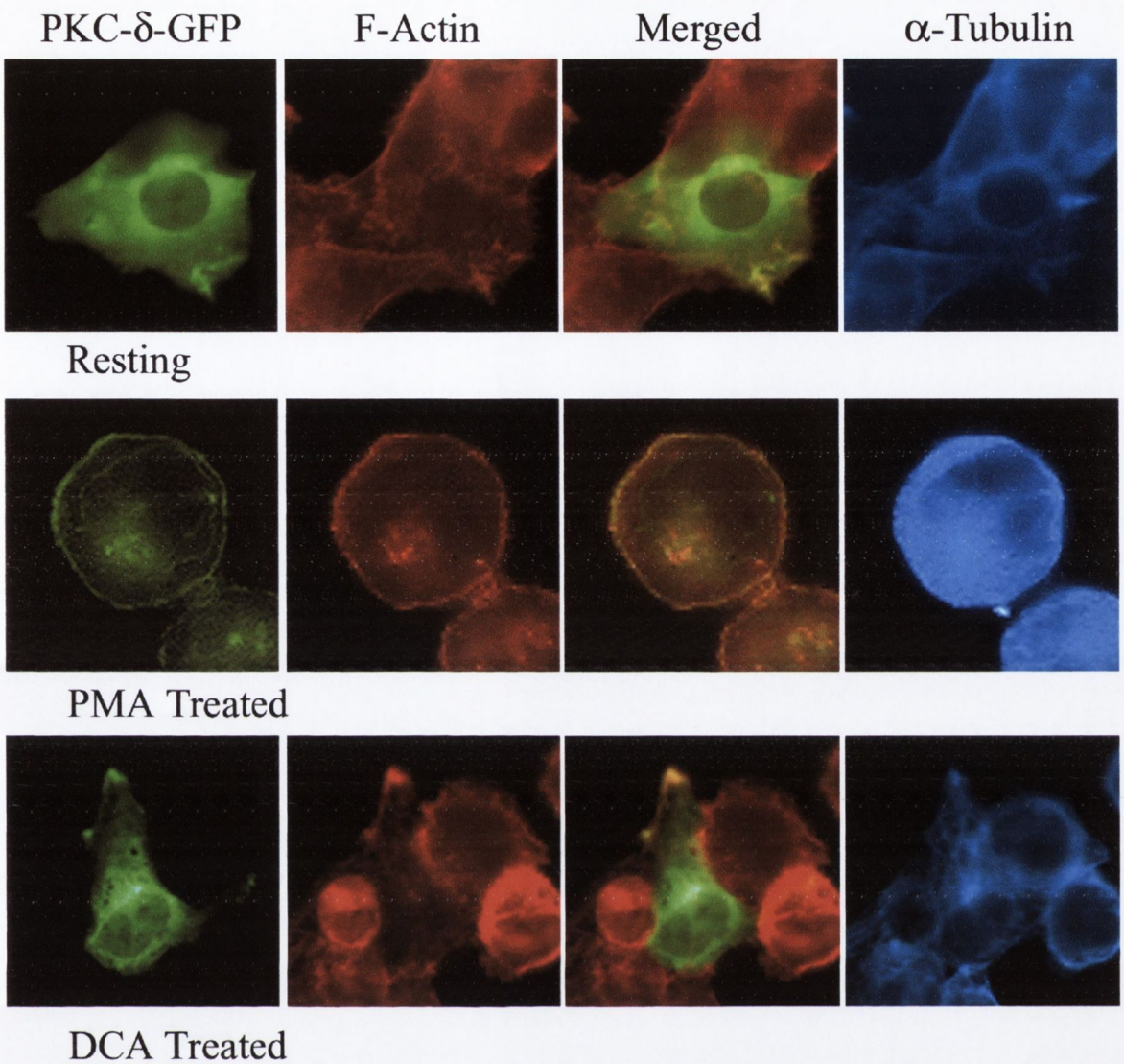


Fig 3.11. PKC-δ-GFP Transfectants treated with DCA Do Not Experience Dramatic Cytoskeletal Rearrangement. PKC-δ-GFP Transfectants were treated for 1 hr with 1 μM PMA and 300 μm DCA. Cells were fixed and stained for microfilament (red) and microtubules (blue) as outlined in chapter two. Yellow overlay regions in merged images reflect partial co-localisation of PKC-δ-GFP with the actin cytoskeletal system. Images are representative of more than three experiments.

cytoskeleton **(Fig 3.11, merged image)**. DCA does not induce a significant morphological change in PKC-δ-GFP transfectants in comparison with PMA. DCA stimulation does not result in disassembly of the actin stress fibres as observed with PMA treatment **(Fig 3.11)**. Some association of PKC-δ-GFP with actin microfilaments adjacent to the plasma membrane is observed after DCA treatment, which is similar to that in resting PKC-δ-GFP cells. Therefore, stimulation of PKC-δ-GFP with DCA does not induce a similar pattern of cytoskeletal rearrangement to that induced by the activation of PKC-β_I-GFP and PKC-ε-GFP by DCA **(Fig 3.11)**. Alterations to the cytoskeletal elements are associated with the rounding of cells following stimulation with DCA. This suggests that remodelling of F-Actin might be a major contributing factor to the altered the morphology of the DCA treated PKC-β_I-GFP and PKC-ε-GFP transfectants.

3.3.9 PKC-β_I, -ε and -δ Association with the Cytoskeletal Element Tubulin

Translocation of PKC isoforms involves extensive reorganisation of the actin cytoskeletal elements, however similar alterations in the microtubule network are observed after treatment with DCA. In the resting PKC-β_I-GFP and PKC-ε-GFP transfectants, the microtubules appear as an intricate network concentrated around the perinuclear region. Following DCA induced PKC-β_I-GFP and PKC-ε-GFP translocation, the microtubules undergo a dramatic redistribution to the periphery of the cell such that they appear to associate with cytoskeletal elements adjacent to the plasma membrane **(Fig 3.9, 3.10)**. Identical structural alterations in the microtubule network in both PKC-β_I-GFP and PKC-ε-GFP cells are observed upon treatment with PMA **(Fig 3.9, 3.10)**. In HCT116 PKC-δ-GFP transfectants, the microtubule network is substantially remodelled from a highly organised network to a more diffuse distribution following PMA treatment **(Fig 3.11)**. Upon DCA stimulation, negligible rearrangement of the microtubule networks is observed in cells transfected with PKC-δ-GFP.

3.4 DISCUSSION

Extensive research has focused on the ability of bile acids to affect multiple signalling pathways. The present study investigated the potential of the bile acid DCA to modulate the expression of PKC isoforms, PKC-β_I, -ε and -δ in colon cancer cells. To date the involvement of the PKC isoforms in bile acid mediated events has been implicated by the use of inhibitors specific for individual PKC isoforms (Glinghammar and Rafter 2001).

The involvement of the PKC isoforms was investigated in a human colonic adenocarcinoma cell line, HCT116. In vivo, during malignancy, a decrease in total PKC levels of expression and activity as compared to adjacent normal mucosa has been noted (Kopp et al. 1991; Doi et al. 1994). In particular, a decrease in the levels of PKC-β_I has been reported in human adenomas (Doi et al. 1994). Western blot analysis of the HCT116 cells shows no endogenous expression of PKC-β_I **(Fig 3.1A, B)**. This is in agreement with results published by other investigators (Hawcroft et al. 2002). Here we demonstrate that transfection of these cells with PKC-β_I-GFP construct restores expression of the PKC-β_I isoenzyme **(Fig 3.1A)**. Endogenous expression of PKC-ε and -δ as well as their GFP counterparts can also be detected in this cell line **(Fig 3.1A)**. No catalytic products of native or transfected PKCs are observed during Western blotting indicating the appropriate expression of both the native and the fluorescent PKC isoforms and preservation of their functional properties **(Fig 3.1A, 3.2A)**. This is in accordance with the findings of Saito and collaborators who have demonstrated that the fusion proteins have intracellular distribution and functional characteristics identical to those of the native PKCs (Ohmori et al. 1998; Shirai et al. 1998b). To test our hypothesis that DCA could modulate PKC expression and translocation, we incubated parental cells with DCA and preformed subcellular fractionation to examine the intracellular expression of PKC-ε and -δ. PMA, as a control for PKC activation, induces membrane translocation of PKC-ε and -δ **(Fig 3.2B),** which has been observed in other cell types (Ohmori et al. 1998; Shirai et al. 1998b). DCA induces translocation of endogenous PKC-ε from the cytosolic fraction to the membrane fraction **(Fig 3.2B)** but does not induce translocation of PKC-δ. Independent studies conducted in our laboratory also demonstrate that PMA and DCA can induce PKC-α membrane translocation **(Fig 3.3)** (Shah Thesis in preparation). DCA has the ability to selectively modulate the intracellular localisation of diverse PKC isoforms. As PKC-ε and -δ are endogenously expressed and functionally distinct isoforms as compared to PKC-β_I, these isoforms in addition to PKC-β_I were assessed in all subsequent experiments.

Advances in GFP technology have enabled the development of GFP-tagged PKCs. These GFP-tagged PKCs allow for direct visualisation of their translocation patterns in living cells in response to different physiological activators. Saito *et al.* have demonstrated unique patterns of PKC-ε-GFP and PKC-γ-GFP translocation in response to distinct fatty acids in CHO-K1 cells (Shirai et al. 1998b). In the same cell type, translocation and

activation of PKC-δ has been reported in response to PMA, ATP and Hydrogen peroxide (H_2O_2) stimulation (Ohmori et al. 1998). Similar fluorescently tagged PKC's were utilised in this study to monitor the dynamics of their translocation in response to treatment with PMA and DCA. The PKC-GFP fusion proteins exhibit a remarkably different pattern of distribution in comparison to the distribution of EGFP alone **(Fig 3.5, 3.6)**. Furthermore the EGFP localisation pattern is unaltered after treatment with either PMA or DCA, remaining uniformly distributed throughout the cytoplasm and the nucleus. PMA stimulates rapid translocation of PKC-β_1-GFP and PKC-ε-GFP from the cytosol to the plasma membrane in 10 min **(Fig 3.5, 3.6)**. In contrast, PKC-δ-GFP translocation in response to PMA evolves slowly, reaching completion after 1 hr **(Fig 3.5, 3.6 middle columns)**. PKC-β_1-GFP and PKC-ε-GFP fluorescence remains at a membrane location at this time point. PMA induces a sustained shift in cytoplasmic PKC-δ-GFP to the perinuclear region as well as the plasma membrane. In contrast, an equal concentration of PMA induces PKC-δ-GFP translocation to the same target sites after 5 min in CHO cells (Ohmori et al. 1998; Wang et al. 1999). These data correlate strongly with previous studies showing that the kinetics of PKC translocation to a given activator is both isoform specific and cell type specific (Sakai et al. 1997; Ohmori et al. 1998; Shirai et al. 1998b; Shirai et al. 1998a).

PKC-β and -ε respond to a physiologically relevant concentration of DCA, 300 μM (Stadler et al. 1988; Allinger et al. 1989), which is optimal for the induction of the transcription factor AP-1 in the same cell line (Qiao et al. 2000). Lower concentrations of DCA such as 100 – 200 μM do not induce PKC translocation, while concentrations at or above 300 μM do **(Fig 3.4A)**. However higher concentrations of DCA in particular 500 μM are associated with apoptotic changes 3 hr after stimulation and were deemed unsuitable for these studies **(Fig 3.4B)**. Similarly in the colonic adenoma cell line RG/C2, concentrations less than 100 μM DCA had no effect on cell growth. Nevertheless at 500 μM DCA significant induction of apoptosis is noted (Hague et al. 1995). DCA, similar to the PMA treatment, induces rapid translocation of PKC-β_1-GFP and PKC-ε-GFP from the cytosol to the plasma membrane in 15 min **(Fig 3.5, 3.6, right column)**. In contrast, DCA does not induce translocation of PKC-δ-GFP **(Fig 3.5, 3.6, right column)** but the isoform reside at the plasma membrane after 18 hr (data not shown). However apoptotic changes are also observed at 18 hr and therefore the PKC-δ-GFP membrane association may not be

a DCA specific effect. Worthy of note is that the endogenously expressed isoforms, PKC-ε and -δ, elicit similar responses to PMA and DCA as their GFP fusion counterparts **(Fig 3.2, 3.5)**, which demonstrates the specificity of the PKC-GFP fusion proteins responses.

These findings demonstrate clearly that DCA has the potential to differentially regulate diverse PKC isoforms subcellular localisation. The delayed response of PKC-δ-GFP to DCA may have more profound indirect effects in the long term such as apoptosis. The early activation of PKC-β_I and PKC-ε emphasises the importance of these two isoforms in early DCA mediated intracellular events. The rapid activation of PKC isoforms by DCA, the levels of which are elevated in colon cancer patients, may result in their subsequent down regulation and depletion from the colonic epithelium although the effects may be differential for each isoform. Down regulation of the PKC isoforms due to persistent activation by the tumour promoter PMA and other stimuli has been reported in several cell types (Tsutsumi et al. 1993; Rivero and Adunyah 1998). Interestingly results from a cDNA microarray study revealed that PKC-β_I mRNA is downregulated in HCT116 cells resistant to DCA-induced apoptosis (Crowley-Weber et al. 2002). Also in Ha-ras transformed colonic epithelial cells, PKC-ε expression is increased five fold. Overexpression of PKC-ε is associated with morphological changes such as anchorage independent colony formation and increased saturation densities (Perletti et al. 1996). In other cancers, prolonged activation of PKC-ε is associated with downregulation of PKC-ε and increased cell survival and clonal expansion (Knauf et al. 2003).

In light of the protective role sodium butyrate is thought to have in colon tumourgenesis, it was of crucial importance to investigate if sodium butyrate could modulate the responses of the PKC isoforms to DCA. McBain and colleagues speculate that activation of PKC by PMA might play a central role in determining the sensitivity of the colon cancer cells to sodium butyrate-induced apoptosis (McBain et al. 1996). The synergistic effect of PKC activation and sodium butyrate has been reported for other biological effects of sodium butyrate, such as enhanced differentiation, in other colonic cancer cell lines (McBain et al. 1997; Rickard et al. 1999). McMillan and colleagues demonstrated that, in AA/C1 colon cancer cells, butyrate-induced apoptosis is dependent upon PKC-δ activation and translocation to the membrane fraction (McMillan et al. 2003). In this study, sodium butyrate alone does not induce translocation of PKC-β_I-GFP, PKC-ε-GFP or PKC-δ-GFP

to any particular subcellular site **(Fig 3.7 left column, 0 min)**. However in the presence of sodium butyrate, DCA is uninhibited and continues to induce translocation of PKC-β-GFP and PKC-ε-GFP but not PKC-δ-GFP in 15 min **(Fig 3.7, middle column)**. Furthermore, no apoptotic changes are observed at these time points and even 1 hr after treatment. Stimulation with sodium butyrate does not modulate the response of the PKC isoforms to DCA, suggesting that DCA might circumvent the action of sodium butyrate, thus highlighting the critical importance of DCA in the pathogenesis of colorectal cancer.

Prior to stimulation by PMA or DCA, HCT116 cells transfected with PKC-β_I-GFP, PKC-ε-GFP or PKC-δ-GFP show no significant morphological differences to HCT116 cells untransfected or cells transfected with EGFP. Upon stimulation, some of the transfectants underwent dramatic morphological changes such as rounding, which is associated with reorganisation of cytoskeletal microfilaments. In NIH3T3 cells overexpressing PKC-γ, a PKC isoform found predominantly in neuronal tissue (Wetsel et al. 1992), PMA treatment causes PKC-γ overexpressers to rapidly round up. In the same cells, PMA stimulation of PKC-β_{II} causes pronounced ruffling at the plasma membrane and the cells flattened out (Goodnight et al. 1995). The co-ordinate translocation of the PKC isoforms to the membrane and the redistribution of the actin and microtubule networks also to the membrane suggest possible functional associations. In T84 intestinal epithelial cells, treatment with PMA is associated with the translocation of PKC-ε from the cytosol to the plasma membrane as well as remodelling of the actin cytoskeleton. This synchronized association facilitates basolateral endocytosis (Song et al. 1999). In the context of the present study, it is likely that remodelling of both cytoskeletal networks by DCA ensures the rapid trafficking of the PKC isoforms to the cell periphery and therefore in closer proximity to their intracellular targets. Previous work has demonstrated that PKC activation by the broad spectrum activator, PMA, induces reorganisation of the actin cytoskeleton, which is associated with alterations in the Rho (Ras homology) signaling pathway (Brandt et al. 2002). Members of the Rho family modulate all aspects of cell morphology and cell proliferation (Schmidt and Hall 1998; Etienne-Manneville and Hall 2002). Bile acid-stimulated invasion of colonic carcinoma cells is dependent upon the Rho pathway; specifically treatment of HCT-8/E11 colon cells with 250 μM DCA for 10 min leads to an 18 fold increase in the amount of active Rho-A and Rac-1 (Debruyne et al.

2002). It is possible that DCA associated cytoskeletal changes in HCT116 cells may involve alterations in the Rho GTPase signaling cascade.

Conversely, DCA does not induce significant reorganisation of either cytoskeletal element in PKC-δ-GFP transfectants as observed with PKC-β_I-GFP and PKC-ε-GFP **(Fig 3.11)**. PKC-δ-GFP expressing cells, after PMA treatment, exhibit a rounded phenotype, which is potentially due to the reorganisation of the actin cytoskeleton. In Baf3 cells, a murine pre-B lymphoid cell line, activation of PKC-δ by PMA results in the dissolution of actin based membrane ruffles (Romanova et al. 1999). Control HCT116 cells, containing EGFP, experience some reorganisation of the cytoskeletal systems in response to stimuli due to the endogenous expression of PKC-ε and PKC-δ in these cells, while HCT116 cells overexpressing these isoforms, PKC-ε-GFP and PKC-δ-GFP transfectants, undergo substantial cytoskeletal reorganisation.

In summary, the present study provides the first direct evidence that in living cells the bile acid DCA differentially activates the PKC isoforms. Alterations in the isoforms activity are likely to have an essential role in the development and maintenance of the abnormal morphogenesis of the colonic epithelium. The fact that a naturally occurring substance, DCA, might trigger PKC translocation in colon cancer cells is compelling evidence for the importance of DCA in this malignant process. As PKC changes are early and DCA levels can be modulated by dietary factors, these mechanisms are likely to be important in mediating the environmentally-induced component of colorectal carcinogenesis.

CHAPTER IV

EFFECTS OF THE BILE ACID DEOXYCHOLATE ON OESOPHAGEAL EPITHELIAL CELLS

CHAPTER IV

4.1 INTRODUCTION

4.1.1 Bile Acids and Oesophageal Cancer

Over the past two decades there has been a dramatic increase in the incidence of Barrett's oesophagus in humans, a premalignant lesion to oesophageal adenocarcinoma, which is associated with gastroesophageal reflux disease (GERD). Reflux of duodenal contents has been consistently associated with increased severity of oesophagitis and Barrett's oesophagus (Gillen et al. 1988; Kauer et al. 1995; Nehra et al. 1999). In addition the concentration of bile acids, in particular unconjugated bile acids, in the refluxate of patients with GERD correlates strongly with the degree of oesophageal mucosal damage (Nehra et al. 1998; Stein et al. 1998; Nehra et al. 1999). Compelling evidence for the involvement of bile acids in contributing to Barrett's oesophagus emerged from animal studies, where reflux of duodenal contents, of which bile acids are a major constituent, led to oesophageal inflammation and increased mucosal thickening (Zhang et al. 2001a). Moreover Barrett's patients on acid suppression therapy still progress to adenocarcinoma highlighting the importance of components other than acid in oesophageal cancer progression (Stein et al. 1998).

Experimental evidence of the contribution of bile acids in the malignant development of the oesophageal epithelium is now emerging. Organ culture experiments with oesophageal biopsies demonstrated that exposure to bile acids results in substantial upregulation of COX-2 expression, which was shown to be dependent on PKC activation (Kaur and Triadafilopoulos 2002). Increased COX-2 expression has been associated with acquisition of resistance to apoptosis and increased invasiveness (Tsujii and DuBois 1995; Tsujii et al. 1997; van der Woude et al. 2002). Furthermore bile acids induce cell proliferation in Barrett's oesophagus, which is mediated in part by PKC activation as judged by the inhibition of cell proliferation by the broad spectrum PKC inhibitor bisindolylmalemide (Kaur et al. 2000). Tselepis *et al* demonstrated that acidified bile induced c-myc expression in oesophageal cells, a protein that is upregulated in more than 50% of Barrett's metaplasia and 90 % of oesophageal adenocarcinomas (Jankowski et al. 1992; Tselepis et al. 2003).

4.1.2 DCA and Apoptosis

Bile acids are normal constituents of the gastrointestinal tract where they act as trophic factors for the gut epithelium and as detergents for the absorption of cholesterol and fat soluble vitamins (Holt 1972; Carlier et al. 1986). While bile acids such as DCA cannot induce tumours, they are generally believed to be tumour promoters. The exact mechanism of their tumour promoting activity is uncertain but it is thought to involve alterations in cellular signalling cascades and gene expression systems, which ultimately affect the biological responses of cells (Debruyne et al. 2001). Bile acids are known mediators of cellular stress (Bernstein et al. 1999b) and have been proposed to induce apoptosis resulting in compensatory hyperproliferation and providing selection for apoptosis resistant cells (Martinez et al. 1998; Bernstein et al. 2000; Qiao et al. 2001a). Furthermore bile acids are known to induce survival pathways in parallel to apoptotic pathways to account for their cytotoxicity (Rust et al. 2000; Washo-Stultz et al. 2002). *In vitro*, short term exposure of colonic cells to DCA induces apoptosis (Martinez et al. 1998) while prolonged exposure is associated with the acquisition of resistance and desensitisation to apoptosis (Bernstein et al. 2000; Crowley-Weber et al. 2002). Similarly *in vivo*, bile acid-induced apoptosis has been linked with compensatory proliferation of crypt epithelial cells (Lapre et al. 1992). In Barrett's epithelium, low rates of apoptosis have been demonstrated (Wetscher et al. 1998). Inhibition of apoptosis in Barrett's epithelium is likely to contribute to this premalignant condition. Decreased levels of apoptosis may be facilitated by overexpression of anti-apoptotic proteins such as Bcl-2 and COX-2 or by mutation of the p53 gene, which frequently occurs in Barrett's oesophagus (Jankowski et al. 1992; Ireland et al. 1997; Katada et al. 1997). In animal models, duodenal reflux led to the development of oesophagitis and mucosal thickening. Increased Ki-67 and cyclin D1 expression was observed in the expanded basilar proliferative zone, indicating that increased cell proliferation is responsible for mucosal thickening (Zhang et al. 2001a). In hepatoma cells, bile acids induce PI-3K and NF-κB activation, which mediate survival signals. Inhibition of either cascade rendered the bile acids cytotoxic (Rust et al. 2000). In oesophageal cell lines, DCA exposure results in NF-κB activation (Jenkins et al. 2004), which in colonic cells is associated with resistance to DCA-induced apoptosis (Crowley-Weber et al. 2002). Therefore the balance between specific anti-apoptotic and pro-apoptotic factors determines the life/death decision of a cell.

The precise mechanisms utilized by duodenal reflux to elicit oesophageal damage and promote tumourgenesis are uncertain and opinion is still divided as to the exact contribution that bile acids and stomach acid make in Barrett's carcinogenesis (Triadafilopoulos 2001). However, it is certain that bile acids such as DCA are contributory. Therefore the effects of DCA were assessed in oesophageal cells in regards to cell proliferation and apoptosis. In summary, exposure to DCA results in inhibition of proliferation with concomitant induction of low levels of apoptosis. Furthermore, DCA induces a dose- and time-dependent increase in COX-2 expression that parallels with PARP cleavage and DNA fragmentation. DCA-induced apoptosis is both dose- and time-dependent and requires caspase-3 activation. Moreover, DCA-induced apoptosis is elevated upon inhibition of DCA-induced COX-2 expression. Our findings strongly suggest that DCA induces pro- and anti-apoptotic signalling cascades and their combined activity determines cell fate.

4.2 OBJECTIVES

The aims of this chapter were to investigate:

1 PKC-β_1-GFP localisation following DCA treatment in two oesophageal cell lines.

2 The effects of DCA on oesophageal cell morphology, proliferation and apoptosis.

3 The involvement of COX-2 in DCA-induced apoptosis.

4.3 RESULTS

4.3.1 DCA Does Not Induce PKC-β_1-GFP Translocation in SKGT4 cells

Activation of PKC-β_1 has been shown to be important in the regulation of cellular processes such as cell growth arrest and apoptosis in colonic cells (Choi et al. 1990; Goldstein et al. 1995). As DCA induces rapid translocation of PKC-β_1 in colonic cells **(Fig 3.4 and 3.5),** we examined whether DCA could induce PKC-β_1 translocation in oesophageal cells. To study the effects of DCA on PKC-β_1 localisation, SKGT4 cells were transfected with EGFP or PKC-β_1-GFP and the appropriate expression of these constructs was verified by Western blotting using antibodies specific for PKC-β_1 and GFP, respectively. SKGT4 cells do not express endogenous PKC-β_1 as confirmed by the absence of a PKC-β_1 band (78 kDa) in lysates of untransfected cells probed with a monoclonal antibody specific for PKC-β_1. Expression of PKC-β_1-GFP was readily detected in cells transfected with PKC-β_1-GFP **(Fig 4.1A)**. No degradation products of PKC-β_1-GFP were

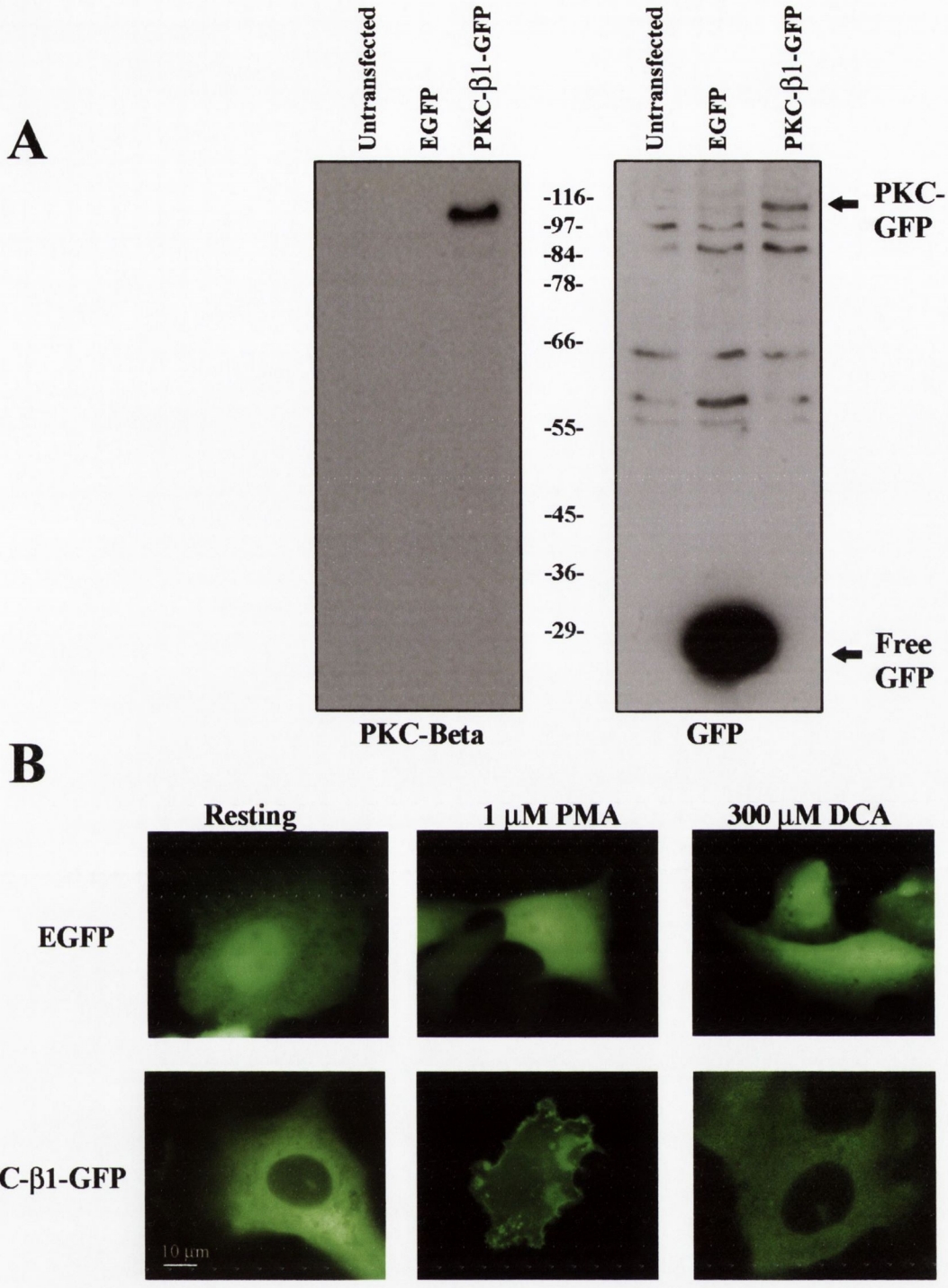


Fig 4.1. DCA Does Not Induce PKC-β_1-GFP Translocation in SKGT4 cells. (**A**) SKGT4 cells transfected either with EGFP or PKC-ε-GFP were examined for the appropriate expression of GFP and PKC-β_1-GFP by Western blotting using antibodies specific for PKC-β_1 and GFP which detected specific bands of the predicted molecular weight 27 kDa and 105 kDa, respectively. Non-specific lower molecular weight bands were observed with the anti-GFP antibody in both untransfected and transfected cells indicating that this is not proteolysis of PKC-GFP fusion proteins. (**B**) SKGT4 cells were transfected with either EGFP or PKC-β_1-GFP as described in chapter two. 24 hr post-transfection, cells were stimulated or not with 1 μM PMA and 300 μM DCA for 1 hr and analysed by fluorescence microscopy. Each experiment was preformed three times and representative results are shown. Scale bar represents 10 μm.

observed, reflecting the expression of the intact fusion protein. GFP and PKC-GFP expression were verified using an anti-GFP antibody, which detected specific bands of the predicted molecular weight 27 kDa and 105 kDa, respectively **(Fig 4.1A)**. Non-specific lower molecular weight bands were observed with the anti-GFP antibody in both untransfected and transfected cells indicating that this is not proteolysis of PKC-GFP fusion proteins.

Localisation of PKC-β_I-GFP was assessed using epifluorescent microscopy and a high-resolution coded CCD camera. SKGT4 cells, transfected with PKC-β_I-GFP, expressed homogenous fluorescence in the cytosol. PMA was used as a positive control for PKC activation and to ensure that the PKC-β_I-GFP was functionally responsive. Incubation with 1 μM PMA induced PKC-β_I-GFP translocation from the cytosol to the plasma membrane within 15 min, with PKC-β_I-GFP remaining at a membrane location for up to 2 hr **(Fig 4.1B)** (data not shown). Stimulation with 300 μM DCA induced no changes in the subcellular localisation patterns of PKC-β_I-GFP transfected cells **(Fig 4.1B)**. Even 2 hr post treatment with DCA, PKC-β_I-GFP remained localised in the cytosol (data not shown). As a control for specificity, SKGT4 cells transfected with EGFP (empty vector) were treated with 1 μM PMA and 300 μM DCA for similar periods of time. EGFP was expressed uniformly throughout the cell and remained unaltered in response to either stimulus **(Fig 4.1B)**. Similar results were observed with another oesophageal cell line, OE-33, demonstrating that these findings are not cell line specific and clearly indicating that DCA does not induce PKC-β_I translocation in oesophageal cells **(Fig 4.2)**.

4.3.2 DCA Induces Morphological Changes in SKGT4 cells

While treatment with DCA did not induce PKC-β_I-GFP translocation in oesophageal cells, morphological changes were observed upon incubation with DCA. Bile acids are known to induce marked morphological changes in colonic cells (LaRue et al. 2000; Washo-Stultz et al. 2002). Therefore alterations in the morphology of oesophageal epithelial cells exposed to DCA were studied. SKGT4 cells were stimulated with DCA and PMA for 1 – 6 hr and their morphology was assessed by light microscopy. Incubation with 300 μM DCA up to 4 hr appeared to have no dramatic effects on SKGT4 morphology **(Fig 4.3A)**. However prolonged exposure to DCA, up to 6 hr, induced shrinkage of the cell body **(Fig 4.3A)**. SKGT4 cells, 6 hr post DCA treatment, were noticeable smaller **(Fig 4.3A, arrow head)**.

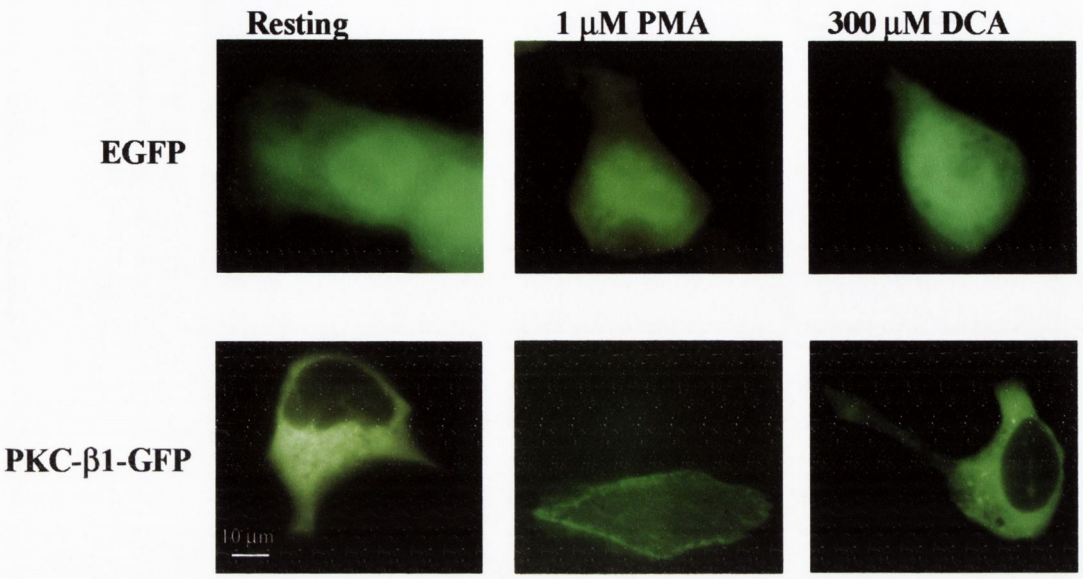


Fig 4.2. DCA Does Not Induce PKC-β_1-GFP Translocation in OE-33 cells. OE-33 cells were transfected either with EGFP or PKC-β_1-GFP as outlined in chapter two. 24 hr post-transfection, cells were stimulated with 1 μM PMA or 300 μM DCA for 1 hr and analysed by fluorescence microscopy. Results are representative of more than eight experiments. The scale bar represents 10 μm.

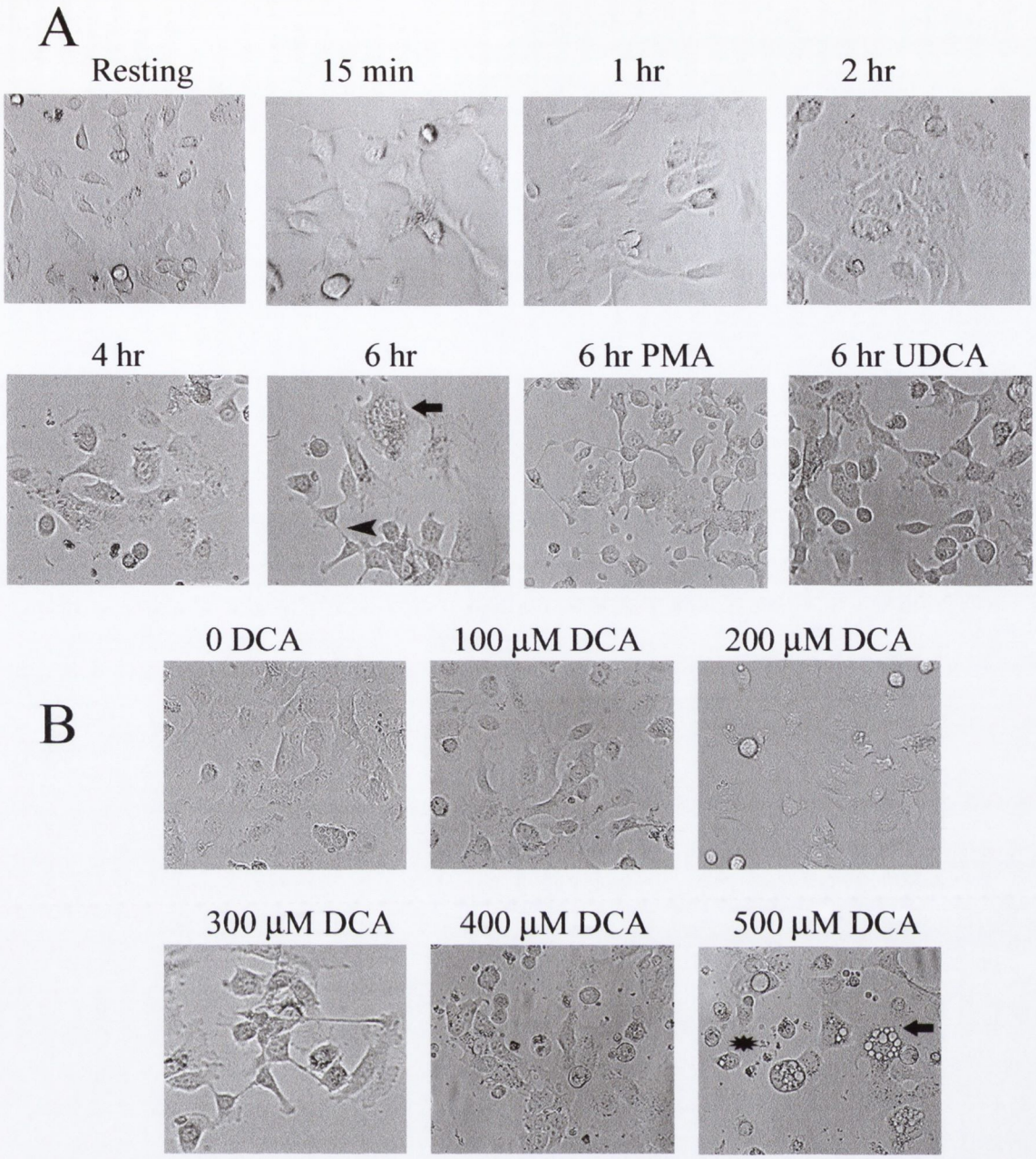


Fig 4.3. DCA Induces Morphological Changes in SKGT4 cells. (A) SKGT4 cells were exposed to 300 µM DCA at different time intervals as indicated. PMA (50 ng/ml) was used as a positive control for PKC activation while the bile acid UDCA (300 µM) was used for comparison purposes. Arrowhead indicates cytoplasmic extensions. Arrow indicates cytoplasmic vacuolisation. (B) SKGT4 cells were stimulated with DCA over a concentration range of 0 – 500 µM for 6 hr. Star indicates detached cells. Cell morphology was assessed by phase contrast under light microscope.

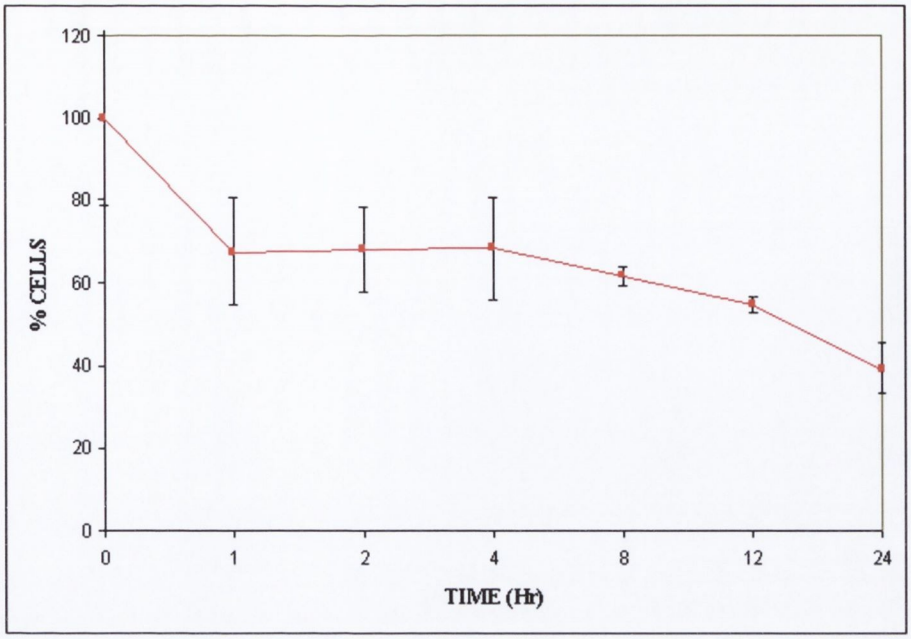
A percentage of cells had large vacuoles in their cytoplasm, which were not observed at the earlier time points **(Fig 4.3A, arrow)**. Cells stimulated with PMA and UDCA have no morphological features indicative of apoptosis at any time point **(Fig 4.3A)**. PMA treated cells acquired a rounded phenotype and long cytoplasmic protrusions were observed. In comparison, UDCA treated cells appear similar to resting cells.

In oesophageal aspirates increasing concentrations of bile acids are associated with increased oesophageal mucosal damage (Nehra et al. 1999). Therefore a DCA concentration curve was performed in order to establish if the morphological changes, observed with 300 μM DCA after 6 hr, were limited to this concentration. Cells treated with low concentrations of DCA (100 - 200 μM) appeared similar to resting cells after 6 hr **(Fig 4.3B)**. However incubation with higher concentrations DCA, in particular 400 - 500 μM, was associated with dramatic morphological changes indicative of apoptosis. A percentage of cells were completely round and had detached from the matrix **(Fig 4.3B, star)**, while membrane blebbing was also evident **(Fig 4.3B, arrow)**. These data, coupled with similar experimental evidence from HCT116 cells treated with DCA (chapter III) (Martinez et al. 1998), demonstrate that this effect is not cell line specific.

4.3.3 DCA Induces a Dose and Time Dependent Decrease in Cell Proliferation

Bile acids, in particular DCA, have been shown to inhibit proliferation and are potent inducers of apoptosis (Martinez et al. 1998; Powell et al. 2001; Qiao et al. 2001b). The morphological changes observed with prolonged DCA stimulation and the concentration dependency of this effect suggested alterations in cellular proliferation. To test for modifications in cell growth, SKGT4 cells were incubated with 300 μM DCA for different periods of time ranging from 0 - 24 hr and proliferation was assessed using the MTT assay. As depicted in **Fig 4.4A**, an initial decrease in cell proliferation was observed within the first hour of treatment, which remained at a similar level up to 8 hr and was more pronounced at 12 and 24 hr **(Fig 4.4A)**. A time point in this plateau (6 hr) was chosen to quantify the dependency of the alterations in cell proliferation on DCA concentration. In comparison to resting cells, DCA induces a dose dependent decrease in cell proliferation, which is maximal at 500 μM **(Fig 4.4B)**. Exposure of oesophageal cells to 300 μM DCA causes a decrease in proliferation that is consistent but not statistically significant. At higher concentrations, 400 – 500 μM, the decrease in cell growth was statistical significant

A



B

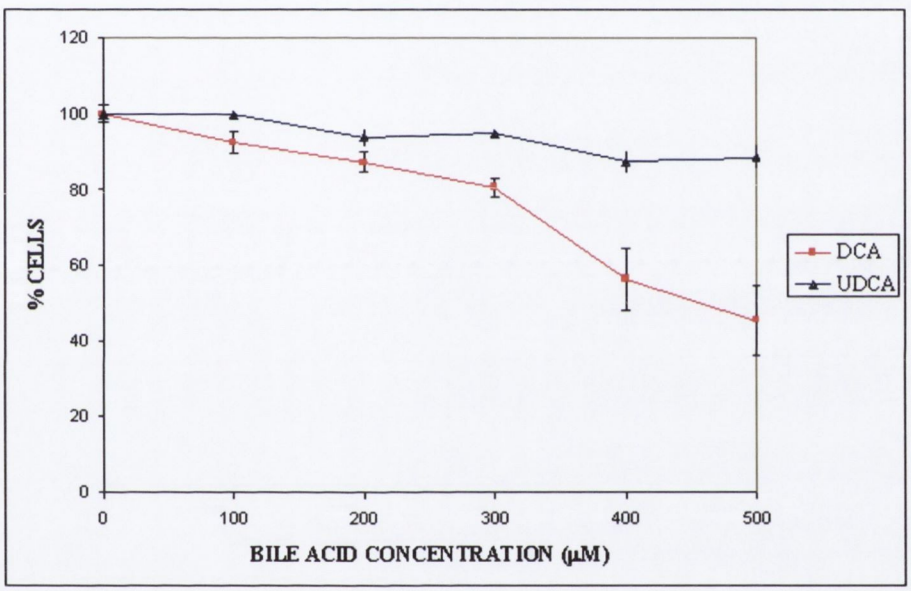


Fig 4.4. DCA Induces a Dose and Time Dependent Decrease in Cell Proliferation. (**A**) SKGT4 cells were incubated with 300 μM DCA at the time intervals indicated. (**B**) SKGT4 cells were simulated with DCA and UDCA at concentrations ranging from 0 – 500 μM for 6 hours. In both panels cellular proliferation was assessed using the MTT assay as described in chapter two. Results are expressed as percentage of proliferating cells relative to untreated controls. Results are representative of at least three independent experiments. Mean ± SD.

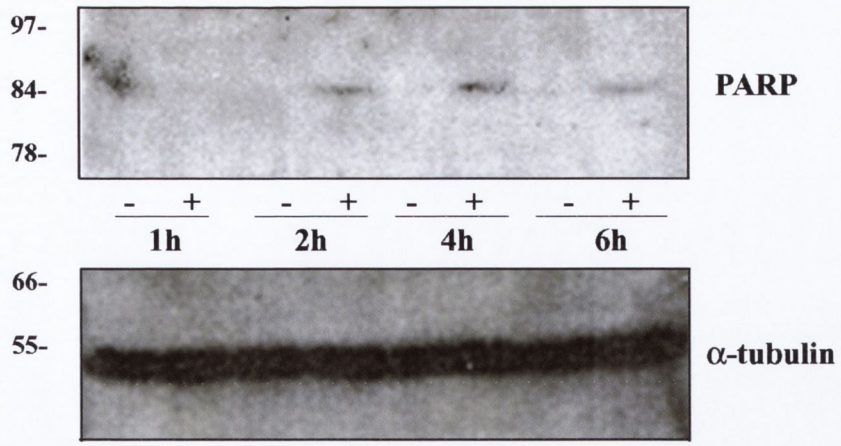
($p < 0.05$) and correlated with the morphological changes observed **(Fig 4.3B)**. UDCA treated SKGT4 cells show identical proliferation patterns to untreated cells at all concentrations tested **(Fig 4.4B)**. DCA was used at 300 μM in all subsequent studies unless otherwise stated. At this concentration, specific gene expression patterns can be analysed without any cytotoxic effects.

4.3.4 DCA Induces Apoptosis in a Time Dependent Manner

Bile acid-induced cytotoxicity and apoptosis have been widely reported in several cell types including colonic and hepatic cells (Washo-Stultz et al. 2002; Qiao et al. 2003). Thus the DCA-induced decrease in SKGT4 cell proliferation together with the morphological changes observed might be due to induction of cell death. Cell death can be caused by necrosis or apoptosis (Nicotera and Melino 2004). Given the cytoplasmic vacuolisation and rounding observed in a percentage of treated cells and strong experimental evidence of DCA's involvement in apoptotic pathways (Martinez et al. 1998; Payne et al. 1998; Bernstein et al. 1999b; Qiao et al. 2002), it was likely that DCA-induced SKGT4 cell death is due to apoptosis. Apoptosis was assessed by two independent methods, firstly by Western blotting through cleavage of PARP to an 85 kDa fragment and secondly by ELISA to detect DNA fragmentation, both of which are hallmarks of apoptosis (Duke and Cohen 1986; Bonfoco et al. 1995; Terui et al. 1995). Firstly we examined the kinetics of DCA-induced apoptosis. SKGT4 cells were stimulated with 300 μM DCA for 15 min to 6 hr. Total cell lysates were prepared and assessed for PARP cleavage and for DNA fragmentation. Degradation of PARP is detected at 2 hr post DCA treatment as seen by the presence of the 85-kDa band that is not present in resting cells **(Fig 4.5A).** DCA-induced PARP persists for up to 6 hr **(Fig 4.5A)**. DCA did not alter α-tubulin protein expression indicating that DCA-induced PARP cleavage is specific **(Fig 4.5A, lower panel)**. Using ELISA, low levels of DNA fragmentation could be detected **(Fig 4.5B)**. Stimulation with 300 μM DCA for 6 hr resulted in a 2-fold increase in DNA fragmentation **(Fig 4.5B)**.

In order to determine whether prolonged activation of DCA would result in increased induction of apoptosis, cells were treated with 300 μM DCA for 6 - 24 hr. As shown in **Fig 4.6A**, DCA-induced PARP cleavage is detected 6 hr following DCA treatment and is sustained for up to 24 hr. Staurosporine, which has previously been documented to induce apoptosis and PARP cleavage in oesophageal cells as well as in several other cell types

A



B

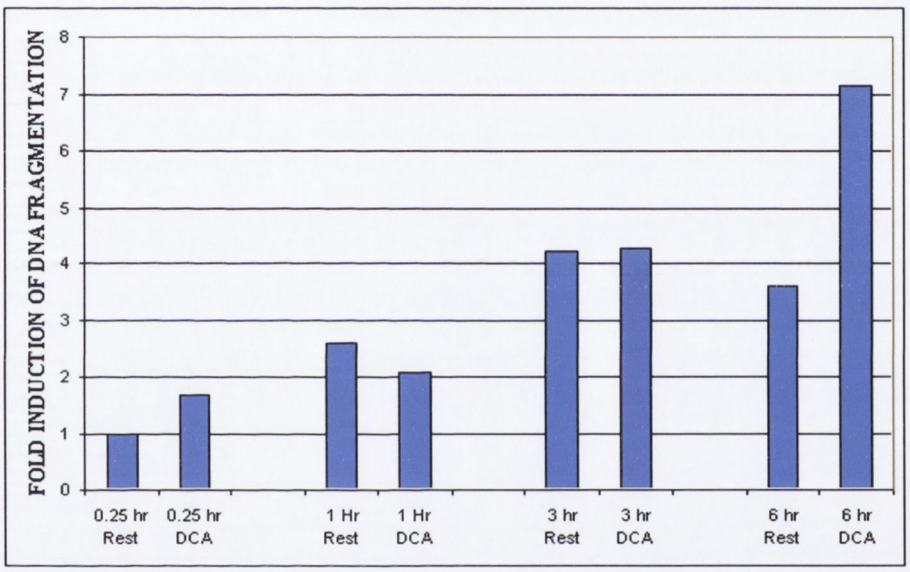


Fig 4.5. DCA Induces a Time Dependent Increase in Apoptosis. (**A**) SKGT4 cells were stimulated with 300 μM DCA for 6 hr. Total cell lysates were standardised to 50μg as described in chapter two and assessed by Western blotting using anti-PARP antibody. The membrane was stripped and reprobed with anti-actin antibody as loading control. (**B**) SKGT4 cells were stimulated with 300 μM DCA for 6 hr. Cell Lysates were assessed for DNA fragmentation as described in chapter two. Results are given as fold induction of DNA fragmentation relative to unstimulated control. Mean ± SD. Results are representative of at least two independent experiments.

(Wang et al. 1995; Shao et al. 1997b; Shao et al. 1997a; Leu et al. 2000), was used as a positive control. Staurosporine induces a higher level of PARP cleavage in comparison to DCA **(Fig 4.6A)**, which is a naturally occurring constituent of the gastrointestinal tract unlike staurosporine. In addition, staurosporine induces apoptosis by acting directly on mitochondria via both caspase dependent and independent mechanisms (Bossy-Wetzel et al. 1998; Daugas et al. 2000) and thereby markedly elevating the degree of apoptosis. DCA induces a two-fold increase in DNA fragmentation after 6 hr, which increases after 24 hr (3-fold) **(Fig 4.6B)**. Similar to DCA-induced PARP cleavage, DCA-induced DNA damage is substantially lower in comparison to that induced by staurosporine (32 fold induction) **(Fig 4.6B)**. These data show that DCA induces constant low levels of apoptosis, which are most likely compensated by other mechanisms as the levels of PARP cleavage and DNA fragmentation remain constant throughout long term exposure to DCA.

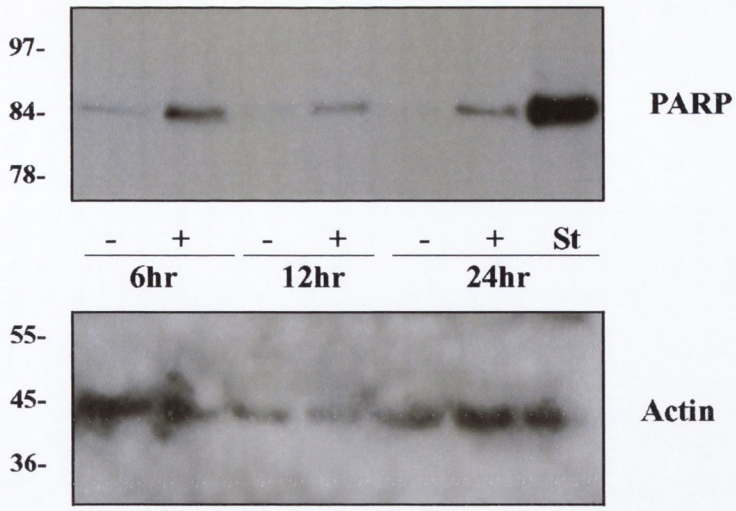
4.3.5 DCA-Induced Apoptosis is Concentration Dependent

In colonic cells, induction of apoptosis by DCA exhibited a strong dependency on the concentration of DCA (Martinez et al. 1998). While DCA-induced apoptosis in SKGT4 cells is time dependent **(Fig 4.6)**, a minimum threshold concentration may exist for DCA to induce apoptosis in SKGT4 cells. Therefore SKGT4 cells were treated with 0 – 500 μM DCA for 6 hr and total cell lysates were assessed for PARP cleavage and DNA fragmentation by Western blotting and ELISA, respectively. No degradation of PARP was observed between 100 – 200 μM DCA, but there was a dose dependent increase in PARP cleavage from 300 – 500 μM **(Fig 4.7A)**. Staurosporine induces a higher level of apoptosis than the maximal concentration of DCA as indicted by the increased intensity of the cleaved PARP fragment **(Fig 4.7A)**. The level of DNA fragmentation in cells treated with low concentrations of DCA (100 – 200 μM) is similar to resting cells **(Fig 4.7B)**. Increasing concentrations of DCA (300 – 500 μM) results in a steady rise in DNA damage **(Fig 4.7B)**, which correlates with the levels of PARP cleavage

4.3.6 DCA-Induced Apoptosis is Caspase Dependent

Bile acids are known activators of death receptors such as Fas and TRAIL resulting in caspase activation and apoptosis (Faubion et al. 1999; Schlottman et al. 2000). As PARP cleavage can occur via caspase dependent and independent mechanisms (Pique et al. 2000), the broad-spectrum caspase inhibitor, Z-Val-Ala-Asp-CH_2F (Z-VAD-FMK), and

A



B

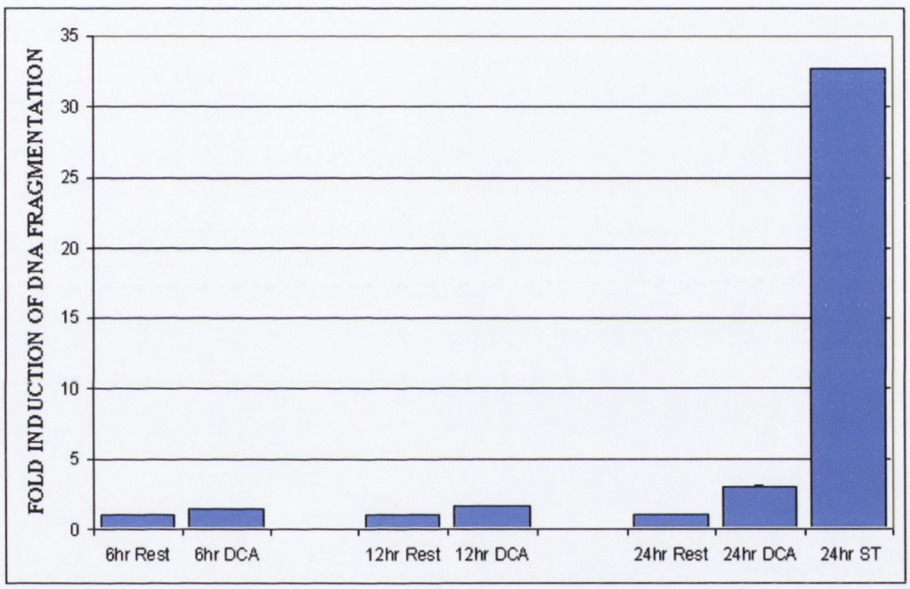


Fig 4.6. Long Term Kinetics of DCA-Induced Apoptosis. (**A**) SKGT4 cells were stimulated with 300 μM DCA for up to 24 hr. Total cell lysates were standardised to 50μg as described in chapter two and assessed by Western blotting using anti-PARP antibody. The membrane was stripped and reprobed with anti-actin antibody as loading control. Staurosporine (ST) was used at 1 μM, which served as positive control for induction of apoptosis (**B**) SKGT4 cells were stimulated with 300 μM DCA for up to 24 hr. Cell Lysates were assessed for DNA fragmentation as described in chapter two. ST was included as a positive control for apoptosis. Results are given as fold induction of DNA fragmentation relative to unstimulated control. Mean ± SD. Results are representative of at least two independent experiments.

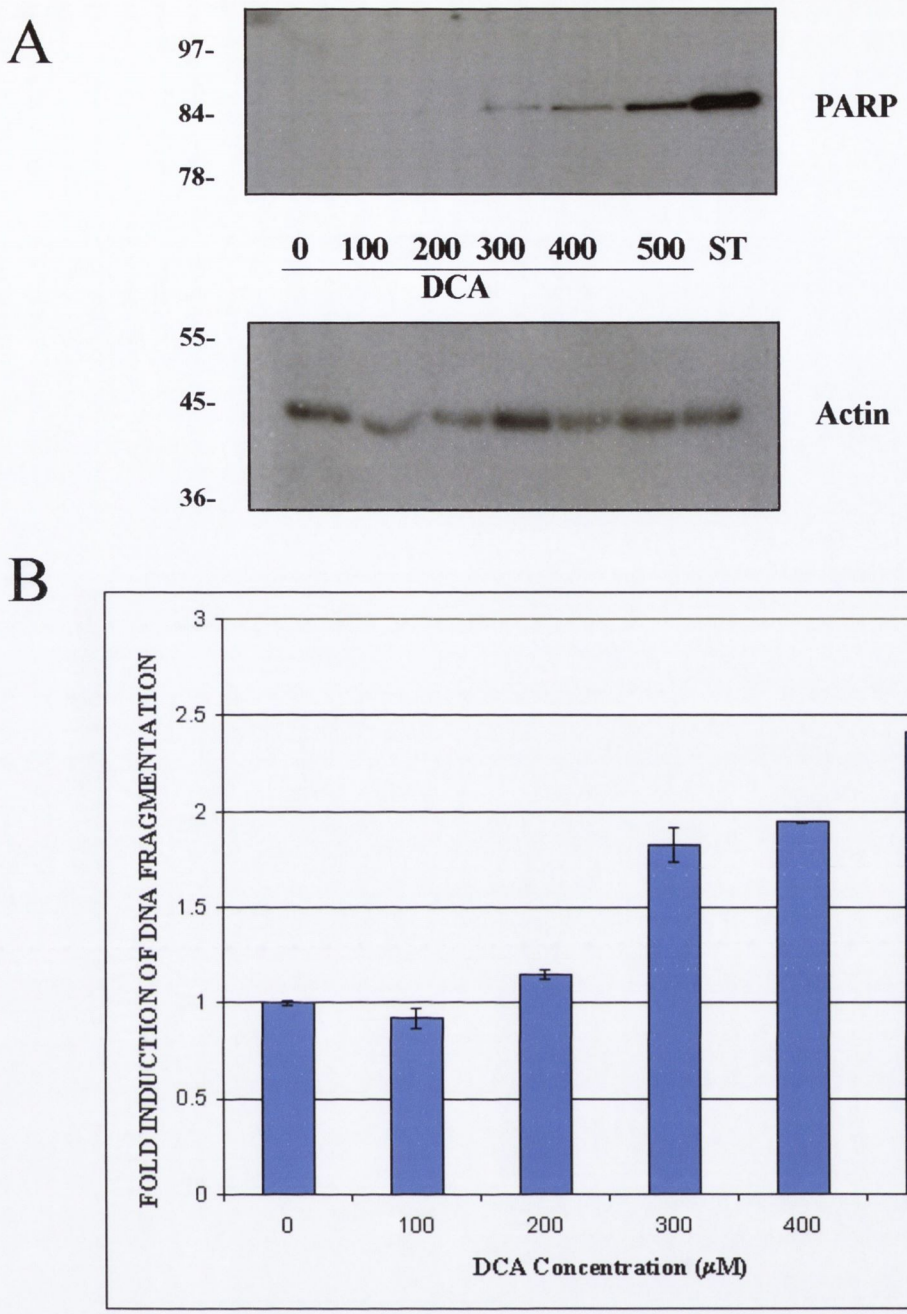


Fig 4.7. DCA Induces Dose Dependent Increase in Apoptosis. (A) SKGT4 cells were stimulated with varying concentrations of DCA ranging from 0 – 500 μM for 6 hr and ST was incorporated as a positive control for PARP cleavage. Total cell lysates were standardised to 50μg as described in chapter two and assessed by Western blotting using anti-PARP antibody. The membrane was stripped and reprobed with anti-actin antibody as loading control. (B) SKGT4 cells were treated with varying concentrations of DCA as indicated and ELISA, as described in chapter two, assessed DNA fragmentation. Results are given as fold induction of DNA fragmentation relative to unstimulated cells. Mean ± SD. Results are representative of at least three independent experiments.

the caspase-3 inhibitor, Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK (Z-DEVD-FMK), were employed to assess whether DCA-induced PARP cleavage is caspase dependent. SKGT4 cells were pretreated for 1 hr with 50 μM Z-VAD-FMK or 50 μM Z-DEVD-FMK and then stimulated with 400 μM DCA for 6 hr. This concentration of DCA was used to ensure significant levels of PARP cleavage. Unstimulated SKGT4 cells with and without Z-VAD-FMK and Z-DEVD-FMK show negligible levels of PARP cleavage **(Fig 4.8A)**. DCA induces marked PARP cleavage that is completely abolished by both Z-VAD-FMK and Z-DEVD-FMK **(Fig 4.8A)** suggesting that DCA-induced PARP cleavage is caspase dependent and more specifically caspase-3 dependent. The DNA fragmentation ELISA was used to verify the effect of Z-VAD-FMK and Z-DEVD-FMK on DCA-induced apoptosis. Resting SKGT4 cells and cells treated with the caspase inhibitors alone induce negligible levels of DNA fragmentation **(Fig 4.8B)**. DCA-induced DNA fragmentation is partially inhibited by both 50 μM Z-VAD-FMK and 50 μM Z-DEVD-FMK **(Fig 4.8B)**. Caspase-independent mechanisms appear to contribute to DCA-induced DNA fragmentation as judged by the partial inhibition seen with the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK. Collectively these data show that DCA-induced PARP cleavage and DNA fragmentation is caspase-3 dependent.

4.3.7 DCA Induces COX-2 Expression in a Time and Dose Dependent Manner

COX-2 is the rate limiting enzyme of arachidonic acid metabolism (Dubois et al. 1998). COX-2 is an inducible protein, which has been shown to be elevated during inflammation in animal models and in human cancers (Tsujii et al. 1997; Zhang et al. 2001a; Subbaramaiah et al. 2002). COX-2 is a potent anti-apoptotic protein in several cell types (Tsujii and DuBois 1995; Elder et al. 1997; Tsujii et al. 1997; Liu et al. 1998). Inhibition of COX-2 expression in colonic cells resulted in increased apoptosis (Washo-Stultz et al. 2002), indicating a role of COX-2 in the balance between pro-apoptotic and anti-apoptotic mechanisms. To assess a possible role for COX-2 in DCA-induced apoptosis, we examined the kinetics and dose dependent response of DCA-induced COX-2 expression and compared it to that of PARP cleavage. SKGT4 cells were stimulated with 300 μM DCA for 1 – 6 hr and Western blot analysis on SKGT4 cell lysates was performed utilising a specific antibody that recognises COX-2. Expression of COX-2 is detected after 4 hr and is more pronounced after 6 hr of DCA stimulation **(Fig 4.9A)**. The kinetics of COX-2 expression correlates with those of PARP cleavage **(Fig 4.5A, 4.9A)**. However DCA-

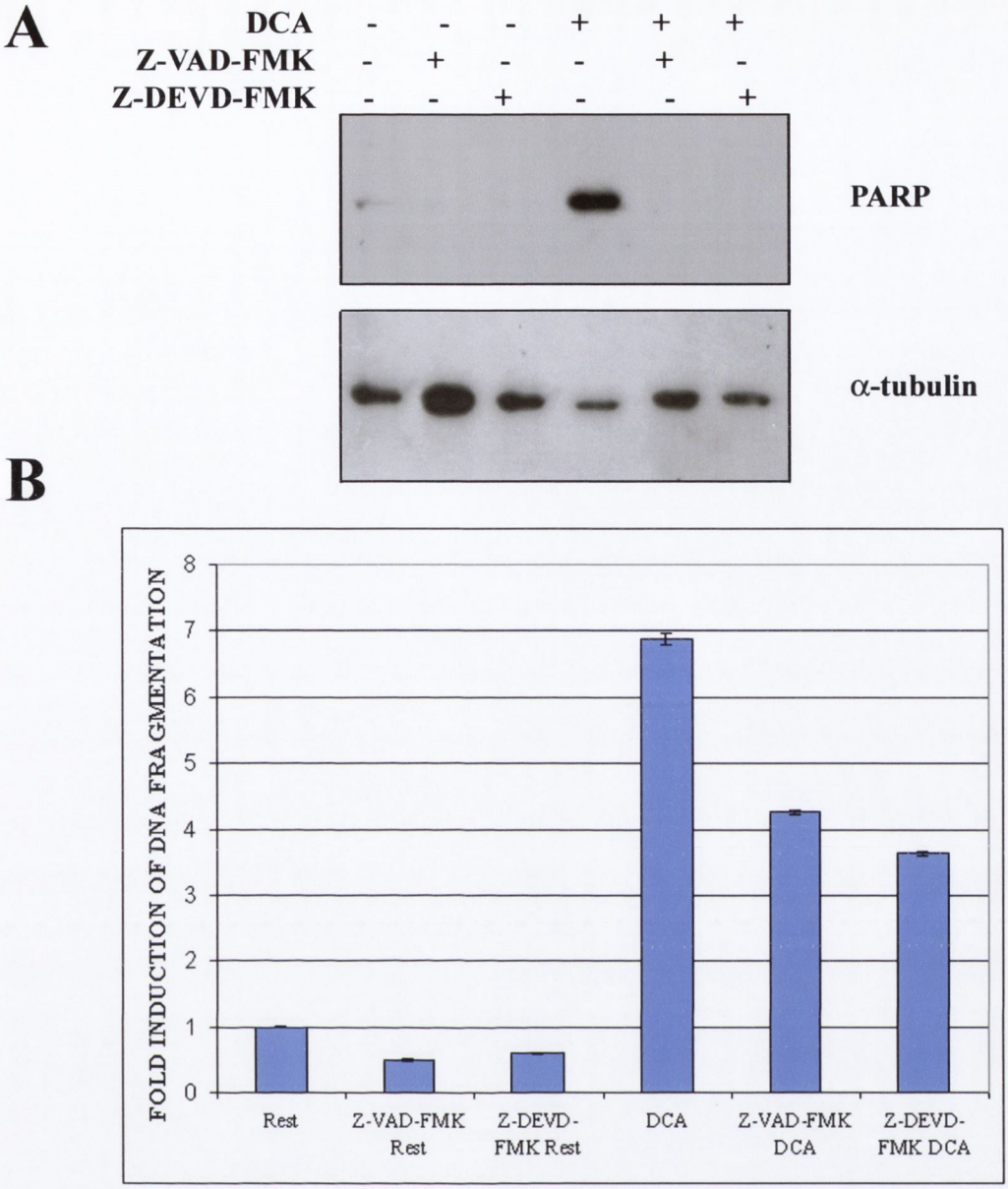


Fig 4.8. DCA-Induced Apoptosis is Caspase Dependent. SKGT4 cells were treated with 50 μM Z-VAD-FMK or 50 μM Z-DEVD-FMK for 1 hr prior to the addition of 400 μM DCA for 6 hr. (**A**) Whole cell lysates were standardised to 50 μg as described in chapter two. PARP cleavage was assessed by immunoblotting with an anti-PARP antibody followed by anti-α-tubulin as loading control. (**B**) DNA fragmentation was assessed by ELISA as outlined in chapter two. Results are given as fold induction of DNA fragmentation relative to unstimulated cells. DCA-induced DNA fragmentation is partially inhibited by both 50 μM Z-VAD-FMK and 50 μM Z-DEVD-FMK Caspase-independent mechanisms appear to contribute to DCA-induced DNA fragmentation as judged by the partial inhibition seen with the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK Mean ± SD. Results are representative of at least two independent experiments.

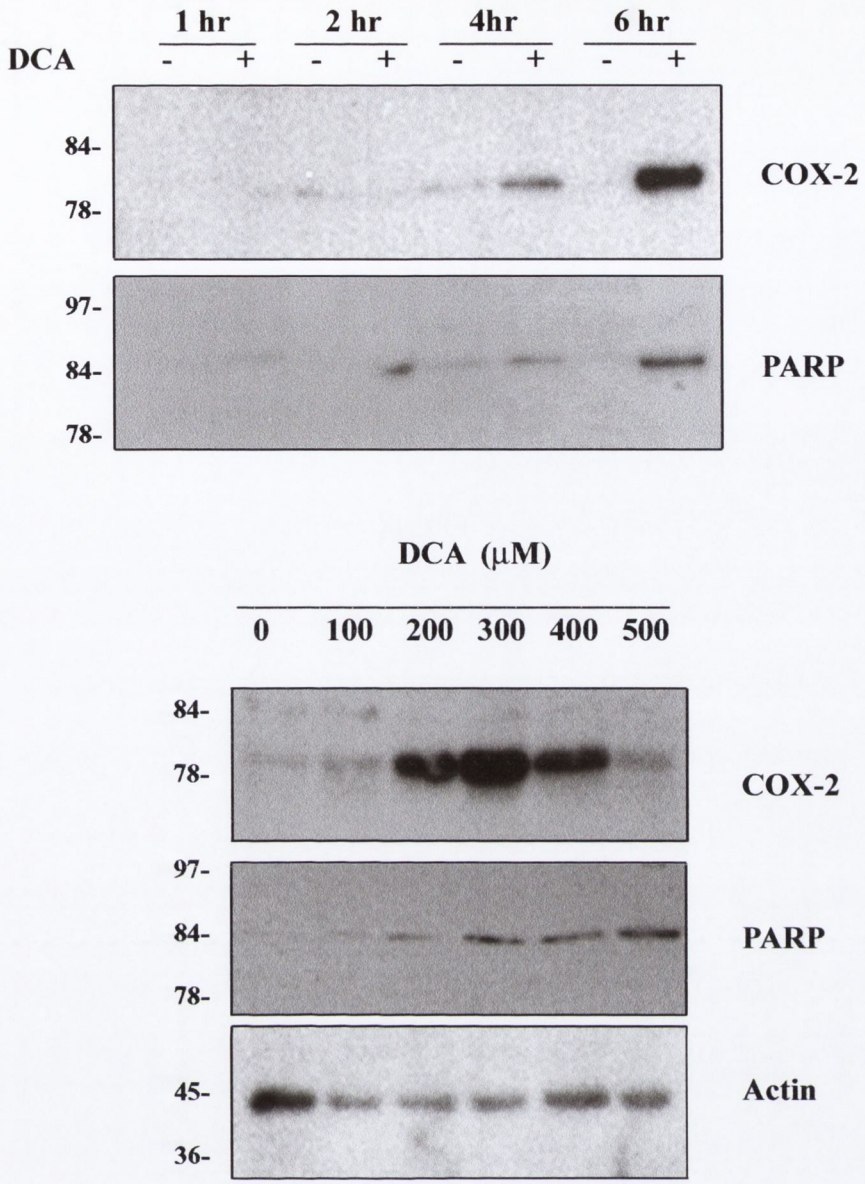


Fig 4.9. DCA Induces COX-2 Expression in a Time and Dose Dependent manner. (**A**) SKGT4 cells were stimulated with 300 μM DCA for 1- 6 hr. (**B**) Cells were stimulated with 0 – 500 μM DCA for 6 hr. In both panels, total cell lysates were standardised to 50 μg as described in chapter two and assessed by Western blotting using anti-COX-2 and anti-PARP antibodies, respectively. The membranes were stripped and reprobed with anti-actin antibody as loading control. Results are representative of at least three independent experiments.

induced COX-2 expression is maximal at 300 μM DCA, while DCA-induced PARP cleavage is maximal at 500 μM **(Fig 4.9B)**.

Specific COX-2 inhibitors (NS398 and aspirin) were used to confirm DCA-induced COX-2 expression. Previous studies have shown that CDCA-induced COX-2 expression and activity can be inhibited by aspirin (Li et al. 2000) while NS398 (N- (2-Cyclohexyloxy-4-nitrophenyl) methanesulfonamide) inhibits COX-2 activity but not expression (Zhang et al. 1998). SKGT4 cells were treated with 10 μM NS398, a non-steroidal anti-inflammatory drug (NSAID), or 2 mM aspirin for 30 min prior to the addition of 300 μM DCA. In agreement with studies of inhibition of CDCA-induced COX-2 expression (Zhang et al. 1998; Li et al. 2000), no inhibition of DCA-induced COX-2 expression was observed with NS398 **(Fig 4.10A)** while 2 mM aspirin partial inhibits DCA-induced COX-2 expression **(Fig 4.10B)**. Aspirin, at the higher dose of 5 mM, results in a pronounced reduction in COX-2 expression **(Fig 4.11)** and was used to inhibit COX-2 in all subsequent studies. However, no inhibition of PARP cleavage is observed with 5 mM aspirin **(Fig 4.11)**. Collectively these data suggest that COX-2 expression occurs in parallel with PARP cleavage through independent upstream pathways responsive to DCA.

4.3.8 Aspirin Potentiates DCA-Induced Apoptosis

Aspirin and other NSAIDs have been shown to have anti-proliferative and apoptosis-inducing effects in colonic, oesophageal and leukaemia cells (Elder et al. 1996; Bellosillo et al. 1998; Li et al. 2000; Pique et al. 2000). We examined whether inhibition of COX-2 expression by aspirin could potentiate DCA-induced apoptosis. SKGT4 cells were pretreated with 5 mM aspirin for 30 min prior to stimulation with DCA for 6 hr. Total cell lysates were assessed by Western blotting using a specific anti-PARP antibody. DCA alone induced clear degradation of PARP in comparison to untreated cells and this effect was markedly enhanced by pretreatment with aspirin **(Fig 4.12A)**. To verify that aspirin potentiates DCA-induced apoptosis, we used the DNA fragmentation ELISA to assess total cell lysates from SKGT4 cells pretreated with 5 mM aspirin and stimulated with 400 μM DCA for 6 hr. Levels of DNA fragmentation are similar in unstimulated cells and cells treated with aspirin alone **(Fig 4.12B)**. DCA induces a four-fold increase in DNA fragmentation that is increased by pre-treatment with aspirin **(Fig 4.12B)**. Together these data show that aspirin enhances the apoptotic effect of DCA.

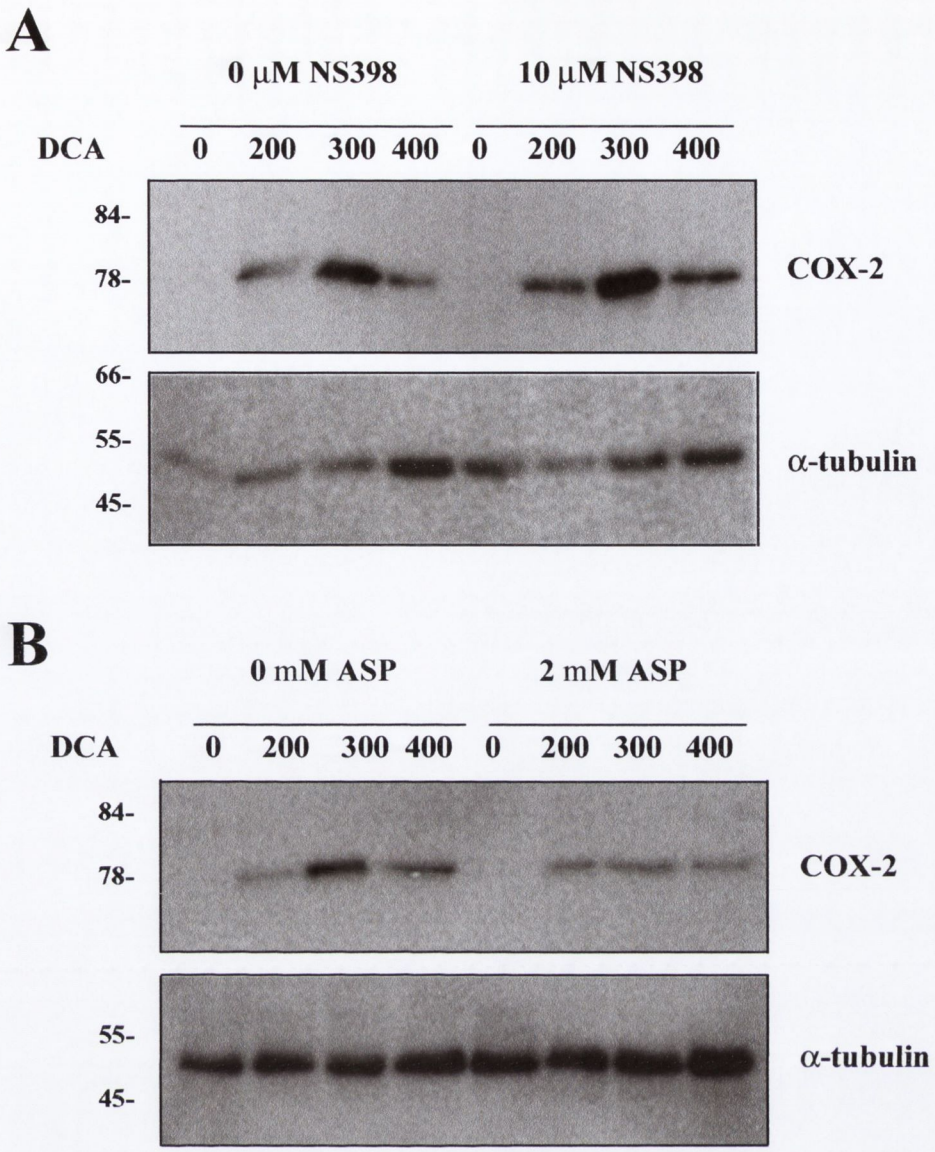


Fig 4.10. DCA-Induced COX-2 is Inhibited by Aspirin But Not By the NS398 Compound. (**A**) SKGT4 cells were treated with 10 μM NS398 for 30 min prior to the addition of 300 μM DCA for 6 hr. (**B**) SKGT4 cells were treated with 2 mM Aspirin (ASP) for 30 min preceding to stimulation with DCA for 6 hr. Total cellular lysates were obtained as described in chapter two and the levels of COX-2 protein were assessed by Western blotting with an anti-COX-2 antibody. The membranes were stripped and reprobed with anti-α-tubulin antibody as loading control. Results are representative of at least two independent experiments

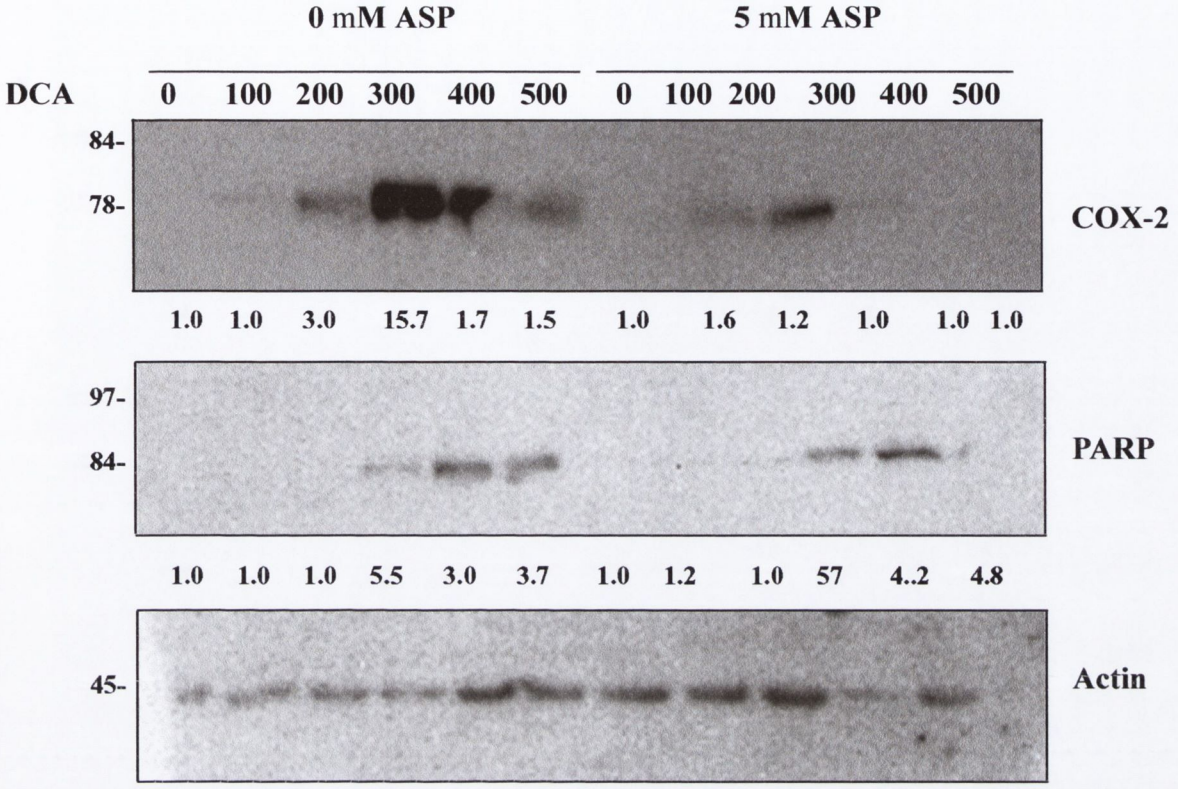
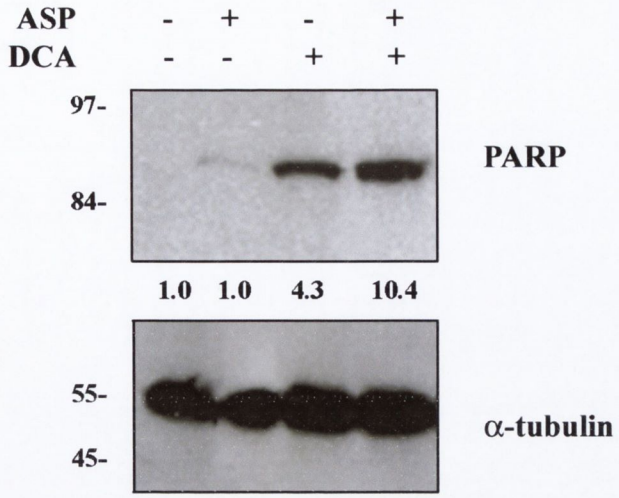


Fig 4.11. Aspirin Inhibits DCA-Induced COX-2 Expression but Not PARP Cleavage. SKGT4 cells were treated with 5 mM Aspirin (ASP) for 30 min preceding stimulation with 300 μM DCA for 6 hr. Total cellular lysates were prepared and the expression of COX-2 and PARP was assessed by Western blotting using anti-COX-2 and anti-PARP antibodies, respectively. The membranes were stripped and reprobed with anti-actin as loading control. COX-2 expression and PARP cleavage were assessed by densitometry and normalised against actin. Fold increases in COX-2 expression and PARP cleavage are then given relative to resting cells. Results are representative of at least two independent experiments

A



B

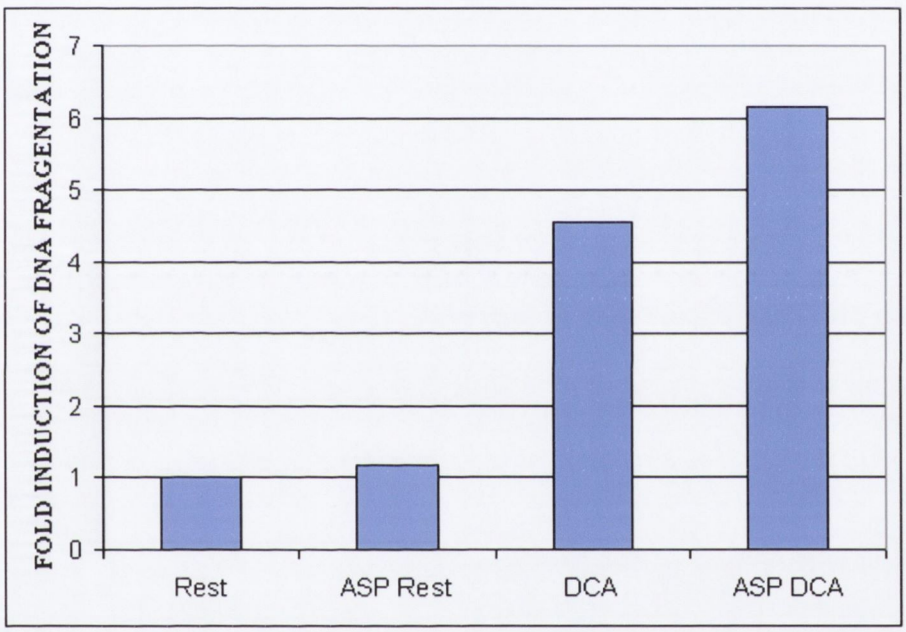


Fig 4.12. Aspirin Potentiates DCA-Induced Apoptosis. SKGT4 cells were treated with 5 mM Aspirin (ASP) for 30 min prior to the addition of 400 μM DCA for 6 hr. (**A**) Western blot analysis was preformed on whole cell lysates, standardised to 50 μg as described in chapter two, using an anti-PARP antibody followed by anti-α-tubulin as loading control. The levels of PARP cleavage was assessed by densitometry and normalised against α-tubulin. Fold increases in PARP cleavage are then given relative to resting cells (**B**) DNA fragmentation was assessed by ELISA as outlined in chapter two. Results are given as fold induction of DNA fragmentation relative to unstimulated cells. Results are representative of three independent experiments.

4.4 DISCUSSION

The precise mechanisms utilized by duodenal reflux to elicit oesophageal damage and promote tumourgenesis are uncertain. Here we show that DCA induces both low levels of apoptosis as reflected by a decrease in cellular proliferation, as well as a survival mechanism. We demonstrate a significant role for COX-2 in mediating survival in these cells, which is dose and time dependent.

PKC-β_I is known to have a negative effect on cellular proliferation and to induce growth arrest (Choi et al. 1990; Goldstein et al. 1995). DCA is able to activate PKC-β_I in colonic cells **(Fig 3.4)**. Colonic cells are physiologically exposed to bile acids, while oesophageal cells are only in contact with bile acids in patients suffering from reflux. Chronic activation of PKCs leads to downregulation of their protein expression (Darbon et al. 1986a; Freire-Moar et al. 1991) and has been proposed as a contributing factor in triggering transformation (Black 2001). Loss of PKC-β_I expression has been found in colonic adenocarcinoma cells (Levy et al. 1993; Doi et al. 1994) and here we show that PKC-β_I is not expressed in SKGT4 cells **(Fig 4.1)**. Absence of PKC-β_I might therefore provide an explanation for the transformed state of these cells.

In colonic cells, physiological concentrations of DCA induce activation of PKC-β_I, leading to actin and tubulin rearrangements **(Fig 3.9)**, while higher doses of DCA induce distinct morphological changes indicative of apoptosis in these cells **(Fig 3.4)**. However in oesophageal cells, DCA does not regulate PKC-β_I but it does induce apoptosis **(Fig 4.5)**. As mentioned above colonic and oesophageal cells are exposed to bile acids during malignant progression. Although DCA evidently regulates apoptosis and transformation of both cell types, the DCA-induced signalling pathways involved in each case are clearly distinct.

DCA induces a time- and dose-dependent decrease in cell proliferation in SKGT4 cells **(Fig 4.4)**, which is associated with clear morphological changes, such as noticeably smaller cell body in comparison to the resting cells. In addition, a high percentage of cells had features indicative of apoptosis (e.g. vacuolisation) **(Fig 4.3)**. Induction of apoptosis by DCA was confirmed by PARP cleavage and DNA fragmentation **(Fig 4.3, 4.4 and 4.5)**. *In vitro*, short term exposure of colonic cells to DCA induces apoptosis (Martinez et al.

1998). However prolonged exposure is associated with the acquisition of resistance and desensitisation to apoptosis (Bernstein et al. 2000; Crowley-Weber et al. 2002). Similarly *in vivo*, bile acid-induced apoptosis has been linked with compensatory proliferation of crypt epithelial cells (Lapre et al. 1992). It has been proposed that bile acids induce DNA damage in cells (Kandell and Bernstein 1991; Venturi et al. 1997), most of which undergo apoptosis, while at the same time selecting for the survival of cells with apoptosis resistant mutations or apoptosis resistant epigenetic changes and thereby contributing to malignant development. The level of DCA-induced apoptosis in SKGT4 cells was low, as that previously observed *in vivo* in Barrett's epithelium (Wetscher et al. 1998). The biological effects of DCA on SKGT4 cells clearly resemble those observed *in vivo*, supporting the use of SKGT4 cells as a relevant model for DCA-induced oesophageal transformation.

The levels of DCA-induced apoptosis in SKGT4 cells are considerably lower as compared to that induced by staurosporine **(Fig 4.6A and 4.6B).** Staurosporine induces apoptosis by acting directly on mitochondria via both caspase dependent and independent mechanisms (Bossy-Wetzel et al. 1998; Daugas et al. 2000). Here we show that caspase-3 is involved in DCA-induced apoptosis **(Fig 4.8)**. However, other caspase-independent mechanisms seem to be required as judged by the partial inhibition of DCA-induced DNA fragmentation by the caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK **(Fig 4.8B)**. DCA has been shown to induce apoptosis via ligand independent activation of the death receptors TRAIL and Fas in hepatocytes and colonic cells (Faubion et al. 1999; Schlottman et al. 2000). The low levels of apoptosis induced by DCA in SKGT4 cells might result from simultaneous stimulation of compensatory mechanisms. For example, in hepatocytes, DCA stimulates ligand independent activation of Fas, which results in low levels of apoptosis due to the concomitant induction of anti-apoptotic proteins such as PUMA and cFLIP (Qiao et al. 2001b). In cholangiocarcinoma cells, bile acids induce the expression of the anti-apoptotic protein Mcl-1, which decreases Fas induced apoptosis (Yoon et al. 2002b). It would of interest to assess the induction of PUMA, cFLIP and Mcl-1 in response to DCA in SKGT4 cells.

Recent studies have demonstrated that DCA can induce NF-κB in SKGT4 cells (data not shown) (Abdel-Latif et al. 2004) and in other oesophageal cells (Jenkins et al. 2004). NF-κB is known to have inhibitory effects on caspases, in particular caspase-3, via the activation of inhibitors of apoptosis (IAPs) (LaCasse et al. 1998). DCA-induced PARP

cleavage is dependent on caspase-3 activation **(Fig 4.8)** (Lazebnik et al. 1994). Therefore, simultaneous activation of caspase-3 and NF-κB explains the observed low levels of PARP cleavage induced by DCA. Glinghammar *et al* observed that in response to DCA, colonic cells undergo apoptosis and have low caspase-3 activation, strong activation of NF-κB and AP-1 transcription factors, and COX-2 expression. Our findings correlate with Glinghammar *et al* as we demonstrate that in SKGT4 cells exposure to DCA results in low levels of caspase-3-dependent PARP cleavage, activation of NF-κB (data not shown) and AP-1 **(Fig 5.1, 5.2 and 5.3)**, and substantial induction COX-2 expression **(Fig 4.9)**.

Overexpression of COX-2 inhibits apoptosis and increases invasiveness of malignant cells (Tsujii et al. 1997), implicating this enzyme in increased resistance to apoptosis. In SKGT4 cells, DCA induces COX-2 expression and PARP cleavage with similar kinetics and dose dependency **(Fig 4.9)**. As demonstrated in colonic cells and hepatocytes (Rust et al. 2000; Qiao et al. 2001a), the concurrent induction of pro- and anti-apoptotic signals by bile acids represents an important mechanism for bile acid-induced tumour promotion. Bile acid-stimulated survival signals such as induction of COX-2 expression coupled with a reduced capacity to undergo apoptosis will shift the balance from pro-apoptotic to anti-apoptotic signalling and facilitate the survival of pre-malignant cells. For example, in intestinal epithelial cells, overexpression of COX-2 leads to elevated levels of the anti-apoptotic protein Bcl-2 and increased cell survival (Tsujii and DuBois 1995). Induction of COX-2 by low concentrations of DCA mediates a survival mechanism. This is lost at higher concentrations due to the inherent cytotoxic effects of DCA as shown by increased PARP cleavage and DNA fragmentation **(Fig 4.9)**. In oesophageal aspirates, the concentration of unconjugated bile acids including that of DCA is usually not in excess of 300 μM (Nehra et al. 1999). This suggests that the induction of COX-2 by DCA that is maximal at 300 μM, is a physiological relevant process and might account for the reduced level of apoptosis observed in Barrett's oesophagus following reflux (Wetscher et al. 1998).

COX-2 is overexpressed in oesophageal cancer and in animal models of reflux (Wilson et al. 1998; Shirvani et al. 2000; Zhang et al. 2001a). Reduced apoptosis is a hallmark of oesophageal cancer (Wetscher et al. 1998; Whittles et al. 1999). Inhibition of DCA-induced COX-2 by aspirin is accompanied by an increase in DCA-induced PARP cleavage **(Fig 4.11 and 4.12)** and DNA fragmentation **(Fig 4.12)** confirming that DCA-induced

COX-2 is involved in SKGT4 cell survival. As mentioned above, other survival pathways beside COX-2 might also be activated in response to DCA. A possible candidate is Akt, as this has been shown to be activated by glycochenodeoxycholic acid in oesophageal cells (Jaiswal et al. 2004) and is also inhibited by aspirin (Gao et al. 2003). Current treatment of Barrett's patients involves acid suppression therapy (e.g. proton pump inhibitors, PPIs), which reduces but does not abolish reflux and therefore does not eliminate exposure to bile acids. PPIs have been shown to increase the relative concentration of unconjugated bile acids such as DCA in the refluxate (Theisen et al. 2000). The present data demonstrates that aspirin is a potent inhibitor of DCA-induced oesophageal cell survival and indicates that administration of aspirin in conjunction with PPIs should be considered in future treatment strategies.

COX-2 is the inducible rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins, which modulate cell growth through the induction of specific genes and cytokines (Dubois et al. 1998; Taketo 1998). Animal models of reflux show increased expression of COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) protein as well as increased mRNA expression of the G-coupled PGE receptors EP_2, EP_3 and EP_4 (Jang et al. 2004). Increased expression of PGE_2 has been shown in response to bile acids such as CDCA and DCA in SKGT4 oesophageal cells (Zhang et al. 1998). It has been proposed that PGE_2 mediates cell survival through induction of IL-6 (Fosslien 2000). Expression of IL-6 can also be regulated by NF-κB (Baeuerle and Henkel 1994). Induction of IL-6 expression via COX-2-PGE_2 and/or NF-κB might explain the survival effect exerted by DCA on SKGT4 cells. This is supported by reports showing increased expression of IL-6 and IL-6 receptor alpha in oesophageal carcinoma tissue (Leu et al. 2003), which is resistant to staurosporine-induced apoptosis through induction of Mcl-1 (Leu et al. 2000).

In summary DCA induces pro-survival mechanisms in tandem with apoptotic programs in oesophageal cells **(Fig 4.13)**, that is similar to that described in other cell types (Rust et al. 2000; Qiao et al. 2001b). Physiological concentrations of DCA mediate a COX-2 dependent survival signal that shifts the balance between anti-apoptotic and pro-apoptotic signals in favour of the survival of pre-malignant cells. This is reflected by the low rates of apoptosis induced by DCA in SKGT4 cells and Barrett's epithelium (Wetscher et al. 1998). The survival advantage mediated by COX-2 is also illustrated in animal models of

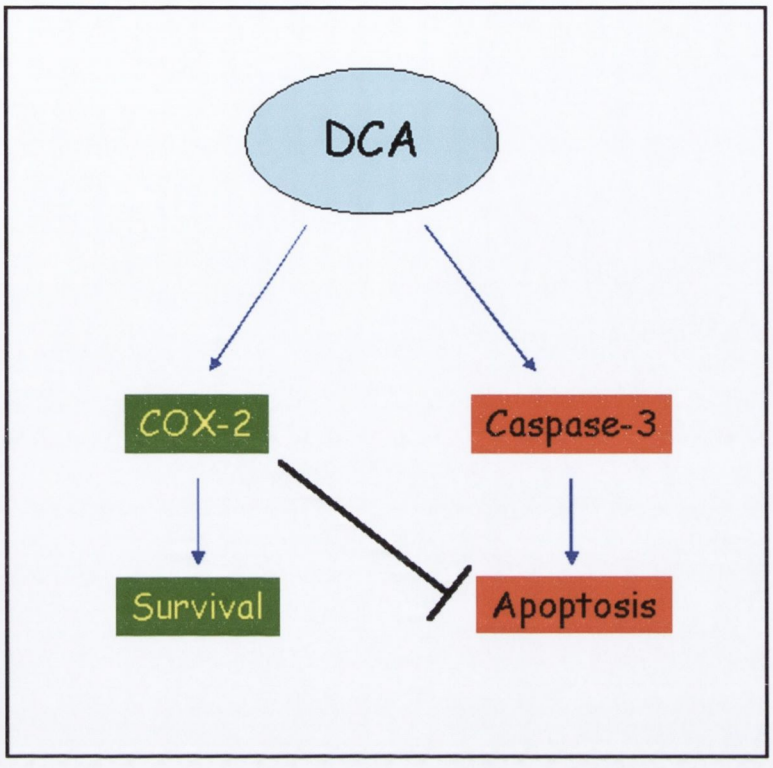


Fig 4.13. DCA-Induced Pro-Survival and Pro-Apoptotic Cascades. DCA induced oesophageal cell survival is mediated by COX-2 expression. In parallel, DCA induces apoptosis via caspase-3 dependent mechanism. Inhibition of DCA-induced COX-2 expression amplifies DCA-induced apoptosis.

reflux, where COX-2 inhibitors prevent oesophageal adenocarcinoma development (Buttar et al. 2002). Repeated exposure to bile acids during reflux, will allow for the accumulation and selection of apoptosis resistant cells resulting in compensatory hyperproliferation, as proposed in colonic cells (Martinez et al. 1998; Bernstein et al. 1999a; Bernstein et al. 2002). This is in agreement in *ex vivo* studies where bile acids induced hyperproliferation in oesophageal biopsies (Kaur et al. 2000). From the data presented in this chapter, we propose that DCA-induced COX-2 expression favours malignant growth and resistance to apoptosis in oesophageal cells. Modulation of the apoptotic cascades by anti-apoptotic signals is likely to promote malignant transformation and account for increased cancer risk.

CHAPTER V

DEOXYCHOLATE INDUCED AP-1 ACTIVATION IS MEDIATED BY MAPKS IN OESOPHAGEAL EPITHELIAL CELLS

CHAPTER V

5.1 INTRODUCTION

5.1.1 Deoxycholate And AP-1

The progression from Barrett's metaplasia to adenocarcinoma is associated with the acquisition of an apoptosis resistant phenotype (van der Woude et al. 2002). Bile acids, such as DCA, are constituents of the refluxate and are regarded to have an important role in malignant development in the oesophagus. The exact molecular mechanism by which bile acids contribute to this process has not been defined. Alterations in gene expression underlie the ability of deoxycholate to deregulate biochemical processes and control the fate of the cells (Qiao et al. 2002). DCA regulates gene transcription by controlling the activity of transcription factors such as AP-1 (Qiao et al. 2000). The AP-1 complex is composed of dimers between the Fos (c-Fos, FosB, Fra-1 and Fra-2) and the Jun (c-Jun, JunB, and JunD) family members. Fos and Jun proteins can form heterodimers while only the members of the Jun family can form homodimers. Fos/Jun heterodimers are more stable than Jun homodimers (Karin et al. 1997). AP-1 dimer composition is critical in determining the functional activity of AP-1 and the target genes induced (Lallemand et al. 1997; Vallone et al. 1997; Bakiri et al. 2000; Shaulian and Karin 2002). AP-1 is a key regulator of cell functions such as proliferation, differentiation and apoptosis (Shaulian and Karin 2002). In addition a correlation between AP-1 and tumourgenesis has been suggested. Significantly, transactivation of AP-1 is required for tumour promotion *in vivo*. Transgenic mice expressing a dominant negative mutant of c-Jun in mouse skin were protected from the tumourigenic effects of 9,10-dimethyl-1,2-benzanthracene (DMBA)/PMA exposure (Young et al. 1999). The involvement of AP-1 in tumourgenesis is also supported by its responsiveness to tumour promoters and its increased activity in transformed cell lines (Eferl and Wagner 2003). During oncogenic transformation the AP-1 components frequently upregulated include Fra-1, Fra-2, c-Jun and JunB while the levels of c-Fos and FosB are generally unaffected (Mechta et al. 1997).

AP-1 is activated by variety of stimuli and can have both anti-apoptotic and pro-apoptotic functions depending on the cellular context (Shaulian and Karin 2002). In colonic cells DCA triggers apoptosis through AP-1-induced expression of the pro-apoptotic gene GADD153 (Qiao et al. 2002). In hepatocytes, DCA-induced AP-1 binding activity is

associated with hepatocytes survival (Qiao et al. 2003). Therefore alterations in the activity of transcriptional complexes such as AP-1 underlie the ability of DCA to deregulate biochemical processes and control cell fate.

5.1.2 DCA and MAPK

AP-1 activity can be regulated by *de novo* protein expression or by posttranslational phosphorylation of pre-existing Fos/Jun proteins through the activation of upstream phosphorylation pathways, principally the MAP kinase pathway (Gruda et al. 1994; Alblas et al. 1998). MAP kinase cascades are ubiquitous signal transduction pathways that regulate a wide variety of cellular functions including proliferation, differentiation and apoptosis (Robinson and Cobb 1997; Wada and Penninger 2004). Phosphorylation of AP-1 components by MAPKs induces transactivation and protein stabilization (Hurd et al. 2002; Vial and Marshall 2003). DCA utilises the MAPK cascade in a variety of cell types. In primary hepatocytes and colonic cells, DCA promotes cell survival through the induction of MAPKs (Qiao et al. 2001a; Qiao et al. 2001b). Deregulation of MAPKs has been observed in colonic cells resistant to DCA-induced apoptosis (Bernstein et al. 2004). Alterations in MAPK signalling are not only associated with bile acid mediated events but have been correlated with malignant progression. For example, constitutive activation of the MAPK Erk has been reported in human renal and breast malignancies (Oka et al. 1995; Sivaraman et al. 1997) and in metastatic oesophagogastric cancer (Barry et al. 2001). Furthermore several upstream regulators of MAPK activity, such as epidermal growth factor receptor (EGFR) and Ras (Robinson and Cobb 1997; Qiao et al. 2001b), are frequently overexpressed in oesophageal adenocarcinoma (Abdelatif et al. 1991; Jankowski et al. 1992; al-Kasspooles et al. 1993; Sorsdahl et al. 1994) implicating deregulation of the MAPK cascades in malignant progression in oesophageal cells. Bile acids are known to contribute to oesophageal carcinogenesis and can deregulate MAPK signalling in other cell types. Abnormal MAPK signalling is a feature of oesophageal malignant progression. These data suggest the possible involvement of bile acids in alterations to the MAPK cascade in oesophageal cancer cells, which to date has not been addressed.

The purpose of this study was to examine the molecular mechanisms underlying bile acid stimulated gene expression in oesophageal adenocarcinoma cells. Using electrophoretic mobility shift assays, we demonstrate that DCA induces persistent AP-1 DNA binding

activity. Further examination identified that the MAPK Erk1/2 and p38 pathways are activated by DCA. Importantly, activation of Erk1/2 and to a lesser extent p38 mediates DCA-induced activity of specific AP-1 components. Furthermore the activation of Erk1/2 and p38 is crucial for DCA-induced COX-2 expression, an AP-1 target gene. These data show an important role of MAPKs in mediating DCA-induced survival via COX-2 (Chapter IV).

5.2 OBJECTIVES

The aim of this chapter was to investigate DCA-induced signalling cascades leading to activation of the transcription factor AP-1 and to define the specific components of the DCA-induced AP-1 complex.

5.3 RESULTS

5.3.1 DCA, CDCA and UDCA Induce AP-1 Binding Activity

Different bile acids exert differential biological effects on colonic cell growth and proliferation (Martinez et al. 1998) as well as on AP-1 activity in colonic cells (Qiao et al. 2000). These differential effects could be explained by the activation of specific signalling pathways in response to each individual bile acid. Deregulation of AP-1 activation has been associated with oncogenic transformation in fibroblasts (Mechta et al. 1997; Cook et al. 1999) In HepG2 cells, incubation with DCA results in a decrease in cell proliferation and induction of multiple stress activated genes including c-Fos, a major component of AP-1 (Karin et al. 1997; Bernstein et al. 1999b). Similar findings have been observed in other intestinal cell lines (Glinghammar et al. 2002). Therefore the effects of different bile acids on AP-1 activation in SKGT4 cells were examined. Chenodeoxycholate (CDCA), a secondary bile acid, has been shown to induce AP-1 binding activity in SKGT4 cells (Zhang et al. 1998; Zhang et al. 1999) and was therefore used as a positive control for these studies. To establish if DCA and UDCA are capable of inducing the formation of AP-1 complexes, SKGT4 cells were treated with DCA or UDCA for 6 hr at 300 μM after which AP-1 binding activity to the AP-1 DNA probe was assessed by electrophoretic mobility shift assay (EMSA). Notably DCA and CDCA caused marked induction, whereas UDCA caused a weak induction of AP-1 binding activity **(Fig 5.1A)**. This effect was not cell line specific as identical results were observed in another oesophageal adenocarcinoma cell line, OE-33 **(Fig 5.1B)**.

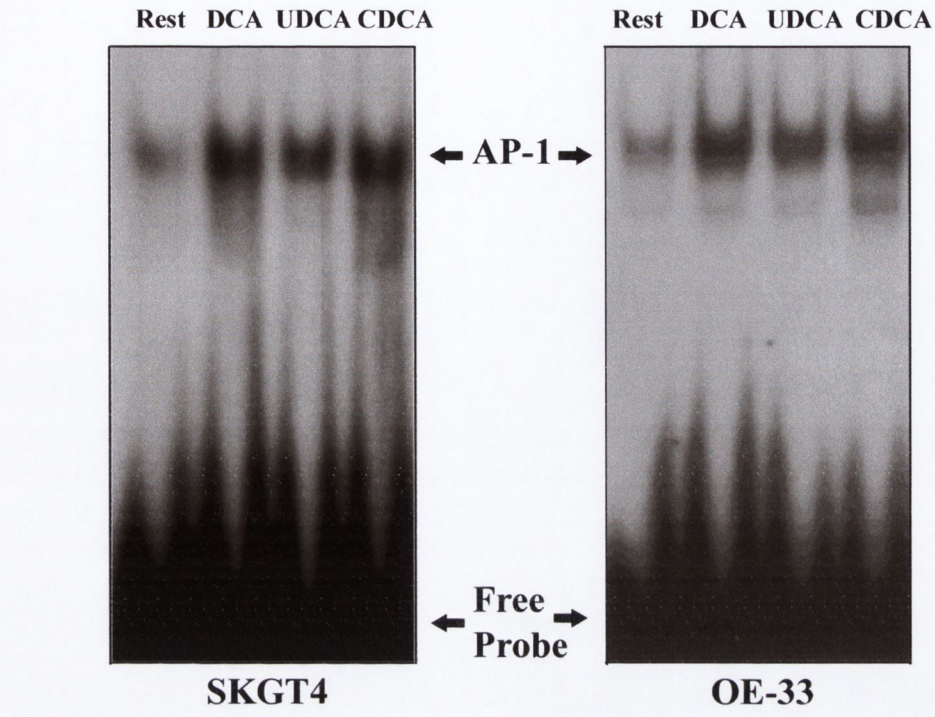


Fig 5.1. DCA, CDCA and UDCA Induce AP-1 Binding Activity. (A) SKGT4 cells were treated for 6 hr with 300 μM DCA, 300 μM UDCA or 400 μM CDCA, which has been previously shown to induce AP-1 activity in SKGT4 cells. (B) OE-33, an esophageal adenocarcinoma cell line was treated for 6 hr with 300 μM DCA, 300 μM UDCA or 400 μM CDCA. In both panels, 4 μg of nuclear extract were analysed by EMSA. Results are representative of at least three independent experiments.

5.3.2 Kinetics of DCA-Induced AP-1 Binding Activity in SKGT4 Cells.

To determine if DCA-induced AP-1 activation was time dependent, the kinetics of DCA-induced AP-1 activation were examined. SKGT4 cells were exposed to 300 μM DCA from 1 – 6 hr and then analysed for AP-1 DNA binding activity by EMSA. As a positive control for AP-1 induction the gastric adenocarcinoma cell line, AGS, was treated with the phorbol ester PMA for 2 hr (Naumann et al. 1999). DCA treatment induced AP-1 DNA binding activity as compared to resting SKGT4 cells, in a time dependent manner **(Fig 5.2)**. DCA-induced AP-1 activation was biphasic. AP-1 activity was markedly induced after 1 hr of stimulation and peaked again at 6 hr. At later time points, 12 hr and 24 hr, AP-1 activity returned to basal levels **(Fig 5.2)**.

5.3.3 Fra-1, c-Jun and JunD are Members of the DCA-Induced AP-1 Complex.

AP-1 dimer composition is crucial in determining the induction of specific target genes and consequent cellular responses. For example, in NIH3T3 fibroblasts, expression of Fra-1 and c-Jun is critical in establishing Ras mediated transformation (Mechta et al. 1997), while in fibroblasts overexpression of JunB antagonises c-Jun mediated expression of cyclin D1. Nuclear extracts, from SKGT4 cells stimulated with DCA, were incubated with respective blocking antibodies prior to incubation with the radiolabelled AP-1 probe. Blocking antibodies to c-Fos, Fra-1, c-Jun and JunD were used which prevent the binding of each transcription factor to the AP-1 complex rather than causing a supershift (Hutchinson and McCloskey 1995). Antibodies against Fra-1, c-Jun and JunD caused a reduction in AP-1 binding activity. The addition of a pool of all the antibodies completely abrogated the DCA-induced AP-1 complex. The specificity of the DCA-induced AP-1 complex was further verified by the addition of unlabeled AP-1 oligonucleotide **(Fig 5.3)**. These studies suggest that Fra-1, c-Jun and JunD are members of the DCA-induced AP-1 complex **(Fig 5.3)**. Since the Fos family members lack the capacity to homodimerise (Karin et al. 1997), the three possible types of AP-1 complexes present in DCA treated cells are either Fra-1/c-Jun, Fra-1/JunD or c-Jun/JunD.

5.3.4 DCA Activates the MAPK Erk1/2

Induction of AP-1 complexes can be achieved through the activation of MAP kinase pathways, key regulators of AP-1 proteins (Cook et al. 1999; Shaulian and Karin 2002; Vial and Marshall 2003). The Erk1/2 family, a subgroup of MAPKs, has been shown to be responsive to bile acids such as DCA in several cell types including colonic cells, primary

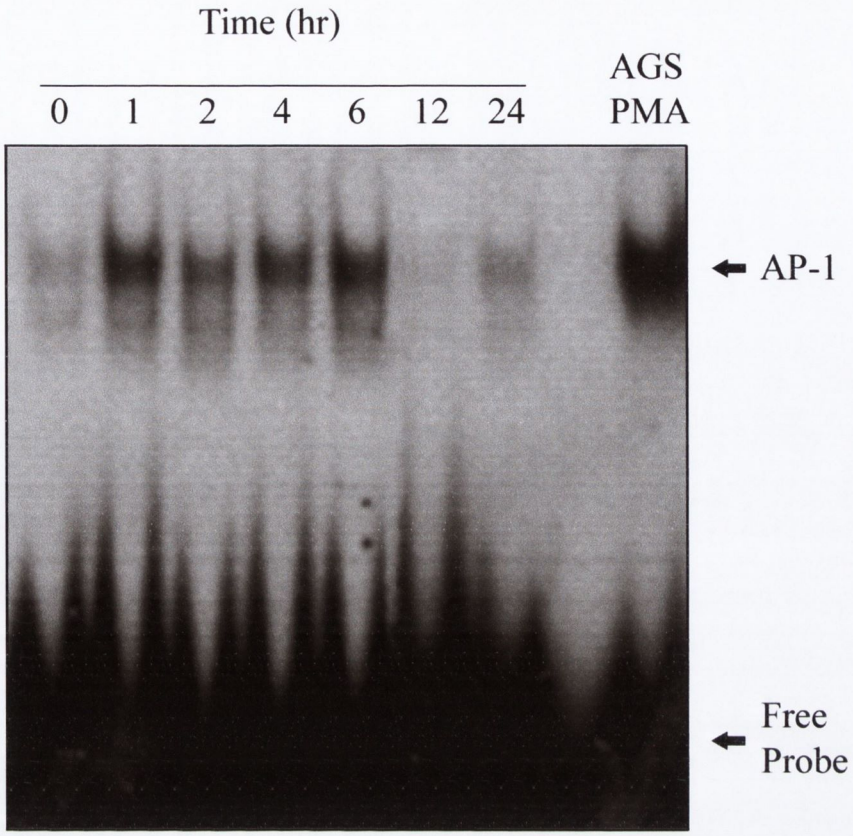


Fig 5.2. DCA Induces AP-1 Binding Activity in SKGT4 Cells. SKGT4 cells were stimulated with 300 μM DCA for 1 - 24 hr. Nuclear extracts (4 μg) were prepared and analysed for AP-1 binding activity by EMSA. Gastric adenocarcinoma cells (AGS) treated with 50 ng/ml PMA were used as a positive control. Results are representative of at least 3 independent experiments.

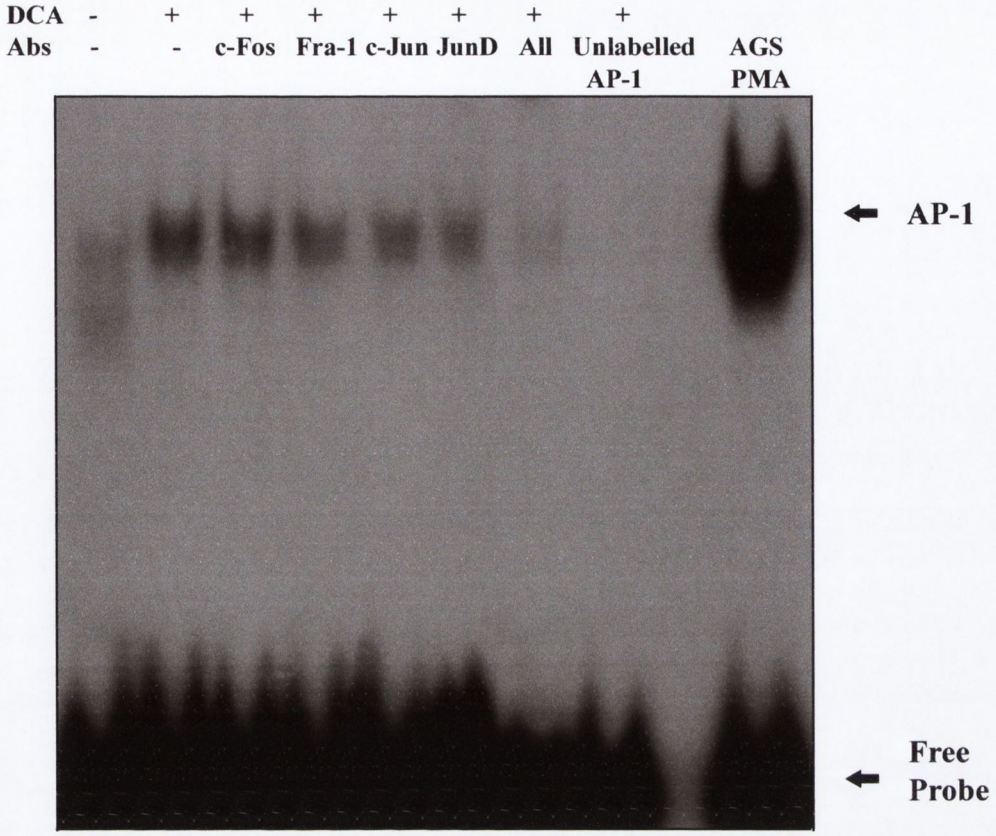


Fig 5.3. Fra-1, c-Jun and JunD are members of DCA induced AP-1 Complex. 4 μg of nuclear extract, derived from SKGT4 cells treated with 300 μM DCA for 6 h, were incubated for 30 min with 450 ng of antibodies against the various Jun and Fos proteins or with all the antibodies pooled as indicated. The extracts were then mixed with the radiolabelled AP-1 probe for another 30 min Unlabelled AP-1 probe was added to the nuclear extracts for 30 min, prior to the addition of radiolabelled probe, to verify the specificity of the DCA induced AP-1 complex. AGS cells treated with PMA (50 ng/ml) were used as a positive control for AP-1 binding. Results are representative of at least three independent experiments.

hepatocytes and cholangiocarcinoma cells, but has not been previously investigated in oesophageal cells (Qiao et al. 2001a; Qiao et al. 2001b; Nakahara et al. 2002). Acute activation of Erk1/2 has been reported in response to growth factor and oxidant stimulation in neuronal and human cervical carcinoma cells, respectively (Guyton et al. 1996; Wang et al. 1998). To analyse the possible role of the MAPK Erk1/2 in DCA-induced AP-1 complexes, the ability of DCA to activate Erk1/2 in SKGT4 oesophageal cells was investigated. Western blot analysis using a specific antibody that recognises active pErk1/2 (p-Tyr204-Erk1/2) was used on SKGT4 cell lysates. As illustrated in **Fig 5.4A**, DCA did not activate Erk1/2 within the first 5 minutes of treatment. Active Erk1/2 could be readily detected after 15 min and persisted up to 60 min. As a control for Erk1/2 activation, SKGT4 cells were treated with 50 ng/ml of phorbol 12, 13-dibutyrate (PdBu) for 30 min, which readily activates Erk1/2 **(Fig 5.4B)**. Cellular levels of Erk1/2 remained unaltered as shown by Western blotting using an anti-Erk1/2 antibody **(Fig 5.4B)**.

5.3.5 DCA Activates Erk1/2 Through Mek1/2

To address the mechanism of DCA-induced activation of Erk1/2, we tested the involvement of Mek1/2, an upstream activator of Erk1/2. SKGT4 cells were incubated with either 10 or 50 μM PD98059, a well known specific inhibitor of Mek1/2 (Davies et al. 2000), prior to the addition of 300 μM DCA. PdBu was included as a positive control for Erk1/2 activation. As previously shown in **Fig 5.4**, DCA induces activation of Erk1/2 at 15 and 60 min. Both basal and DCA-induced Erk1/2 activities were inhibited by both concentrations of PD98059 at 15 and 60 min **(Fig 5.5A)**. In addition 10 μM PD98059 effectively inhibited PdBu-induced Erk1/2 activity at 15 min, however the higher concentration of the inhibitor (50 μM) is required to inhibit PdBu-induced Erk1/2 activity at 60 min **(Fig 5.5A)**. Inhibition of DCA-induced Erk1/2 was further verified using the U0126 compound, a potent inhibitor of Mek1/2 (Davies et al. 2000; English and Cobb 2002). SKGT4 cells were pretreated with 13 μM U0126 prior to the addition of DCA (300 μM) or PdBu (50 ng/ml). DCA and PdBu induce rapid activation of Erk1/2 after 15 min, which is completely blocked by U0126 **(Fig 5.5B)**, verifying that DCA utilises Mek1/2 to induce Erk phosphorylation.

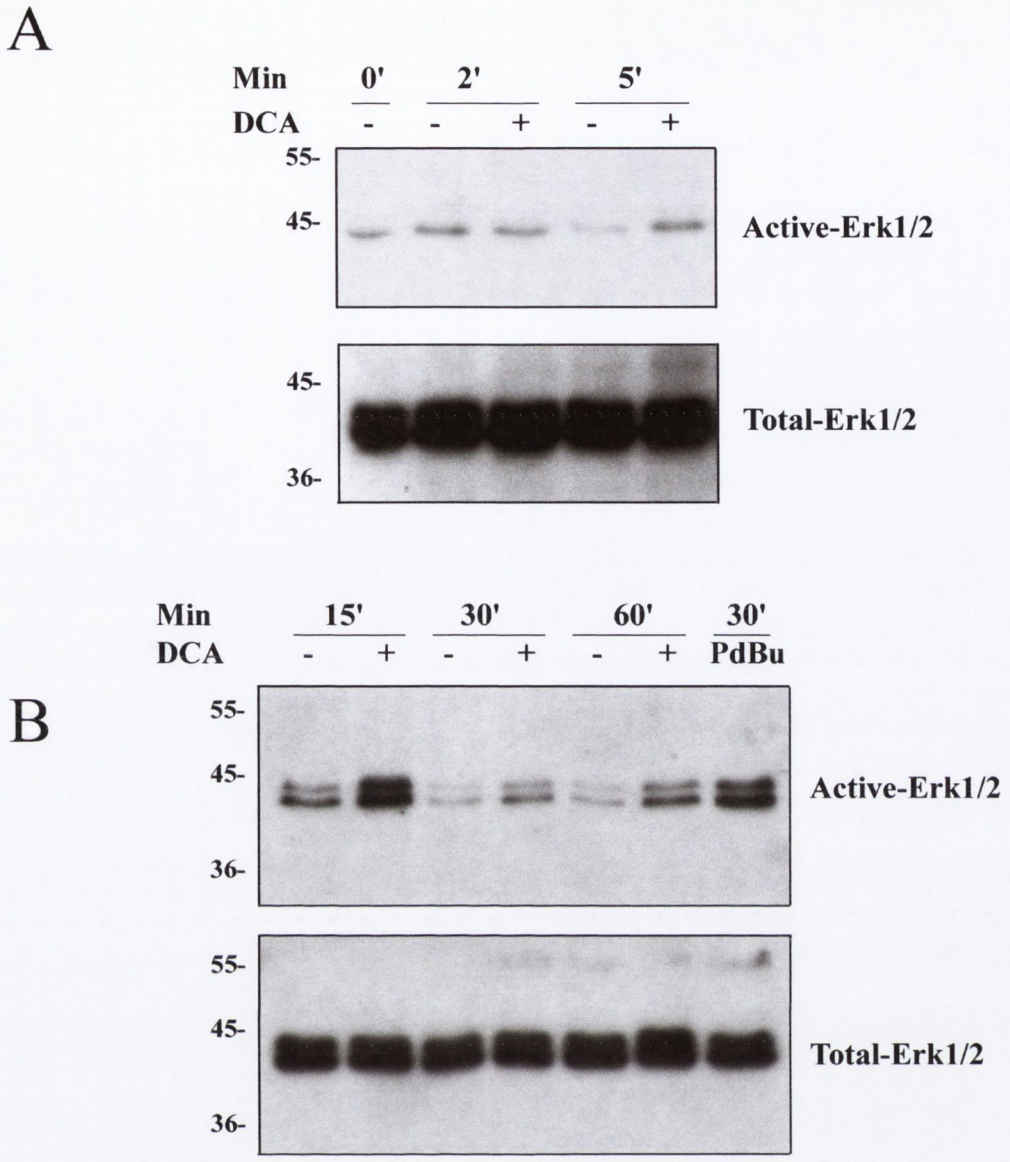


Fig 5.4. DCA Activates the MAPK Erk1/2. (**A**) SKGT4 cells were treated with 300 μM DCA over the time interval 0 – 5 min. Proteins were resolved by SDS-PAGE and immunoblotted with a phospho-specific Erk1/2 antibody followed by stripping and reprobing with an Erk1/2 antibody. (**B**) Cells were treated with 300 μM DCA for up to 60 min as indicated. As a control for Erk activation, SKGT4 cells were treated for 30 min with 50 ng/ml PdBu. Erk1/2 activities were assessed by Western blotting as above. Results are indicative of at least 3 independent experiments.

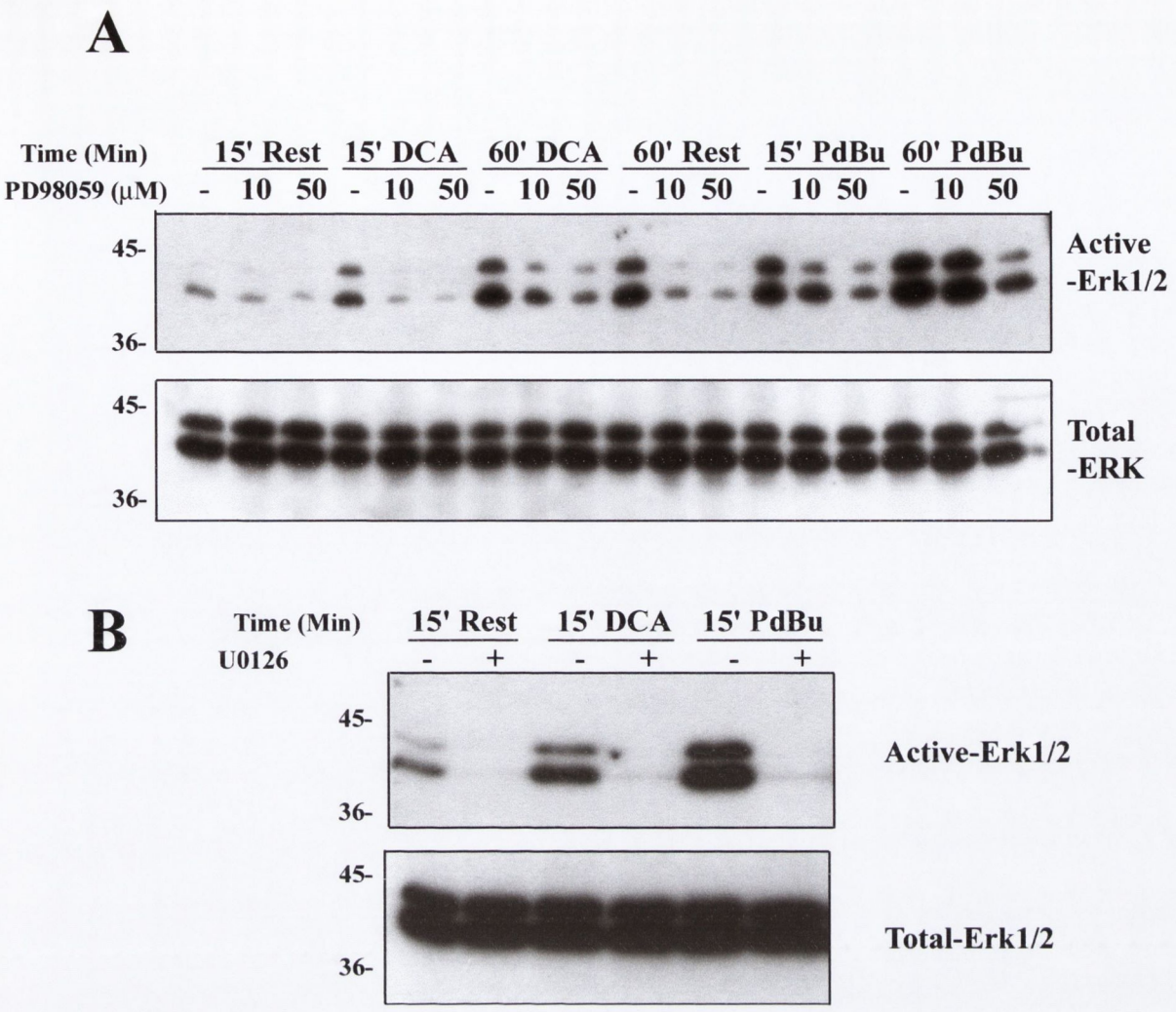


Fig 5.5. DCA Activates Erk1/2 Through Mek1/2. (**A**) SKGT4 cells were treated with 10 μM or 50 μM PD98059 for 30 min prior to the addition of either 300 μM DCA or 50 ng/ml PdBu at the indicated times. (**B**) SKGT4 cells were treated for 30 min with 13 μM UO126 prior to the addition of either DCA (300 μM) or PdBu (50 ng/ml) for 15 min. Total cellular proteins were standardised to 50 μg as described in chapter two and assessed for Erk1/2 activation by Western blotting using an anti-phospho-Erk1/2 antibody followed by stripping and reprobing with an Erk1/2 antibody. A representative experiment of three is shown.

5.3.6 DCA Induces Persistent Activation of Erk1/2 in a Dose Dependent Manner

The duration of MAPK activation is a key determinant in cell fate decisions. In PC12 cells, nerve growth factor stimulates sustained activation of Erk, which is required for cell cycle arrest and terminal differentiation (Cowley et al. 1994). While factors that stimulate transient activation of Erk do not promote differentiation (Marshall 1995). The extent of DCA-induced Erk activity was examined to establish if treatment with DCA results in transient or persistent activation. In SKGT4 cells, time course analysis shows that 300 μM DCA induces persistent activation of Erk1/2 for at least 6 hr **(Fig 5.6A)**. PD98059 completely abolishes both basal and DCA-induced Erk1/2 activity in these cells at all the time points tested **(Fig 5.6A)**. The persistent activation of Erk1/2 by DCA is dose dependent. Lower concentrations of DCA (100 – 200 μM) induce a mild increase in Erk1/2 phosphorylation above the basal level, while higher concentrations of DCA (300 – 500 μM) lead to strong activation of Erk1/2 as reflected by the levels of phosphorylation **(Fig 5.6B)**.

5.3.7 DCA Induces Sustained Activation of p38

Another member of the MAPK family, p38, is activated in response to a variety of cellular stress signals such as cisplatin and oxidant injury (Guyton et al. 1996; Wang et al. 2000b). Bile acids, including DCA, have been previously shown to activate p38 in colonic and hepatoma cell lines (Qiao et al. 2001b; Glinghammar et al. 2002). However, a link between DCA and p38 activation has never been addressed in oesophageal epithelial cells. Therefore, the status of p38 activation in SKGT4 cells after DCA stimulation was examined. SKGT4 cells were stimulated with DCA for 15 min to 6 hr. Activation of p38 was assessed on total cell lysates by Western blotting using a specific antibody that recognises active p38 (p-Thr180/Tyr182-p38). DCA induces sustained activation of p38, which is detected at 15 min and is still present after 6 hr of stimulation **(Fig 5.7A)**. The level of p38 activation is lower as compared to a well-known p38 activator, anisomycin (ANIS). Although DCA induces p38 activation, it does not influence its protein expression levels **(Fig 5.7A lower panel)**.

p38 is activated via the MAPK MKK3/6 (Robinson and Cobb 1997; Pearson et al. 2001). The SB203580 compound (4-[4'-fluorophenyl]-2[4'-methylsulfinylphenyl]-5-[4'-pyridyl] imidazole), a MKK3/6 inhibitor, was used to verify activation of p38 in response to DCA.

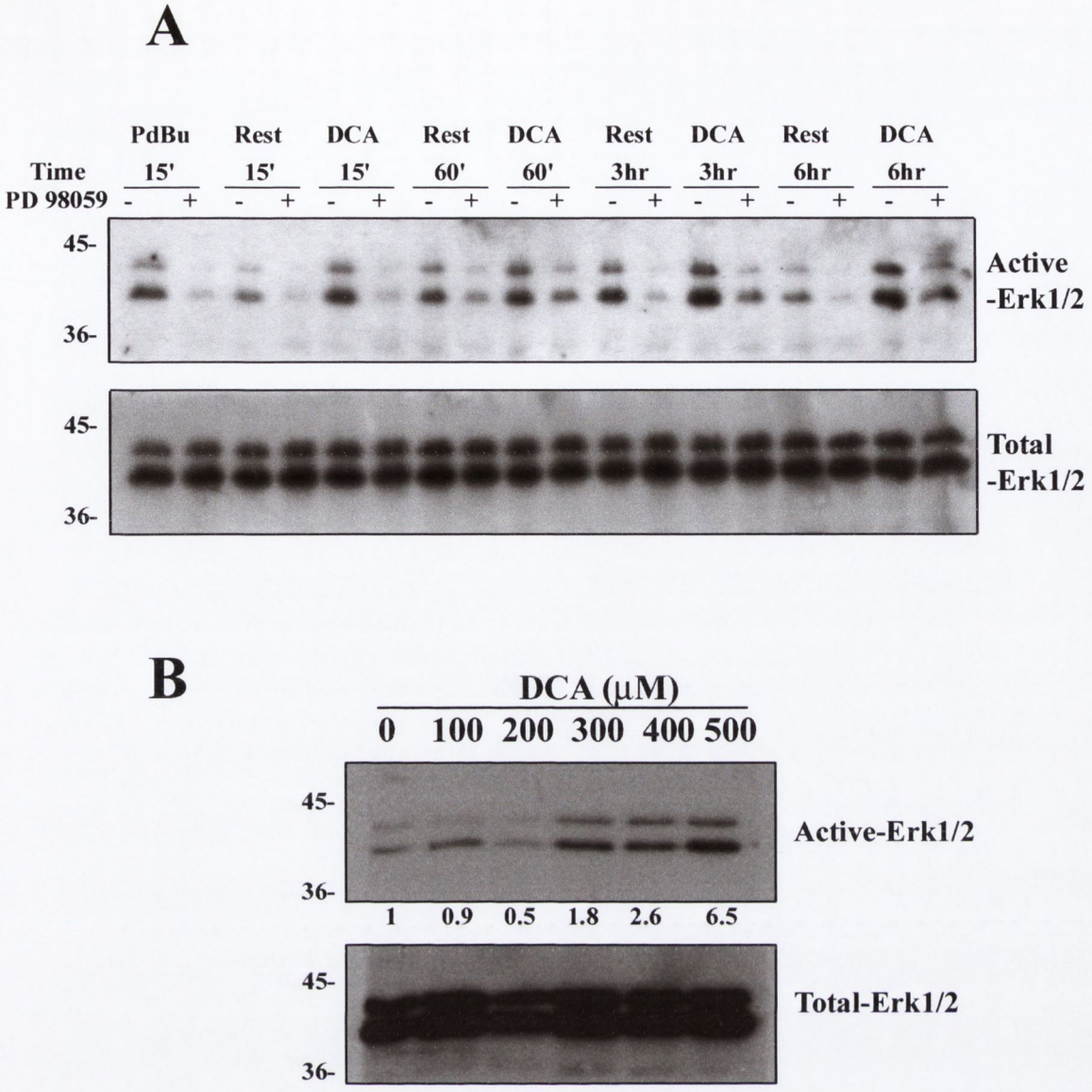


Fig 5.6. DCA Induces Persistent Activation of Erk1/2 in a Dose Dependent Manner. (**A**) DCA induces persistent activation of Erk1/2, which can be blocked by 50 μM PD98059. SKGT4 cells were treated with or without 50 μM PD98059 for 30 min prior to the addition of 300 μM DCA at the times indicated. PdBu was included as a control for ERK activation. (**B**) SKGT4 cells were treated with different concentrations of DCA, as indicated, for 6 hr. In both panels total cellular proteins were standardised to 50 μg as described in chapter two and assessed by Western blotting using antibodies specific for phospho-Erk1/2 or total Erk1/2, respectively. The levels of p-Erk1/2 were assessed by denisitometry and normalised with total Erk. Fold changes in p-Erk1/2 activity are given relative to the resting cells. Results are representative of at least three independent experiments.

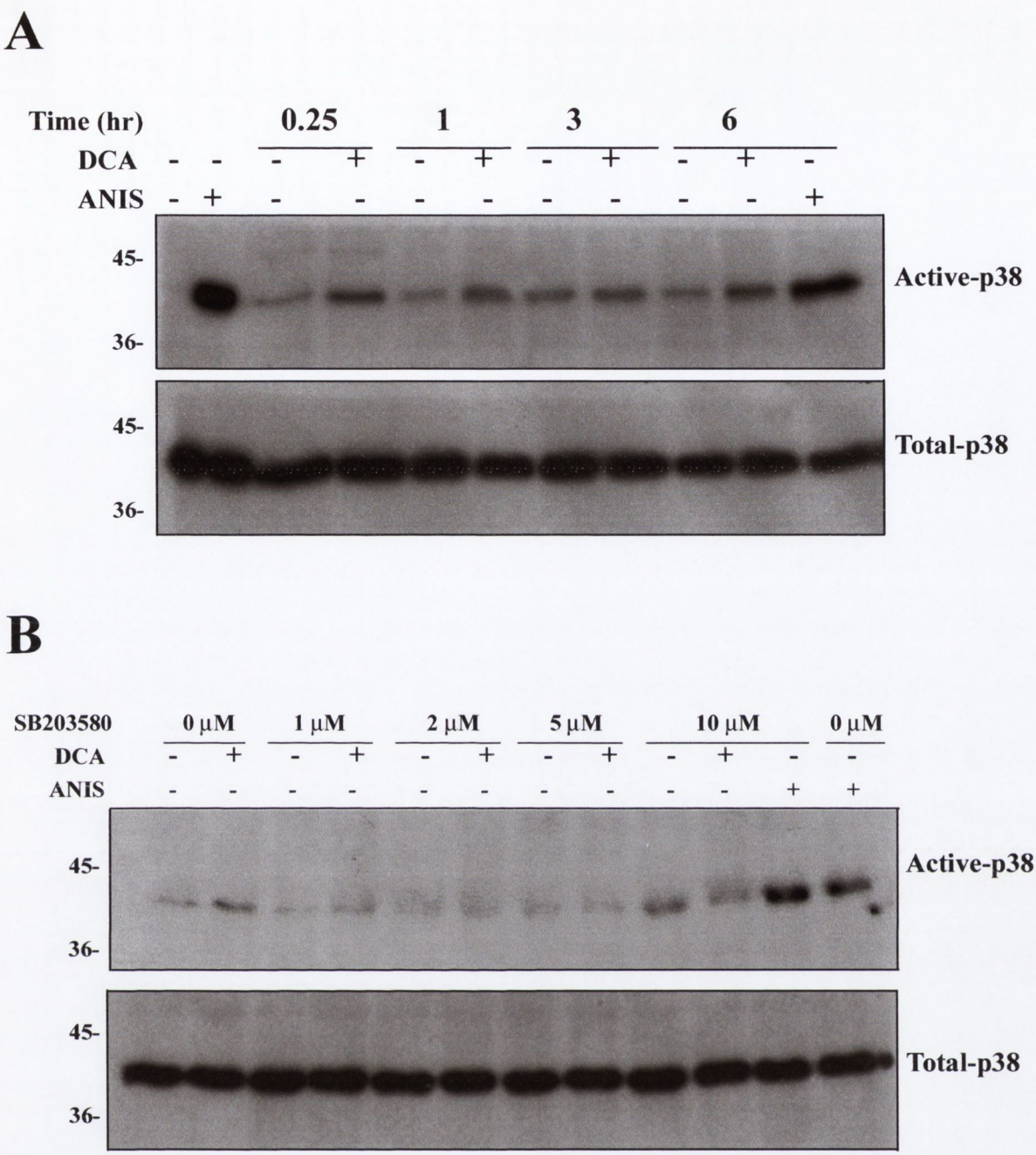


Fig 5.7. DCA Induces Sustained Activation of p38 (**A**) SKGT4 cells were treated with 300 μM DCA at the times indicated. The first two lanes contain control cell extracts. First lane contains resting C-6 glioma cells, which act as a negative control, and the second lane contains C-6 glioma cells activated with anisomycin (ANIS). As an internal control for p38 activation, SKGT4 cells were treated with anisomycin (10 μg/ml) for 6 hr. (**B**) SKGT4 cells were treated with varying concentrations of SB203580 for 30 min prior to addition of DCA or anisomycin for 60 min. In both panels, whole cell lysates were standardised to 50 μg and assessed by Western blotting using antibodies specific to the activated phosphorylated form of p38 or to total p38, respectively. A representative experiment of two is shown.

Previous reports have shown dose specific effects of the SB203580 compound against different stimuli (Lali et al. 2000). Therefore, a dose response of the SB203580 compound was performed to establish the optimal concentration that would inhibit p38 in response to DCA. SKGT4 cells were treated with the SB203580 compound at a concentration range of 0-10 μM, for 30 min prior to stimulation with 300 μM DCA for 60 min. Concentrations of 1, 2 and 5 μM effectively inhibit both basal and DCA-induced levels of p38 activity **(Fig 5.7B)**. The highest dose of the SB203580 compound (10 μM) does not inhibit p38 activation induced by DCA or anisomycin **(Fig 5.7B)**. Therefore, the SB203580 compound was used at 2 μM in all subsequent studies. SKGT4 cells were treated with 2 μM of the SB203580 compound for 30 minutes prior stimulation with DCA for 15 or 60 min. As shown above, this concentration of the SB203580 compound inhibits the activation of p38 by DCA at tested time points **(Fig 5.8)**.

5.3.8 DCA Stimulation Does Not Induce JNK Activation.

JNK, another member of the MAPK family, can be activated in response to cellular stress as well as bile acids. For example, in KMBC cells, a human cholangiocyte cell line, bile acids induce JNK activation, which mediates COX-2 protein expression (Yoon et al. 2002a) while in colonic cells, DCA treatment induces ERK and p38 but not JNK activation (Qiao et al. 2000). Since JNK activity has been implicated in carcinogenesis and apoptosis (Kyriakis et al. 1994; Gupta et al. 1996), the activation status of JNK after DCA stimulation was assessed. SKGT4 cells were stimulated with 300 μM DCA for 15 min to 6 hr and activation of JNK was assessed on total cell lysates by Western blotting using a specific antibody that recognises active JNK (p-Thr-183/Tyr-185-JNK). DCA does not stimulate JNK phosphorylation even after prolonged treatment, 6 hr, **(Fig 5.9)** while treatment with anisomycin readily induces sustained activation of JNK **(Fig 5.9)**. JNK protein levels remain unaltered following DCA stimulation **(Fig 5.9, lower panel)**. These results indicate that DCA is unable to induce JNK phosphorylation and consequently activation in SKGT4 cells.

5.3.9 DCA Induces the Expression of Fra-1, JunB and c-Jun Proteins.

Induction of AP-1 binding activity can be achieved not only through phosphorylation by activation of upstream signalling pathways but also by inducing *de novo* protein expression of the relevant Fos/Jun proteins. Initial results from EMSA assays suggested that Fra-1, c-

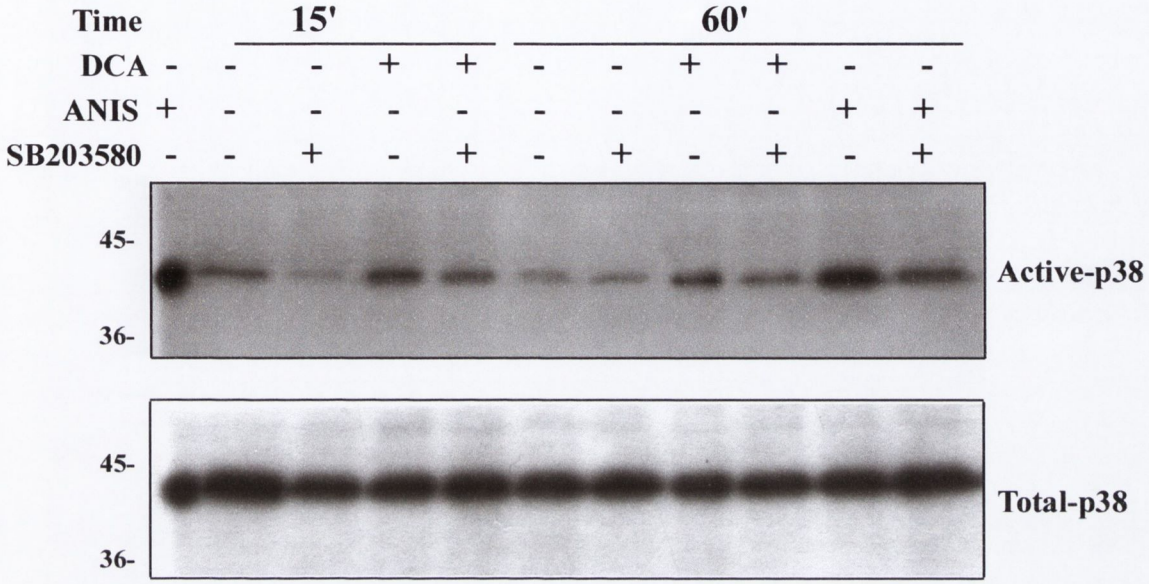


Fig 5.8. DCA Induces p38 Through MKK3/6. SKGT4 cells were treated with 2 μM SB203580 for 30 min prior to addition of 300 μM DCA or 10 μg/ml anisomycin (ANIS). Total cell lysates were prepared as described in chapter two and assessed by Western blotting using antibodies specific to the phosphorylated form of p38 or to total p38, respectively.

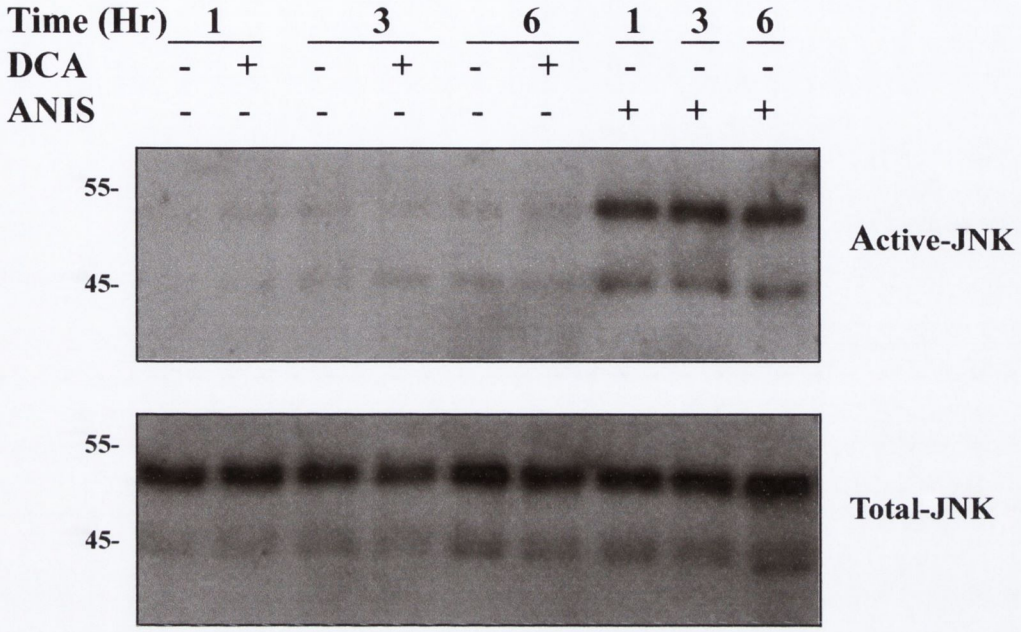


Fig 5.9. DCA Stimulation Does Not Induce JNK Activation. SKGT4 cells were treated with 300 μM DCA at the times indicated. As an internal control for JNK activation, cells were treated with 10 μg/ml anisomycin (ANIS) for identical periods of time. Whole cell lysates were standardised to 50 μg and examined by Western blotting using antibodies specific to the activated phosphorylated form of JNK or to total JNK. Similar experiments rendered identical results.

Jun and JunD were possible components of the DCA-induced AP-1 complex **(Fig 5.3)**. Therefore the protein expression of these molecules following DCA treatment was assessed to determine if AP-1 induction was achieved through posttranslational phosphorylation of pre-existing Fos/Jun proteins or through newly synthesised proteins. In addition to these components, the Jun protein, JunB, was also assessed as it has been shown that sustained MAPK activity, as that found in response to DCA **(Fig 5.6A and Fig 5.7A)**, induces expression of several AP-1 components (Cook et al. 1999; Gallo et al. 2002; Young et al. 2002; Casalino et al. 2003; Vial and Marshall 2003). SKGT4 cells were left untreated or treated with 300 μM DCA for 1 – 6 hr. DCA induces an increase in Fra-1 and JunB protein levels within 1 hour of stimulation, which remain constant throughout **(Fig 5.10)**. JunB is detected as three distinct bands while c-Jun is generally found as a doublet. Multiple electrophoretic mobility forms of JunB and c-Jun have previously been reported and have been attributed to different protein phosphorylation status (Papavassiliou et al. 1995; May et al. 1998; Cook et al. 1999; Gallo et al. 2002). DCA induces a smaller increase in c-Jun protein expression as compared to Fra-1 and JunB, which is diminished by 6 hr **(Fig 5.10)**. No change in the protein levels of JunD was detected in response to DCA (data not shown). These data show that DCA induces the protein expression of Fra-1, JunB and c-Jun, but does not have an effect on JunD protein levels, suggesting that if JunD is indeed a component of the DCA-induced AP-1 complex observed by EMSA **(Fig 5.3)**, this is possibly regulated through phosphorylation of a pre-existing protein pool of JunD.

5.3.10 DCA Induces Sustained Fra-1, JunB and c-Jun DNA Binding Activity

The AP-1 complex is composed of dimers between the Fos (c-Fos, FosB, Fra-1 and Fra-2) and the Jun (c-Jun, JunB, and JunD) family members. Fos and Jun proteins can form heterodimers while the only the members of the Jun family can form homodimers (Karin et al. 1997). Dimer composition is a key determinant of the specific target genes induced (Cook et al. 1999). To analyse the components of the DCA-induced AP-1 complex, total cell lysates were prepared from DCA treated SKGT4 cells and incubated with streptavidin-agarose beads linked to a double stranded biotinylated oligonucleotide containing the AP-1 consensus sequence (5'- CGC TTG ATG AGT CAG CCG GAA-3'), which specifically binds to active Fos/Jun proteins. Western blotting using antibodies specific to Fra-1, JunB and c-Jun identified the members of the DCA-induced AP-1 complex. DNA affinity purification experiments showed that DCA stimulated a time dependent increase in Fra-1, JunB and c-Jun DNA binding activity **(Fig 5.11)**. DCA strongly induces Fra-1 binding

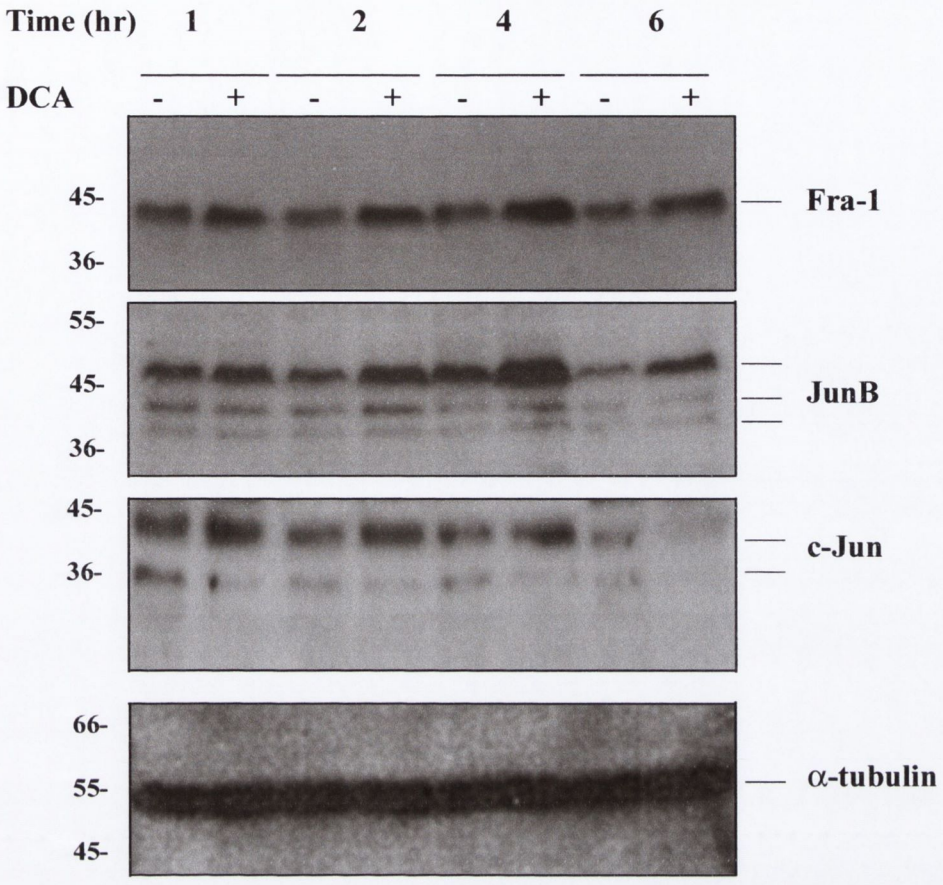


Fig 5.10. DCA Induces the Expression of Fra-1, JunB and c-Jun Proteins. SKGT4 cells were treated with 300 μM DCA from 1 – 6 hr. Whole cell lysates were standardised to 50 μg and examined by Western blotting using anti-Fra-1, anti-JunB and anti-c-Jun antibodies followed by anti-α-tubulin as a loading control. Equivalent experiments gave identical results.

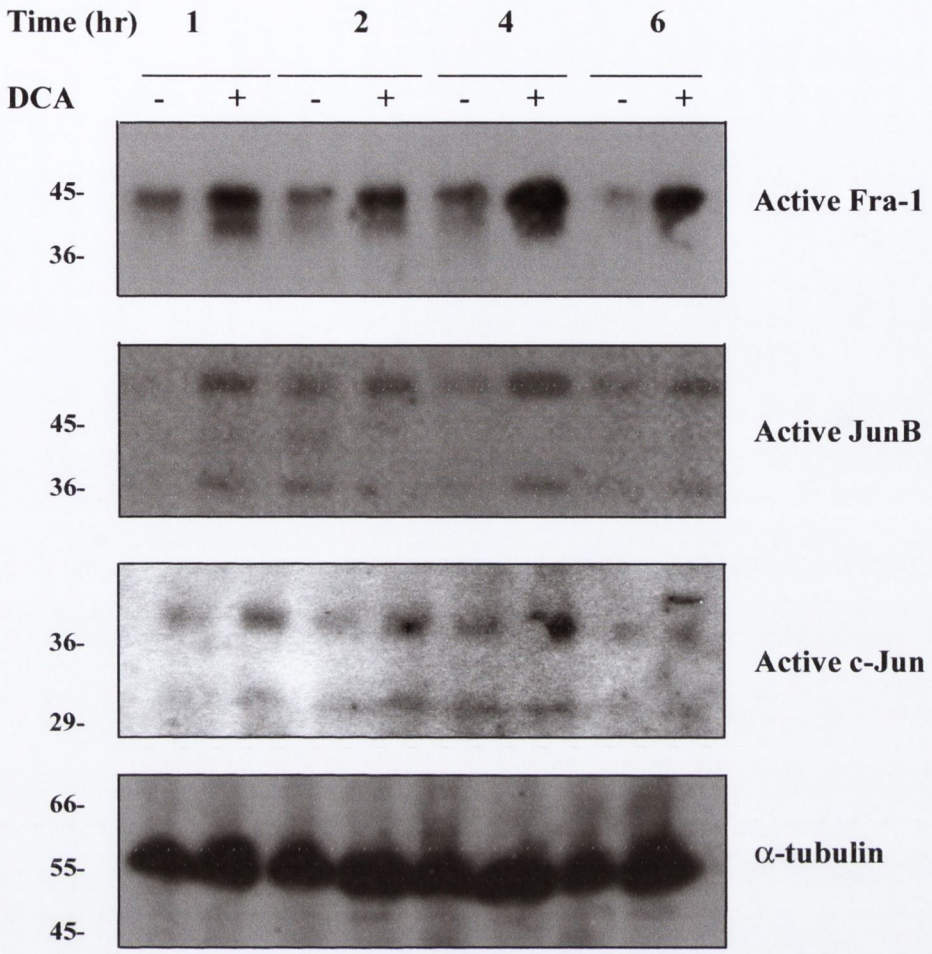


Fig 5.11. DCA Induces sustained Fra-1, JunB and c-Jun DNA binding activity. SKGT4 cells were treated with 300 μM DCA at the times indicated. Total cell lysates were prepared as described in chapter two and standardised to 350 μg. DNA affinity purification was performed by linking a biotinylated oligonucleotide containing the AP-1 consensus sequence to streptavidin-agarose beads. Purified proteins were resolved by SDS-PAGE and immunoblotted with anti-Fra-1, anti-JunB or anti-c-Jun antibodies, respectively. Unbound proteins were immunoblotted with anti-α-tubulin as loading control. Results are representative of at least three independent experiments.

activity after 1 hr, which is sustained for at least 6 hr. Fra-1 is detected as two bands with distinct electrophoretic mobility: a slower migrating, more prominent band and a fainter faster migrating band **(Fig 5.11)**. After prolonged stimulation, the slower species is stabilised while the faster migrating species is no longer detected **(Fig 5.11).** These two electrophoretic mobility forms of Fra-1, which most likely correspond to different phosphorylation states, have been previously reported in other cell types (Gruda et al. 1994; Cook et al. 1999; Casalino et al. 2003; Vial and Marshall 2003). Similarly, DCA stimulates strong JunB activity at 1 hr of stimulation **(Fig 5.11)**. JunB activity remains elevated for up to 6 hr **(Fig 5.11).** DCA induces weak activation of c-Jun in comparison to untreated cells, which is maximal at 4 hr (Fig 5.11). Since c-Jun activation at 6 hr is consistently weak and sometimes negligible, the AP-1 components Fra-1 and JunB were assessed in all subsequent studies. Collectively these data show that the kinetics of the DCA-stimulated Fra-1, JunB and c-Jun DNA binding activity correlates strongly with the kinetics of protein induction of these components. Furthermore the expression and activation of Fra-1 and JunB correlates with the duration of the MAPK activation.

5.3.11 DCA-Induced Fra-1 and JunB Activation is Dose Dependent

Increased concentrations of bile acids in oesophageal aspirates have been associated with increased severity of disease. In patients with erosive oesophagitis and Barrett's oesophagus, increased concentrations of DCA, in excess of 200 μM, have been observed (Nehra et al. 1998; Nehra et al. 1999). Therefore the contribution of increased DCA concentrations on Fra-1 and JunB DNA binding activity was examined. SKGT4 cells were stimulated with 0 - 500 μM DCA for 6 hr. Total cell lysates were prepared from DCA treated SKGT4 cells and incubated with streptavidin-agarose beads linked to a double stranded biotinylated oligonucleotide containing the AP-1 consensus sequence, which specifically binds active Fos/Jun proteins. DCA induces a dose dependent increase in the DNA binding activity of Fra-1 and JunB **(Fig 5.12).** Low concentrations of DCA stimulate a modest increase while stronger activation of Fra-1 and JunB is detected at and above 300 μM DCA **(Fig 5.12)**. Activation of Fra-1 and JunB is consistent with Erk1/2 activation, which exhibited a similar dose dependency on DCA stimulation **(Fig 5.6B)**.

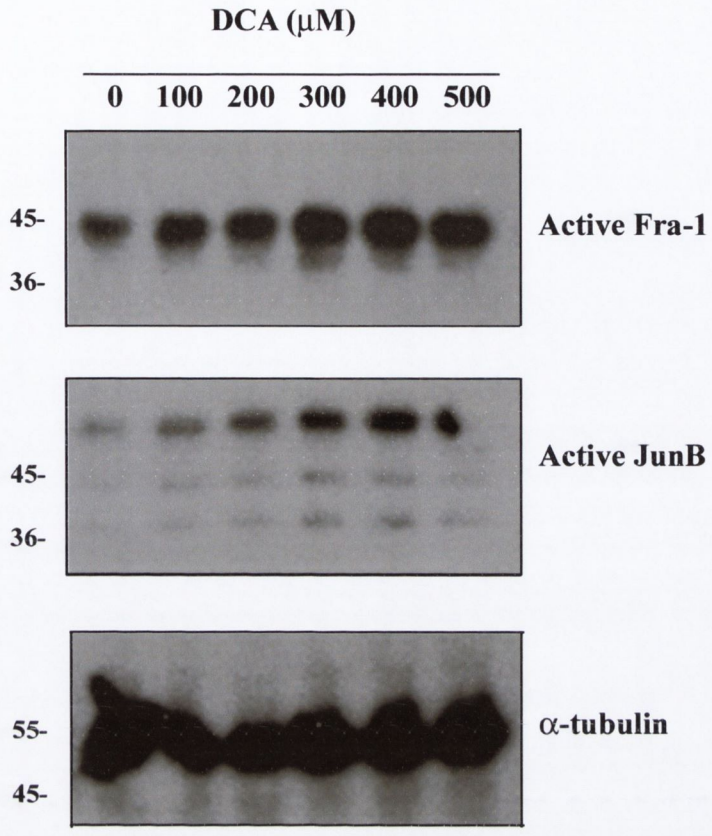


Fig 5.12. DCA-induced Fra-1 and JunB Activation is Dose Dependent. SKGT4 cells were treated with DCA over a concentration range of 0 – 500 μM for 6 hr. Total cell lysates were prepared as described in chapter two and standardised to 350 μg. DNA affinity purification was performed by linking a biotinylated oligonucleotide containing the AP-1 consensus sequence to streptavidin-agarose beads. Purified proteins were resolved by SDS-PAGE and immunoblotted with anti-Fra-1 or anti-JunB, respectively. Unbound proteins were immunoblotted with anti-α-tubulin as loading control. Results are representative of at least three independent experiments.

5.3.12 Fra-1 And JunB Activation in Response to DCA is Differentially Regulated Through Specific MAPK Pathways

The transcriptional activity and half life of some Fos and Jun proteins is regulated by upstream signalling pathways such as MAPKs giving rise to AP-1 dimers of different transcriptional specificity (Karin 1995; Whitmarsh and Davis 1996). In response to DCA, the kinetics of MAPK activation correlates with that of Fra-1 and JunB. The pharmacological inhibitors PD98059 and SB203580 were respectively used to evaluate the contribution of the Raf-Mek-MAPK and the MKK3/6-p38 pathways (Hommes et al. 2003) in DCA-induced DNA binding of Fra-1 and JunB. SKGT4 cells were pretreated with 10 μM PD98059 or 2 μM SB203580 for 30 min prior to addition of 300 μM DCA for 6 hr. Total cell lysates were prepared and proteins were purified by DNA affinity purification and assessed by Western blotting as described in chapter II. Pretreatment of SKGT4 cells with 10 μM PD98059 impairs and diminishes DCA-induced activation of Fra-1 and JunB, respectively **(Fig 5.13)** suggesting that activation of these transcription factors by DCA is dependent on the activity of the Raf-Mek-Erk cascade. The SB203580 compound completely abolishes DCA-induced Fra-1 while having no effect on DCA-induced JunB **(Fig 5.13)**. These data suggest that both Raf-Mek-Erk and MKK3/6-p38 are involved in DCA-induced Fra-1 activation, while only Raf-Mek-Erk is upstream of JunB activation.

As seen in chapter III, DCA can modulate the PKC-β_I and -ε in colonic cells and has been shown to require PKC activation to induce AP-1 DNA binding activity in the same cell line, HCT116 cells (Qiao et al. 2000). Studies in COS-1 cells and in NIH3T3 fibroblasts, demonstrated that PKC can induce Mek-Erk activation in response to PMA and EGF (Ueda et al. 1996). Expression of constitutively active PKC-α and PKC-β results in the activation of the c-Fos promoter and TPA response element (TRE) sites in rat fibroblasts and Jurkat cells (Hata et al. 1989; Muramatsu et al. 1992). PKC-α can activate MEKK1 (Soh et al. 1999) as well as Ras (Ueda et al. 1996). The data presented in chapter IV show that PKC-β_I is not activated by DCA in oesophageal cells; however it could still activate other PKC isoforms. Hence the PKC inhibitor GÖ6978, which inhibits the conventional PKCs (Davies et al. 2000), was utilised to assess any possible PKC contribution to the activation of members of the DCA-induced AP-1 complex. SKGT4 cells were treated with 1 μM GÖ6976 for 30 min prior to stimulation with 300 μM DCA. Proteins from total cell lysates were purified using a biotinylated AP-1 oligonucleotide and assessed by Western

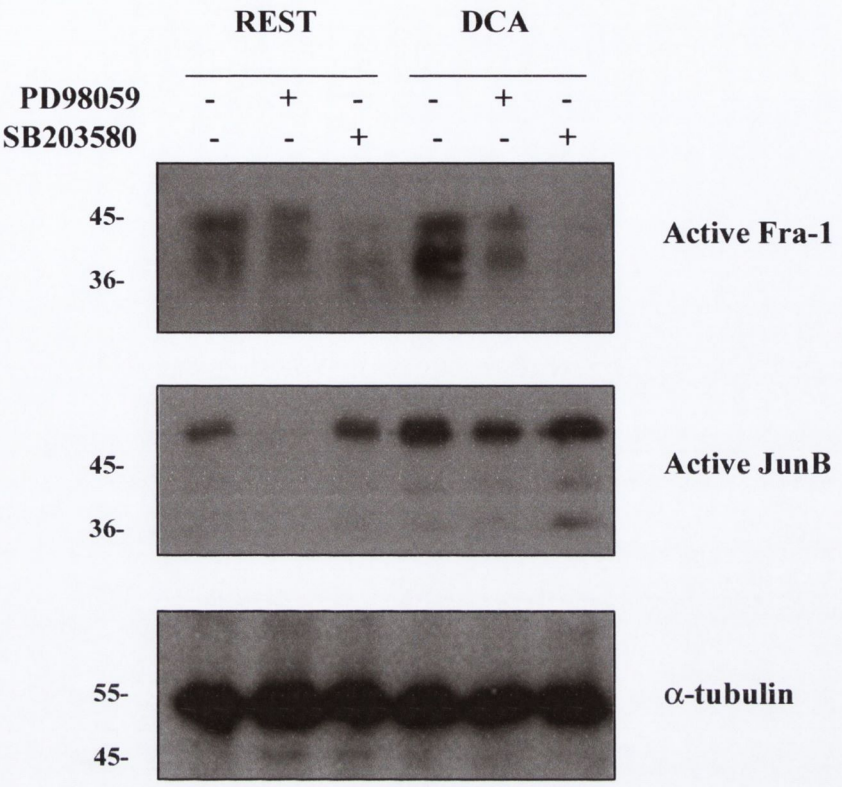


Fig 5.13. DCA-Induced Fra-1 and JunB are Differentially Regulated by Specific MAPK Pathways. SKGT4 cells were treated with PD98059 (10 μM) and SB203580 (2 μM) for 30 min prior to treatment with 300 μM DCA for 6 hr. Total cell lysates were prepared as described in chapter two and standardised to 350 μg. DNA affinity purification was conducted using a biotinylated oligonucleotide containing the AP-1 consensus sequence linked to streptavidin-agarose beads. Purified proteins were resolved by SDS-PAGE and immunoblotted with anti-Fra-1 or anti-JunB antibodies, respectively. Unbound proteins were immunoblotted with anti-α-tubulin as loading control. Representative results are shown.

blotting as above. DCA induced marked Fra-1 DNA binding activity in comparison to untreated cells, as previously observed **(Fig 5.11 and 5.12)**. Pretreatment with GÖ6976 reduces DCA-induced Fra-1 activity to basal levels **(Fig 5.14)** while activation of JunB is not inhibited (data not shown). Taken together these data indicate that DCA-induced Fra-1 activation is dependent on the activation of the conventional PKCs. Since SKGT4 cells, lack PKC-β_I **(Fig 4.1)** and PKC-γ (data not shown), the conventional PKC isoform, PKC-α, is a possible candidate for DCA-activated PKC.

5.3.13 Fra-1, JunB and c-Jun are Differentially Activated By CDCA and UDCA

DCA markedly induces Fra-1 and JunB DNA binding activity concomitant with weak activation of c-Jun **(Fig 5.11)**. As shown previously, CDCA can induce significant AP-1 DNA binding activity while UDCA stimulates only low levels of AP-1 activation **(Fig 5.1)**. The DNA binding activity of Fra-1, JunB and c-Jun in response to CDCA and UDCA was assessed by DNA affinity purification experiments in order to determine if composition of the active AP-1 dimer varied with the different bile acid stimulus. SKGT4 cells were stimulated with 400 μM CDCA and 300 μM UDCA for 6 hr and total cell lysates were purified using the biotinylated AP-1 oligonucleotide. Western blot analysis using antibodies specific to Fra-1, JunB and c-Jun identified the members of the induced AP-1 complexes. CDCA treatment results in strong activation of Fra-1 and JunB but not c-Jun in comparison to unstimulated SKGT4 cells **(Fig 5.15).** UDCA induces a mild increase in Fra-1 DNA binding, while stimulating a substantial increase in c-Jun activity but does not activate JunB **(Fig 5.15)**. Interestingly CDCA induces a similar pattern and level of activation as DCA while UDCA-induced pattern is distinctly different. These data indicate that CDCA induces Fra-1/JunB, or JunB/JunB dimers. On the other hand UDCA induces c-Jun/c-Jun or c-Jun/Fra-1 dimers.

5.3.14 DCA-Induced COX-2 Expression is Dependent on Erk1/2, p38 and PKC

AP-1 complexes can drive COX-2 protein expression by binding to the CRE site contained in the COX-2 gene promoter (Subbaramaiah et al. 2002). MAPKs can regulate COX-2 expression at a transcriptional and posttranscriptional level in intestinal epithelial cells (Dean et al. 1999; Guo et al. 2001). We and others have shown that DCA induces COX-2 protein expression in SKGT4 cells **(Fig 4.9-4.11)** and (Zhang et al. 1998). However the exact mechanisms responsible for DCA induced COX-2 expression has not been

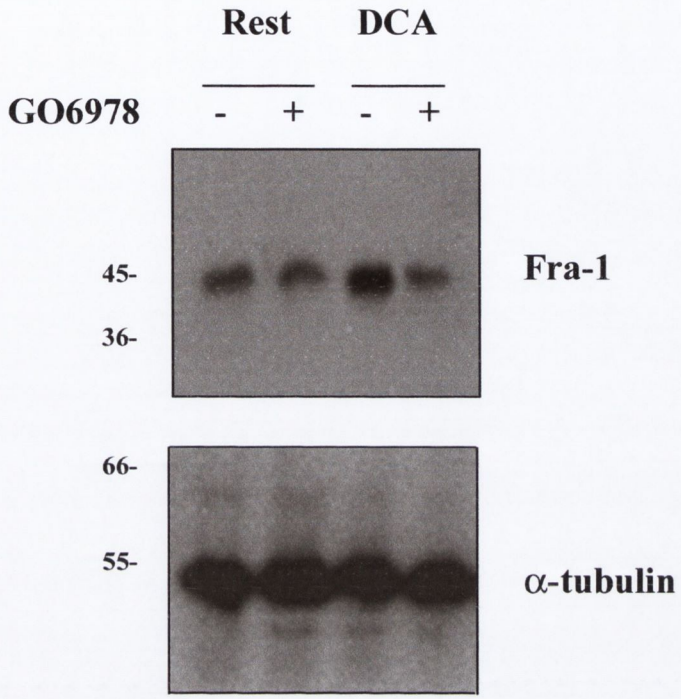


Fig 5.14. The GÖ6976 Compound Inhibits DCA-Induced Fra-1 Activation. SKGT4 cells were treated with GÖ6976 (1 μM) for 30 min prior to treatment with 300 μM DCA for 6 hr. Total cell lysates were prepared as described in chapter two and standardised to 350 μg. DNA affinity purification was conducted using a biotinylated oligonucleotide containing the AP-1 consensus sequence linked to streptavidin-agarose beads. Purified proteins were resolved by SDS-PAGE and immunoblotted with anti-Fra-1 antibody. Unbound proteins were probed with anti-α-tubulin as loading control. Representative results are shown.

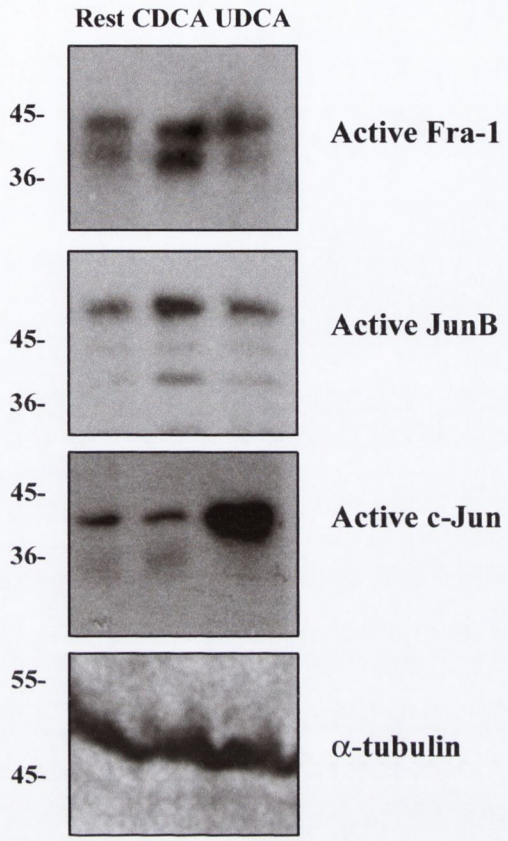


Fig 5.15. Fra-1, JunB and c-Jun are Differentially Activated by CDCA and UDCA. SKGT4 cells were treated with CDCA (400 μM) or UDCA (300 μM) for 6 hr at 37°C. Total cell lysates were prepared as described in chapter two and standardised to 350 μg. DNA affinity purification was performed and the purified proteins were resolved by SDS-PAGE and immunoblotted with anti-Fra-1, anti-JunB or anti-c-Jun antibodies, respectively. Unbound proteins were immunoblotted with anti-α-tubulin as loading control. Representative results of two independent experiments are shown.

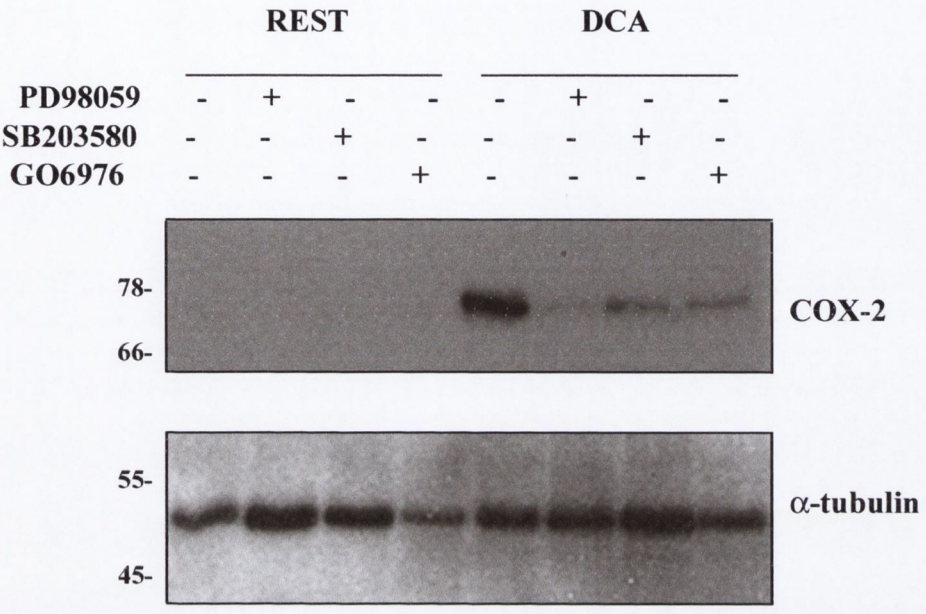


Fig 5.16. DCA-Induced COX-2 Expression is Dependent on Erk1/2, p38 and PKC. SKGT4 cells were treated with 10 μM PD98059, 2 μM SB203580 or 1 μM GÖ6976 for 30 min prior to the addition of 300 μM DCA for 6 hr. Total cell lysates were standardised to 50μg as described in chapter two and assessed by Western blotting using anti-COX-2 antibody and stripped and reprobed with anti-α-tubulin antibody as loading control. Results are representative of at least three independent experiments.

determined. Since we have shown that DCA induces AP-1 activity through the activation of Erk1/2 and p38 **(Fig 5.2, 5.6, 5.7, 5.11)** as well as PKC, we examined if DCA could drive COX-2 protein expression through these pathways. SKGT4 cells were pretreated with 10 μM PD98059, 2 μM SB203580 or 1 μM GÖ6976 for 30 min prior to addition of 300 μM DCA for 6 hr. Induction of COX-2 was assessed on total cell lysates by Western blotting using a specific anti-COX-2 antibody. DCA induces substantial COX-2 expression in comparison to untreated cells **(Fig 5.16)**. Pretreatment with the PD98059, SB203580 or GÖ6976 compounds inhibit DCA-induced COX-2 expression **(Fig 5.16).** These data suggest that the Erk and p38 pathways induced by DCA are not only responsible for Fra-1 and JunB activation but also for COX-2 induction. In addition DCA-induced COX-2 expression is also mediated by the conventional PKCs, most likely PKC-α, as suggested by the inhibition of DCA-induced COX-2 expression by GÖ6976 **(Fig 5.16)**. No cytotoxicity was observed with any of the inhibitors used (data not shown), confirming that the observed inhibition of COX-2 is a specific event.

5.4 DISCUSSION

Reflux of duodenal contents, of which DCA is a constituent, appears to contribute to the development of oesophagitis and Barrett's adenocarcinoma (Gillen et al. 1988; Kauer et al. 1995). This could be potentially mediated by bile acid deregulation of phosphorylation cascades leading to alterations in transcription factor expression and activity and consequently changes in gene expression patterns. In the present chapter, we show that the DCA activates the Raf-Mek-Erk and MKK3/6-p38 pathways, which in turn differentially regulate specific members of the AP-1 family of transcription factors. Persistent activation of Erk1/2 and p38 results not only in activation of specific members of the DCA-induced AP-1 complex but also in induction of COX-2 expression.

The specific receptor for DCA has not been precisely defined. Different sets of data have proposed the G protein-coupled receptor TGF-5 (Kawamata et al. 2003), the death receptors TRAIL and Fas (Schlottman et al. 2000), as well as EGFR as possible candidates (Yoon et al. 2002a; Im and Martinez 2004). EGFR expression and that of its downstream target H-Ras, is increased in oesophageal adenocarcinoma (Abdelatif et al. 1991; Jankowski et al. 1992; al-Kasspooles et al. 1993; Sorsdahl et al. 1994). In colonic adenocarcinoma cells and primary hepatocytes, DCA induces persistent activation of EGFR and Raf-1 resulting in activation of Erk1/2, p38 and AP-1 (Qiao et al. 2000; Qiao et

al. 2001b; Im and Martinez 2004). As DCA treatment results in EGFR upregulation in oesophageal cells (Jenkins et al. 2004), we rationalised that DCA might induce MAPK activation in these cells. Here we show that in response to 300 μM DCA, strong activation of Erk1/2 and p38 is detected after 15 min **(Fig 5.4 - 5.7)** while no activation of JNK is detected at any time **(Fig 5.9)**. These data were confirmed by effective inhibition of DCA-induced Erk1/2 and p38 activities using two highly selective inhibitors, PD98059 and SB203580, respectively **(Fig 5.5- 5.7)**. Similar findings have been reported in colonic cells, where treatment with 300 μM DCA induced Erk1/2 and p38 but not JNK activation (Qiao et al. 2000). These results demonstrate that DCA signals through the Raf-Mek-Erk and MKK3/6-p38 pathways and suggests the possible utilisation of EGFR by DCA in oesophageal cells. It would be of interest to confirm the role of EGFR by assessing direct activation of this receptor by DCA.

The data presented in Chapter IV demonstrates that DCA induces signalling cascades leading to both apoptosis and cell survival, and propose that the balance between these two opposing biological outcomes dictates the cell fate in response to DCA. We show that induction of COX-2 expression is involved in DCA-induced survival in oesophageal cells. However, other DCA-regulated pathways might also participate. Activation of MAPKs is involved in cell survival mechanisms through upregulation of anti-apoptotic proteins such as Mcl-1 and cFLIP. In oesophageal cells, EGF induces Mcl-1 via Raf/Mek/Erk (Leu et al. 2000). DCA has been shown to upregulate cFLIP through Raf/Mek/Erk in hepatocytes (Qiao et al. 2001b; Qiao et al. 2003) and in oesophageal cells (Jenkins et al. 2004). Taken together these data suggest that DCA might regulate the expression of both Mcl-1 and cFLIP in oesophageal cells via Raf/Mek/Erk.

The duration of MAPK activation is important for the biological responses of the cells. In general, sustained activation is required for cell differentiation, while a mitogenic response can be achieved by either transient or sustained activation of MAPK depending on the cell type. For example, in PC12 cells, nerve growth factor-stimulated differentiation is associated with prolonged MAPK activation (Cowley et al. 1994). In the same cells, epidermal growth factor induces transient activation of MAPK, which is accompanied by a mitogenic response (Cowley et al. 1994; Marshall 1995; Alblas et al. 1998). Here we show that DCA induces sustained activation of both Erk1/2 and p38 for at least 6 hr **(Fig 5.6 and Fig 5.7)**. Long-term intermittent exposure of oesophageal tissue to DCA as that caused by

duodenal reflux leading to sustained MAPK activation might be responsible for driving the DCA-regulated apoptosis/survival balance towards the acquisition of an anti-apoptotic phenotype, which has been associated with the progression from Barrett's metaplasia to adenocarcinoma (van der Woude et al. 2002)

The MAPKs are primarily responsible for transducing signals from the cell membrane to the nucleus where they modulate the activity of transcription factors including the AP-1 family. AP-1 plays a central role in the reprogramming of gene expression (Shaulian and Karin 2001) and can be activated by bile acids in hepatocytes and colonic cells (Qiao et al. 2000; Qiao et al. 2003). Here we show that DCA induces sustained activation of AP-1 for at least 6 hr in SKGT4 cells **(Fig 5.2)**. AP-1 induction by DCA is not cell line specific as elevated levels of AP-1 activity in response to DCA are also observed in another oesophageal adenocarcinoma cell line, OE-33 **(Fig 5.1)**. Sustained AP-1 activation by DCA is likely to have important implications in oesophageal tumourigenesis considering that blockage of DMBA/PMA-induced AP-1 activity in transgenic mice prevents neoplastic transformation (Young et al. 1999). Initial studies using EMSA suggested that Fra-1, c-Jun and JunD are possible members of the DCA-induced AP-1 complex **(Fig 5.3)**.

AP-1 dimer composition is a key determinant of the specific target genes. AP-1 results from dimerisation of either pre-existing or newly synthesised phosphorylated proteins of the Fos and Jun families. Phosphorylation of these proteins increases protein stability and enhances transactivation activity (Eferl and Wagner 2003). We initially examined the protein expression of Fos/Jun proteins after DCA treatment. Western blot analysis revealed increased protein expression of Fra-1 and JunB after stimulation with DCA with weak induction of c-Jun **(Fig 5.10)**. These results suggest that *de novo* protein synthesis of Fra-1, JunB and to a lesser extent c-Jun contribute to the increase in AP-1 activity observed by EMSA **(Fig 5.2)**. The increased protein expression of Fra-1, JunB and c-Jun might be due to stabilisation of these proteins through phosphorylation by the MAPKs. In HCT116 cells, elevated Erk activity protected Fra-1 from proteasomal degradation (Vial and Marshall 2003), which has also been reported in Ras transformed thyroid cells (Casalino et al. 2003). We detected constitutive expression of JunD, which is unaltered after DCA treatment (data not shown). The JunD component, present in the DCA-induced AP-1 complex that was observed by EMSA **(Fig 5.3)**, must be due to the phosphorylation of the

pre-existing JunD proteins as has been shown in human embryonic kidney cells and NIH3T3 cells, where Erk directly phosphorylates JunD (Gallo et al. 2002).

The kinetics of Fra-1, JunB and c-Jun DNA binding activity in response to DCA was assessed to determine if the observed increases in protein expression correlated with increased DNA binding activity. Increased Fra-1 and JunB activity is readily detectable for at least 6 hr while c-Jun activity is weak **(Fig 5.11)**, which is in parallel with the protein expression patterns. These data indicate that the possible dimers induced by DCA are Fra-1/JunB, Fra-1/c-Jun, JunB/JunB and c-Jun/c-Jun.

Fra-1 and c-Jun are regarded as being positive regulators of cell proliferation and have been shown to mediate oncogenic transformation in fibroblasts (Mechta et al. 1997). JunD is known to have both positive and negative effects on cell growth depending on the cellular context (Shaulian and Karin 2001; Eferl and Wagner 2003). JunD can antagonises the growth promoting effects of c-Jun and reduce proliferation of immortalised fibroblasts (Bakiri et al. 2000). JunD is also essential for the survival of primary embryonic fibroblasts (Passegue and Wagner 2000) and to inhibit apoptosis by modulating p53 activity (Weitzman et al. 2000). In MCF-7 cells, TPA induced strong activation of JunB while weak c-Jun activity was detected (Alblas et al. 1998). JunB can oppose c-Jun transformation and transactivation possibly through JunB binding preferentially to c-Jun and thereby preventing DNA binding to c-Jun (Deng and Karin 1993; Mechta-Grigoriou et al. 2001). We observe strong JunB protein expression and DNA binding activity in comparison to c-Jun in response to DCA in SKGT4 cells, which agrees with a repressing role of JunB over c-Jun in our system. Our data indicate that Fra-1 and JunB are the main components of the DCA-induced AP-1 complexes. The balance between the contrasting activities of DCA-induced AP-1 components will ultimately determine cell fate. The Fos family members and c-Jun are regarded as tumour promoters whereas the functions of JunB are complex and dependent on the stimulus and cellular context. DCA induces cell survival and low rates of apoptosis in SKGT4 cells (Chapter IV). Taken together these data support a balance between the activity of DCA-induced Fra-1 and JunB. Disruption of this fine balance by other DCA-regulated pathways and/or during repeated exposure to DCA during reflux might explain the role of DCA in oesophageal malignant development.

The individual MAPK pathways responsible for Fra-1 and JunB activation in SKGT4 cells were identified through the use of well-known pharmacological inhibitors selective for the Erk and p38 pathways. Our findings demonstrate that DCA-induced activation of Fra-1 is mediated by both the Mek-Erk and p38 pathways in oesophageal cells **(Fig 5.13)**, while JunB activation is mediated solely through the Erk cascade. These data are in agreement with others who have shown that sustained activation of Erk results in Fra-1 and JunB activation with negligible induction of c-Jun (Cook et al. 1999). These findings show that sustained Erk activation mediates both Fra-1 and JunB activation by DCA whereas the p38 is solely responsible for Fra-1 activation.

The conventional PKC inhibitor, GÖ6976, reduced DCA-induced Fra-1 activity to basal levels **(Fig 5.14)**. Our data implicates the conventional PKCs, more specifically PKC-α, in DCA-induced Fra-1 activity. This is supported by previous reports showing that in NIH3T3 cells PKC-α dependent activation of SRE transcription was attenuated by dominant negative MEKK1 (Soh et al. 1999), which can interact and phosphorylate with Mek via its catalytic domain (Yan and Templeton 1994; Xu et al. 1995). In addition MEKK1 can activate p38 through SEK (English et al. 1999a; Ichijo 1999). On the other hand PKC can regulate β-catenin protein stabilization (Orford et al. 1997; Murray et al. 1999; Chen et al. 2000) of which Fra-1 is a target gene (Mann et al. 1999). Microarray analysis of oesophageal cells exposed to DCA showed substantial upregulation of β-catenin at 1 - 24 hr post DCA treatment (Jenkins et al. 2004). Therefore the observed DCA-induced Fra-1 activation mediated by PKC might be regulated directly by the Mek/Erk and p38 pathways or by ensuring β-catenin stabilization.

The major AP-1 components upregulated by DCA include Fra-1, JunB and c-Jun albeit the latter at a much lower level. Cyclin D1 is a key regulator of the G1/S phase transition (Wang et al. 2004). Previous studies have demonstrated that the ratio of JunB/c-Jun is an important parameter in regulating cyclin D1 expression (Mechta-Grigoriou et al. 2001). The cyclin D1 promoter can be repressed by JunB and activated by c-Jun (Bakiri et al. 2000). In lung epithelial cells Fra-1 activation was associated with cyclin D1 expression (Burch et al. 2004). In CCL39 fibroblast cells, thrombin stimulates persistent MAPK activation that is required for the expression of cyclin D1, Fra-1 and JunB (Balmanno and Cook 1999). Given that the cyclin D1 promoter requires Mek activation (Lavoie et al.

1996), the combined activity of Erk, Fra-1 and JunB induced by DCA will regulate the levels of cyclin D1 expression and consequent cell cycle progression in these cells. Cyclin D1 overexpression is an early event in malignant progression in the oesophagus as shown *in vitro* (Arber et al. 1996) and *in vivo* using animal models of reflux (Zhang et al. 2001a). DCA-induced sustained activation of ERK, p38, Fra-1 and JunB represent a plausible mechanism for cyclin D1 deregulation in oesophageal tissue during reflux (Shintani et al. 2002).

Deregulation of the expression of target genes such as COX-2, in response to sustained activation of Erk1/2, p38 and AP-1, will affect the biological responses of oesophageal cells. COX-2 expression can be regulated by MAPKs both directly by mRNA stabilization in intestinal epithelial cells (Subbaramaiah et al. 1998; Zhang et al. 2000) and through activation of AP-1 complexes in mammary epithelial cells and colonic cells (von Knethen and Brune 2000; Subbaramaiah et al. 2001; Glinghammar et al. 2002). MAPKs can also regulate COX-2 posttranscriptional in intestinal epithelial cells (Dean et al. 1999; Guo et al. 2001). COX-2 expression is frequently upregulated in Barrett's oesophagus, oesophageal cancer and in animal models of reflux (Zimmermann et al. 1999; Shirvani et al. 2000; Zhang et al. 2001a). We used specific pharmacological inhibitors of the MAPK cascades to identify the pathways mediating DCA-induced COX-2 expression in SKGT4 cells. COX-2 expression is completely blocked by the PD98059 and SB203580 compounds **(Fig 5.16)**, demonstrating the involvement of Raf-Mek-Erk and MMK3/6-p38 pathways in DCA-induced COX-2 expression. These data are in agreement with reports in human monocytes and intestinal epithelial cells, where Erk and p38 have been shown to regulate COX-2 expression through regulation of transcription and stabilisation of COX-2 mRNA (Dean et al. 1999) (Subbaramaiah et al. 1998; Zhang et al. 2000). As the MAPK inhibitors used in this study respectively block Erk1/2 and p38 and consequently AP-1 activation **(Fig 5.17A)**, it is not possible to differentiate between the specific contribution of MAPK and AP-1 in the regulation of DCA-induced COX-2 expression. It is possible that Erk1/2 and p38 are regulating COX-2 expression in response to DCA either directly or indirectly by ensuring sustained expression and activation of Fra-1 and JunB.

COX-2 expression can be regulated by PKC. In colonic cells, PKC-β_2 induces COX-2 expression by both increasing COX-2 gene transcription and stabilising COX-2 mRNA (Yu et al. 2003). Here we demonstrate that in SKGT4 cells, DCA-induced COX-2

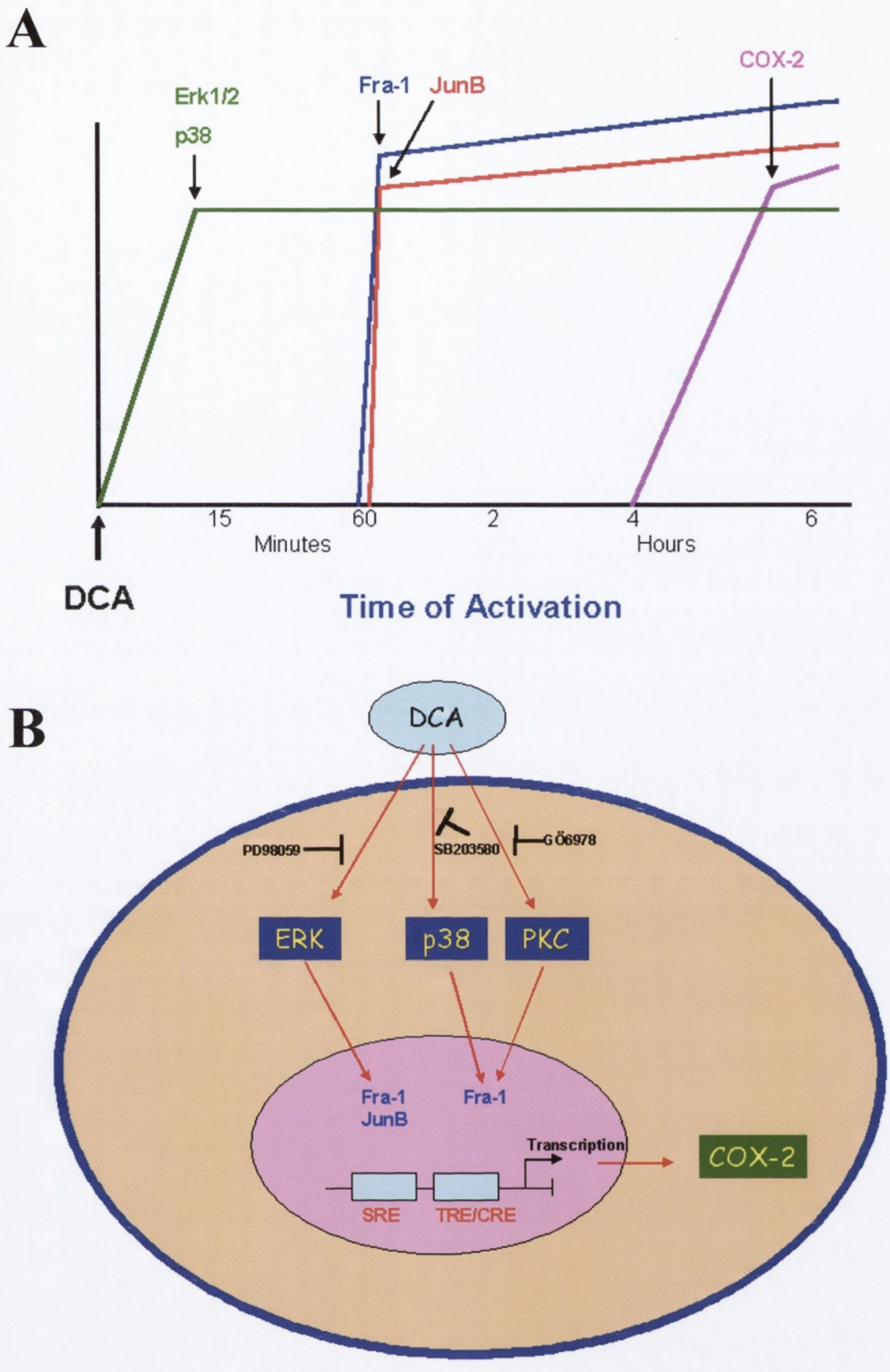


Fig 5.17. The Intracellular Signalling Cascades Induced by DCA in Oesophageal Cells. (**A**) A time sequence of DCA-induced Erk1/2, p38, Fra-1, JunB and COX-2. (**B**) Intracellular signalling pathways activated by DCA in SKGT4 cells. DCA induces Erk1/2, p38 but not JNK activation. Activation of these MAPK cascades results in persistent activation of the AP-1 components, Fra-1 and JunB, with subsequent induction of COX-2 expression. DCA-stimulated Fra-1 activation is additionally regulated by PKC, which in turn also regulates COX-2 induction. Inhibitors of Mek-Erk (PD98059), MKK3/6 (SB203580) and the conventional PKCs (GÖ6978) block induction of AP-1 components and consequently COX-2 expression.

expression is dependent on the activation of the conventional PKCs as judged by the inhibition of DCA-induced COX-2 expression using the PKC inhibitor GÖ6976 **(Fig 5.16)**. Since SKGT4 cells do not express PKC-β **(Fig 4.1)** or -γ (data not shown), these findings suggest that PKC-α mediates DCA-induced COX-2 expression. This is in agreement with studies in mammary and oral epithelial cells where PKC-α mediates COX-2 expression (Subbaramaiah et al. 1998). In primary keratinocytes, activation of PKC-α results in the coordinate regulation of cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation/arachidonic acid release and COX-2 induction, which is Mek dependent (Wang and Smart 1999; Wang et al. 2001). These data suggest that DCA would activate PKC-α in SKGT4 cells, leading to COX-2 expression.

In vivo DCA and CDCA are regarded as potent tumour promoters (Jankowski et al. 1992; Kauer et al. 1995; Nehra et al. 1998; Nehra et al. 1999) while UDCA is thought to be cytoprotective (Tung et al. 2001). CDCA, in comparison to UDCA, induces stronger AP-1 binding activity **(Fig 5.1)** and this might be explained in the AP-1 components induced by each bile acid **(Fig 5.15)**. The pattern of Fos/Jun activity induced by CDCA is similar to DCA. Fra-1 and JunB activity is markedly upregulated in response to CDCA with weak c-Jun activation. Interestingly UDCA causes marked induction of c-Jun with a modest increase in Fra-1 activity and no activation of JunB detectable **(Fig 5.15)**, the opposite of DCA and CDCA. Increased expression of Fra-1 has been associated with oncogenic transformation (Vallone et al. 1997), increased motility (Vial et al. 2003) and invasion (Kustikova et al. 1998). While overexpression of JunB correlates with an even more malignant phenotype in colonic adenocarcinoma (Wang et al. 2000a), ovarian cancer (Mao et al. 2003) and T-cell lymphomas (Mao et al. 2003). In some instances, c-Jun can have a negative effect on cell proliferation depending on the cellular context and the nature of the inducing signal. In lymphocytes undergoing apoptosis and also during neuronal differentiation, c-Jun expression is elevated (Cowley et al. 1994; Preston et al. 1996). The pattern of AP-1 components induced by DCA and CDCA as compared to UDCA is in agreement with their respective known tumour promoting and cytoprotective activity. Therefore, *in vivo*, the AP-1 components induced in oesophageal epithelial cells exposed to a pool of bile acids as in the case of reflux will depend on the ratio of DCA: CDCA: UDCA.

From the data presented in this chapter, summarised in **Fig 5.17**, we propose a model for the activation of COX-2 through the upstream regulators Erk1/2, p38 and AP-1. Sustained activation of MAPK and AP-1 by DCA will alter gene expression patterns and ultimately the biological response of oesophageal cells. Persistent activation of Erk1/2 and p38 has been shown to lead to induction of cyclin D1, which in turn induces cell proliferation (Lavoie et al. 1996). Furthermore sustained activation of AP-1 and COX-2 is associated with increased invasion (Subbaramaiah et al. 1996; Tsujii et al. 1997) and oncogenic transformation (Young et al. 1999; Young et al. 2002). DCA is capable of inducing gene expression patterns that correlate with enhanced survival and proliferation of oesophageal cells and therefore strengthens the argument that bile acid reflux is important in malignant progression in Barrett's patients

CHAPTER VI

GENERAL DISCUSSION

CHAPTER VI

GENERAL DISCUSSION

6.1. The Contributory Role of Bile Acids in the Development of Gastrointestinal Cancer

The structural resemblance of bile acids to carcinogenic polycyclic aromatic hydrocarbons led Cook and Kennaway to suspect that bile acids were involved in tumourigenesis. This was confirmed by subcutaneous injection of DCA into mice, which resulted in "malignant spindle-celled tumours" (Cook and Kennaway 1940). It is now clear that bile acids such as DCA are important factors in the development of gastrointestinal cancer especially since they are modulated by diet. Diet, in particular dietary fat, has long been regarded as the most important nutritional influence on gastrointestinal cancer and several epidemiological studies have demonstrated that typical Western diets, containing a high proportion of fat, are associated with increased incidences of gastrointestinal cancer in Western countries (Woutersen et al. 1999). The amount of dietary fat influences bile acid secretion (Cummings et al. 1978; Reddy 1981; Nagengast et al. 1995; Reddy et al. 1996) as well as the composition of gut bacteria, which determines the amount of secondary bile acids produced (Cummings et al. 1978; Mastromarino et al. 1978).

Bile acids have been implicated in the development of colon cancer and oesophageal cancer (Jankowski et al. 2000; Debruyne et al. 2001). The importance of bile acids in colon cancer is emphasised by several epidemiological studies. Natives from countries with high incidence of colon cancer have increased concentrations of secondary bile acids in the gut, as compared to individuals from countries where the incidence of colon cancer is lower. This is explained by an altered intestinal microflora with overgrowth of bacteria capable of dehydroxylating bile acids (Hill 1995). Patients with colorectal adenomas have higher serum levels of DCA as well as elevated levels of faecal secondary bile acids in comparison to healthy volunteers (Bayerdorffer et al. 1995; Kishida et al. 1997). Bile acids are also important in the pathogenesis of oesophageal adenocarcinoma given that toxic secondary bile acids are significantly elevated in oesophageal aspirates from patients with erosive oesophagitis and Barrett's oesophagus as compared to controls (Nehra et al. 1998; Stein et al. 1998; Nehra et al. 1999). Malignant progression of Barrett's metaplasia has been reported in patients who had bile reflux without any pathological acid reflux

(Jankowski et al. 1993). Increased exposure of the oesophageal epithelium to bile acids is associated with increased mucosal damage, the appearance of Barrett's metaplasia and increased severity of disease (Gillen et al. 1988). Although in colonic and oesophageal adenocarcinoma similar genes and regulatory systems are altered, the timing of these events is different. However, the role of bile acids in the development of both malignancies is unquestionable. In both diseases, bile acids promote malignant development and progression through early cellular alterations that result in the aberrant expression of specific proteins such as COX-2 and MAPKs.

6.2 Bile Acids Alter PKC Isoforms in Colon Cancer Cells

Bile acids are thought to promote carcinogenesis through alterations in both gene expression and intracellular signalling cascades such as those involving the different PKC isoforms. Alterations in the activity of the PKCs have been noted in the malignant progression from colonic adenoma to carcinoma (Levy et al. 1993; Doi et al. 1994). A growing body of evidence implicates increased bile acid secretion and the alterations in PKC activity in contributing to colon carcinogenesis. To date the involvement of the PKC isoforms in bile acid mediated events in colon cancer cells has been implied by the use of inhibitors specific for individual PKC isoforms (Glinghammar and Rafter 2001) or from PKC alterations detected in cancer tissue (Pongracz et al. 1995; Reddy et al. 1996). We investigated the role of bile acids in colonic adenocarcinoma cells. Translocation of the different PKC isoforms from the cytosol to the plasma membrane is a hallmark of activation. We show for the first time that DCA can differentially modulate PKC isoform localisation in living colonic cells; specifically DCA induces PKC-β_I-GFP and PKC-ε-GFP but not PKC-δ-GFP translocation (Chapter III). Using Western blotting, we demonstrate that the translocation patterns of endogenous PKC-ε and PKC-δ are identical to those observed with the transfected PKC isoforms. Persistent activation of PKC leads to downregulation and loss of expression of the PKC isoforms (Darbon et al. 1986a; Freire-Moar et al. 1991; Strulovici et al. 1991). Previous studies demonstrated that total PKC activity is decreased in colonic carcinoma tissue in comparison to normal colonic mucosa (Guillem et al. 1987; Kopp et al. 1991). Therefore chronic activation of PKC isoforms by bile acids such as DCA is likely to facilitate their selective downregulation and even loss from the colonic epithelium.

Much interest has been focused on the role of dietary fibre in particular sodium butyrate, the by-product of dietary fibre fermentation, and its putative protective role in the colonic epithelium. Epidemiological studies have suggested that a high fibre diet has a protective effect against colon cancer development (Harris and Ferguson 1999). In colonic cancer cell lines, sodium butyrate has been shown to be cytoprotective as it increases alkaline phosphatase activity, via PKC activation (Rickard et al. 1999). As demonstrated by real time microscopy analysis, sodium butyrate does not prevent DCA-induced PKC-β_I or PKC-ε translocation (Chapter III). These data suggest that if sodium butyrate is indeed preventing DCA-induced alterations, this would occur via PKC-independent mechanisms. Alternatively, sodium butyrate might exert a direct cytoprotective effect by either inducing apoptotic programmes or inhibiting survival mechanisms.

This is the first report showing that DCA-induced PKC isoform translocation in colonic cells is accompanied by rearrangements in microfilament and microtubule assembly (Chapter III) The actin cytoskeleton is linked to the cadherin family of transmembrane cell-cell adhesion receptors via adherens junctions of which β-catenin is a key component (Ben-Ze'ev and Geiger 1998). Recently, DCA has been shown to induce nuclear accumulation of β-catenin resulting in the loss of E-cadherin binding in colonic cancer cells and increased invasiveness (Pai et al. 2004). Thus the reorganisation of the actin cytoskeleton observed in this study could be due in part to DCA-induced phosphorylation of β-catenin and altered E-cadherin binding. Furthermore DCA-induced reorganisation of the actin cytoskeleton is likely to affect the Rho (Ras homology) signalling family (Brandt et al. 2002), key regulators of cytoskeletal organisation that control epithelial cell integrity, migration and invasion. Studies by Qiao *et al* demonstrated that DCA induces AP-1 activity via a PKC dependent pathway, as judged by the use of a broad spectrum PKC inhibitor, in HCT116 cells (Qiao et al. 2000). Fra-1 is a member of the DCA-induced AP-1 complex (Qiao et al. 2000) and promotes cell motility and invasiveness by inactivating beta1-integrin and altering RhoA and Rac activity in colonic cells (Vial et al. 2003). Therefore the DCA-induced cytoskeletal rearrangements observed in our study, which are associated with PKC translocation, might be mediated by Fra-1. Alterations to the cytoskeleton have been associated with changes in migratory activity (Masur et al. 2001). The observed DCA-induced cytoskeletal changes most likely contribute to alterations in cell motility and invasion, leading to the promotion of cancer.

6.3 DCA Modulates Oesophageal Cell Proliferation and Apoptosis

Recently attention has been focused on the process of apoptosis as being crucial in the homeostasis of cell renewal and turnover in both the colon and oesophagus. Evidence now shows that deregulation of apoptosis contributes to the development of neoplasia in both organs (Katada et al. 1997; Wetscher et al. 1998; Bernstein et al. 1999a; Bernstein et al. 2002; van der Woude et al. 2002). Bile acid-induced alterations in signalling cascades such as the MAPK pathway will perturb cellular processes such as proliferation and apoptosis. Extensive studies in colonic cells have demonstrated that stimulation with DCA inhibits proliferation and induces apoptosis (Martinez et al. 1998; Bernstein et al. 1999b; LaRue et al. 2000). The biological responses of oesophageal cells following treatment with DCA were the focus of this study. As demonstrated by the MTT and DNA fragmentation assays, DCA induces a time and dose dependent decrease in cell proliferation with concomitant induction of apoptosis (Chapter IV). Further confirmation of the apoptotic response induced by DCA was provided by immunoblotting for the cleaved fragment of PARP. In addition the upstream signals leading to PARP cleavage were dissected using caspase inhibitors, which demonstrated that PARP cleavage in response to DCA is dependent on caspase activation, specifically caspase-3. Similarly findings have been reported in hepatocytes and colonic cells (Qiao et al. 2001b; Glinghammar et al. 2002). However, DCA-induced DNA fragmentation is not completely blocked by caspase inhibitors suggesting that other mechanisms are also involved. For example, the release of cytochrome C and apoptosis inducing factor (AIF) can lead to cell death via caspase independent mechanisms (Daugas et al. 2000; Joseph et al. 2002). Bile acid-induced apoptosis in cultured rat hepatocytes has been shown to involve the release of cytochrome C (Webster et al. 2002). Therefore bile acids activate both caspase dependent and independent apoptotic cascades in gastrointestinal cells including oesophageal cells (Schlottman et al. 2000; Qiao et al. 2001b) (Chapter IV).

A balance between proliferation, cell survival and apoptosis regulates normal cell growth. Pre-neoplastic and neoplastic conditions are characterised by an increase in cell proliferation and a decrease in apoptosis. This is illustrated *in vivo* during oesophageal cancer progression by high rates of apoptosis during reflux oesophagitis, which are decreased in Barrett's metaplasia (Wetscher et al. 1998). It is of paramount importance to understand how apoptosis is regulated and can be modulated in individuals suffering from

these conditions (Wetscher et al. 1998; Bernstein et al. 2000). The data presented in this thesis demonstrates that DCA, an important constituent of oesophageal refluxate, induces survival mechanisms in tandem with apoptotic cascades. DCA induces significant upregulation of COX-2 expression with low levels of caspase-dependent PARP cleavage. Inhibition of DCA-induced COX-2 expression potentiates DCA-induced apoptosis as demonstrated by immunoblotting and ELISA. Interestingly severe reflux oesophagitis and Barrett's metaplasia are mediated at least in part by increased production of reactive oxygen species (ROS) (Wetscher et al. 1995b; Wetscher et al. 1995a; Wetscher et al. 1997). ROS can be generated by numerous metabolic processes including mitochondrial electron transport chain (Sohal 1997) and by arachidonic acid metabolism (Roy et al. 1994). ROS, generated by cyclooxygenases (COX-1, COX-2) and lipoxygenases (LOX) **(Fig 6.1)**, can activate NF-κB and MAPKs, both of which are activated by DCA in oesophageal cells. Taken together, these studies suggest that in response to DCA, ROS might act downstream of COX-2 to regulate activation of NF-κB and MAPK and consequent cell survival **(Fig 6.1)**. ROS has been shown to induce DNA damage and apoptosis **(Fig 6.1)** . The similarities between the biological effects exerted by ROS and DCA also support a role for ROS downstream of DCA. This is the first report showing that DCA regulates both COX-2 mediated cell survival and apoptosis in oesophageal cells, and suggests that the balance between these two opposing signals will determine the transformation potential of oesophageal cells **(Fig 6.1)**.

6.4 Bile Acids Alter Transcriptional Regulation in Oesophageal Cells

Aberrant expression of genes induced by environmental stress such as that caused by bile acids can result in an altered phenotype and tissue function contributing to oesophageal pathogenesis. Abnormal expression and/or activation of transcription factors in response to bile acid reflux can deregulate downstream target genes thereby altering normal injury and repair processes resulting in pathogenesis. A better understanding of the mechanisms regulating the activation of transcription factors in response to bile acids in oesophageal cells, is crucial for developing effective strategies to modulate disease. DCA has been shown to activate the transcription factor AP-1 via MAPK activation in colonic cells and in primary hepatocytes (Qiao et al. 2000; Qiao et al. 2001b). Here we demonstrate that DCA induces sustained activation of Erk and p38 as well as AP-1 in oesophageal adenocarcinoma cells, which has not been previously reported. Sustained activation of Erk and p38 in response to DCA is extremely important in terms of the functional responses of

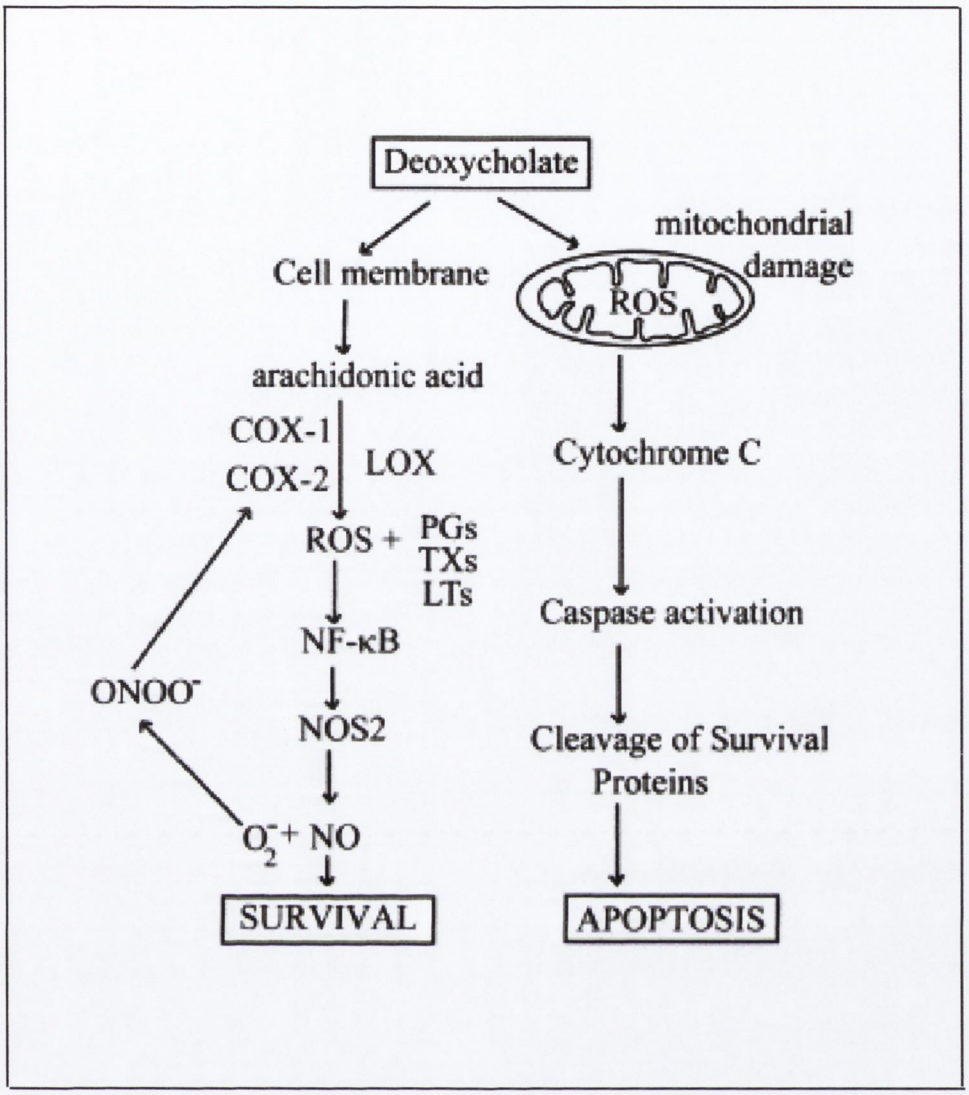


Fig 6.1. Pro-survival and Pro-apoptotic Mechanisms Induced by DCA in Oesophageal Cells. The pro-survival pathway, involving COX-2 induction, and the pro-apoptotic pathway, involving the cleavage of survival proteins such as PARP and DNA fragmentation, is the focus of this study. The NF-κB, and PG production elements of the pro-survival pathway have been previously reported (Zhang et al. 1998; Abdel-Latif et al. 2004). Adapted from Washo-Stultz et al. 2002.

the oesophageal cells, as persistent activation of MAPK has been associated with a malignant progression. Constitutive activation of Erk has been reported in several human malignancies (Oka et al. 1995; Sivaraman et al. 1997; Towatari et al. 1997; Kim et al. 1999) including oesophagogastric rib bone marrow cells (Barry et al. 2001) and has been shown to mediate tumourigenic (Welch et al. 2000) and metastatic potential as well as regulate cell motility (McCawley et al. 1999). IL-8 is a key regulator of inflammatory processes and is primarily responsible for the recruitment and activation of leucocytes (Herlaar and Brown 1999). Activation of p38 has been shown to mediate IL-8 production (Knall et al. 1996), and both p38 and IL-8 are upregulated in response to DCA in oesophageal cells (Jenkins et al. 2004) **(Fig 5.7A)**. Therefore chronic activation of p38 by DCA in the oesophageal epithelium is likely to contribute to IL-8 production and consequent recruitment of lymphocytic cells. This is of particular importance as an inflammatory cell infiltrate has been proposed to be necessary for the maintenance and progression of Barrett's metaplasia (Jankowski et al. 2000).

Chronic activation of MAPK by bile acids is likely to influence the local environment to favour malignant progression. For example, sustained activation of Erk results in its nuclear accumulation and consequent sustained phosphorylation of transcription factors (Marshall 1995). In this study, we establish that sustained activation of Erk and p38 results in persistent activation of Fra-1 and JunB as demonstrated by the use of specific inhibitors in conjunction with DNA affinity purification experiments. Clearly, modulation of the expression of AP-1 components such as Fra-1 and JunB will result in marked changes in target gene expression. Active AP-1 complexes regulate the transcription of immunomodulatory cytokines such as IL-6 (Zerbini et al. 2003) and cell adhesion molecules (Vial et al. 2003). Increased concentrations of these molecules due to chronic stimulation are likely to be instrumental in the carcinogenic process. Sustained activation of Fra-1 and JunB by DCA in oesophageal adenocarcinoma cells is extremely important considering that increased expression of Fra-1 has been associated with oncogenic transformation (Vallone et al. 1997), increased motility (Vial et al. 2003) and invasion (Kustikova et al. 1998). Increased expression of JunB correlates with an even more malignant phenotype. JunB is overexpressed in colonic adenocarcinoma (Wang et al. 2000a), ovarian cancer (Mao et al. 2003) and T-cell lymphomas (Mao et al. 2003). Specific inhibition of DNA binding of Fra-1 and JunB could potentially be utilised to prevent bile acid-induced alterations in gene expression.

COX-2 overexpression enhances prostaglandin production in particular PGE_2 (Zhang et al. 2001a), which is involved in induction of cytokines such as IL-6. Studies in oesophageal cells demonstrated that IL-6 protects against staurosporine-induced apoptosis through the activation of Erk, STAT3 and Mcl-1 (Leu et al. 2003). Interestingly bile acids induce expression of COX-2 and the anti-apoptotic protein Mcl-1 in cholangiocarcinoma cells (Yoon et al. 2002a). DCA induces a survival mechanism in oesophageal cells **(Fig 4.12)**. This could be achieved by inducing the expression of anti-apoptotic proteins of the Bcl-2 family either directly through activation of MAPK (Chapter V) or indirectly through an amplification loop via COX-2-mediated induction of IL-6. Ultimately the combined activity of the MAPK, AP-1 components and COX-2 will determine cell fate. Modulation of apoptotic cascades, as a result of significant alterations to gene expression, is likely to have a key role in contributing to oesophageal tumourigenesis and resistance to chemotherapy.

6.5 Bile Acids and Chemoprevention

Barrett's oesophagus, a complication of GERD, would be of lesser importance were it not for its well-known association with oesophageal adenocarcinoma. Given that Barrett's oesophagus has the most severe pathophysiological abnormalities of GERD, acid suppression therapy involving proton pump inhibitors have been used in the management of this disease. Proton pump inhibitors consistently alleviate symptoms and heal inflammation in Barrett's oesophagitis patients. However even at high doses, they lead only to a modest regression of Barrett's oesophagus (Sampliner 1994; Peters et al. 1999), which is characterised by an increase in islands of normal squamous cells. Nonetheless, analysis of biopsies from these islands demonstrates the presence of underlying intestinal metaplasia (Sharma et al. 1998). Similarly, anti-reflux surgery alleviates GERD symptoms in Barrett's patients but complete regression of Barrett's epithelium is uncommon (Brand et al. 1980). Even though surgery provides an excellent means of symptom control it does not equate to normalisation of the oesophageal epithelium. New multi-agent chemo-preventive combinations targeting leading to the amplification of pro-apoptotic cascades and/or elimination of survival cascades are likely to be effective in the treatment of Barrett's oesophagus associated with bile acid reflux.

Reflux contains a mixture of secondary (DCA and CDCA) and tertiary bile (UDCA) acids. Secondary bile acids are regarded as being potent tumour promoters. While, the tertiary bile acid UDCA is known to act as a cytoprotective agent. *In vivo* studies using AOM treated animals demonstrated that UDCA inhibited the growth of chemically-induced tumours (Earnest et al. 1994). In patients with ulcerative colitis, the use of UDCA is associated with lower prevalence of colonic neoplasia (Tung et al. 2001). In colonic cells, UDCA inhibits DCA-induced MAPK and AP-1 activation (Im and Martinez 2004). UDCA appears to inhibit early events such as receptor activation that leads to MAPK and AP-1 activation **(Fig 6.2)**. In this study, we show that UDCA does not induce either Fra-1 or JunB activation unlike the secondary bile acids DCA and CDCA. UDCA does not alter oesophageal cell proliferation even at high doses. Structural differences between UDCA as compared to DCA and CDCA are thought to account for their opposing actions. The orientation of the hydroxyl group at C-7 is important for their cytotoxicity. In DCA and CDCA, the hydroxyl group is located in the α-position at C-7 whereas in UDCA the hydroxyl group is located in the β-position at C-7 (Hino et al. 2001). These structural differences are thought to provide steric hindrance and thereby prevent receptor activation **(Fig 6.2)**. For example, unlike UDCA, DCA and CDCA can activate the orphan nuclear receptor FXR (Parks et al. 1999). Therefore the chemical structure of UDCA could be exploited for the design of new therapeutic drugs for future chemo-preventive strategies.

The carcinogenic process is typified by the aberrant expression of specific proteins. Constitutive activation of Mek has been demonstrated in several human malignancies including breast cancer (Sivaraman et al. 1997), renal cell carcinoma (Oka et al. 1995), colon cancer (Lee et al. 2004) and metastatic oesophagogastric cancer (Barry et al. 2001). Inhibition of Mek has proven to be effective in the management of other malignancies such as head and neck squamous cell carcinomas where it attenuated the invasiveness of these carcinomas (Simon et al. 1999). Studies in colonic cells have demonstrated that DCA-induced Erk activation is dependent on Mek (Qiao et al. 2000). Furthermore Mek2 is known to be overexpressed in colonic cells resistant to DCA-induced apoptosis *in vitro* (Bernstein et al. 2004). Interestingly, Mek inhibition suppresses the growth of colonic tumour cells *in vivo* (Sebolt-Leopold et al. 1999). In our system, DCA induces sustained activation of Erk in oesophageal cells, which requires Mek activity as judged by the use of two structurally different Mek inhibitors. Given the extensive experimental evidence for the involvement of Mek in tumourigenesis and the fact that bile acids activate Mek-Erk, it

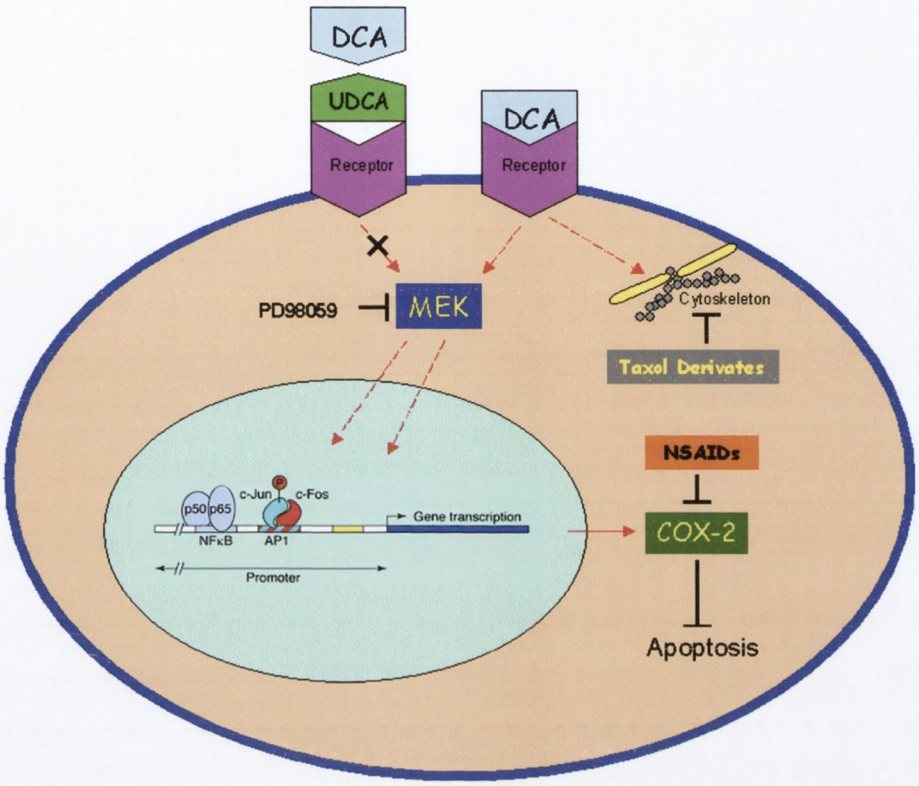


Fig 6.2. Schematic Representation of Potential Chemopreventative Mechanisms Directed Against Bile Acid-Induced Modifications. UDCA could be used as a model for the design of drugs that specifically interact with and inhibit bile acids such as DCA. Inhibitors, directed against bile acid-induced alterations in the cytoskeleton, could prevent the acquisition of an invasive phenotype. Inhibition of bile acid-induced target genes such as MEK and COX-2 might prove effective in halting the progression of the carcinogenic process. Adapted from Herlaar and Brown 1999.

is highly likely that deregulation of this process contributes to oesophageal tumourigenesis. Inhibitors of Mek, in conjunction with proton pump inhibitors, could be utilised as therapeutic agents in the management of Barrett's patients and could even eliminate the contribution of bile in malignant progression **(Fig 6.2)**. The long-term use of Mek inhibitors in chemopreventative strategies might be limited by their cytotoxicity as Mek is activated in response to a wide variety of physiological stimuli. Development of cell specific derivatives of the Mek inhibitors with minimised cytotoxicity would represent a powerful tool in the prevention of colonic and oesophageal malignancies.

Malignant progression involves alterations in the cytoskeleton of cells resulting in an altered phenotype. As demonstrated in colon cancer cells, DCA induces marked alterations in the cytoskeleton, with dramatic rearrangements observed in the microtubule network **(Fig 6.2)**. Taxol, a well-known anti-microtubule agent that stabilises mitotic spindles, has proven clinical efficacy in a variety of human neoplasms including breast cancer (Sledge et al. 2003), in which bile acids have been implicated. Taxol, in combination with cisplatin, can induce apoptosis in human ovarian cells that are resistant to cisplatin (Zaffaroni et al. 1998). However, taxol is highly toxic and some patients can develop resistance. Less toxic derivatives such as BMS-247550, a semisynthetic analogue of the natural product epothilone B, with improved anti-neoplastic activity should be considered. BMS-247550 has significant anti-tumour activity and its mode of action is analogous to that of taxol (*i.e.*, microtubule stabilization) (Lee et al. 2001). Less toxic derivatives of taxol or alternative microtubule stabilisers could be considered as possible chemopreventative agents in future treatment strategies in colon cancer and Barrett's metaplasia in which bile acids are implicated.

Multiagent combinations using NSAIDs such as aspirin together with acid suppression therapy could provide a promising new approach to oesophageal cancer chemoprevention. Several studies using animal models of reflux, in addition to organ culture experiments, have conclusively demonstrated that increased expression of COX-2 is an early and specific event in the Barrett's metaplasia-dysplasia-carcinoma sequence (Shirvani et al. 2000; Zhang et al. 2001a; van der Woude et al. 2002). Souza *et al* demonstrated that selective COX-2 inhibitors block the growth of oesophageal adenocarcinoma cell lines after 48 hr of treatment. In this study we demonstrate that aspirin sensitises oesophageal cells to DCA-induced apoptosis within 6 hr **(Fig 6.2)**. Acid suppression therapy is known

to increase the relative concentration of unconjugated bile acids in the refluxate while reducing bile acid reflux (Theisen et al. 2000). The use of NSAIDS in combination with proton pump inhibitors might therefore prove highly effective in potentiating bile acid-induced apoptosis.

6.6 Future Prospects

The data presented in this thesis demonstrates an important role for bile acids mainly DCA, in activating the PKC and MAPK signalling cascades in gastrointestinal cells and provides insights into DCA-induced transcriptional deregulation. However, further work to expand our knowledge of the role of bile acids as epigenetic factors in the pathogenesis of oesophageal adenocarcinoma could be envisaged. Some of these experimental studies would complement the data presented in this thesis while others represent a significant departure from the described studies.

In animal models, reflux of duodenal contents leads to inflammation and mucosal thickening. Bile reflux is thought to impair oesophageal motility through increased mucosal thickening, enabling mucosal damage by decreasing acid and bile clearance (Freedman et al. 2002). In animal models of reflux, cyclin D1 is exclusively localised to the abnormally expanded basilar proliferative zone of the oesophageal mucosa (Zhang et al. 2001a). Immunoblotting and DNA affinity purification experiments demonstrate that DCA activates Erk, which is essential for Fra-1 activation (Chapter V). Expression of cyclin D1 is a consistent feature of oesophageal cancer (Arber et al. 1996). Since both Erk and Fra-1 regulate cyclin D1 expression (Lavoie et al. 1996; Mechta et al. 1997), a detailed investigation of the regulation of cyclin D1 expression in response to DCA would provide invaluable information in oesophageal proliferation and cancer progression, is a consistent feature of oesophageal cancer.

Barrett's oesophagus is characterised by a Th2 cytokine profile (Fitzgerald et al. 2002). Increased expression and secretion of IL-6 in patients with Barrett's oesophagus has recently been demonstrated (Dvorakova et al. 2004). As IL-6 is thought to be involved in malignant transformation, tumour progression and inhibition of apoptosis (Hirano 1998), an interesting study would be to compare the cytokine profile of oesophageal cells following bile acid stimulation with the cytokine profile of tissue from patients with Barrett's oesophagus. IL-6 warrants specific attention especially given DCA induces

persistent activation of Fra-1 in oesophageal cancer cells and constitutive activation of Fra-1 and JunD has been shown to be essential for IL-6 expression in prostate cancer cells (Zerbini et al. 2003). This would further substantiate the importance of bile acid reflux in the malignant development of Barrett's oesophagus.

Acquisition of apoptosis resistance is a feature of Barrett's oesophagus (van der Woude et al. 2002). This is most likely associated with elevated expression of STAT3 and the anti-apoptotic proteins Mcl-1 and Bcl-x_L in tissue from Barrett's oesophagus (Dvorakova et al. 2004). Considering that Erk regulates STAT3 activation (Chung et al. 1997; Zhang et al. 2001b) and that Erk is activated in response to DCA, an investigation of the transcriptional regulation of STAT3 by DCA will be a fruitful exercise. Inhibition of STAT activation would clearly represent a novel therapeutic strategy. Bile acids are known to activate Mcl-1 and Bcl-x_L in cholangiocarcinoma cells and hepatocytes (Qiao et al. 2001b; Yoon et al. 2002b). The role of the Bcl-2 family in bile acid-mediated events in oesophageal cells might represent an interesting avenue of research and would help to elucidate other mechanisms utilised by bile to contribute to apoptosis resistance in Barrett's epithelium.

In the future, a greater understanding of the cellular and molecular alterations in Barrett's oesophagus will enable the development of a more effective therapeutic approach. In the advent of the genomic and proteomic revolution, high throughput and site specific analysis will allow detection of modifications in specific survival and apoptotic pathways, which will enable the identification of novel candidates for biomarker development and cancer prevention.

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APPENDICES

Appendix A

Reagents and Buffers

1- *Cell Culture Reagents*

Complete RPMI 1640 Medium

RPMI 1640	500 ml
FCS	50 ml
Culture Cocktail	10 ml

McCoys Modified 5A Complete Medium

McCoys modified 5A	500 ml
FCS	50 ml
Culture Cocktail	10 ml

CO_2 Independent Medium

CO_2 Independent Medium	500 ml
FCS	50 ml
Culture Cocktail	10 ml

Hanks Balanced Salts Solution

HBSS	500 ml
Hepes (1 M)	10 ml

Cryogenetic Medium

FCS	9.0 ml
Dimethyl Sulfoxide	1.0 ml

Ethidium Bromide (EB) Stock

EB	100 mg
PBS	20 ml

Acridine Orange (AO) Stock Solution

AO	20 mg
PBS	20 ml

EB/AO Working Solution

EB Solution	40 µl
AO Solution	40 µl

Make up to 10 ml with PBS.

2- Cell Protein Preparations

1X Phosphate Buffered Saline (PBS)
One tablet dissolved in 500 ml distilled water.

1X Tris Buffered Saline (TBS)
One sachet dissolved in 500 ml distilled water.

Buffer C

Tris-HCL (pH 7.5)	41.4 ml
SDS	0.5 g
1 M NaCl	7.5 ml
100 mM EGTA	500 µl
0.5 M EDTA	100 µl
0.1 M PMSF	250 µl
2 mg/ml Leupeptin	250 µl

Hypotonic Buffer

100 mM $NaHCO_3$	50 µl
1 M $MgCL_2$	25 µl
0.1 M PMSF	50 µl
5 mg/ml leupeptin	10 µl
Complete Inhibitors	200 µl
Distilled Water	4665 µl

Buffer B

20 mM Tris-HCL (pH 7.5)	3880 µl
NP-40	50 µl
1 M NaCl	750 µl
0.1 M EGTA	50 µl
0.5 M EDTA	10 µl
0.1 M PMSF	50µl
5 mg/ml leupeptin	10 µl
Complete Inhibitor	200 µl

25X Protease Inhibitor Cocktail
One tablet dissolved in 2 ml distilled water.
Store 200 µl Aliquots in -20°C.

2X Cell Lysis Stock

1 M Tris-HCL pH 7.9	50 ml
0.5 M Sodium Fluoride pH 8.0	20 ml
Glycerol	100 ml
0.5 M EDTA	0.2 ml

Make up to 500 ml with distilled water.
Store at 4°C.

Lysis Buffer

2X Cell lysis stock	2.5 ml
10 % NP-40	500 µl
0.5 M NaCl	150 µl
0.1 M Sodium Orthovandate	50 µl
0.1 M PMSF	50 µl
25X Protease Inhibitor Cocktail	200 µl

Make up 5 ml with distilled water.
Make up fresh.

High Salt Lysis Buffer

2X Cell lysis stock	2.5 ml
10 % NP-40	500 µl
2.5 M NaCl	300 µl
0.1 M Sodium Orthovandate	50 µl
0.1 M DTT	50 µl
0.1 M PMSF	50 µl
25X Protease Inhibitor Cocktail	200 µl

Make up 5 ml with distilled water.
Make up fresh

Salt Free Lysis Buffer

2X Cell lysis stock	2.5 ml
10 % NP-40	500 µl
0.1 M Sodium Orthovandate	50 µl
0.1 M DTT	50 µl
0.1 M PMSF	50 µl
25X Protease Inhibitor Cocktail	200 µl

Make up 5 ml with distilled water.
Make up fresh

BSA Buffer Solution

Bovine Serum Albumin	20 mg

Make to 1 ml with cell lysis buffer.

3- Polyacrylamide Gel Electrophoresis

2X Sample Buffer

Glycerol
SDS
0.5 M Tris-HCL pH 6.8
0.2 % Bromophenol blue
Store as 450 µl aliquots in -20°C.
On day of use, aliquots were thawed and 50 µl β-Mercatoethanol and 500 µl water were added.

Resolving Gel Buffer

Tris Base	36.3 g
Distilled Water	100 ml

Adjust to pH 8.8 with conc. HCL.

Stacking Gel Buffer

Tris Base	6 g
Distilled Water	100 ml

Adjust to pH 6.8 with conc. HCL.

10 % Ammonium Persulphate (APS)

APS	0.1 g
Distilled Water	1.0 ml

Make up fresh before use.

Acrylamide/Bisacrylamide Mix

Acrylamide	30 g
Bisacrylamide	0.8 g
Distilled Water	100 ml

Water-Saturated Butanol

Butanol	10 ml
Distilled Water	10 ml

Mixed well and allowed to settle. Top layer was used.

10X Electrode Buffer

Tris Base	30 g
Glycine	114 g
SDS	5 g

Dissolved in 1 L of distilled water.
Dilute 1:10 in distilled water before use.

4- *Polyacrylamide Gel Electrophoresis*

Transfer Buffer

Tris Base	2.9 g
Glycine	1.45 g
SDS	0.185 g

Dissolved in 400 ml distilled water.
Once dissolved, 100 ml of Methanol was added.

Coomassie Blue Gel Stain

Coomassie Brilliant Blue R	0.5 g
Methanol	200 ml
Glacial Acetic Acid	35 ml
Distilled Water	265 ml

5- *Immunoblotting*

Blocking Solution

Skimmed Dried Milk (Marvel)	5 g
PBS/TBS	100 ml

Make up fresh before use.

5.0 % BSA/TBS

30% BSA	833 µl
TBS	4167 µl

0.05 % PBS-Tween Washing Solution

PBS	500 ml
Tween 20	250 µl

Primary Antibody Solution

Primary Antibody	10 µl
Blocking Solution	10 ml

Secondary Antibody Solution

Secondary Antibody peroxidase conjugated Ig	10 µl
Blocking Solution	10 ml

6- *Immunostaining*

4 % Paraformaldehyde

Paraformaldehyde	0.4 g
PBS	10 ml

Solution was heated to 65°C to dissolve contents and allowed to cool.
Once cool, solution was filtered.
Make up fresh before use.

Permeabilisation Buffer

Trition X-100	10 µl

Make up to 10 ml with PBS and store at 4°C.

Antibody Buffer

BSA	0.1 g
10 % Azide	100 µl

Make up to 10 ml with PBS and store at 4°C.

Primary Antibody Solution

Primary Antibody	4 µl
Antibody Buffer	196 µl

Secondary Antibody Solution

Secondary Antibody	2 µl
Antibody Buffer	198 µl

Stripping Buffer

14.3 M β-Mercapoethanol	350 µl
SDS	1 g
1 M Tris pH 6.8	3.125 ml

Make up to a final volume of 50 ml with distilled water.

7- *Nuclear Extract Preparation*

Extractions Buffers

All stock solutions should be sterile.

Buffer A

1 M Hepes pH 7.9	100 µl
1 M Magnesium Chloride	15 µl
1 M Potassium Chloride	100 µl
0.1 M PMSF	50 µl
1 M DTT	5 µl

Make up to 10 ml with sterile water.

Buffer C

1 M Hepes pH 7.9	200 µl
1 M Magnesium Chloride	15 µl
1 M Sodium Chloride	4.2 ml
0.5 M EDTA	4 µl
25 % Glycerol	2.5 ml
0.1 M PMSF	50 µl

Make up to 10 ml with sterile water.

Buffer D

1 M Hepes pH 7.9	100 µl
1 M Potassium Chloride	500 µl

0.5 M EDTA	4 µl
20 % Glycerol	2 ml
0.1 M PMSF	50 µl
1 M DTT	5 µl

Make up to 10 ml with sterile water.

8- *Electrophoretic Mobility Shift Assay*

10X Tris Borate Buffer (TBE)

Tris Base	108 g
Boric Acid	54 g
0.5 M EDTA	40 ml

Make up to 1 L with distilled water.

1X TE Buffer

1 M Tris-HCL pH 8.0	100 µl
0.5 M EDTA	20 µl

Make up to 10 ml with distilled water and autoclave.

10X Binding Reaction Buffer

1 M Tris pH 7.5	50 µl
5 M NaCL	100 µl
Glycerol	200 µl
0.5 M EDTA	10 µl
1 M DTT	25 µl
10 mg/ml Nuclease Free BSA	50 µl

Make up to 500 µl with sterile water.

9- *Plasmid Preparation*

All Stock Solutions were autoclaved.

Luria-Bertani (LB) Broth Medium

Gibco® LB Powder	20 g
Distilled water	1000 g

L-Broth Agar

L-Broth	20 g
Agar	15 g

Make up to 1 L with distilled water and autoclave immediately.

Antibiotic Selection

Amplicillin	100 mg/ml
Kanamycin	30 mg/ml
Kanamycin	50 mg/ml

10- *Agarose Gel Electrophoresis*

10X TAE Buffer

Tris Base	48.8 g
Glacial Acetic Acid	11.42g
0.5 M EDTA pH8.0	20 ml

Electrode Buffer

10X TAE Buffer	50 ml
Distilled Water	450 ml

0.8% Agarose Gel

Agarose	0.32 g
Electrode Buffer	40 ml

Solution was heated to dissolve agarose.

Gel Loading Dye

Bromophenol Blue	0.125 g
Xylene Cyanol	0.125 g
Glycerol	15 ml

Make up to 50 ml with distilled water.

11- *Affinity Precipitation*

TE/NaCl Buffer

Tris-HCL pH 8.0	10 mM
0.5 M EDTA	1 mM
NaCL	75 mM

Appendix B

Publications

Bile Acid Deoxycholate Induces Differential Subcellular Localisation of the PKC Isoenzymes β_1, ε and δ in a Sodium Butyrate Insensitive Manner in Colonic Epithelial Cells.
E. Looby, A. Long, D. Kelleher, Y. Volkov. *International Journal of Cancer*. 2005 May 10; 114(6): 887-95.

Sustained MAPK Activation Stimulates the Transcription Factor AP-1 in Oesophageal Cancer Cells: Induction by Bile Acid Exposure.
E. Looby, V. Athié Morales, M. M. Abdel-Latif, A. Long, D. Kelleher. *(Manuscript in preparation)*

Differential Effects of the Tumour Promoting Bile Acid Deoxycholic Acid and the Chemoprotective Ursodeoxycholic Acid on Protein Kinase C Beta1, Epsilon and Delta in Human Colon Cancer Cells.
S. A. Shah, **E. Looby**, Y. Volkov, A. Long and D. Kelleher. *(Manuscript under Review).*

The Scaffolding Protein CG-NAP/AKAP450 is a Component of LFA-1 Induced Cytoskeleton/Signalling Complex in Locomotory T Cells.
B. S. El Din El Homasany, Y. Volkov, M. Takahashi, Y. Ono, G. Keryer, A. Delouvée, **E. Looby**, A. Long, D. Kelleher. *(Manuscript under Review)*

CG-NAP: An Essential Component of LFA-1 Induced Cytoskeleton/Signaling Complex.
B. S. El Homasany, Y Volkov, M Takahashi, Y Ono, G Keryer, **E Looby**, A Long, and D Kelleher (2003). Abstract. *Molecular Biology of the cell. Volume 14, p.437a, no.2447, Nov.2003 Supplement*

Presentations

Differential Activation of The PKC Isoenzymes To The Bile Acid Deoxycholate Using GFP-Fusion Constructs.
Eileen Looby, Yuri Volkov, Sinead McGrath, Dermot Kelleher.
Keystone Symposium 2001, Taos, New Mexico, US.

Differential Response of The PKC Isoenzymes To The Bile Acid Deoxycholate and Short Chain Fatty Acid Sodium Butyrate Using GFP-Fusion Constructs.
Eileen Looby, Yuri Volkov, Sinead McGrath, Dermot Kelleher.
UK Biochemical Society 2001, Brighton, UK

Real Time Imaging Using GFP-Tagged Isoenzymes Reveals Differential Activation Of PKC Isoenzymes By The Bile Acid Deoxycholate And The Short Chain Fatty Acid Sodium Butyrate.

Eileen Looby, Yuri Volkov, Sinead McGrath, Dermot Kelleher.
Digestive Disease Week (DDW) 2001, Atlanta, Georgia, US.
Awarded Poster of Distinction.

Bile Acid Exposure Induces Activation of the Extracellular Signal-Regulated Kinase and the Transcription Factor AP-1 in Esophageal Cancer Cells.
Eileen Looby, Mohamed M. M. Abdel-Latif, V. Athié Morales, Dermot Kelleher.
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