INVESTIGATION INTO THE INTRACELLULAR MECHANISMS WHEREBY LONG-CHAIN FATTY ACIDS PROTECT THE HEART IN ISCHAEMIA/REPERFUSION

by

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Dissertation presented for the Degree of

DOCTOR OF PHILOSOPHY

(Medical Physiology)

in the

Department of Medical Physiology

Faculty of Health Sciences

at

Stellenbosch University

STELLENBOSCH

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April 2005

DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

Date:

ABSTRACT

Although there is evidence for a protective role of long-chain polyunsaturated fatty acids (PUFAs) in cardiovascular disease, their mechanism of action as well as their participation in intracellular signalling processes remain to be elucidated. Therefore the aims of this study were twofold: (i) to characterize the roles of the mitogen-activated protein kinases (MAPKs) and protein kinase B (PKB/Akt) in ischaemia/reperfusion-induced apoptosis of neonatal cardiomyocytes and (ii) to establish whether long-chain PUFAs protect the heart via manipulation of these kinases.

Rat neonatal ventricular myocytes exposed to simulated ischaemia and reperfusion (SI/R) were used to characterize the role(s) of extracellular signal-regulated kinase (ERK), p38 and c-Jun NH_2-terminal protein kinase (JNK), as well as PKB/Akt in apoptosis. The effects of an omega-3 fatty acid (eicosapentaenoic acid – EPA) and an omega-6 fatty acid (arachidonic acid – ARA) on the response of neonatal rat cardiomyocytes to SI/R with regard to the above parameters were determined.

Exposure of the myocytes to SI (energy depletion induced by KCN and 2-deoxy-*D*-glucose) reduced cell viability, as measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, and stimulated apoptosis (increased caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage). However, morphological evidence of increased apoptosis (Hoechst 33342 staining) occurred only after reperfusion. A rapid activation of p38 and PKB/Akt Ser^{473} occurred during SI, while significant activation of ERK and JNK was observed during reperfusion only. Myocytes pre-treated with SB203580, a p38-inhibitor, displayed a significant increase in cell viability and attenuation of apoptosis during SI/R, while SP600125, a specific JNK inhibitor, significantly increased both caspase-3 activation and the apoptotic index. However, PD98059, an ERK inhibitor, was without effect. Wortmannin, a PI3-kinase inhibitor, reduced PKB/Akt Thr^{308} but not Ser^{473} phosphorylation during SI/R and caused a significant increase in

PARP cleavage during reperfusion, but had no effect on caspase-3 activation or the apoptotic index.

EPA and ARA (20 μM, present before and after SI) significantly reduced caspase-3 activation, PARP-cleavage and the apoptotic index during reperfusion. This was associated with increased ERK- and decreased p38 phosphorylation. Vanadate (a tyrosine phosphatase inhibitor), but not okadaic acid (a serine-threonine phosphatase inhibitor), significantly reduced ARA-induced inhibition of p38 phosphorylation, suggesting involvement of tyrosine phosphatases during SI/R. MKP-1, a dual-specificity phosphatase, was targeted and a significant induction of MKP-1 by ARA and EPA was observed. An *in vitro* dephosphorylation assay confirmed that this phosphatase might be responsible for the inhibition of p38 activation. It was also demonstrated that the protective actions of ARA are PI3-K dependent.

The results suggest that p38 has a pro-apoptotic role while JNK phosphorylation is protective and that these kinases act via caspase-3 to prevent or promote cell survival in response to SI/R-induced injury. It was demonstrated for the first time that EPA and ARA protect neonatal cardiac myocytes from ischaemia/reperfusion-induced apoptosis through induction of a dual-specific phosphatase, MKP-1, causing dephosphorylation of the pro-apoptotic kinase, p38. These beneficial effects of ARA and EPA were also reflected by improvement in functional recovery during ischaemia/reperfusion of the isolated perfused rat heart model.

OPSOMMING

Dit word algemeen aanvaar dat lang-ketting poli-onversadigde vetsure teen kardiovaskulêre siektes beskerm, maar hul meganisme van aksie sowel as hul invloed op intrasellulêre seinoordragpaaie is egter onbekend. Die doelwitte van hierdie studie is dus tweevoudig: (i) om die belang van mitogeen-geaktiveerde proteïen kinases (MAPKs) en protein kinase B (PKB/Akt) in isgemie/herperfusie-geïnduseerde apoptose vas te stel en (ii) om te bepaal of lang-ketting poli-onversadigde vetsure die hart, deur manipulering van hierdie kinases, beskerm.

Rot neonatale ventrikulêre miosiete, blootgestel aan gesimuleerde isgemie en herperfusie (SI/H), is gebruik om die aktivering van ekstrasellulêre sein-gereguleerde kinase (ERK), p38, c-Jun NH_2-terminale protein kinase (JNK) asook PKB/Akt tydens apoptose, te karakteriseer. Die effek van 'n omega-3 vetsuur (eikosapentaenoësuur – EPA) en 'n omega-6 vetsuur (aragidoonsuur – ARA) op die respons van bogenoemde kinases in neonatale kardiomiosiete tydens SI/H, is ondersoek.

Blootstelling van miosiete aan SI (energie-uitputting geïnduseer deur kaliumsianied en 2-deoksi-*D*-glukose) het 'n afname in die vermoë van die sel om te oorleef, soos gemeet deur die MTT (3-[4,5-dimetieltiazol-2-yl]-2,5-difeniel tetrazolium bromied) bepaling, tot gevolg gehad. 'n Toename in apoptose (kaspase-3 aktivering en poli(ADP-ribose) polimerase (PARP) kliewing) is ook waargeneem. Morfologiese bewyse van apoptose (Hoechst 33342 kleuring) was egter eers tydens herperfusie sigbaar. SI is gekenmerk deur vinnige aktivering van p38 en PKB/Akt Ser^{473}, terwyl ERK en JNK fosforilering slegs tydens herperfusie waargeneem is. Vooraf-behandeling met SB203580, 'n p38 inhibitor, het 'n beduidende toename in sellewensvatbaarheid asook 'n afname in die apoptotiese indeks tydens SI/H teweeggebring, terwyl SP600125, 'n spesifieke JNK inhibitor, apoptose bevorder het. PD98059, 'n ERK inhibitor, het geen invloed op apoptose tydens SI/H gehad nie. Wortmannin, 'n PI3-kinase inhibitor, het Thr^{308} (nie Ser^{473}) fosforilering onderdruk, gepaargaande met 'n toename in PARP

kliewing, maar dit het geen invloed op kaspase-3 aktivering of die apoptotiese indeks gehad nie.

EPA en ARA (20 μM, teenwoordig voor en na SI) het kaspase-3 aktivering en PARP kliewing asook die apoptotiese indeks tydens herperfusie beduidend verminder. Beide vetsure het ook 'n beduidende toename in ERK en afname in p38 fosforilering veroorsaak. Vanadaat ('n serien-threonien fosfatase inhibitor), maar nie "okadaic" suur ('n tirosien fosfatase inhibitor), kon die ARA-geïnduseerde inhibisie van p38 ophef nie. Induksie van MKP-1, 'n tweeledige-spesifieke fosfatase, is beduidend deur ARA en EPA tydens herperfusie verhoog. 'n *In vitro* defosforileringbepaling het bevestig dat hierdie fosfatase wel betrokke by die inhibisie van p38 kan wees. Daarbenewens is gevind dat die beskermende aksie van ARA PI3-K afhanklik is.

Hierdie resultate wys dat fosforilering van p38 pro-apoptoties is, terwyl JNK beskermend is en dat hierdie kinases via kaspase-3 seldood of oorlewing tydens SI/H-geïnduseerde beskadiging bemiddel. In hierdie model is daar vir die eerste keer getoon dat EPA en ARA neonatale kardiale miosiete teen isgemie/herperfusie-geïnduseerde apoptose beskerm deur induksie van MKP-1, wat defosforilering van die pro-apoptotiese kinase, p38 teweegbring. Hierdie voordelige effekte van EPA en ARA is ook sigbaar in die funksionele herstel tydens isgemie/herperfusie van die geïsoleerde rothart model.

ACKNOWLEDGEMENTS

I sincerely wish to express my gratitude to:

Prof Amanda Lochner, my sincerest appreciation, not only for the many hours she spent in reviewing this thesis, but also for the encouragement, guidance and advice she so freely and willingly gave me at all times. I would like to thank her for giving me the freedom to pursue avenues not originally intended and for being an exceptional mentor and a source of great inspiration.

Dr Carine Page, my warmest gratitude for reviewing the thesis as co-promotor, and guiding me through the initial experimental work; and for the preparation of fibronectin.

The members of the heart lab, especially Petro Engelbrecht, Sonia Genade and Johanna van Wyk for technical assistance. Dr Barbara Huisamen, Dr Erna Marais, Dr Hans Strijdom and Suzél Hattingh for providing practical help and encouragement and for the laughter and pleasant atmosphere which pervades in the lab.

Dr Carola Niesler for assistance with the fluorescence microscopy work and Mary Mattheyse for encouragement and proof-reading at short notice.

My husband, Natie and daughter, Retha for their seemingly endless encouragement and patience during the preparation of this manuscript. My father, for checking the references. Both my parents and sisters, for their love, immense support and steadfast belief in my abilities. This thesis is dedicated to all of you.

My Heavenly Father for showing me the way; He provided me with knowledge, guidance and perseverance in abundance.

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LIST OF ABBREVIATIONS

AIF	Apoptosis inducing factor
Apaf	Apoptotic protease inducing factor
ARA	Arachidonic acid
ARC	Apoptosis repressor with caspase recruitment domain
ASK	Apoptosis signal-regulation kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CT-1	Cardiotrophin-1
CED	C.*elegans* death
cFLIP	Cellular FADD-like inhibitory protein
cIAP	Cellular inhibitor of apoptosis
COX-2	Cyclooxygenase-2
CPT-1	Carnitine palmitoyltransferase 1
CREB	cAMP-response element binding protein
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DSPs	Dual specificity phosphatases
EGF	Epidermal growth factor
EPA	Eicospentaenoic acid
ERK	Extracellular signal-regulated kinase
FABP	Fatty acid binding protein
FADD	Fas-associated death domain protein
FasL	Fas Ligand
FCS	Fetal calf serum
FFA	Free fatty acid
GLUT 4	Glucose transporter 4
IAP	Inhibitor of apoptosis protein
ICE	Interleukin-1β-converting enzyme

IL-3	Interleukin-3
JNK	c-Jun N-terminal kinase
LCAS	Long-chain acyl-CoA synthase
LCFA	Long-chain fatty acid
MAPK	Mitogen-activated protein kinase
MEF-2	Myocyte enhancer factor-2
MEK	MAP kinase kinase
MKP-1	MAPK phosphatase-1
MLK	Mixed lineage kinase
MUK	MAPK upstream kinase
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
PAK	p21-activated kinase
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PI3-K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PP2A	protein phosphatase 2A
PT	Permeability transition
PTPs	Protein tyrosine phosphatases
PUFAs	Polyunsaturated fatty acids
RAIDD	RIP associated ICH/Ced-3-homologous death domain protein
RPTK	Receptor protein tyrosine kinase
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SAPK	Stress activated protein kinase
SH2	Src homology domain type 2
SI/R	Simulated ischaemia and reperfusion
TAK	TGF-β activated kinase 1

TAO	Thousand and one amino acid protein kinase
TGF	Transforming growth factor
TNF	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
Tpl-2	tumor progression locus-2
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor associated factor
UV	Ultraviolet

CHAPTER 1 INTRODUCTION

1.1 MOTIVATION FOR STUDY

There is increasing evidence that fatty acids are important role players in cardiovascular disease. Fatty acids, released from membrane phospholipids by cellular phospholipases or taken up by the cell from the extracellular environment, play an important role in many steps in cytosolic signalling pathways, initiated by triggers at the level of the cell membrane (Speizer *et al.*, 1991). In this regard, fatty acids can either act as second messengers, i.e. molecules involved in the transduction of external signals to the cytosol in a rapid and transient fashion, or they can act as modulators, i.e. molecules acting in a reversible manner at a precise intracellular location for a very short time to amplify, attenuate or deviate a signal. It has indeed been shown that fatty acids can substitute for classical second messengers of the inositide phospholipid and cAMP signal transduction pathways. Since fatty acids can modulate the composition of the membrane phospholipids, the structural unit of biological membranes, they can modify the activities of phospholipases, protein kinases, G-proteins, adenylate and guanylate cyclases, as well as ion channels (Graber *et al.*, 1994) and thus also affect the response of the heart to ischaemia/reperfusion.

During the past ten years it has become clear that, in addition to necrosis, apoptosis plays an important role in ischaemia/reperfusion injury. Apoptosis or programmed cell death is a physiological process critical for organ development, tissue homeostasis and elimination of defective or potentially dangerous cells in complex organisms – it permits cell death without a concomitant inflammatory response in the surrounding tissues. The process of apoptosis depends on the reception of multiple extracellular and intracellular signals, integration and amplification of these signals by second messengers and finally, activation of the death effector proteases. Defects in control of the apoptotic pathways may contribute to a variety of diseases, including coronary heart disease.

Myocardial ischaemia/reperfusion activate several protein kinase pathways, which potentially regulates the onset of cellular injury and apoptosis. The primary protein kinases that are activated under these circumstances include the mitogen-activated protein kinases (MAPKs) and protein kinase B (PKB/Akt).

MAPKs are proline-directed Ser/Thr protein kinases activated by dual phosphorylation on both a tyrosine and a threonine residue (Waskiewicz & Cooper, 1995). These enzymes are critical components of a complex intracellular signalling network that ultimately regulates gene expression in response to a variety of extracellular stimuli. The three well-known mammalian MAPK families are: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and the p38 MAP kinases (p38 MAPKs). Each of these enzymes is a target for discrete but closely related phosphorylation cascades in which the sequential activation of three kinases constitutes a common signaling module. The best characterized MAPK pathway is the Ras/Raf/MEK cascade leading to the activation of ERK1/2 in response to growth factors (Davis, 1993). JNK and p38 MAPK are key mediators of stress signals and inflammatory responses evoked by a variety of agents such as UV- and γ-irradiation, heat shock, osmotic stress, and inflammatory cytokines (Hibi *et al.*, 1993; Han *et al.*, 1994). Many studies have suggested an important role for the MAPKs in apoptosis in ischaemia/reperfusion, but the reports on their specific roles are conflicting (reviewed by Steenbergen, 2002).

Studies conducted over the last few years also showed that PKB/Akt (a serine/threonine protein kinase) is critical for cell survival. For example, dominant negative alleles of PKB/Akt reduce the ability of growth factors and other stimuli to maintain cell survival whereas over-expression of wild type or activated PKB/Akt can rescue cells from apoptosis induced by various stress signals (Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997). Although there

can be little doubt that PKB/Akt promotes cell survival, the mechanisms involved have only recently begun to emerge.

In addition, although there is ample evidence for the role of polyunsaturated fatty acids (PUFAs) as second messengers in signal transduction cascades, there is no evidence for their participation in or effect on the MAPK or PKB/Akt signal transduction pathways during stressful conditions such as myocardial ischaemia and reperfusion.

Using cultured neonatal cardiomyocytes as model, the aims of this study are:

- To characterize the effects of simulated ischaemia and reperfusion on the phosphorylation status of the MAPKs and PKB/Akt as well as their role in apoptosis.
- To determine the effect of long-chain PUFAs on cell viability and apoptosis during simulated ischaemia and reperfusion.
- To determine the importance of the MAPK family and PKB/Akt in fatty acid-induced protection during simulated ischaemia and reperfusion.
- To unravel some of the signalling mechanisms in the above-mentioned cascades.
- To determine the effects of long-chain PUFAs on mechanical recovery of the isolated rat heart subjected to ischaemia/reperfusion.

Before a study of this nature can be attempted, a thorough knowledge and insight into the processes of necrosis, apoptosis and the MAPK- as well as PKB/Akt signalling pathways are required. In addition, fatty acid metabolism and β-oxidation as well as the participation of these processes in apoptosis and signal transduction need to be outlined for the purpose of this study. The current understanding and knowledge of these aspects will now be addressed in the literature discussion.

CHAPTER 2 LITERATURE DISCUSSION

2.1 Apoptosis in cardiovascular disease

2.1.1 Introduction

Cardiovascular disease remains one of the major killers in modern society. It is believed to account for ~12 million deaths annually (Gill *et al.*, 2002). To enable the development of viable treatment strategies, the complex pathogenesis of the disease must first be understood.

In the human heart, cardiac failure is characterized by the progressive death of myocytes (Olivetti *et al.*, 1997). Until recently, it was believed that all adult cardiomyocyte death is caused by necrosis (unprogrammed cell death as a result of massive injury). However, Narula *et al.* (1996) and Olivetti *et al.* (1997) provided the first evidence that apoptosis (programmed cell death) occurs in the myocardium of patients with end-stage dilated cardiomyopathy. Similarly, Saraste *et al.* (1997) demonstrated the presence of apoptotic cells in the infarct and peri-infarct regions of myocardium obtained from patients dying from a recent myocardial infarction. Probably the best information about apoptosis in the heart has been derived from studies of experimental ischaemia/reperfusion. For example, Kajstura and co-workers have shown that apoptosis is the predominant mode of cell death in ischaemia-reperfused tissue (1996).

The significance of cell death by apoptosis in cardiac pathology recently gained great interest as, unlike necrosis, which is thought to be an essentially irreversible process, the step-by-step nature of apoptosis suggests it may be amenable to therapeutic intervention.

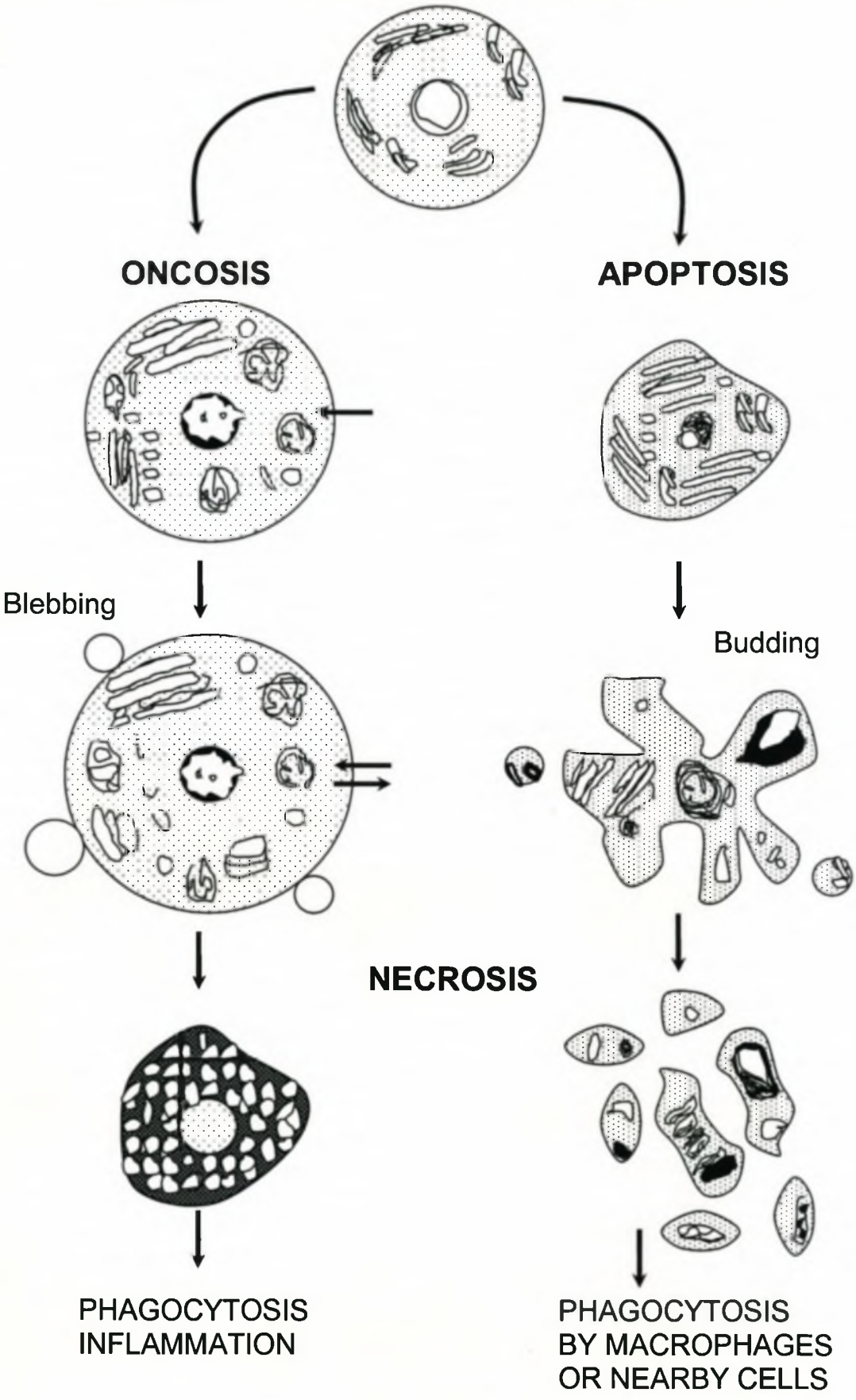
2.1.2 A morphologic description of the apoptotic and necrotic cell

Apoptosis was first reported by Kerr and co-workers (1972), a group of pathologists studying cell population regulation. In this paper, the authors described a form of cell death marked by its singularity, unique morphology and resolution without apparent traces of inflammation in the tissue of origin. Apoptosis may be considered a mechanism that counterbalances the effects of cell proliferation by mitotic division. In fact, deregulated apoptosis has been implicated as a fundamental pathogenic mechanism in a variety of human diseases. Excessive apoptotic cell death may cause organ atrophy and organ failure, as suggested for neurodegenerative diseases and viral hepatitis. On the other hand, inefficient elimination of malignant, autoreactive, infected, or redundant cells may lead to the development of neoplasia, autoimmunity, viral persistence, and congenital malformations (Haunstetter & Izumo; 1998).

Apoptosis is an energy-dependent process, which is characterized by preservation of mitochondria and sarcolemmal integrity, nuclear chromatin condensation and removal by macrophages and neighbouring cells. On the other hand, prominent features of oncosis (accidental cell death), induced by depletion of intracellular ATP stores, include swelling, disruption of the sarcolemma and the mitochondria, chromatin clumping, blebbing and removal by inflammation (fig 2.1). The term "necrosis" often used to describe cell death other than apoptotic cell death, is imprecise, because it should be reserved for changes that occur after cell death irrespective of whether cell death is apoptotic or oncotic (Levin *et al.*, 1999). However, the term oncosis as proposed by Manjo and Joris (1995) has not been widely accepted even though the nomenclature would be more distinct. The term necrosis to indicate acute ischaemic cell death will, therefore, be used throughout this thesis.

Apoptosis is an active and physiological mode of cell death, in which the cell itself designs and executes the program of its own demise and subsequent

Figure 2.1 Schematic representation of typical characteristics of oncosis and apoptosis (Manjo and Joris, 1995)



disposal. A multistep complex mechanism regulates the cell's propensity to respond to various stimuli by apoptosis. The complexity of this mechanism has become apparent over the last decade (Oltvai & Korsmeyer SJ, 1994). The regulation system involves the presence of at least two distinct checkpoints, one controlled by the bcl-2/bax family of proteins (Hockenberry; 1995; Reed, 1994), another by cysteine-proteases (caspases) (Fernandes-Alnemri *et al.*, 1995; Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995) and possibly by serine-proteases (Bruno *et al.*, 1992; Hara *et al.*, 1996; Weaver *et al.*, 1993).

A cell undergoing apoptosis activates a series of molecular and biochemical events which lead to its total physical disintegration. Because many of these changes are very characteristic and appear to be unique to apoptosis, they have become markers used to identify this mode of cell death biochemically, by microscopy or cytometry. One of the early events is cell dehydration. Loss of intracellular water leads to condensation of the cytoplasm resulting in a change in cell shape and size. Another change, perhaps the most characteristic feature of apoptosis, is condensation of nuclear chromatin. The condensation starts at the nuclear periphery and the condensed chromatin often takes on a concave shape resembling a half-moon, horseshoe or sickle (Manjo & Joris; 1995). The condensed chromatin has a uniform, smooth appearance, with no evidence of any texture normally seen in the nucleus. DNA in condensed (pycnotic) chromatin exhibits hyperchromasia, staining strongly with fluorescent or light absorbing dyes. The nuclear envelope disintegrates, lamin proteins undergo proteolytic degradation, followed by nuclear fragmentation (karyorrhexis). Many nuclear fragments, which stain uniformly with DNA dyes and thereby resemble DNA droplets of different sizes, are scattered throughout the cytoplasm. The nuclear fragments, together with constituents of the cytoplasm (including intact organelles), are then packaged and enveloped by fragments of plasma membrane. These structures, called "apoptotic bodies", are then shed from the dying cell (Liu *et al.*, 1995a; Elsasser *et al.*, 1997). When apoptosis occurs *in vivo*, apoptotic bodies are phagocytosed by neighbouring cells, including those of

epithelial (Bennett *et al.*, 1995) or fibroblast origin (i.e. not necessarily by machrophages), without triggering an inflammatory reaction in the tissue (Cohen, 1993; Compton, 1992; Wyllie *et al.*, 1992a).

Activation of endonuclease(s) preferentially cleaving DNA between the nucleosomes is another characteristic event of apoptosis (Arends *et al.*, 1990; Compton, 1992; Wyllie *et al.*, 1992b). The products of DNA degradation are nucleosomal and oligonucleosomal DNA fragments (180 bp and multiples of 180 bp) which generate a characteristic "ladder" pattern during agarose gel electrophoresis.

Another characteristic feature of apoptosis is the preservation, at least during the initial phase of cell death, of the structural integrity and most of the plasma membrane transport function. Also, cellular organelles, including mitochondria and lysosomes remain preserved during apoptosis. The mitochondrial transmembrane potential, however, is markedly decreased (Petit *et al.*, 1995; Zamzami *et al.*, 1995a). Release of cytochrome c from mitochondria appears to be an earlier important event in apoptosis, triggering activation of caspases and other downstream apoptotic effectors (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997; Yang *et al.*, 1997, Kluck *et al.*, 1997a). Other features of apoptosis include mobilization of intracellular ionized calcium (McConkey *et al.*, 1989), activation of transglutaminase which crosslinks cytoplasmic proteins (Piacentini *et al.*, 1995), loss of microtubules (Endersen *et al.*, 1995), and loss of asymmetry of the phospholipids on the plasmamembrane. In most cell membranes, an asymmetry of phospholipids is maintained, with phosphatidylserine and phosphatidylethanolamine being confined to the inner leaflet and phosphatidylcholine and sphingomyelin are exposed on the external surface of the lipid bilayer. This asymmetry of membrane composition is lost during apoptosis, leading to exposure of phosphatidylserine on the cell surface, which can be recognized by specific receptors (Zwaal & Schroit, 1997).

While apoptosis is characterized by an active participation of the affected cell in its own demise, even to the point of triggering (in some cell systems), the *de novo* synthesis of the effectors, necrosis, on the other hand, is a passive, catabolic and degenerative process. Necrosis generally represents a cell's response to gross injury and can be induced, for example by an overdose of cytotoxic agents or exposure to ischaemia. The early events of necrosis are mitochondrial swelling followed by rupture of the plasma membrane and release of cytoplasmic constituents, which include proteolytic enzymes (Manjo & Joris, 1995; Wyllie, 1992a). Nuclear chromatin shows patchy areas of condensation and the nucleus undergoes slow dissolution (karyolysis). Necrosis triggers an inflammatory reaction in the tissue and often results in scar formation. DNA degradation is not so extensive during necrosis as in the case of apoptosis, and the products of degradation are heterogenous in size, failing to form discrete bands on elecrophoretic gels.

The pattern of cell death may not always have the classical features of either apoptosis or necrosis. Numerous examples of cell death have been described where the morphological and/or biochemical changes resembled neither typical apoptosis nor necrosis, but often had features of both (Ueda *et al.*, 1995; Schwartz *et al.*, 1993). In some cases, the integrity of the plasma membrane was preserved but DNA degradation was random, without evidence of internucleosomal cleavage. In other situations, DNA degradation was typical of apoptosis but nuclear fragmentation and other features of apoptosis were not apparent.

2.1.3 The molecular pathways through which apoptosis is induced

2.1.3.1 Introduction

One of the most widely recognized biochemical features of apoptosis is the activation of a class of cysteine proteases known as **caspases** (Thornberry &

Lazebnik, 1998). Caspases are present in the cell as inactive procaspases that are cleaved and activated in response to apoptotic stimuli. Initial activation of caspases may involve transduction of a signal from **membrane death receptors** belonging to the tumor necrosis factor (TNF) receptor family, such as Fas and TNF receptor 1 (TNFR1) (Ashkenazi & Dixit, 1998), or may be mediated by a **mitochondrial pathway** (Green & Reed, 1998).

Stimulation of death receptors results in the activation of caspase-8, which goes on to activate caspase-3, a key effector protein of the apoptotic machinery. Release of cytochrome c from the mitochondria, on the other hand, leads to activation of caspase-9 through formation of a cytosolic complex and subsequent activation of caspase-3.

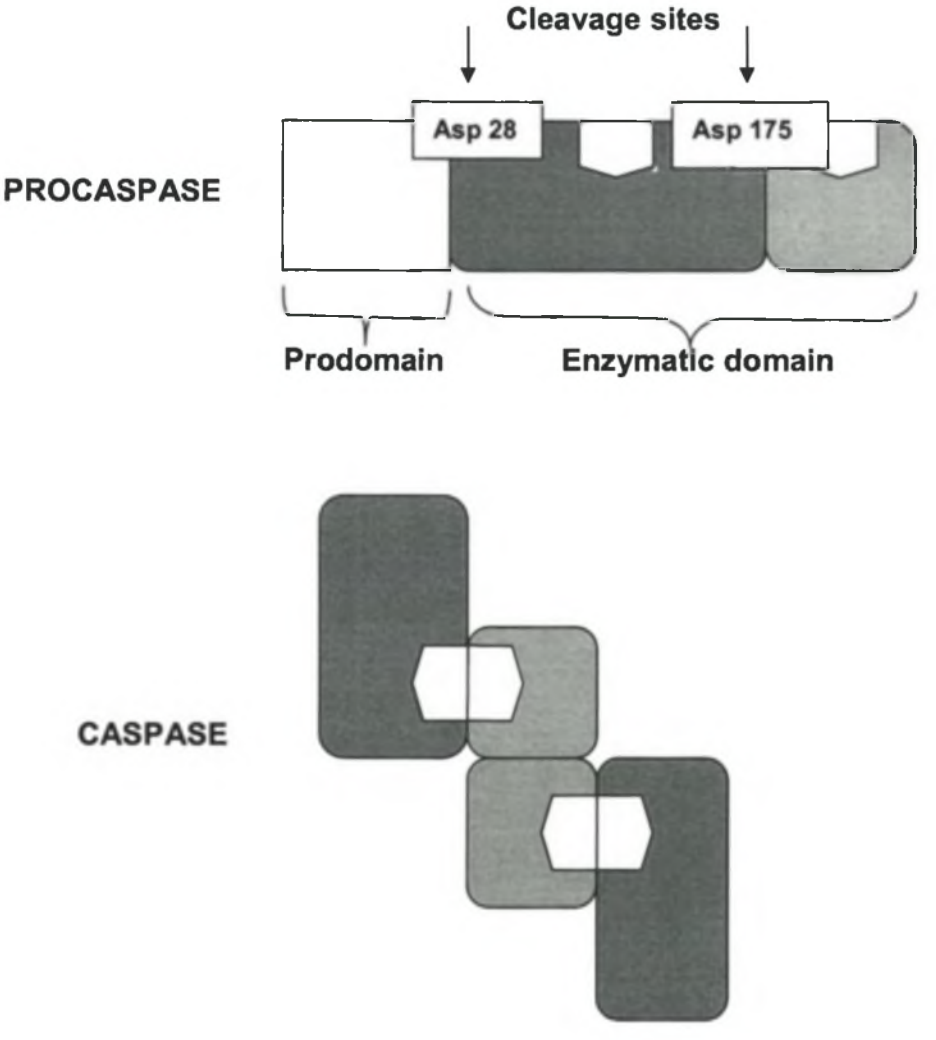
Unlike necrosis, which is a passive, unregulated process, apoptosis is both energy dependent and highly regulated. Apoptosis is controlled by the complex interaction of numerous prosurvival and prodeath signals. These include the **Bcl-2 family of proteins**, which may be antiapoptotic (Bcl-2, Bcl-X_L) or pro-apoptotic (Bad, Bid), and exerts its effects primarily at the level of mitochondria (Adams & Cory, 1998). Other important regulators of apoptosis act at the level of caspases. Such proteins include **cellular FADD-like inhibitory protein (cFLIP)** and the **inhibitor of apoptosis (IAP) family** (Rasper *et al.*, 1998; Uren *et al.*, 1998). There are also proteins that counteract the effect of the caspase inhibitors themselves such as the recently characterized Smac/DIABLO, a mitochondrial protein that, when released, binds and neutralizes IAPs, thus promoting caspase activity (Srinivasula *et al.*, 2000). Several other factors are also believed to be involved in the regulation of apoptosis including, growth factors, **mitogen activated protein kinases (MAPKs)**, **PKB/Akt,** calcium, and oxidants. The complex interaction of all these molecules determines the ultimate fate of the cell: life or death.

2.1.3.2 Caspases, the proteases of the apoptotic pathway

As stated before, a key phenomenon of apoptotic cell death is the activation of a unique class of aspartate-specific proteases known as caspases. Until now, at least 14 members have been identified in this subclass of proteases (Nicholson & Thornberry, 1997). In order to simplify the confusing terminology of the known aspartate-specific proteases, a new nomenclature has been adopted, classifying all aspartate-specific proteases as caspases (Alnemri *et al.*, 1996). Crystal structure analysis of the prototypical caspase ICE (caspase-1) revealed that the serine residue common to many proteases is replaced by a cysteine residue within a highly conserved pentameric sequence in the catalytic center (Walker *et al.*, 1994; Wilson *et al.*, 1994). All caspases are composed of a prodomain and an enzymatic region. Heterogeneity among the proteases exists regarding the structure of the prodomain, suggesting that this region may define important functional differences between caspases. Caspase-1, -2, -4, -5, -8, -9, and –10 contain a long prodomain of ≈15 to 25 kDA compared with <5 kDA in caspases-3, -6, and –7. For activation, the caspase proform has to be cleaved into a large subunit and a small subunit within the enzymatic domain that finally reassociate to form a complex comprising two small and two large subunits. The prodomain is not necessary for the proteolytic activity once the caspase is activated. Interestingly, all activating cleavages occur behind an aspartate residue (fig 2.2). Because this cleavage site is a unique characteristic of caspases (with the serine protease, granzyme B, being the only exception), activation can occur only through auto activation or cleavage by another caspase or granzyme B.

Cells possess multiple caspases, which may work in a cascade fashion. The redundancy may serve to amplify and accelerate the response, as well as to provide multiple mechanisms to complete the process. Indeed, the redundancy is apparent in the phenotypes of knockout mice in which the deletion of a single caspase is associated with a relatively unimpressive phenotype. Deletion of

Figure 2.2 Structural characteristics of caspases (Tran and Milller, 1999)



caspase-1 or caspase-11 does not result in dramatic phenotypic changes; thus the roles for these caspases are less clear (Li *et al.*, 1997; Wang *et al.*, 1998). However, deletion of caspase-3 results in a failure of neuronal apoptosis, and the mice are born with overlarge brains and die soon after birth (Kuida *et al.*, 1996).

Substrates for caspases comprise many different proteins, including nuclear proteins, proteins involved in signal transduction, and cytoskeletal targets (Cardone *et al.*, 1997; Kothakota *et al.*, 1997; Sakahira *et al.*, 1998). Most of these protein substrates appear to be cleaved by caspases-3 and –7. However, lamin is selectively cleaved by caspase-6 (Orth *et al.*, 1996; Takahashi *et al.*, 1996). Although many of the target proteins defined to date have a nuclear localization, apoptotic cell death does not depend on the presence of a cell nucleus, as the characteristic cytoplasmic features of apoptosis can be observed in anucleate cytoplasts (Jacobson *et al.*, 1994). Therefore, proteolytic cleavage of nuclear proteins may be important in eliciting the nuclear features of apoptosis, like chromatin margination induced by lamin B cleavage, but does not constitute a critical event for the apoptotic death of the entire cell (Liu *et al.*, 1997; Lazebnik *et al.*, 1995). Interestingly, internucleosomal DNA fragmentation requires the prior cleavage of a cytoplamic inhibitor of the apoptosis-specific endonuclease (Enari *et al.*, 1998; Sakahira *et al.*, 1998; Liu *et al.*, 1997). Only after cleavage of the inhibitor can the endonuclease translocate to the nucleus and degrade genomic DNA (Enari *et al.*, 1998; Sakahira *et al.*, 1998). For most of the downstream targets, the overall contribution to the final cell fate remains to be determined.

In summary, caspases can be grouped into an upstream (caspases -1, -2, -4, -5, -8, -9, -10) and a downstream (caspases -3, -6, -7) subgroup. Upstream caspases are characterized by long prodomains that appear to contain essential regulatory proteins. Most of the activity that finally leads to the lethal proteolytic breakdown of cellular target proteins is exerted by downstream caspases sensitive to DEVD (aspartate-glutamine-valine-aspartate) oligopeptides (caspase-3 and caspase-7).

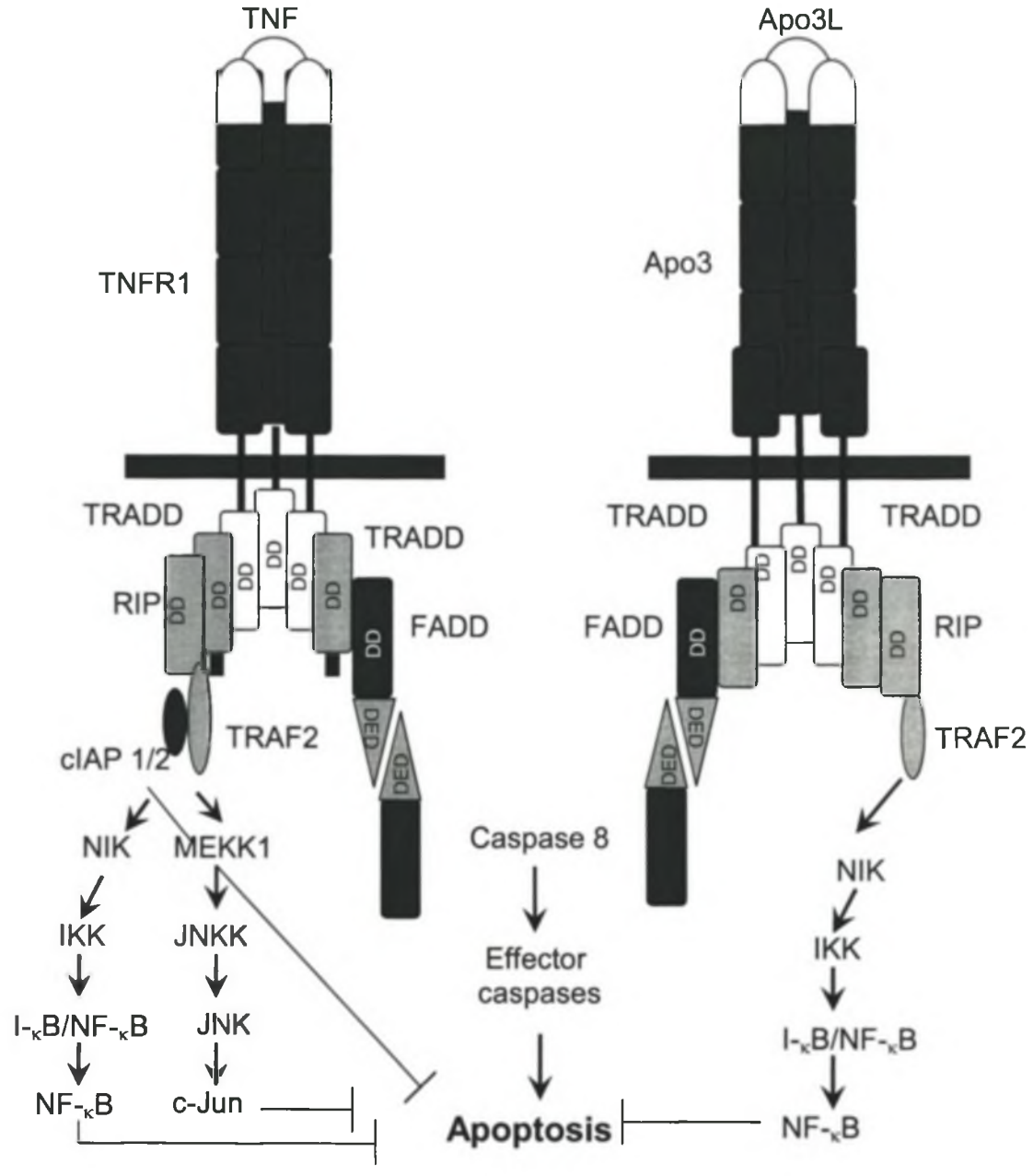
2.1.3.3 Mechanisms of caspase activation

Once downstream caspases, that execute the lethal cuts to vital cellular components, are activated, cell death appears to be inevitable. Therefore, understanding the mechanisms that initiate proteolytic activation of caspases is a crucial step in defining targets that allow for the modulation of apoptotic cell death. Recent data suggest that activation of caspases may take place either within **death receptor complexes** of the cytoplamic membrane or by a **mitochondrion-dependent mechanism** within the cytosol.

2.1.3.3 (a) Death- and decoy receptor pathway (fig 2.3)

The best characterized pathways for the initiation of apoptosis involve the binding of extracellular death signal ligands (FasL and TNF) to receptors that belong to the TNFR gene superfamily (Smith *et al.*, 1994). The cytoplasmic sequence divides the TNFR superfamily into two main subgroups of receptors that either possess or lack a 'death domain' (DD) (Tartaglia *et al.*, 1993; Itoh & Nagata, 1993). The death-domain-containing receptors, or 'death receptors' (DR) include TNFR1, Fas (Apo1/CD95), DR3 (Apo3), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and DR6. The death receptors interact via their DD with intracellular DD-containing adaptors, such as FADD (Fas-associating protein with DD) and TRADD (TNF receptor-associated death domain), and recruit these adaptors to the cell membrane. Thus, binding of Fas ligand to the Fas receptor leads to clustering of the Fas receptor's DD. The adapter, FADD, then binds through its own DD to the cluster receptor DD. FADD also contains a 'death effector domain' (DED) that binds to an analogous domain within the pro-caspase-8 protein. Upon recruitment by FADD, pro-caspase-8 oligomerization drives its own activation (to caspase-8) through self-cleavage. Caspase-8 then activates downstream effector caspases, such as caspase-3, thereby initiating apoptosis. Signaling induced by

Figure 2.3 Death receptor complexes



DD – death domain; DED – death effector domain; NIK – NF-κB-inducing kinase; IKK – inhibitor of κB kinase complex; TRADD – TNFR-associated death domain; RIP – receptor interacting protein; TNF – tumor necrosis factor; TNFR – tumor necrosis factor receptor; TRAF2 – TNFR-associated factor-2 (Ashkenazi & Dixit, 1998)

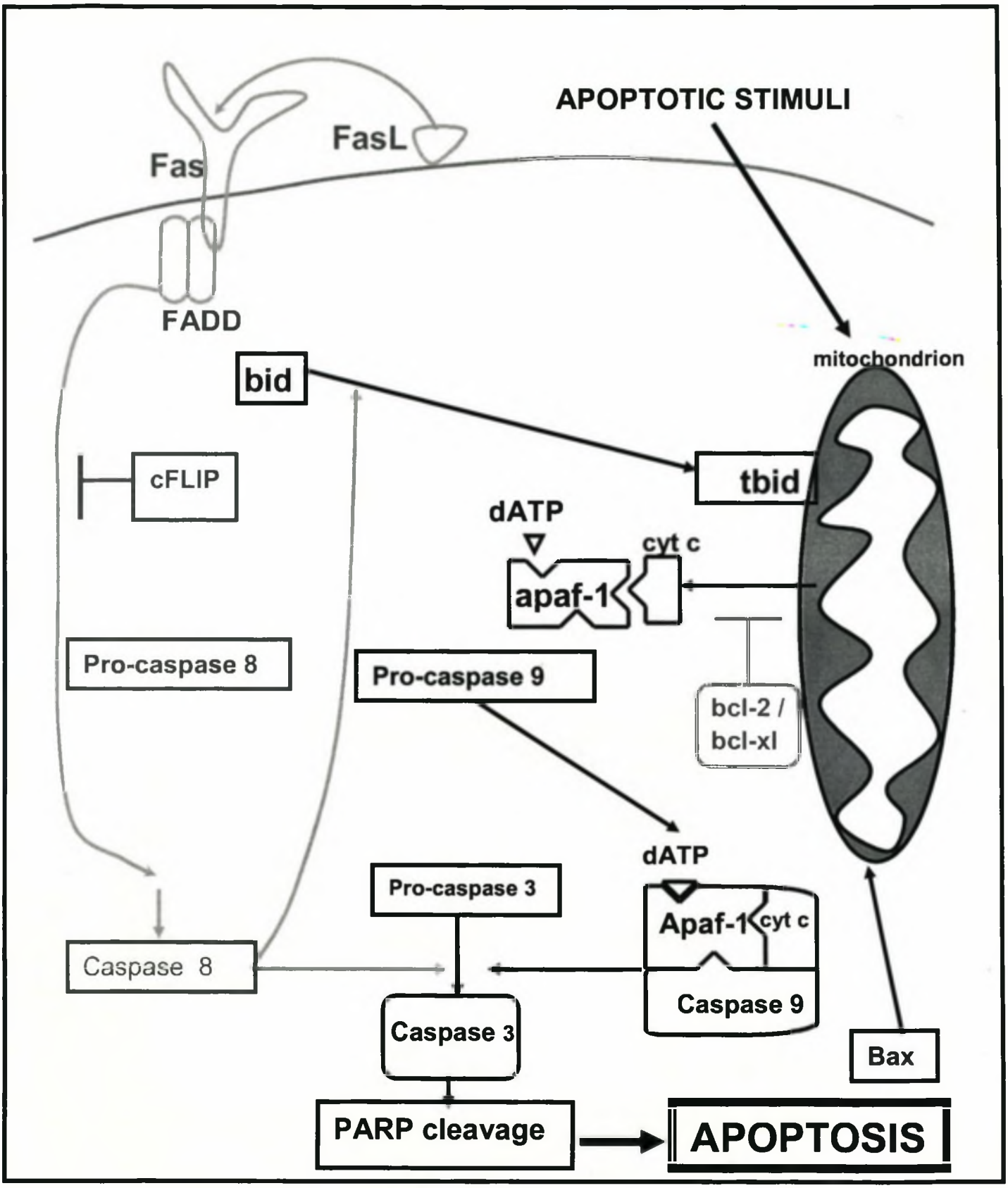
activation of TNFR1 or DR3 diverges at the level of TRADD (Hsu *et al.*, 1995). On the one hand, nuclear translocation of the transcription factor nuclear factor κB (NFκB) and activation of c-Jun N-terminal kinase (JNK) are initiated (Chinnaiyan *et al.*, 1996; Hsu *et al.*, 1996b; Yeh *et al.*, 1997). On the other hand, TNF-α signaling is linked to Fas signaling pathways through interaction of TRADD with FADD. Surprisingly, FADD knockout mice exhibit a phenotype of ventricular thinning and poorly developed trabeculation of the heart (Yeh *et al.*, 1998). An additional death domain-containing protein, receptor-interaction protein (RIP), was also shown to interact with the cytoplasmic domain of TNFR1 (Stanger *et al.*, 1995; Hsu *et al.*, 1996a). Caspase-2 can be recruited to RIP through an adaptor protein called RIP-associated ICH/Ced-3-homologous death domain protein (RAIDD) (Duan *et al.*, 1997).

Another subgroup of TNFR-homologues consists of decoy receptors, which function as inhibitors, rather than transducers of signalling. This subgroup includes decoy receptor (DcR)1 and DcR2 (both of which are cell-surface molecules) as well as osteoprotegerin (OPG) and DcR3 (both of which are secreted, soluble proteins) (reviewed by Ashkenazi & Dixit, 1999).

2.1.3.3 (b) Mitochondrial pathway (fig 2.4)

The other well-characterized pathway to caspase activation involves participation of the mitochondria. In 1997, Zou and co-workers showed that cytochrome c participates in caspase activation, in concert with other proteins that were subsequently identified as caspase-9 and Apaf-1. Apaf-1 contains a region shared with other caspases and may be required for protein-protein interaction. In addition, it has a binding site for ATP (or dATP), and a series of 12 WD-40 repeats, which may be involved in binding cytochrome c (Zou *et al.*, 1997). In addition, Apaf-1 has a region of homology to the *Caenorhabditis elegans* death gene 4 (Ced-4), one of the three genes controlling developmental programmed cell death in the nematode. It is now thought that Apaf-1 represents the

Figure 2.4. The mitochondrial pathway of apoptosis
(modified from Kang *et al.*, 2002)



mammalian homolog of Ced-4 and interacts with cytochrome c and dATP to activate caspase-9 (Li *et al.*, 1997a). The activated and processed caspase-9 then cleaves caspase-3 to generate the active enzyme, which is the effector protease that proceeds to degrade most of the cellular targets.

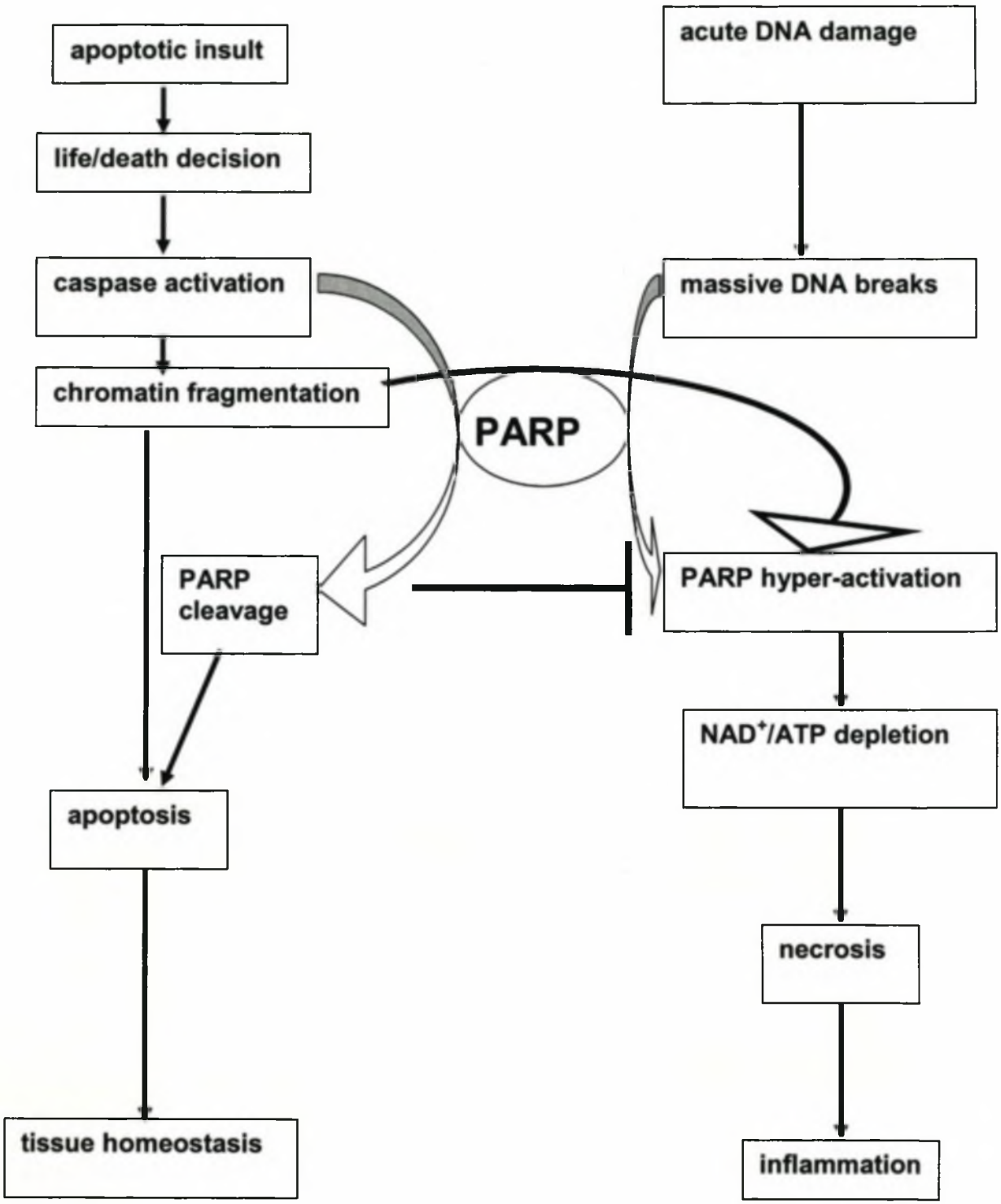
One problem with this model is that it depends upon cytochrome c being released from the mitochondria, where it is normally sequestered in the intermembrane space. Thus, agents affecting mitochondrial integrity may lead to cytochrome c release and activation of caspases. Little information is available explaining how cytochrome c release is controlled, or even if this is a nonspecific event accompanying general loss of mitochondrial integrity or a regulated release of a single protein. It appears to occur before loss of mitochondrial membrane potential (Kluck *et al.*, 1997b), but may represent generalized leakage of intermembrane space constituents. Adachi and co-workers (1998) have shown that loss of outer membrane integrity is a rather late event, but far earlier, cytochrome c has become unavailable for electron transport, although still present in the intermembrane space. This suggests its interaction with other protein(s) that eventually may all be released from the mitochondria when the outer membrane ruptures. One postulated mechanism for the rupture of the outer mitochondrial membrane to occur is swelling of the matrix (Vander Heiden *et al.*, 1997). Since the inner membrane is heavily folded, it can tolerate swelling in response to ion fluxes without rupture, however, as it swells, it will stretch and eventually rupture the outer membrane. Disruption of mitochondrial calcium homeostasis, leading to the formation of the permeability transition pore, may also result in mitochondrial matrix swelling and rupture (Crompton, 1999).

A widely recognized target of caspases is the enzyme poly-(ADP-ribose) polymerase (PARP). PARP was the first cellular protein to be identified as being specifically cleaved in apoptosis (Kaufmann, 1989), and its cleavage subsequently was shown to be a universal phenomenon occurring in apoptosis induced by a variety of stimuli (Kaufmann *et al.*, 1993). Specific proteolysis of

PARP occurs in the DNA binding domain and as a result, two polypeptides of 24 and 89 kDa are formed, thereby abolishing PARP's catalytic activity (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). An apparent active role for PARP in apoptosis came from studies using human osteosarcoma cells that undergo spontaneous apoptosis. Smulson and co-workers (1997) observed transient PARP activation in the early phase of apoptosis, prior to caspase-mediated cleavage and inactivation of PARP protein. This transient burst of poly-(ADP-ribosyl)ation of nuclear proteins was subsequently detected at an early stage of induced apoptosis in other cell types (Simbulan-Rosenthal, 1998). These findings suggest that PARP and poly-(ADP-ribosyl)ation may trigger an important step of early apoptosis. However, treatment of $PARP^{-/-}$ fibroblasts with TNF-α, anti-Fas and IL-3 withdrawal (Menissier-de Murcia *et al.*, 1997; Wang *et al*, 1997), and of thymocytes with γ-irradiation, dexamethasone, ceramide and etoposide induces normal apoptotic responses (Leist *et al.*, 1997; Wang *et al.*, 1997). Moreover, $PARP^{-/-}$ primary neurons and hepatocytes undergo apoptosis normally in response to a variety of stimuli, including potassium withdrawal, staurosporine, and colchicine (Leist et al., 1997). These results indicate that at least in certain cell types, PARP does not actively participate in the apoptotic cascade. Despite the above-mentioned discrepancies, most studies point out that PARP is specifically cleaved during apoptosis, and that massive DNA damage activates PARP, before or in parallel to caspase activation, leading to necrosis. Therefore, it remains to be elucidated whether the activation and/or cleavage of PARP has a specific role in the "decision-making" phase or activating phase of programmed cell death.

An important question, which is often raised, is why must PARP be cleaved in cells destined to die? It has been postulated that PARP cleavage occurs in order to prevent depletion of energy pools required for later stages of apoptosis (Earnshaw, 1995). Transient transfection of cells with non-cleavable PARP delays apoptosis induced by anti-Fas, as judged by morphological criteria such as cell shrinkage and nuclear condensation. The authors claimed that PARP

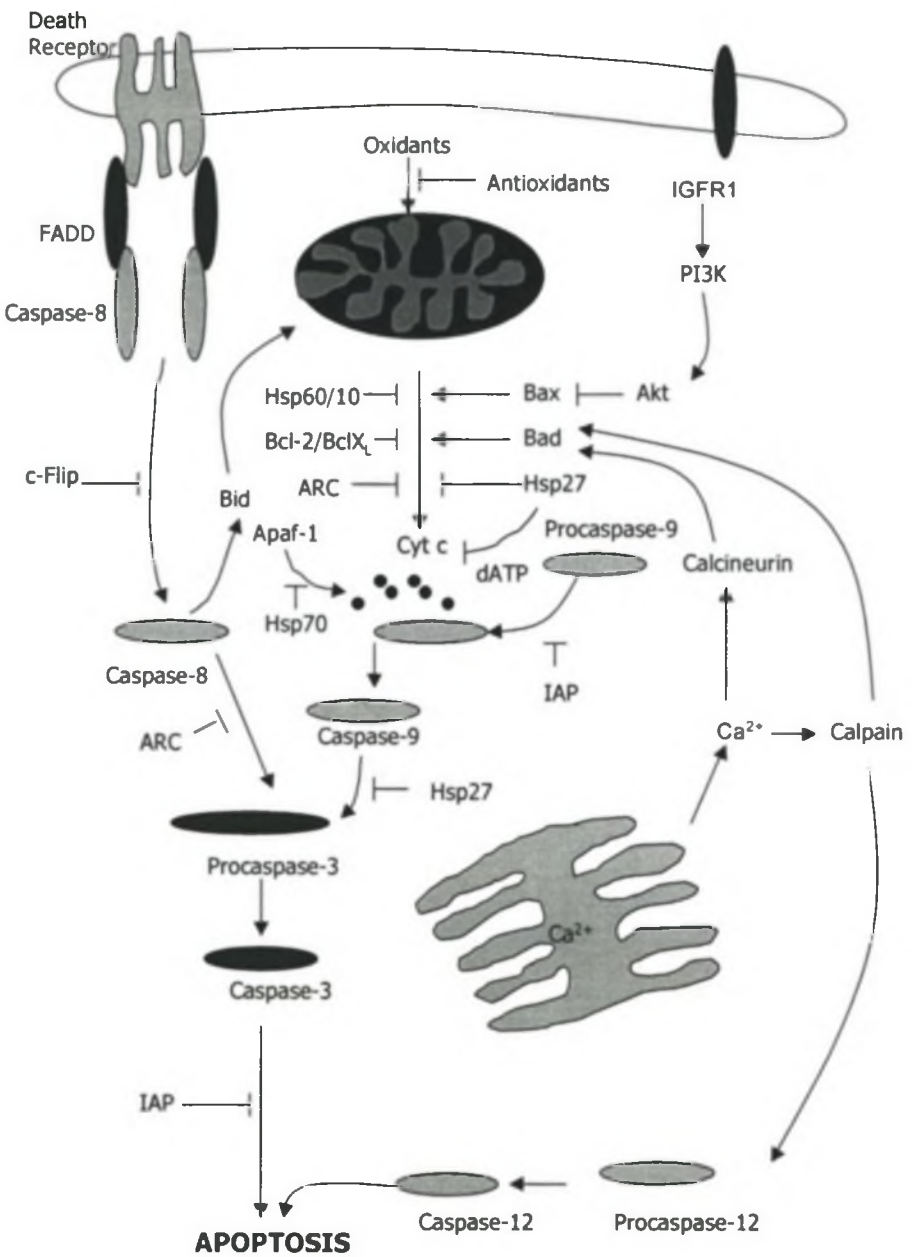
Figure 2.5. A hypothetical model of PARP as determinator in cell death



A cell can respond to physiological or genotoxic stimuli by activating apoptosis that involves activation of caspases and cleavage of chromatin DNA and PARP. Cleavage and inactivation of PARP prevents energy depletion and induction of necrosis. Acute and massive DNA damage induces hyper-activation of PARP leading to NAD^+/ATP depletion, which eventually causes necrosis (modified figure from Herceg & Wang, 2001).

cleavage facilitates cellular disassembly ensuring cell death completion (Olivier, 1998). However, in two other studies, cell lines stably expressing non-cleavable PARP exhibit an increased apoptosis in response to TNF-α and staurosporine, as determined by extensive morphological analyses (Herceg & Wang, 1999; Boulares *et al.*, 1999). It was hypothesized that mutant PARP accelerates apotosis through compromised energy metabolism and the mitochondrial permeability transition (PT) (Nicholson & Thornberry, 1997), or by shifting caspase-3 activation to an earlier time point (Boulares *et al.*, 1999). Alternatively, poly(ADP-ribosyl)ation of histones and other nuclear proteins by non-cleavable PARP could also substantially contribute to unwinding of chromatin structures thereby facilitating access to apoptotic DNAase(s). The apparent discrepancy of the results between the above-mentioned studies using non-cleavable PARP can be attributed to the technical means by which the mutant PARP was introduced into the target cells, i.e. transient versus stable transfection. More importantly, the observed induction of massive necrosis coupled with NAD^+ and ATP depletion in mutant PARP expressing cells (Herceg & Wang, 1999), strongly argues that PARP cleavage plays an important role in ensuring the normal speed and order of apoptotic events, and prevention from necrosis in cells undergoing apoptosis. Since massive activation of PARP leads to necrotic cell death, cells that die via apoptosis must block PARP-mediated necrosis by cleaving PARP (fig 2.5). Together, these findings fit well with the proposal that PARP cleavage has a function in the prevention of necrotic death that would otherwise lead to pathological inflammatory responses (Earnshaw, 1998). This theory is also supported by several other studies demonstrating that PARP activation is involved in inflammatory responses (Szabo *et al.*, 1997, 1998; Cuzzocrea *et al.*, 1999; Olivier *et al.*, 1999). Moreover, streptozotocin-induced disruption of pancreatic β-cells underlines PARP activation and energy depletion as a cause of necrotic cell death in this cell type (Burkart *et al.*, 1999).

Figure 2.6 Regulation of cardiomyocyte apoptosis (Gill *et al.*, 2002)



Apoptotic pathways include those initiated by death receptor ligation, mitochondrial stress or ER stress. These pathways may be downregulated at several levels by anti-apoptotic bcl-2 family members, heat shock proteins and inhibitors of apoptosis such as cFLIP, IAP and ARC. In contrast, they may be up-regulated by pro-apoptotic bcl-2 family members such as Bax, Bad and Bid. The balance between these molecules determines the ultimate fate of the cell: life or death.

2.1.3.4 Regulatory proteins

In apoptosis, protein-protein interactions are the underlying theme in both mitochondria and death receptor pathways (fig 2.6). A sophisticated and tightly controlled network of protein-protein interactions exists to ensure the accuracy of the cell-death machinery.

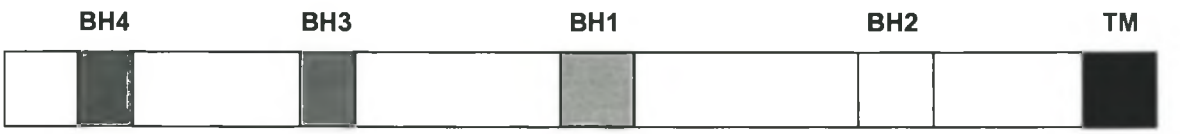
The **Bcl-2 family** is a large key group of apoptosis regulators which, through the diverse interactions among themselves and with other proteins, control the release of apoptogenic factors needed for caspase activation (Adams & Cory, 1998; Chao & Korsmeyer, 1998). The **IAP (Inhibitor of apoptosis protein)** family is another family of proteins that inhibits apoptosis through physically interacting with caspases and thereby directly inhibiting their function.

2.1.3.4 (a) Bcl-2 Protein Family

Members of the Bcl-2 family include both anti-apoptotic proteins, exemplified by Bcl-2, Bcl-X_L, Mcl-1, Bcl-w and A-1, and pro-apoptotic proteins exemplified by Bax, Bak, Bik, Bad and Bid. In terms of sequence, Bcl-2 family proteins share at least one of four homologous regions termed Bcl homology (BH1 to BH4) (fig 2.7). Based on sequence homology, a subclass of pro-apoptotic proteins termed "BH3-only" can be classified that share sequence homology only in the BH3 domain. While all of the pro-apoptotic members use the BH3 domain to interact with anti-apoptotic proteins, BH3-only proteins, including Bad and Bid, appear to act mainly as antagonists of anti-apoptotic members such as Bcl-2 and Bcl-X_L. In contrast to the opposing biological functions and wide differences in amino-acid sequences, experimentally determined crystal structures of Bcl-2 (Petros *et al.*, 2001) and Bcl-X_L (Muchmore *et al.*, 1996; Aritomi *et al.*, 1997), vs Bax (Suzuki *et al.*, 2000) and Bid (McDonnel *et al.*, 1999; Chou *et al.*, 1999) are surprisingly similar.

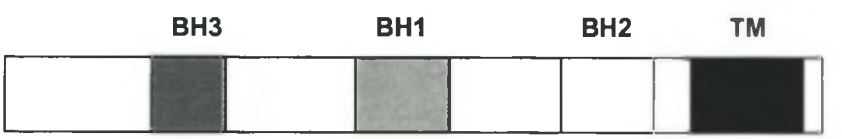
Figure 2.7 Structural features of anti-apoptotic and pro-apoptotic members of the bcl-2 protein family (Haunstetter and Izumo, 1998)

Anti-apoptotic



Bcl-2
Bcl-X_L
Mcl-1
Bcl-w
A1

Pro-apoptotic



Bax
Bak
Bad (TM domain missing)

Bik
Hrk
Bid (TM domain missing)
Bcl-Xs (contains BH4 domain)

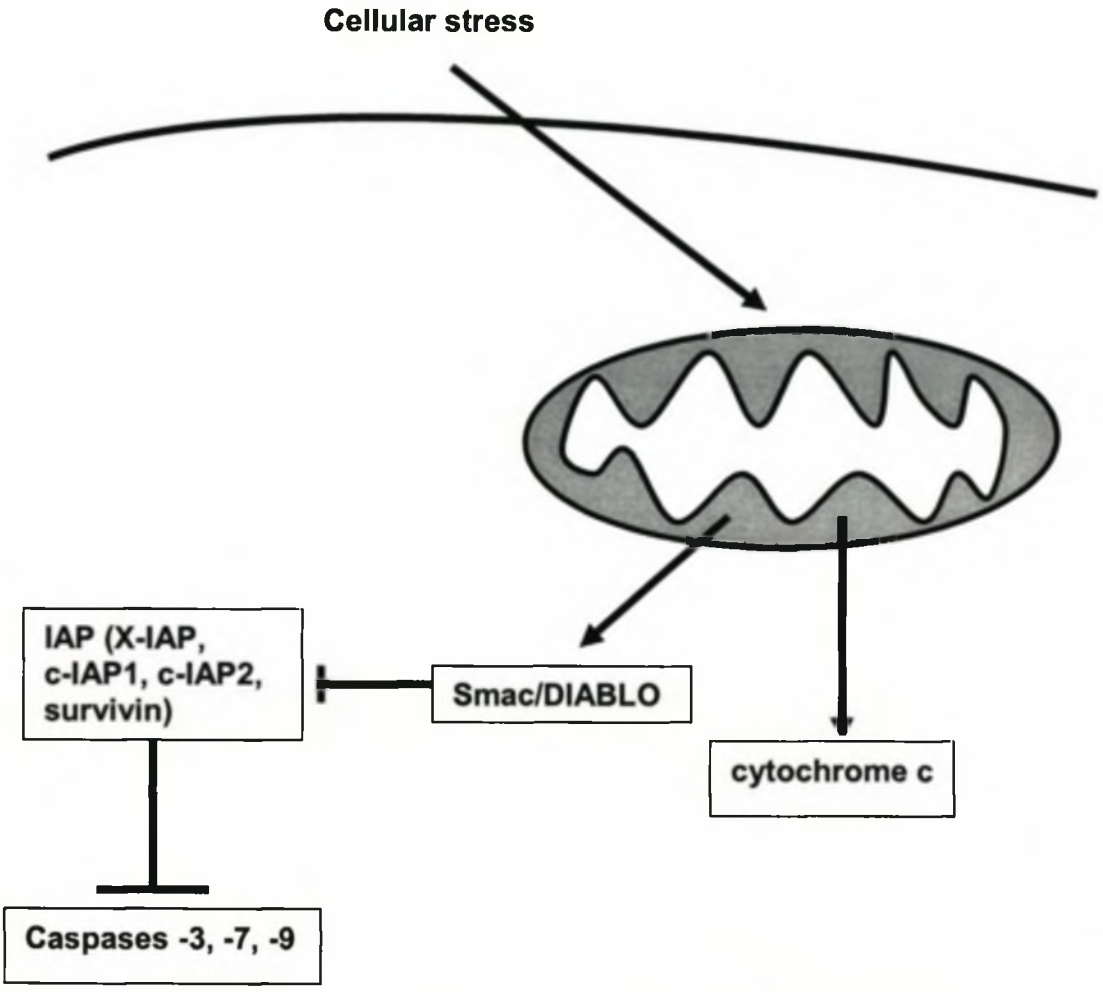
BH – bcl-2 homology domain; TM – transmembrane domain

The mechanism by which Bcl-2 family proteins regulate apoptosis has been a subject of intensive research. Currently it remains controversial and several models have been proposed. An attractive hypothesis for the mode of action is the heterotrimerization between anti-apoptotic and pro-apoptotic Bcl-2 family members (Reed *et al.*, 1996; Yang *et al.*, 1995; Oltvai *et al.*, 1993). Some information about the structural basis of these interactions is provided by the three-dimensional structure of Bcl-X_L in complex with a peptide derived from the BH3 domain of Bak (Sattler *et al.*, 1997). The structure reveals a hydrophobic surface pocket on Bcl-X_L formed by the BH1-3 domains bound by the Bak BH3 domain peptide in helical conformation. Since the BH3 domain is buried in the structure of pro-apoptotic proteins Bid (McDonnel *et al.*, 1999; Chow *et al.*, 1999) and Bax (Suzuki *et al.*, 2000), this raises the speculation that conformational changes are necessary for the exposure of the BH3 domain of a pro-apoptotic protein and its inhibition of the functional pocket on the anti-apoptotic partner. In the cell environment, pro-apoptotic Bcl-2 family members are suggested to undergo such conformational changes (Desagher *et al.*, 1999) triggered by dephosphorylation (Zha *et al.*, 1996) or proteolytic cleavage by caspases (such as the cleavage of Bid to generate tBid) (Slee *et al.*, 2000; Li *et al.*, 1998; Luo *et al.*, 1998).

2.1.3.4 (b) IAP (Inhibitor of apoptosis protein) family

The Bcl-2 and IAP families are regulators of caspases at two different levels: the Bcl-2 family controls signaling events upstream of caspases, while the IAP family directly binds and inhibits caspases. The human IAP family contains eight distinct cellular members that were just discovered in the past 5-6 years, including X-IAP (X-linked IAP), c-IAP1, c-IAP2, and survivin (Devereaux *et al.*, 1999). In humans, IAPs such as X-IAP, c-IAP1, and c-IAP2 selectively inhibit caspase-3, -7 and –9 through direct molecular interactions but not caspase-1, -6, -8, -10. In addition, IAPs can interact with Smac/DIABLO, which is released from the mitochondria

Figure 2.8 Inhibitor of apoptosis (IAP) proteins



together with cytochrome c upon death stimuli. The binding of Smac/DIABLO removes IAPs from their association with caspases and thus relieves their caspase-inhibiting function (Devereaux *et al.*, 1997; Roy *et al.*, 1997) (fig 2.8).

2.1.4 Regulators of apoptosis with therapeutic potential

Apoptosis is a sequential, multistep process and there are many different points at which it can be regulated (fig 2.6). This is of particular importance in fully differentiated cardiomyocytes in order to avoid unnecessary death of salvageable cells and to promote apoptosis in response to irreversible cellular damage, as opposed to necrosis, which could further harm the myocardium. Although most of our knowledge of regulation of apoptosis is of that occurring in non-myocytes, emerging evidence suggests that similar controls are present in the heart.

2.1.4.1 Caspase inhibitors

Apoptosis may be initiated by a variety of different signals, but the ultimate result is the same in most cells. Thus, it appears that the final steps of apoptotic death are highly conserved and likely to be mediated by a similar set of caspases. As described earlier, various regulatory mechanisms exists within cells that target caspases. These include cFLIP and the IAP family, inhibitors present in a variety of cell types that may also play an important role in the heart. An inhibitor of apoptosis that is expressed almost exclusively in skeletal muscle and heart has been characterized. ARC (apoptosis repressor with caspase recruitment domain) was first shown to interact with caspase-8 and –2 and to attenuate apoptosis induced by stimulation of death receptors (Koseki *et al.*, 1998). More recently it was demonstrated that ARC inhibits cytochrome c release from mitochondria and protects against hypoxia-induced apoptosis (Ekhterase *et al.*, 1999), suggesting that ARC can exert its effect at different levels in the apoptotic pathway and may

be a key regulator of apoptosis in the heart (fig 2.6). As over-expression of ARC had a protective effect compared with cells expressing only endogenous levels of ARC, up-regulation of this protein *in vivo* could provide a means of attenuating cell death in certain conditions.

The use of synthetic inhibitors of apoptosis presents another potential therapeutic avenue. The broad range caspase inhibitor zVAD-fmk was effective in reducing myocardial reperfusion injury in rats, which was attributed in part to the attenuation of cardiomyocyte apoptosis (Yaoita *et al.*, 1998). The same inhibitor is reported to attenuate apoptosis in rabbit cardiomyocytes (Gottlieb *et al.*, 1996). Unfortunately, any potential beneficial effect this inhibitor may have *in vivo* could far be outweighed by its side effects, since caspase inhibition may increase ROS production resulting in secondary toxicity as observed in other cell types (Yaoita *et al.*, 2000).

Others have investigated the effect of specific inhibitors of caspases. One group found that cardiomyocyte DNA fragmentation and caspase activation were prevented by inhibitors of caspase-1 and –3 without reduction of infarct size in ischemia/reperfused rat hearts (Okamura *et al.*, 2000). This is in contrast with another study where it was found that, in addition to zVAD-fmk, inhibitors of caspase-8, -9, and –3 all limited infarct size as a result of reperfusion injury (Mocanu *et al.*, 2000). The different outcomes of these studies may be due to the time of administration. In the former study, inhibitors were administered before ischemia; in the latter, they were administered during early reperfusion. This underscores the importance of establishing the optimum time for therapeutic intervention. Like zVAD-fmk, specific caspase inhibitors might not be of major therapeutic value. They do, however, provide a starting point for the development of more sophisticated inhibitors for use in treating cardiac disease.

2.1.5 Signaling pathways and apoptosis

2.1.5.1 Introduction

Only recently have efforts been made to disentangle the intricate relationships between signal transduction and apoptosis. Analysis is complicated by the fact that receptor agonists may activate several signal transduction mechanisms with opposing effects on apoptosis regulation. In the recent past it has become evident that the **MAPK-** as well as the **PKB/Akt signalling pathways** play major roles in the regulation of apoptosis in the heart.

2.1.5.2 Mitogen-activated protein kinase (MAPK) pathways

2.1.5.2.1 The major MAPK subfamilies

Regulatory mechanisms controlling proliferation, differentiation, or apoptosis of cells involve intracellular protein kinases that can transduce signals detected on the cell's surface into changes in gene expression. Most prominent amongst the known signal transduction pathways that control these events are the MAPK cascades. MAPKs represent a family of serine-threonine kinases with the potential to phosphorylate other cytoplasmic proteins and to translocate from the cytoplasm to the nucleus, where they can directly regulate the activity of transcription factors controlling gene expression. Their components are evolutionary highly conserved in structure and organization, each consisting of a module of three cytoplasmic kinases: a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK), and MAP kinase kinase (MAPKK), and the MAP kinase (MAPK) itself (fig 2.9). The MAPKKK (Fanger *et al.*, 1997) is a serine-threonine kinase that receives activating signals from a membrane-spanning receptor and then phosphorylates and activates its substrate, a MAPKK (Siow *et al.*, 1997). This enzyme is a dual-specificity kinase with the potential to phosphorylate critical threonine and tyrosine residues in its substrate protein, the MAPK (Gartner *et al.*, 1992). As illustrated in Figure 2.10, the mammalian

Figure 2.9 Basic assembly of mitogen-activated protein kinase (MAPK) pathways is a three-component cascade conserved from yeast to humans. Divergent stimuli (activators) feed into core MAPK kinase kinase (MKKK), MAPK kinase (MKK) and MAPK pathways that are sequentially activated by phosphorylation (Marais, PhD dissertation, 2001)

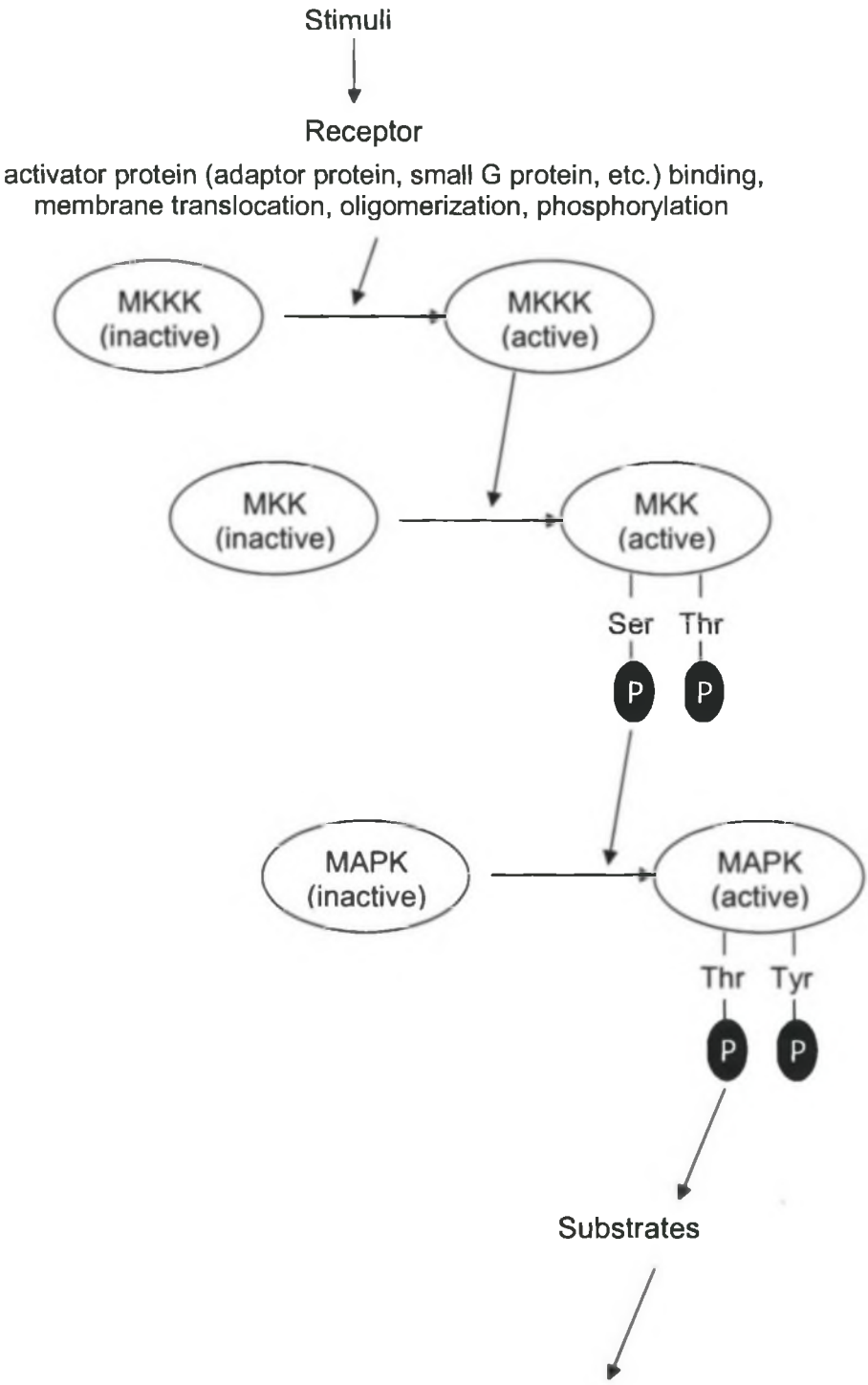
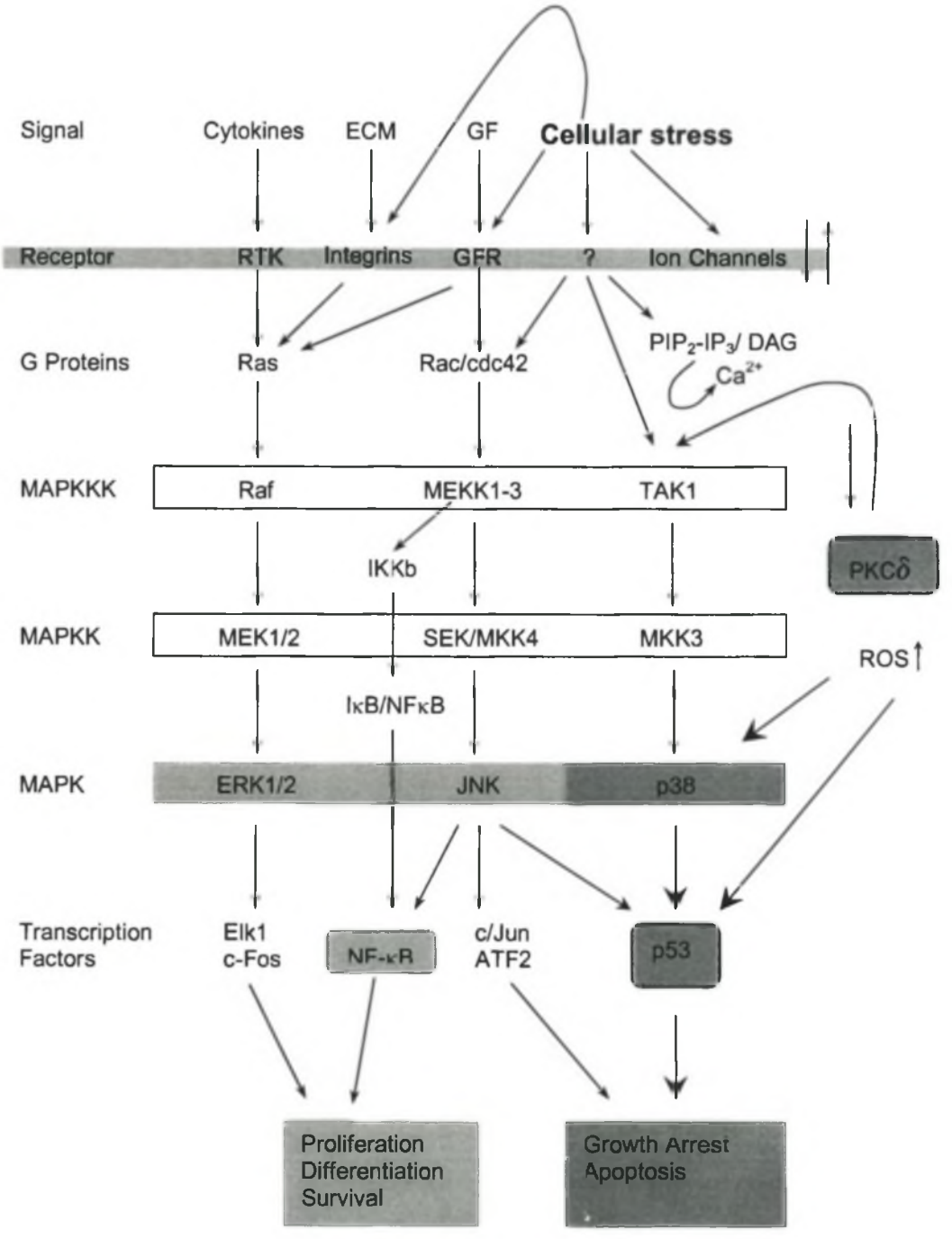


Figure 2.10 MAPK activation in stress-induced signaling
(modified from Wernig and Xu, 2002)



MAPKs fall into three main families, the ERKs (extracellular regulated kinases), JNK (c-Jun N-terminal protein kinase) and p38-MAPKs. Each family contains multiple isoforms encoded by different genes and splice variants, and differs from other family members in the amino acid X in the threonine-X-tyrosine activation motif and the size of the loop that contains it.

2.1.5.2.1 (a1) Extracellular signal-regulated kinases (ERKs)

Extracellular signal-regulated kinase-1 (ERK1) and ERK2 also known as p44 and p42 MAPKs, represent the prototypical MAPKs in mammalian cells. A wide variety of growth promoting or hypertrophic agents activate these kinases in cardiac myocytes, fibroblasts, smooth muscle cells and endothelial cells (Bogoyevitch, 2000). Activation of ERK may also follow exposure to a variety of extracellular stresses. Such activation of a potent growth factor–stimulated pathway by stress may seem contradictory, but if stress-activated ERK is chemically inhibited, cell death is accelerated (Wang *et al.*, 1998). Therefore activation of the ERKs may contribute to cardiovascular cell survival.

Although ERK1 and ERK2 were the first MAPK described, a closely related member BMK1 (ERK5) has also been identified (Zhou *et al.*, 1995; Lee *et al.*, 1995). BMK1/ERK5 is activated in vascular smooth muscle cells and aortic endothelial cells exposed to oxidative stress, osmotic shock, and serum but not by angiotensin II, PDGF or TNF (Abe *et al.*, 1996; Yan *et al.*, 1999). BMK1/ERK5 shows similarities to the ERKs with a Thr-Glu-Tyr motif within its activation loop. The significant differences within this activation loop sequence confirm that BMK1/ERK5 is activated by a distinct upstream kinase MEK5 (Zhou *et al.*, 1995). BMK1/ERK5 has also been proposed to be anti-apoptotic based on results of MEK5 inhibition. (Kato *et al.*, 1998). MEK5-dependent BMK1 activation results in the phosphorylation of MEF2A and MEF2C, pro-survival transcription factors that belong to the myocyte enhancer factor-2 (MEF2) family (Kato *et al.*, 1997), which are important regulators of cardiac gene expression. Although the expression of

BMK1/ERK5 in the heart is high (Zhou *et al.*, 1995), its activation in cardiac myocytes remains uncharacterized. It is however important to note that BMK1/ERK5 can be activated in the ischaemic myocardium (Takeishi *et al.*, 1999).

2.1.5.2.1 (a2) Structure and basic regulation of the Ras-Raf-MEK-ERK pathway

Proliferation signals like growth factors cause activation and autophosphorylation of their cognate receptor protein tyrosine kinases (RPTK). The phosphorylated tyrosine receptor allows the binding of adaptor proteins like Shc to the receptor. This association of Shc permits phosphorylation thereof by the receptor itself or by intracellular tyrosine kinases such as Src. This phosphorylation allows the binding of another adaptor protein Grb2, which also results in the recruitment of SOS. The latter stimulates the exchange of GDP bound to Ras for GTP (Downward, 1996) and functions as an adaptor that binds to Raf kinases with high affinity and causes their translocation to the cell membrane, where Raf activation takes place (Moodie & Wolfman, 1994). Raf genes encode serine/threonine-specific kinases that integrate the upstream input signals, and hence feature a complex regulation. Mammals possess 3 Raf proteins: Raf-1, A-Raf and B-Raf (Morrison & Cutler, 1997; Hagemann & Rapp, 1999). Although all 3 Raf isoforms interact with Ras, there are important differences. Activation of B-Raf is obtained after binding to Ras alone, whereas for activation of A-Raf and Raf-1, additional signals are required (Hagemann & Rapp, 1999).

Phosphatases such as protein phosphatase 2A (PP2A) remove inhibitory phosphorylation that keeps Raf-1 in the inactive state (Morrison & Cutler, 1997; Hagemann & Rapp, 1999). Several kinases, including PKC (Kolch *et al.*, 1993; Morrison *et al.*, 1997), PAK and the Src-family tyrosine kinases phosphorylate and activate Raf-1 (Morrison & Cutler, 1997). All three Raf isoforms share Ras as a common upstream activator and MEK as the only common accepted

downstream substrate (Schaeffer & Weber, 1999; Morrison & Cutler, 1997). MEK is activated by phosphorylation of two serine residues. Although other kinases such as MEKK1 can phosphorylate the same serines, the predominant MEK activators in most cell types are Raf kinases (Schaeffer & Weber, 1999). Raf can activate both MEK1 and MEK2 (also called MKK1 and MKK2) with similar efficacy *in vitro*. Experimental data suggest a preferential coupling between certain Raf and MEK isoforms, but the biological significance is still unclear (Schaeffer & Weber, 1999). MEK activates ERK1 and ERK2 via phosphorylation of a Thr-Glu-Tyr-motif in the activation loop.

Although more than 50 substrates for ERK have been identified (Lewis *et al.*, 1998), special attention should be given to p90 ribosomal S6 kinase (p90RSK) in the heart, which is a ubiquitous and versatile mediator of ERK signal transduction (Frodin *et al*, 1999). Functions of p90RSK include: (1) regulation of gene expression via phosphorylation of transcription factors including c-Fos and cAMP response element-binding (CREB) protein; (2) regulation of protein synthesis by phosphorylation of polyribosomal proteins and glycogen synthase kinase-3; and (3) stimulation of Na^+-H^+ exchanger by phosphorylating serine 703 of NHE-1 (Takahashi *et al.*, 1999).

2.1.5.2.1 (a3) The role of ERK in apoptosis

The ERK family of MAPKs have been implicated in survival signaling in response to ischaemia/reperfusion, oxidative stress, β-adrenergic stimulation, and anthracycline exposure. Several studies have shown that the MEK-ERK pathway may be protective or neutral against apoptosis. Insulin-like growth factor-1 (IGF-1), cardiotrophin-1 (CT-1), and catecholamines were each shown to exert their antiapoptotic effects, in part, by inducing ERK signalling (Sheng *et al.*, 1997; Parrizas *et al.*, 1997; De Windt *et al.*, 2000). Ischaemia/reperfusion and oxidative stress induced by anthracyclines activate ERK. Inhibition of ERK signalling was demonstrated to increase daunomycin-induced apoptosis in cultured

cardiomyocytes (Zhu *et al.*, 1999), whereas in a model of ischaemia/reperfusion in the intact heart, ERK1/2 activation was shown to attenuate the extent of apoptosis subsequent to reperfusion injury (Yue *et al.*, 2000). MEK1 transgenic mice were also shown to be partially resistant to ischaemia/reperfusion-induced DNA laddering, suggesting a cardioprotective function for ERK1/2 signalling (Bueno *et al.*, 2000).

Although these various reports discussed have shown that stress- or agonist-induced ERK1/2 activation is associated with protection from apoptosis, little is known as to how ERK signalling results in cellular protection. Cyclooxygenase-2 (COX-2) has been identified as a possible downstream mediator of protection in association with ERK1/2 signalling in cardiomyocytes (Adderley *et al.*, 1999). In T cells, ERK1/2 activation has been associated with induction of expression of FLICE (FADD-like interleukin 1β-converting enzyme) inhibitory protein, a known inhibitor of the caspase cascade (Yeh *et al.*, 1998). It is unknown whether protection induced by ERK signalling is mediated by increasing FLICE-inhibitory protein expression in the heart.

The cardioprotective effects of estrogen have been extensively investigated. In addition to the classical genomic effects, estrogens have also been shown to have rapid nongenomic effects, which include NO release and ERK1/2 activation (Chen *et al.*, 1999; de Jager *et al.*, 2001). In the heart, ERK signalling induced by estrogens results in the rapid expression of early growth response gene-1 (Erg-1) and ANF, which have been shown to have cardioprotective effects (de Jager *et al.*, 2001; Jankowski *et al.*, 2001).

Another mechanism whereby ERK1/2 may function in a cardioprotective manner is through the association with protein kinase Cε (PKCε), which is a well-characterized mediator of cardiomyocyte protection (Dawn & Bolli, 2002). Indeed, Baines and co-workers (2002) recently demonstrated that the ERKs form a complex with PKCε in the mitochondria resulting in the phosphorylation and

inactivation of BAD (Baines *et al.*, 2002). As a final mechanism, it was also shown that the ERK substrate, p90RSK, phosphorylated the pro-apoptotic protein BAD at serine 112, which specifically suppressed BAD-mediated apoptosis (Bonni *et al.*, 1999; Tan *et al.*, 1999).

These findings suggest that p90RSK and ERK promote cell survival by both inhibiting components of the cell death machinery (e.g. BAD) and increasing transcription of pro-survival genes (e.g. CREB). Future studies using MEK1, MEK2, ERK1 or ERK2 gene-targeted mice (or dominant-negative-expressing transgenic mice) subjected to cardiac ischaemia/reperfusion injury will ultimately establish the overall importance of MEK-ERK signaling as an anti-apoptotic effector pathway *in vivo*.

2.1.5.2.1 (b1) JNK and p38 MAPK (Stress activated protein kinases – SAPKs)

Two other MAPK subfamilies that are activated by a diverse range of extracellular stresses such as irradiation, ultraviolet light, mechanical stress, hyperosmotic shock, heat shock, hypoxia/reoxygenation, reactive oxygen species (ROS), pro-inflammatory cytokines (such as interleukin-1 and TNF-α) and to a lesser degree by growth factors (Sugden & Clark, 1998; Bogoyevitch, 2000) were identified as c-Jun NH_2-terminal kinases (JNK) and p38 MAPK. Molecular cloning revealed three genes that encode the 46- and 54-kDa isoforms of JNK: JNK1 (SAPKγ), JNK2 (SAPKα) and JNK3 (SAPKβ) (Kyriakis *et al.*, 1994). These kinases selectively phosphorylate the NH_2-terminal transactivation domain of the transcription factor c-Jun (Hibi *et al.*, 1993), which results in gene expression of *c-jun* (Karin, 1994). JNKs are important for cytokine biosynthesis and are involved in cell transformation, stress responses and apoptosis (Kyriakis & Avruch, 1996; Dong *et al.*, 1998; Kuan *et al.*, 1999).

To date, six members of the p38 MAPK group have been cloned and characterized: p38 α1/α2 (Han *et al.*, 1994; Lee *et al.*, 1994), p38 β1/β2 (Jiang *et*

al., 1996), p38γ (Lechner *et al.*, 1996; Li *et al.*, 1996; Cuenda *et al.*, 1997) and p38δ (Jiang *et al.*, 1997; Kumar *et al.*, 1997). Like the JNKs, they are strongly activated by environmental stress and inflammatory cytokines and therefore also referred to as stress-activated protein kinases. In almost all instances, the same stimuli that recruit the JNKs also recruit p38-MAPKs (Kyriakis & Avruch, 1996). One exception is myocardial ischaemia/reperfusion. JNKs are not activated during ischaemia, but rather during reperfusion, whereas p38-MAPKs are activated during ischaemia and remain active during reperfusion (Pombo *et al.*, 1994; Bogoyevitch *et al.*, 1996; Kyriakis & Avruch, 1996). Activation of p38 MAPKs has been implicated in transcription, protein synthesis, cell surface receptor expression and cytoskeletal integrity, ultimately affecting cell survival or leading to apoptosis (Obata *et al.*, 2000).

2.1.5.2.1 (b2) Stucture and basic regulation of the JNK and p38 MAPK pathway

Members of the MAPKKK superfamily responsible for activating JNK and p38 MAPK pathways that have been identified, include MEKK-1, MEKK-2, MEKK-3, MEKK-4 (MTK), MUK (MAPK upstream kinase), MLK (mixed lineage kinase), Tpl-2 (tumor progression locus-2), TAK-1 (TGF-β activated kinase 1), ASK-1 (apoptosis signal-regulation kinase) and TAO (thousand and one amino acid protein kinase) (reviewed in Obata *et al.*, 2000; Gutkind, 2000; Widmann *et al.*, 1999). These MKKK are regulated by Rac and Cdc42, two small guanosine 5'-phosphate (GTP)-binding proteins of the Rho family, in a manner similar to that of Ras acting on Raf (Minden *et al.*, 1995). At least a portion of this JNK/p38 MAPK activation by small GTP-binding proteins involves activation of a serine/threonine kinase called PAK (p21-activated kinase) (Bagrodia *et al.*, 1995). The relative contribution of each MKK to either JNK or p38 MAPK pathways has not yet been clearly defined. MEKK-1, MEKK-2, MEKK-3, MUK, MLK and Tpl-2 seemed to preferentially activate JNK, rather than p38 MAPK (Fanger *et al.*, 1997; Obata *et al.*, 2000; Gutkind 2000), through two MAPKK,

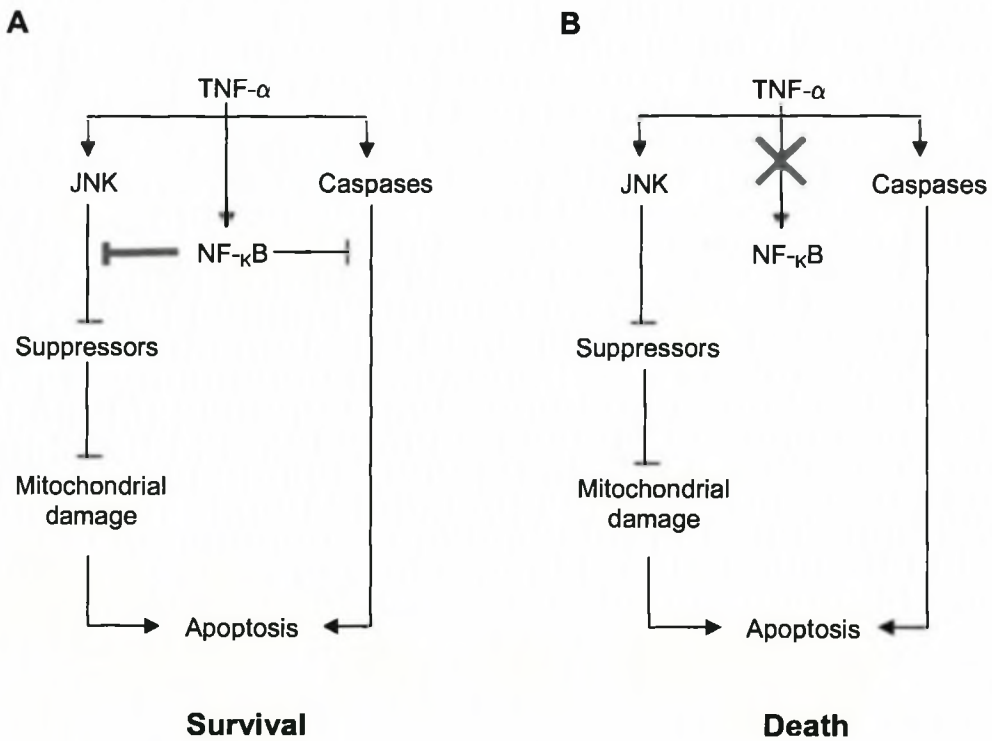
namely MKK4 (Sanchez *et al.*, 1994) and MKK7 (Tournier *et al.*, 1997). On the other hand, MEKK-4, TAK-1, ASK-1 and TAO appear to be more effective activators of p38 MAPK (Obata *et al.*, 2000), through MKK6 and MKK3 (Moriguchi *et al.*, 1996). MKK6 appears to phosphorylate all p38 MAPK isoforms, whereas MKK3 phoshorylate only p38α, γ and δ (Enslen *et al.*, 1998). Crosstalk between the JNK and p38 MAPK pathways has also been observed, since MEKK-1 can activate p38-MAPK through MKK-4 in NIH 3T3 cells (Guan *et al.*, 1998), and similarly, MEKK-2 or MEKK-3 can couple to the p38 MAPK pathway via MKK6 (Deacon & Blank, 1999).

2.1.5.2.1 (b3) The role of JNK in apoptosis

JNK has been shown to be activated before or after apoptotic stimulation, and is a potential candidate for apoptotic signalling in the heart (Davis, 2000). Reoxygenation and reperfusion are known to activate JNK in the heart (Bogoyevitch *et al.*, 1996; Laderoute & Webster, 1997; Yue *et al.*, 1998) and inhibition of this pathway has been shown to abrogate apoptosis induced by these stressors (Laderoute & Webster, 1997; Yue *et al.*, 2000). Furthermore, it has been demonstrated in the rat heart that JNK is translocated to the nucleus during ischaemia where it is phosphorylated during the reperfusion period (Yue *et al.*, 1998). Hreniuk and co-workers (2001) also demonstrated that treatment of rat cardiac myocytes with antisense oligonucleotides that specifically targeted either JNK1 or JNK2 and significantly reduced both mRNA and protein expression of the target isoforms, resulted in almost complete attenuation of reperfusion-induced apoptosis.

Several mechanisms have been proposed for JNK-mediated apoptosis, one of which is the activation of *c-Jun* transcriptional activity by phosphorylation. In that case, the Fas ligand is upregulated in a MEKK-1 and c-Jun-dependent manner (Faris *et al.*, 1998; Behrens *et al.*, 1999). Also, JNK phosphorylation of Bcl-2 and Bcl-X_L has been implicated (Yamamota *et al.*, 1999; Kharbanda *et al.*, 2000).

Figure 2.11 **A: While caspase activation initiates and executes apoptosis, prolonged JNK activation promotes apoptosis by inactivating suppressors of the mitochondrial dependent death pathway. Activation of NF-κB by TNF-α blocks caspase activation and prevents prolonged JNK activation, thereby inhibiting TNF-α-induced apoptosis. B: Inactivation of NF-κB removes inhibition on both caspases and JNK, allowing TNF-α to induce apoptosis (Lin, 2003).**



However, this has been challenged by the demonstration of an anti-apoptotic effect of Bcl-2 phosphorylation by JNK (Deng *et al.*, 2001). Recently, the critical role of JNK in ultraviolet (UV)-induced apoptosis was shown using $JNK1^{-/-}JNK2^{-/-}$ deficient double null embryonic fibroblasts (Tournier *et al.*, 2000). In this study, the JNK pathway was found to be dispensable for Fas-induced receptor-mediated apoptosis, but a prerequisite for UV-induced Bid translocation, cytochrome c release, mitochondrial dysfunction and apoptosis. However, no phosphorylation of Bcl-2 family proteins was detected (Tournier *et al.*, 2000). These findings and those of Aoki and co-workers (2001) in cardiac myocytes are consistent in that the JNK pathway activates mitochondrial apoptotic signaling which does not require phosphorylation of Bcl-2 family, suggesting the presence of as yet unidentified JNK target in the mitochondrial pathway.

It has also been proposed by Lin (2002) that JNK might be a modulator rather than an intrinsic component of the apoptotic machinery. Thus, JNK facilitates but does not induce apoptosis. They hypothesized that activated JNK inactivates suppressors of the apoptotic machinery. According to their model, JNK activation contributes to apoptosis only if the apoptotic process has already been activated. This can explain how prolonged JNK activation alone does not induce apoptosis, but is able to promote TNF-α-induced apoptosis in the absence of NF-κB activation (fig 2.11). TNF-α is not typically a killer, since activation of NF-κB inhibits caspases and prevents prolonged JNK activation (Tang *et al.*, 2001; Karin & Lin, 2002). In the absence of NF-κB-mediated inhibition, such as in *RelA*$^{-/-}$ fibroblasts, TNF-α induces apoptosis through activation of caspases and release of apoptotic pathway inhibtion by prolonged JNK activation (fig 2.11). This model may also explain how activation of JNK alone does not induce apoptosis, but inhibition of JNK activation blocks UV-induced cell death. UV induces apoptosis by activating the mitochondrial-dependent death pathway and it also induces prolonged JNK activation by an unknown mechanism (Tournier *et al.*, 2000). Nevertheless, prolonged JNK activation may function to inactivate inhibitors of the mitochondrial-dependent death pathway, there contributing to

activation of the death pathway. Since TNF-α activates both receptor- and mitochondrial-dependent death pathways, while UV activates only the mitochondrial-dependent death pathway, it is likely that prolonged JNK activation inactivates inhibitors involved in blocking the mitochondrial-dependent death pathway.

In summary, the role of the JNK pathway in apoptosis is complex, as it is able to both promote and suppress the process. Although activation of the JNK pathway can contribute to apoptosis, JNK may not be an intrinsic component of the apoptotic machinery. Instead, activation of JNK may modulate the apoptotic process in a cell-type and stimulus-dependent manner.

2.1.5.2.1 (b4) The role of p38-MAPK in apoptosis

The role of p38-MAPK in myocyte apoptosis is intriguing because p38 MAPK can also mediate cardiac hypertrophy. One of the ways that p38 MAPK can induce apoptosis is through its effect on cyclin D1 expression during the cell cycle. It has been demonstrated that the coexpression of MKK3 along with p38 MAPK inhibits mitogen-induced cyclin D1 expression (Lavoie *et al.*, 1996).

Studies using chemical inhibitors have led to the conclusion that activation of p38 MAPK promote cardiac myocyte death during extended periods of ischaemia (Mackay & Mochly-Rosen, 1999; Saurin *et al.*, 2000; Barancik *et al.*, 2000). In a cultured neonatal rat cardiac myocyte model, inhibition of p38 MAPK protects against ischaemic injury as evidenced by a reduction in LDH release (Mackay & Mochly-Rosen, 1999; Saurin *et al.*, 2000). In addition, Barancik and co-workers (2000) reported that a specific inhibitor of p38 MAPK, SB203580, reduced infarct size in ischaemic pig hearts *in vivo*. Several studies indicated that p38 MAPK activation also plays a pivotal role in promoting myocardial apoptosis (Ma *et al.*, 1999; Mackay & Mochly-Rosen, 1999; Yue *et al.*, 2000). Ma and co-workers (1999) demonstrated that in isolated perfused rabbit hearts, ischaemia alone

caused a moderate but transient increase in p38-MAPK activity. Reperfusion further activated p38-MAPK, which remained elevated throughout 20 minutes of reperfusion. Administration of SB203580 before ischaemia and during reperfusion completely inhibited p38 MAPK activation and exerted significant cardioprotective effects, characterized by decreased myocardial apoptosis and necrosis as well as improved post-ischaemic function. In contrast, administering SB203580 10 minutes after reperfusion (a time point when maximal MAPK activation had already been achieved), failed to convey significant cardioprotection. Mackay and Mochly-Rosen (1999) observed two distinct phases of p38 MAPK activation in ischaemic neonatal rat cardiomyocytes: the first phase began within 10 minutes and lasted less than 1 hour, and the second began after 2 hours and lasted throughout the ischaemic period. They demonstrated that SB203580 also protected cardiac myocytes against ischaemia by reducing activation of caspase-3, a key event in apoptosis. However, the protective effect was seen even when the inhibitor was present during only the second, sustained phase of p38 MAPK activation. Subsequent studies by Yue and co-workers (2000), exposing rat neonatal cardiomyocytes to ischaemia showed a rapid and transient activation of p38-MAPK and JNK. On reoxygenation, further activation of the SAPKs was noted. With pretreatment of the cells with SB203580 apoptotic cells were reduced, suggesting p38 MAPK activation mediates apoptosis in rat cardiac myocytes subjected to ischaemia/reoxygenation. In addition, Yue and co-workers (2000) also showed that SB203580 improved cardiac contractile function in isolated ischaemic rat hearts. Thus, in a large number of studies, inhibition of p38 MAPK activation, is associated with cardioprotection against ischaemia/reperfusion injury in myocytes as well as in isolated hearts.

On the other hand, a number of authors also showed that p38 MAPK activation is protective in the heart. Weinbrenner and co-workers (1997) demonstrated that phosphorylation of tyrosine 182 of p38 MAPK correlates with protection of preconditioning in the rabbit heart and Nakano and co-workers correlated the

protective effect of ischaemic preconditioning with the activation of MAPKAPK2 (a substrate of p38 MAPK) in the isolated rabbit heart. Zechner and co-workers (1998) reported that overexpression of MKK6, an upstream activator of p38 MAPK, resulted in protection of cardiac myocytes from apoptosis. In addition, expression of MKK6 elicited a hyperthrophic response, which was enhanced by co-infection with p38β (Wang *et al.*, 1998). It appears that a distinct isoform of p38 MAPK, p38β, participates in mediating cell survival. In contrast, over-expression of MKK3 in mouse cardiomyocytes led to apoptosis, which was increased by co-infection with p38α (Wang *et al.*, 1998). Therefore, differential activation of p38 MAPK isoforms may exert opposing effects: p38α is implicated in cell death, while p38β may mediate myocardial survival.

These suggestions were subsequently confirmed by Saurin and co-workers (2000): rat neonatal cardiomyocytes were infected with adenovirus encoding either wild-type p38α or p38β, which was differentially activated during sustained ischaemia, with p38α remaining activated but p38β deactivated. Furthermore, cells expressing a dominant negative p38α, which prevented ischaemia-induced p38 MAPK activation, were resistant to sustained ischaemic injury. It was concluded from these observations that activation of p38α MAPK isoform is detrimental during ischaemia.

2.1.5.2.2 MAPK inactivation by phosphatases

Dephosphorylation of either the threonine or tyrosine residue within the MAPK activation loop T*x*Y motif alone can result in their inactivation. In intact cells, dephosphorylation and inactivation of MAPK occur, within minutes to several hours depending on the cell type and activating stimulus. In endothelial cells, exposure to serum leads to ERK activation that is sustained at high levels for over 2 h. In contrast, different patterns can be observed in the a PC12 cell line where EGF-(epidermal growth factor) stimulated ERK activation is transient, with inactivation initiated within 5 min and nearly completed within 15-30 min,

whereas this MAPK displays prolonged activation for several hours on stimulation with NGF (nerve growth factor) (Alessi *et al.*, 1995). It is believed that different patterns of ERK activation elicited by EGF and NGF underlie their differential effects to drive either cellular proliferation of differentiation, respectively (Marshall, 1995). Using PC12 cells as a model system to identify key phosphatases suppressing ERK activation, biochemical studies revealed that early rapid inactivation of these MAPKs reflects, in part, threonine dephosphorylation by the serine/threonine protein phosphatase PP2A (Alessi *et al.*, 1995).

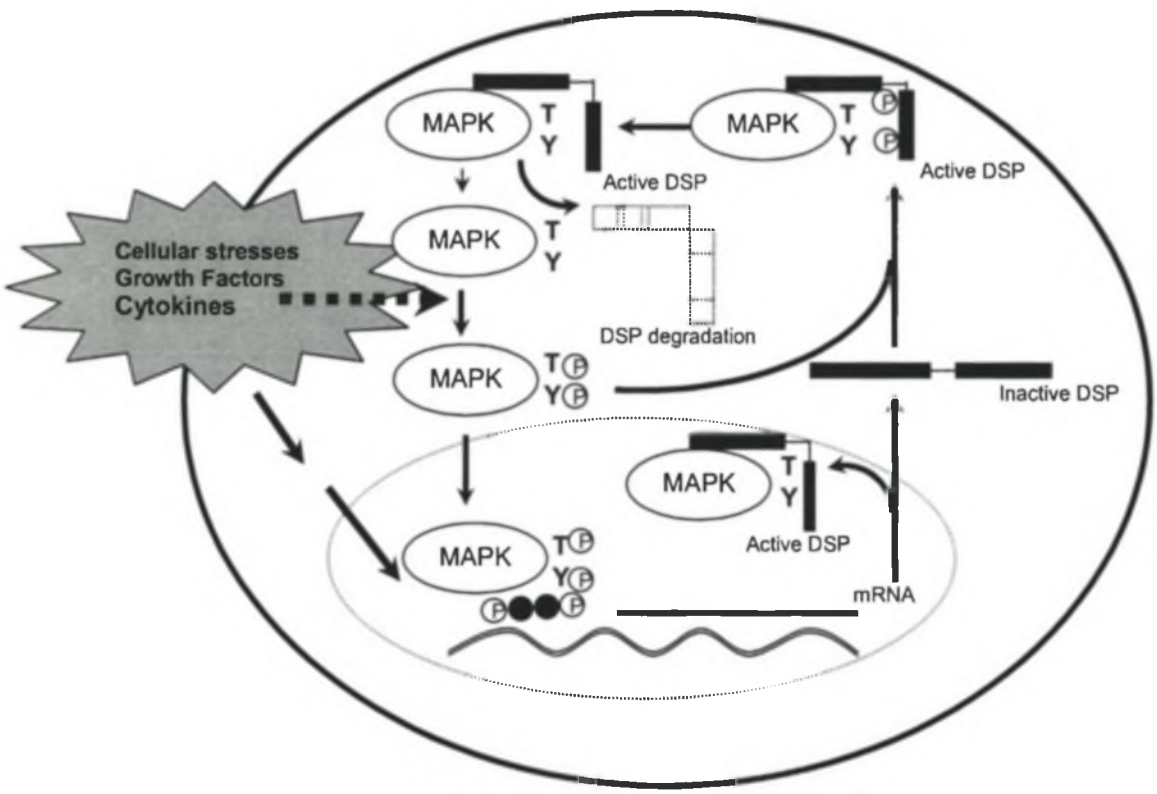
In addition to threonine dephosphorylation, these studies also indicated that the tyrosine-specific protein phosphatases (PTPs) also contribute to ERK inactivation (Alessi *et al.*, 1995). Currently, 50 or more PTPs have been characterized (Neel & Tonks, 1997; Hooft van Huisduijnen; 1998; Denu & Dixon, 1998), and although the PTPs in PC12 cells have not been identified molecularly (Alessi *et al.*, 1995), recent studies in other cell types have identified a possible role for three related PTP gene family members (Wurgler-Murphy *et al.*, 1997; Shiozaki & Russel, 1995, Millar *et al.*, 1995; Zhan *et al.*, 1997). Notwithstanding the importance of these early reports on PP2A and tyrosine-specific protein phosphatases inactivating ERKs, little is known about their general importance in terminating MAPK signaling and the molecular mechanisms that may control phosphatase catalytic activity, or of their specificity for inactivating different MAPK isoforms.

In contrast to these protein phosphatase classes, there has been significant and rapid progress in our understanding of the role played by a subclass of PTP which can dephosphorylate both phosphotyrosine and phosphothreonine residues, known as the dual specificity phosphatases (DSPs). The first mammalian DSP was identified as the mouse immediate early gene 3CH134 or its human orthologue CL100, which is induced rapidly after exposure to growth factors, heat shock, or oxidative stress (Keyse & Emslie, 1992; Charles *et al.*, 1992; Noguchi *et al.*, 1993). Recombinant CL100/3CH134 was shown to

dephosphorylate threonine and tyrosine residues of ERK, leading to its inactivation. These studies showed that CL100/3CH134 was specific for dephosphorylation of ERK when compared to a number of other unrelated phosphoproteins (Alessi *et al.*, 1993). A correlation between 3CH134 levels and ERK inactivation was also found in mammalian cells, leading to its renaming to MAPK phosphatase-1 (MKP-1) (Sun *et al.*, 1993). Despite this important early work, the relevance of MKP-1 in ERK inactivation remains to be elucidated. Firstly, ERK activity is apparently normal after deletion of the MKP-1 gene in mice (Dorfman *et al.*, 1996). Secondly, it has also become evident that MKP-1 is at least as effective in inactivating JNK and p38 when compared to the ERKs (Chu *et al.*, 1996; Franklin & Kraft, 1997). Thirdly, newly identified members of the DSP gene family appear highly selective for ERK and may represent the true physiological regulators of this MAPK isoform. Since the initial cloning of MKP-1, eight additional mammalian DSP gene family members have been identified and characterized, which include MKP-2, MKP-3, MKP-4, MKP-5, MKP-X, PAC1, M3/6 and B59. These DSPs all appear to be effective in mediating inactivation of MAPKs.

The following model for MAPK inactivation by DSP is suggested (fig 2.12). Stimulation by growth factors, cytokines, cellular stresses or some active oncogenes leads to rapid transcription of one or a subset of DSP genes. Increased DSP transcription may reflect activation of specific MAPK, although alternative pathways are not excluded. After translation of the DSP mRNA into protein, the catalytically inactive DSP translocates to a specific subcellular compartment within either the nucleus or the cytosol. Upon encountering its target MAPK, the DSP binds tightly to its amino terminus, which in turn triggers activation of the phosphatase catalytic domain. If the bound MAPK is already activated, this will result in its rapid inactivation. Conversely, if the MAPK is not active, then its tight interaction with an active DSP is expected to block any possibility of kinase activation by a subsequent stimulus. MAPKs that fail to bind the DSP within its amino terminus remain active or susceptible to activation after

Figure 2.12 Model for MAPK inactivation by DSPs
(Camps *et al.*, 1999)



Cell exposure to growth factors, cytokines and cell stresses leads to induction of a subset of DSP genes. Increased expression is likely to reflect activation of transcription factors (black circles) via both MAPK-dependent and independent pathways. Newly synthesized DSPs translocate to specific subcellular compartments as dictated by anchorage and/or localization motifs not yet identified. Specific binding to target MAPKs through regions within the DSP amino terminus then triggers activation of the phosphatase catalytic domain. Bound MAPKs are in turn inactivated by dephosphorylation on threonine and tyrosine residues localized within the "activation loop" motif of TxY. Inactive MAPKs then dissociate, leaving the DSP free to bind and inactivate another MAPK molecule. In the absence of continued DSP gene transcription and protein synthesis, rapid degradation may limit their duration of activity in cells.

extracellular stimulation. Depending on their cellular localization, these regulatory effects allow for selected inhibition of MAPK activities in specific subcellular compartments. Some DSPs have been shown to possess short half-lives (Alessi *et al.*, 1993; Noguchi *et al.*, 1993), suggesting that in the absence of continued gene transcription and protein synthesis, their rapid turnover limits their duration of action in cells. Overall, tight control of DSP gene induction, combined with their differential binding and catalytic activation by a specific repertoire of MAPKs, provides a sophisticated mechanism for rapid targeted inactivation of selected MAPK activities.

2.1.5.2.3 Protein kinase B (PKB)/Akt pathway

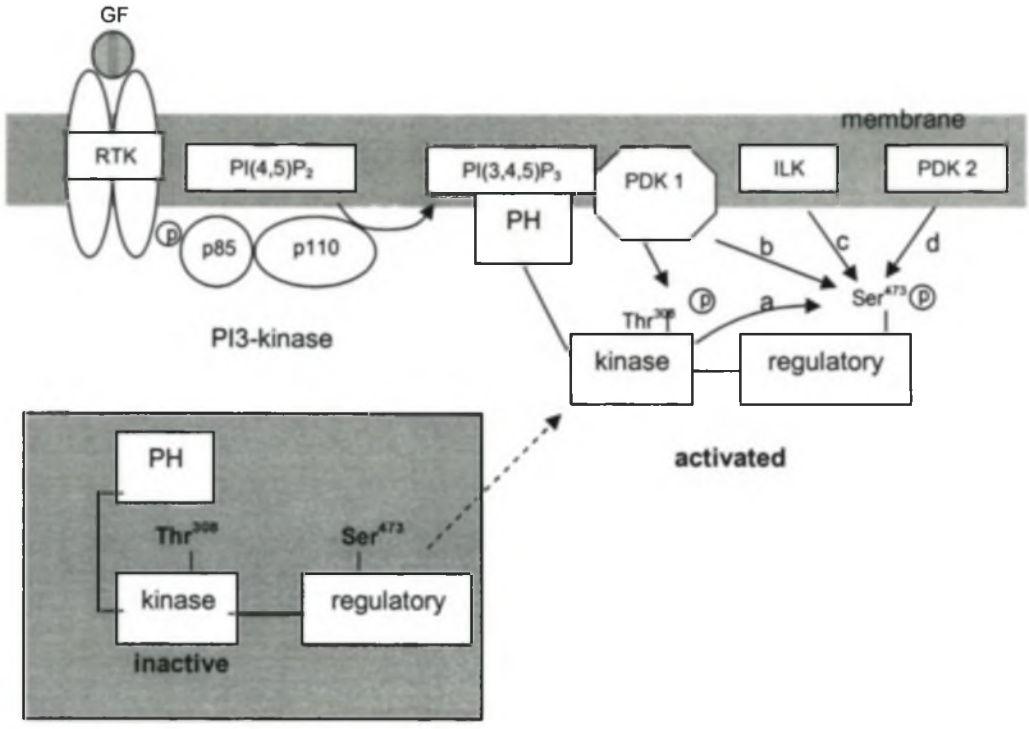
The serine/threonine protein kinase, protein kinase B or Akt (PKB/Akt), has emerged as a crucial regulator of widely divergent cellular processes including apoptosis, proliferation and differentiation. The PKB/Akt story began with the isolation of two genes, named *Akt1* and *Akt2*, which were identified as human homologues of the viral oncogene v-akt, previously known to cause a form of leukaemia in mice (Staal, 1987). Subsequently, three independent studies revealed that v-akt and its mammalian homologue, encoded a protein kinase with some similarities to protein kinase C (PKC) and protein kinase A (PKA) (Bellacosa *et al.*, 1991; Coffer & Woodget, 1992; Jones *et al.*, 1991). Its relation to PKA and PKC led to it being named PKB by the authors of one of these studies. To date, three members of the family have been isolated and these are now referred to as PKBα (Akt 1), PKBβ (Akt 2), and PKBγ (Akt 3). They are products of distinct genes but are highly related, exhibiting greater than 80% homology at amino acid level. The three genes are expressed differentially, with PKBα/Akt 1 and PKBβ/Akt 2 displaying fairly broad and PKBγ/Akt3 more restricted tissue distribution.

Although originally suggested to be involved in the regulation of cell growth, its central role in signaling only became apparent when PKB/Akt was shown to be a

downstream target for PI-3K (Burgering & Coffer, 1995; Franke *et al.*, 1995). The cellular activation of PKB/Akt is dependent upon the generation of inositol-containing membrane lipids phosphorylated by PI-3K at the D3-OH group on the inositol ring. A number of studies have also documented PI-3K-independent activation of PKB/Akt in various cell systems (Vanhaesebroeck & Alessi, 2000), but the physiological significance of these findings remains uncertain. PKB/Akt is activated downstream of Class 1_A and Class 1_B PI-3K, which are activated by tyrosine kinases and G-protein coupled receptors, respectively. Following its recruitment to these receptors in the plasma membrane, PI-3K is activated and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) on the 3-OH group generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 levels are tightly regulated by the action of phosphatases such as PTEN, which removes phosphate from the 3-OH position, and SHIP, which dephosphorylates at the 5-OH position. PIP_3 does not activate PKB/Akt directly but instead appears to recruit PKB/Akt to the plasma membrane and to alter its conformation to allow subsequent phosphorylation by the phosphoinositide-dependent kinase-1 (PDK-1) (fig 2.13). PDK-1 is a 63-kDa serine/threonine kinase containing a C-terminal PH domain that binds with high affinity to 3-phosphoinositides. PDK-1 phosphorylates PKB/Akt in the activation loop, which regulates access to the catalytic site of PKB/Akt. Phosphorylation of this site (Thr^{308} in PKBα/Akt 1) *in vitro* is enhanced by 3-phosphoinositides and it has been suggested that the lipids induce both a favourable conformation of PKB/Akt (and possibly PDK-1) allowing access to the acceptor phosphorylation site and also co-localization of the two proteins in the lipid micro-environment (Alessi *et al.*, 1997; Stokoe *et al.*, 1997).

Although phosphorylation at Thr^{308} partially activates PKB/Akt (Alessi *et al.*, 1996), full activation of PKB/Akt requires phosphorylation on a second site (Ser^{473} in PKBα/Akt 1) located in the regulatory tail. The mechanism mediating Ser^{473} phosphorylation remains controversial. Balendran and co-workers (1999) showed that, following phosphorylation of PKB/Akt at Thr^{308}, PDK-1 converts to a

Figure 2.13 Mechanism of activation of PKB/Akt
(Nicholson and Anderson, 2002)



In unstimulated cells PKB/Akt is not phosphorylated on Thr^{308} or Ser^{473} and resides mainly in the cytosol. Following growth factor (GF) activation of receptor tyrosine kinases (RTKs, or other cell surface receptors, not shown), PI-3K is recruited to the receptor and activated, resulting in the production of PIP_3. This recruits PKB/Akt to the membrane where it is phosphorylated on Thr^{308} within the catalytic domain by PDK-1 and on Ser^{473} within the regulatory domain by an ill-defined mechanism, possibly involving (a) autophosphorylation, (b) PDK-1, (c) ILK, or (d) an unidentified PDK-2. PKB/Akt is then released from the membrane and translocates to other subcellular compartments.

Ser^{473} kinase as a result of its interaction with the singly phosporylated PKB/Akt and a fragment of a protein known as PRK-2. Modification of Ser^{473} has also been shown to occur through autophosphorylation (Toker & Newton, 2000).

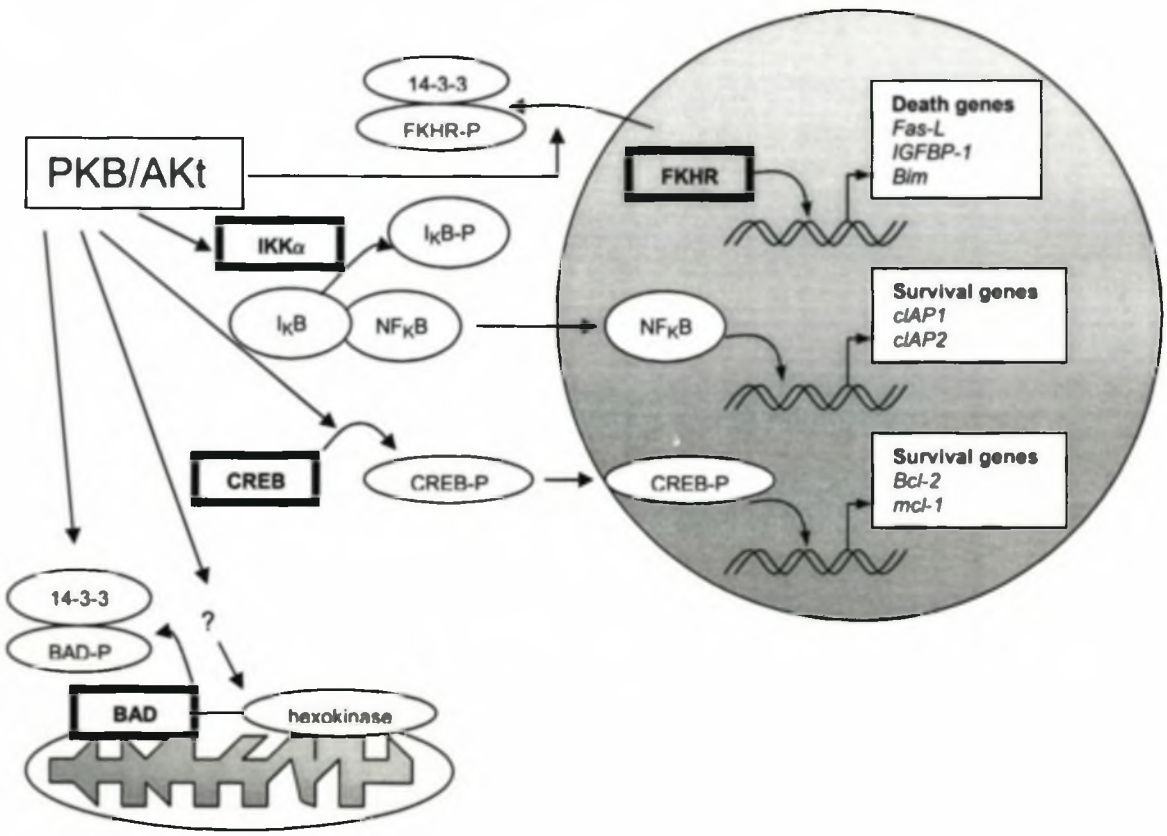
Other findings suggest that Ser^{473} is modified by a distinct kinase or PDK-2. Although in most situations, phosphorylation of PKB/Akt at Ser^{473} in intact cells, occurs in tandem with that at Thr^{308}, a number of studies have shown that phosphorylation at the two sites can occur independently (Alessi *et al.*, 1996; Kroner *et al.*, 2000). Furthermore, PDK-1 null embryonic stem cells maintain the ability to undergo Ser^{473} phosphorylation (Williams *et al.*, 2000) and a recent study showed that Ser^{473} phosphorylation could be stimulated by insulin in the absence of Thr^{308} phosphorylation and PKB/Akt activation, indicating the existence of a ligand-activatable PDK-2 (Hill *et al.*, 2001). The integrin-linked kinase (ILK) was shown to phosphorylate Ser^{473} (Delcommenne et al., 1998) but a subsequent report suggested that ILK acts only as a facilitator and does not phosphorylate PKB/Akt directly (Lynch *et al.*, 1999). A more recent study showed that ILK could phosphorylate PKB/Akt on Ser^{473} and that the kinase activity of ILK was essential for Ser^{473} phosphorylation in cells. A novel ILK-specific inhibitor also blocked Ser^{473} phosphorylation of PKB/Akt (Persad *et al.*, 2001). These studies strongly suggest that ILK plays some role in the activation process but whether it phosphorylates PKB/Akt directly remains an open question. Clearly, the regulation of PKB/Akt is complex, and not fully understood. Although there is evidence for independent regulation of Ser^{473}, phosphorylation at this site alone has not been shown to increase PKB/Akt activity, therefore the physiological significance of these findings is unclear. A recent report documented evidence that PKB/Akt activation requires the phosphorylation of two tyrosine residues in the activation loop, Y315 and Y326 in PKBα/Akt 1 (Chen *et al.*, 2001). These modifications were shown to be dependent on src family tyrosine kinases. If confirmed then these findings add further to the complexity of PKB/Akt regulation.

2.1.5.2.3 (a) Involvement of PKB/Akt in anti-apoptotic mechanisms (fig 2.14)

The intrinsic capacity of all cells to undergo apoptosis is suppressed by survival signals induced by factors within their immediate microenvironment. Studies conducted over the last few years showed that PKB/Akt is critical for cell survival. For example, dominant negative alleles of PKB/Akt reduce the ability of growth factors and other stimuli to maintain cell survival whereas over-expression of wild type or activated Akt can rescue cells from apoptosis induced by various stress signals (Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997). Although there can be little doubt that PKB/Akt promotes cell survival, the mechanisms involved have only recently begun to emerge. Progress in this regard has come mainly through the identification of PKB/Akt substrates that either participate directly in the apoptotic cascade, or regulate the transcription of pro- and anti-apoptotic genes. Prosurvival substrates of PKB/Akt signaling include ASK1, Bad, CREB, Forkhead family (FKHR, FKHRL1, AFX), Iκ-B kinase and procaspase-9.

One means by which PKB/Akt may promote cell survival is through direct phosphorylation of transcription factors that control the expression of pro- and anti-apoptotic genes. PKB/Akt appears to both negatively regulate factors that promote the expression of death genes and positively regulate factors that induce survival genes. An example of the former is the forkhead family of transcription factors. The three identified mammalian members of the forkhead family, FKHR, FKHRL1, and AFX, all contain consensus PKB/Akt phosphorylation sequences, which can be effectively phosphorylated by PKB/Akt *in vitro* (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Rena *et al.*, 1999). Phosphorylation of forkhead proteins by PKB/Akt appears to alter their subcellular location. Stimulation of cells with factors that increase PKB/Akt activity leads to the export of FKHRL1 from the nucleus (Biggs *et al.*, 1999) and its accumulation and sequestration by 14-3-3 proteins in the cytoplasm (Brunet *et*

Figure 2.14 **Regulation of cell survival by PKB/Akt (Nicholson and Anderson, 2002)**



PKB/Akt promotes cell survival by multiple mechanisms: (1) decreasing the transcription of death genes by phosphorylating forkhead family transcription factors such as FKHR, which promotes their sequestration by 14-3-3 proteins in the cytoplasm, (2) increasing the transcription of survival genes by activating NF-κB and CREB transcription factors, (3) phosphorylating and inactivating the pro-apoptotic protein BAD, and (4) maintaining mitochondrial integrity by activating hexokinase (the direct PKB/Akt substrates that mediate these events are represented in black boxes).

al., 1999). In addition to negatively regulating forkhead activity, PKB/Akt appears to positively regulate at least two other transcription factors, viz NF-κB and CREB. NF-κB is involved in the regulation of cell proliferation, apoptosis, and survival by a wide range of cytokines and growth factors. Its survival-promoting activity is mediated through its ability to induce prosurvival genes such as *c-IAP-1* and *c-IAP-2*. NF-κB is regulated through its association with an inhibitory cofactor I-κB, which sequester NF-κB in the cytoplasm. Phosphorylation of I-κB by upstream kinases, known as IKKs, promotes its degradation, allowing NF-κB to translocate to the nucleus and induce target genes (fig 2.14). PKB/Akt has been shown to interact with and activate IKKα (Romashkova & Makarov, 1999). Data suggest that PKB/Akt phosphorylates IKKα directly, but more importantly, PKB/Akt is believed to be essential for IKK-mediated destruction of I-κB and activation of NF-κB (Romashkova & Makarov, 1999; Ozes *et al.*, 1999). These findings indicate that PKB/Akt is a critical regulator of NF-κB-dependent gene transcription and may play a critical role in promoting cell survival.

The PKB/Akt pathway has been shown in some cell types to increase expression of the anti-apoptotic gene *bcl-2* (Skorski *et al.*, 1997; Pugazhenthi *et al.*, 2000). Induction of bcl-2 promotor activity by IGF-1 was shown to occur via a PKB/Akt pathway involving the cyclic AMP (cAMP)-response element binding protein (CREB) transcription factor. CREB is a direct target for phosphorylation by PKB/Akt (Du *et al.*, 1998) and this phosphorylation occurs on a site that increases binding of CREB to accessory proteins necessary for induction of genes containing cAMP response elements (CREs) in their promotor regions.

Panka and co-workers (2001) also indicated a role for PKB/Akt in regulating the expression of c-FLIP, a caspase-8 homologue that acts as dominant negative inhibitor of TNF receptor family-induced apoptosis. The mechanism by which PKB/Akt induces c-FLIP has not been characterized, but work by Micheau and

co-workers (2001) showed that NF-κB promotes c-FLIP expression and suggested that regulation of this transcription factor by PKB/Akt may be critical.

In addition to influencing the transcription of pro- and anti-apoptotic genes, numerous studies indicate that PKB/Akt promotes survival by directly phosphorylating key regulators of the apoptotic cascade. The most widely studied example of this type of regulation involves Bad, a member of the Bcl-2 family, which promotes apoptosis by binding to and antagonizing the actions of prosurvival members of the family such as Bcl-2 and Bcl-X_L. PKB/Akt can phosphorylate Bad at residue S136 and this modification promotes the sequestration of Bad by 14-3-3 proteins in the cytosol, thus preventing Bad from interacting with Bcl-2 and Bcl-X_L at the mitochondrial membrane (del Peso *et al.*, 1997; Datta *et al.*, 1997). PKB/Akt-induced phosphorylation of Bad may also occur indirectly, through intervening protein kinases such as Raf-1 (Majewski *et al.*, 1999) and $p65^{PAK}$ (Schurmann *et al.*, 2000; Tang *et al.*, 2000).

Stress-activated protein kinases (SAPKs) such as JNK are critically involved in the induction of apoptosis following exposure of cells to stimuli such as ionizing radiation, heat shock, or osmotic stress. A recent report has provided the first evidence that PKB/Akt may interfere with SAPK signaling and thereby inhibit apoptosis. PKB/Akt was shown to phosphorylate and inactivate ASK1, a kinase that transduces stress signals to the JNK and p38 MAP kinase pathways (Kim *et al.*, 2001). To this point, all of the PKB/Akt targets mentioned regulating apoptosis prior to the release of cytochrome c from the mitochondria and activation of the caspase cascade that characterizes the terminal execution phase of apoptosis. However, data suggesting that PKB/Akt also influences postmitochondrial events have been reported. Procaspase-9, the initiator caspase in the caspase cascade, was shown to be a substrate for PKB/Akt (Cardone *et al.*, 1998). In these experiments, phosphorylation of human procaspase-9 by PKB/Akt blocked its intrinsic protease activity and was shown to be critical for overall regulation of the apoptotic process. In a more recent study

by Zhou and co-workers (2000), exogenous PKB/Akt was shown to inhibit the activation of caspase-9 and –3 induced by cytochrome c in a cell-free system. However, these authors reported that procaspase-9 was not a direct substrate for PKB/Akt, suggesting that PKB/Akt inhibits caspase activation by modifying an unknown cytosolic factor.

2.1.5.2.4 Negative regulation of PKB/Akt activity

The activation status of PKB/Akt in cells will depend upon the balance between the "on" signals generated by elevated PIP_3 levels and the influence of "off" signals that lead to PKB/Akt dephosphorylation. Several lines of evidence indicated that PKB/Akt phosphatases participate in both the positive and negative regulation of PKB/Akt. The facts that key phosphoserine and phosphothreonine residues in PKB/Akt have a relatively short half-life and that phosphatase inhibitors such as vanadate and okadaic acid were shown to augment PKB/Akt kinase activity, indicate that the enzyme is negatively regulated by dephosphorylation (Andjelkovic *et al.*, 1996; Meier *et al.*, 1997). Protein phosphatase 2A (PP2A) may be the key enzyme associated with dephosphorylation of PKB/Akt *in vitro* and *in vivo* (Andjelkovic *et al.*, 1996; Meier *et al.*, 1997; Meier *et al.*, 1998).

Attenuation of PI-3K activation leads to a rapid dephosphorylation of Ser^{473} and (more slowly) Thr^{308}, accompanied by a loss of PKB/Akt activity, indicating that PIP_3 constrains phosphatase action as well as promotes PDK-1 activation (Anjelkovic *et al.*, 1999). The inactivation of PKB/Akt by ceramide (Schubert *et al.*, 2000) and osmotic stress (Meier *et al.*, 1998; Chen *et al.*, 1999) occurs predominantly via Ser^{473} dephosphorylation by an okadaic acid-sensitive phosphatase. Dephosphorylation of Thr^{308} appears to occur independently (Schubert *et al.*, 2000) and a recent study suggests, controversially, that PDK-1 participates in the dephosphorylation of Thr^{308} by an unknown mechanism (Yamada *et al.*, 2001).

A 3-phosphoinositide-specific phosphatase activity was found to reside in the tumor suppressor PTEN (Phosphatase and tensin homologue deleted from chromosome 10), which is mutated or deleted in a wide range of human cancers (reviewed in Cantley & Neel, 1999). PTEN shares homology with DSP that can dephosphorylate serine, threonine and tyrosine residues. However, attempts to confirm PTEN as a protein phosphatase revealed only relatively weak activity (Li & Sun, 1997; Myers *et al.*, 1997), suggesting that PTEN is a specialized phosphatase for certain proteins and/or possesses a different activity. Indeed, it was found that PTEN is a potent lipid phosphatase (Maehama & Dixon, 1998; Myers *et al.*, 1998). Overexpression of PTEN significantly reduced $PI(3,4,5)P_3$ production induced by insulin, and PTEN-null cells have higher levels of $PI(3,4,5)P_3$ (Haas-Kogan *et al.*, 1998; Maehama & Dixon, 1998; Stambolic *et al.*, 1998). A recombinant PTEN dephosphorylates 3-phosphoinositides specifically at position 3-OH of the inositol ring and has highest specificity for $PI(3,4,5)P_3$ (Maehama & Dixon, 1998). Since PTEN antagonizes the PI 3-kinase activity, it may also affect PKB/Akt. Experiments with inactive PTEN and in PTEN-null fibroblasts showed that these cells exhibit high basal activity of PKB/Akt (Myers *et al.*, 1998; Li & Sun, 1998; Wu *et al.*, 1998). These results, together with genetic studies in *C elegans* demonstrating that PTEN resides in the same pathway as PI 3-kinase/PKB and inhibits PKB/Akt (Ogg & Ruvkun, 1998), established PTEN as a negative regulator of PKB/Akt.

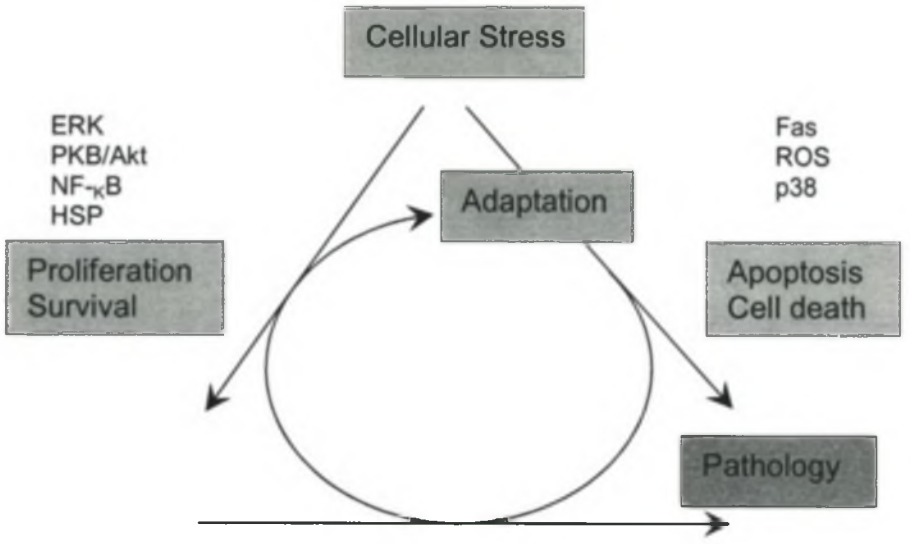
Another lipid phosphatase that can negatively regulate PKB/Akt is SHIP – an inositol 5' phosphatase that hydrolyzes $PI(3,4,5)P_3$ to $PI(3,4)P_2$. Over-expression of SHIP was shown to inhibit PKB/Akt activity and SHIP-null cells exhibit prolonged activation of PKB/Akt upon stimulation (Liu *et al.*, 1995; Aman *et al.*, 1998).

2.1.5 Conclusion

It is clear from the previously discussed pathways that apoptosis is a highly regulated and complex process, controlled by numerous checkpoints and communication networks. In recent years, however, cell biologists have recognized that the regulation of cell death is much more than the fatal result from acute cellular injury. Moreover, proliferation and cell death seem to be highly connected and considerable overlap exists between the components that execute both processes. Apoptosis often represents an immediate response to external stimuli. The following adaptive changes leading to a remodelled structure are usually beneficial and protective. Overwhelming stress, however, may lead to disturbed remodelling processes (fig 2.15).

Although the extent to which apoptosis is involved in cardiac disease remains to be established, the evidence that has emerged clearly supports a role for this mode of cell death. A better understanding of the underlying pathways may lead to the design of a new class of therapeutic agents aimed at preventing myocyte death and attenuating the progression of cardiac disease. Indeed, some treatments already in use may work in part by inhibition of apoptosis. β-Blockers such as carvedilol are known to have anti-apoptotic properties (Yue *et al.*, 1998). Similarly, NO donors used in the treatment of myocardial infarction have both pro-apoptotic and anti-apoptotic properties, although it has yet to be shown that this is part of the therapeutic effect of these agents (Kim *et al.*, 1999). It may turn out that other conventionally used pharmaceuticals exert their effect via apoptotic pathways. However, there is still controversy whether apoptosis or necrosis is the main form of cardiomyocte death involved in the pathogenesis of cardiac disease or whether there is a third, as yet uncharacterised, form of cell death that combines aspects of both apoptosis and necrosis. Further research should provide answers to these questions and determine the therapeutic value of anti-apoptotic intervention in the treatment of cardiovascular disease. The challenge

Figure 2.15 Schematic representation of cellular stress-induced effects in the cardiovascular system (modified from Wernig and Xu, 2002)



of future therapeutic concepts will be to develop a therapy, which is short acting and localized with minimum or no side effects.

2.2 Fatty acids in cardiovascular disease

2.2.1 Introduction

The concept that dietary polyunsaturated fatty acids (PUFAs) affect the incidence of coronary heart disease (CHD) is widely accepted, based on both epidemiological and experimental evidence. The effects of dietary PUFAs on serum lipoprotein levels and membrane phospholipid composition and its (patho)physiological and biochemical consequences have extensively been studied in the cardiovascular system, however, the mechanisms underlying the beneficial effects of PUFAs, of either n-6 or n-3 family, are largely unclear.

Long-chain PUFAs, taken up by the cell from the extracellular environment or released from cellular triglycerides or membrane phospholipids play an important role in various biological processes in the heart. Not only can they be **(a) metabolized to other longer chain fatty acids and eicosanoids,** or **(b)** do they serve as **fuel through the β-oxidation pathway**, but they are also involved in **(c) apoptosis** and **(d) cellular signal transduction.**

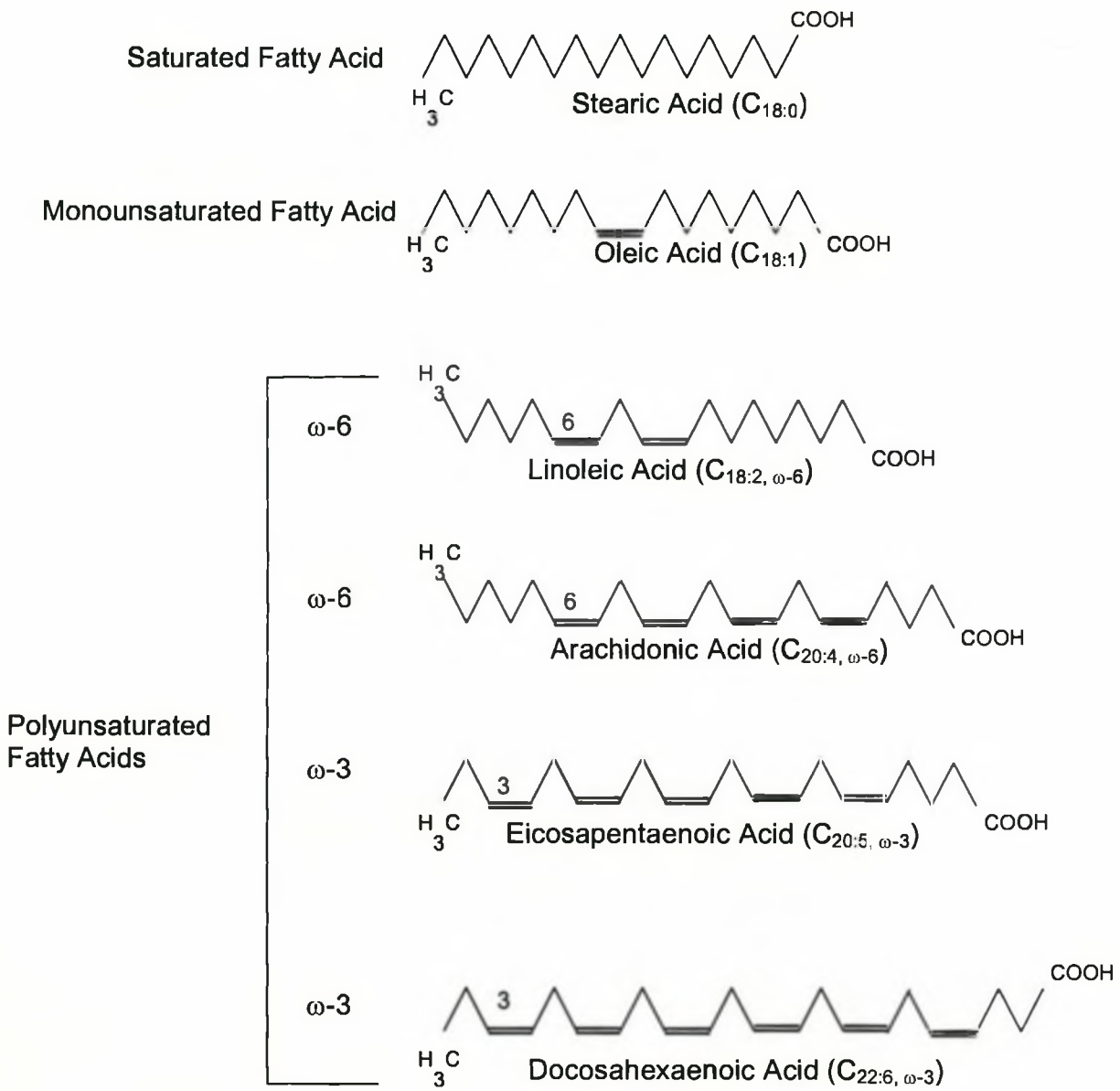
2.2.2 Fatty acid metabolism

Fatty acids are commonly classified according to a shorthand nomenclature which designates the chain length, number of double bonds, and position of double bond nearest to the methyl group (Willis, 1987). This terminal carbon atom is often referred to as the omega (ω) carbon atom, as it occurs at the opposite end of the molecule to carbon-1, which bears the carboxyl function. The term n-x is used instead of omega-x to describe the position of the double bond nearest to the methyl group. According to the number of double bonds, fatty acids can be classified as saturated-, monounsaturated-, and polyunsaturated fatty acids (fig 2.16). Saturated fatty acids are straight chain structures with no double bonds and an even number of carbon atoms. In general, human

monounsaturated fatty acids have an even number of carbon atoms, a chain length of 12-22C and a double bond with the *cis* configuration. Polyunsaturation is common, but largely confined to the C18 and C20 acids.

All mammals can synthesise fatty acids *de novo* from acetyl-coenzyme A. The end product of the fatty acid synthesised enzyme is palmitic acid (16:0), which in turn can be elongated to stearic acid (18:0). There is little need for the synthesis of saturated fatty acids in Western man, since the diet normally supplies adequate amounts. However, cell membranes require unsaturated fatty acids to maintain their structure, fluidity and function. Therefore a mechanism for the introduction of double bonds (i.e. desaturation) exists. The introduction of a single double bond between carbon atoms 9 and 10 is catalysed by the enzyme Δ9-desaturase, which is universally present in both plant and animals. This enzyme results in the conversion of stearic acid to oleic acid (18:1n-9). Plants, unlike animals can insert additional double bonds into oleic acid between the existing double bond at the 9-position of the methyl terminus of the carbon chain; a Δ12-desaturase converts oleic acid into linoleic acid (18:2n-6), while a Δ15-desaturase converts linoleic acid into α-linolenic acid (18:3n-3). Since animal tissues are unable to synthesise linoleic acid and α-linolenic acid, these fatty acids must be consumed in the diet and are termed essential fatty acids. Using the pathway outlined in figure 2.17, animal cells can convert dietary α-linolenic acid into eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), by a similar series of reactions dietary linoleic acid is converted via γ-linolenic acid (18:3n-6) and dihomo-γ-linolenic acid (20:3n-6) to arachidonic acid (ARA; 20:4n-6). The n-9, n-6 and n-3 families of polyunsaturated fatty acids (PUFAs) are not metabolically interconvertible in mammals. Many marine plants, especially the unicellular algae in phytoplankton, also carry out chain elongation and further desaturation of α-linolenic acid to yield the long-chain n-3 PUFAs, EPA and DHA. It is the formation of these long-chain n-3 PUFAs by marine algae and their transfer through the food chain to fish that account for their abundance in some marine fish oils.

Figure 2.16 **The basic structures of saturated-, monounsaturated- and polyunsaturated fatty acids**

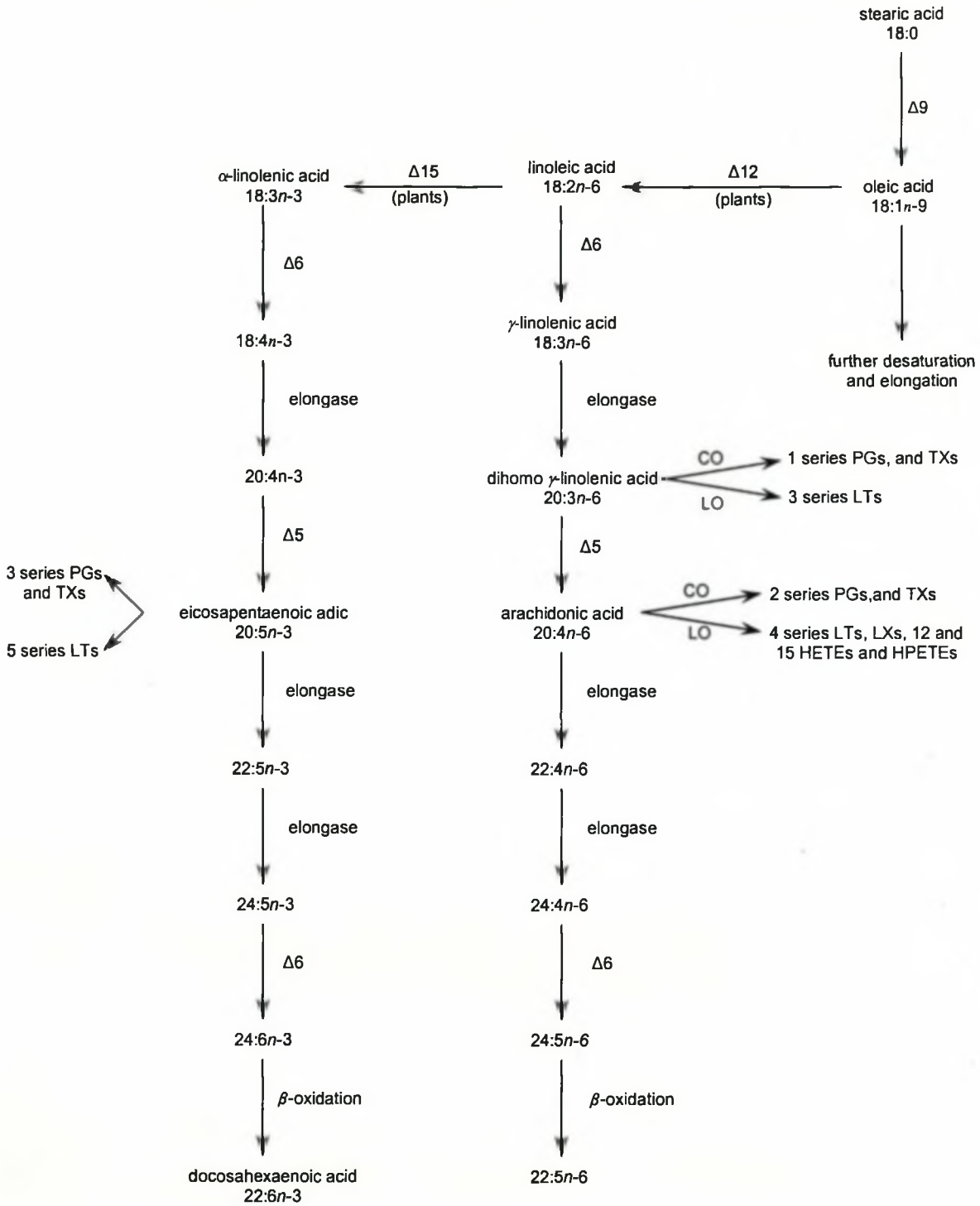


Both n-3 and n-6 essential fatty acid families have distinct nutritional and metabolic effects, and each has a direct precursor relationship with specific classes of eicosanoids. Eicosanoids are a family of oxygenated derivatives of dihomo-γ-linolenic, arachidonic and eicosapentaenoic acids. Eicosanoids include prostaglandins (PGs) and thromboxanes (TXs), which together are termed prostanoids, and leukotrienes (LTs), lipoxins, hydroperoxyeicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs). In most conditions, the principal precursor for these compounds is arachidonic acid and the eicosanoids produced from ARA appear to have more potent biological functions than those released from dihomo-γ-linolenic acid or EPA. PUFAs are released from membrane phosphatidylcholine by the action of phospholipase A_2 or from membrane phosphatidylinositol-4,5-bisphosphate by the actions of phospholipase C and a diacylglycerol lipase. The pathways of eicosanoid synthesis begin with cyclooxygenase, which yields the PGs and TXs, or with the 5-, 12-, or 15-lipoxygenases, which yield the LTs, HPETEs, HETEs and lipoxins (fig 2.18). A third pathway, which operates through the microsomal cytochrome P-450, results in the formation of epoxides, which are converted to HETEs. The amounts and types of eicosanoids synthesized are determined by the availability of arachidonic acid, by the activities of phospholipases A_2 and C and by the activities of cyclooxygenase and lipoxygenases. Eicosanoids usually have a short half-life and act locally on the cell by which they are produced. Their production is initiated by particular stimuli (e.g. cytokines, growth factors, oxygen free radicals, bradykinin, thrombin) and, once produced, they themselves are able to modify the response to the stimulus.

2.2.3 β-oxidation in the aerobic heart

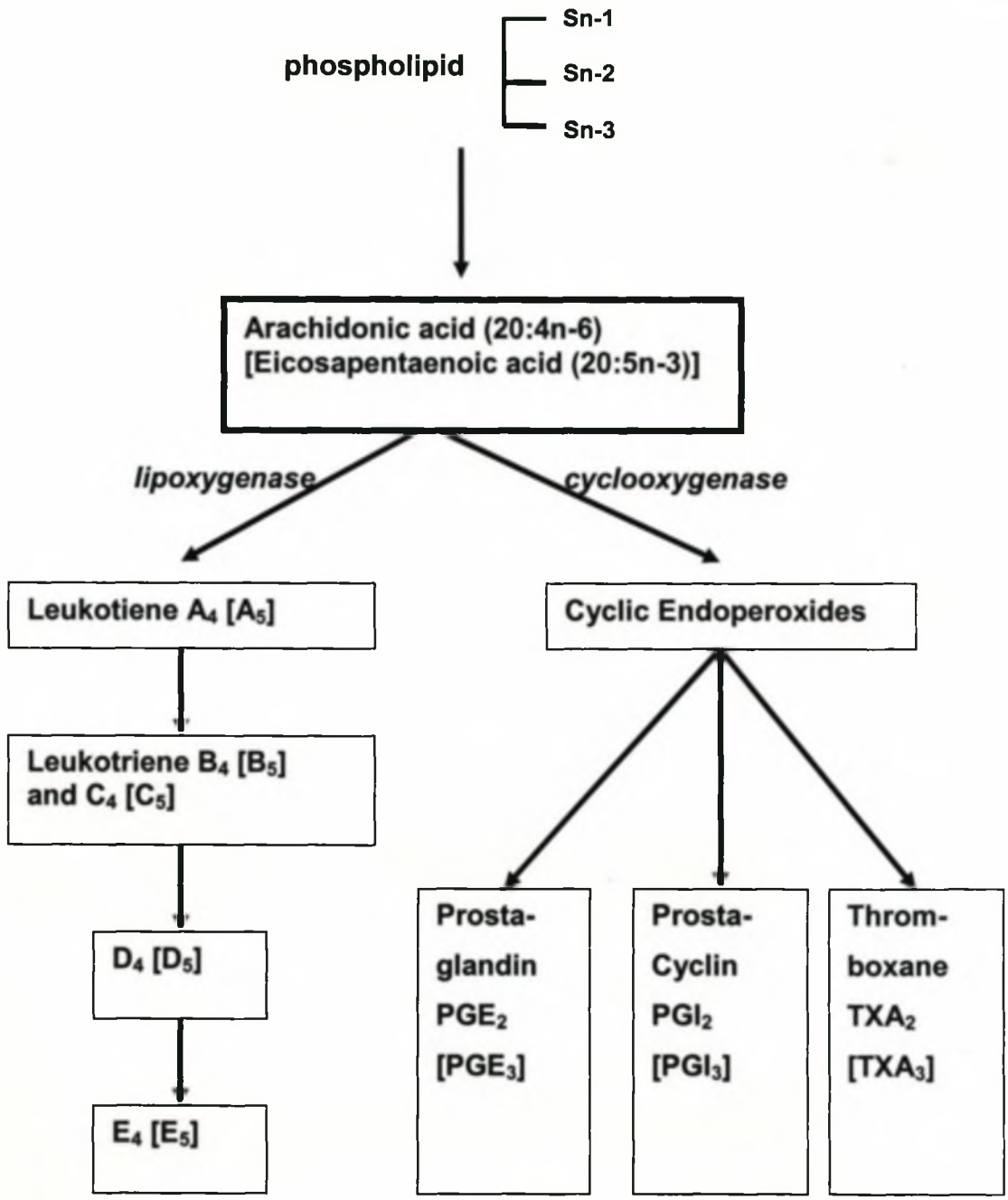
Within the circulation, fatty acids are tightly bound to albumin and represent an important, readily available energy source for certain organs like the heart. Entry of fatty acids into the myocardial cell is presently thought to be mediated by

Figure 2.17 Polyunsaturated fatty acid metabolism



Δ5, 6, 9, 12 and 15 indicate desaturase enzymes; CO – cyclooxygenase; LO – lipoxygenase; LX - lipoxins

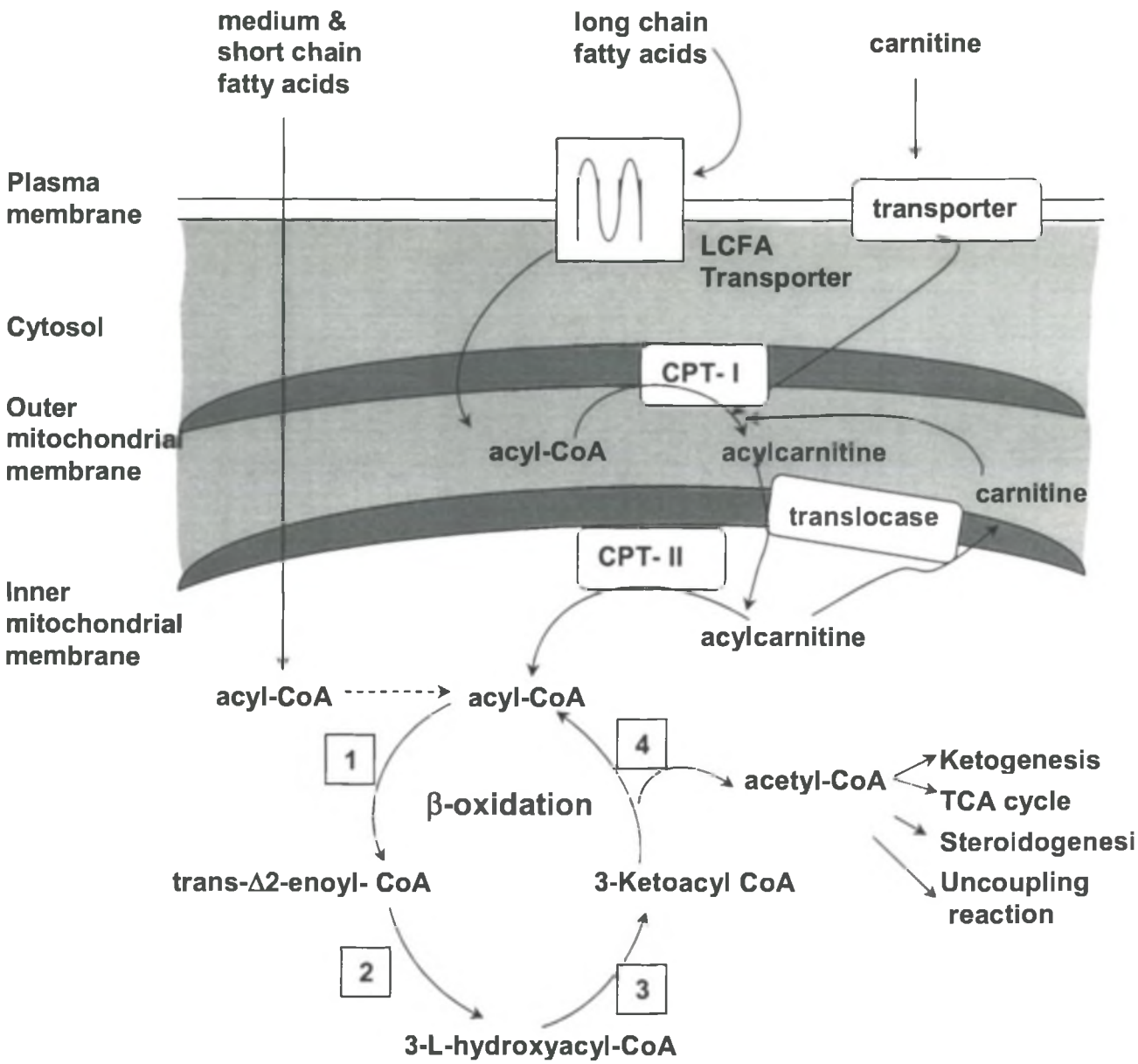
Figure 2.18 **The two major pathways of arachidonic acid and eicosapentaenoic acid metabolism to eicosanoids**



several proteins including fatty acids binding proteins (FABPs) and a myocardial-specific integral membrane transporter (fatty acid translocase or FAT). The non-enzymatic FABP also serves as a facilitator of intracellular transport of relatively insoluble long-chain fatty acids to sites of metabolic utilization (e.g. mitochondria). In mammals, the FABP content in skeletal and cardiac muscle is related to the fatty acid oxidation capacity of the tissue (Glatz & Storch, 2001).

Once in the cytoplasm, long-chain fatty acids (LCFAs) may be transported by heart-type fatty acid binding protein (H-FABP) (Binas *et al.*, 1999) and are rapidly esterified to acyl-CoA by long-chain acyl-CoA synthase (LCAS). Transport of LCFAs into the mitochondria occurs via transesterification by carnitine palmitoyltransferase I (CPT-I) and subsequent translocation across the inner mitochondrial membrane facilitated by carnitine/acyl-carnitine translocase. CPT-I catalyzes a key rate-limiting step in mitochondrial fatty acid flux and, as such, is highly regulated at gene transcriptional level (Brown *et al.*, 1995; Brandt *et al.*, 1998; Steffen *et al.*, 1999) as well as through post-translational control via reversible binding of the inhibitor, malonyl-CoA, the first committed intermediate in the pathway of fatty acid synthesis (McGarry *et al.*, 1989). Upon translocation across the inner mitochondrial membrane, long-chain acylcarnitines are re-esterified to acyl-CoA derivates by CPT-II and enter the β-oxidation spiral. The initial step in the oxidative spiral is catalysed by: (1) a family of acyl-CoA dehydrogenases (AD) specific for very long-chain (VLCAD), long-chain (LCAD), medium-chain (MCAD) and short-chain (SCAD) acyl-CoA substrates. Subsequent steps involve: (2) hydration by enoyl-CoA hydratase; (3) a second oxidation by a 3-hydroxyacyl-CoA dehydrogenase; and then (4) a thiolytic cleavage by 3-ketoacyl-CoA thiolase to yield acetyl-CoA and shortened acyl-CoAs destined for additional rounds of oxidation. Steps 1 and 3 of the fatty acid oxidation (FAO) cycle generate electrons which are ultimately transferred to the electron transport chain where ATP is produced in the presence of oxygen (i.e. oxidative phosphorylation). The acetyl-CoA end-product is oxidized via the tricarboxylic acid (TCA) cycle (fig 2.19).

Figure 2.19 **Fatty Acid Oxidation Pathway (Barger & Kelly, 2000)**



1 – first enzymes of β-oxidation spiral: very long chain (VLCAD), long-chain (LCAD), and medium-chain (MCAD) acyl-CoA dehydrogenases
2 – second enzyme: enoyl-CoA hydratase
3 – third enzyme: 3-hydroxyacyl-CoA dehydrogenase
4 – fourth enzyme: 3-ketoacyl-CoA thiolase

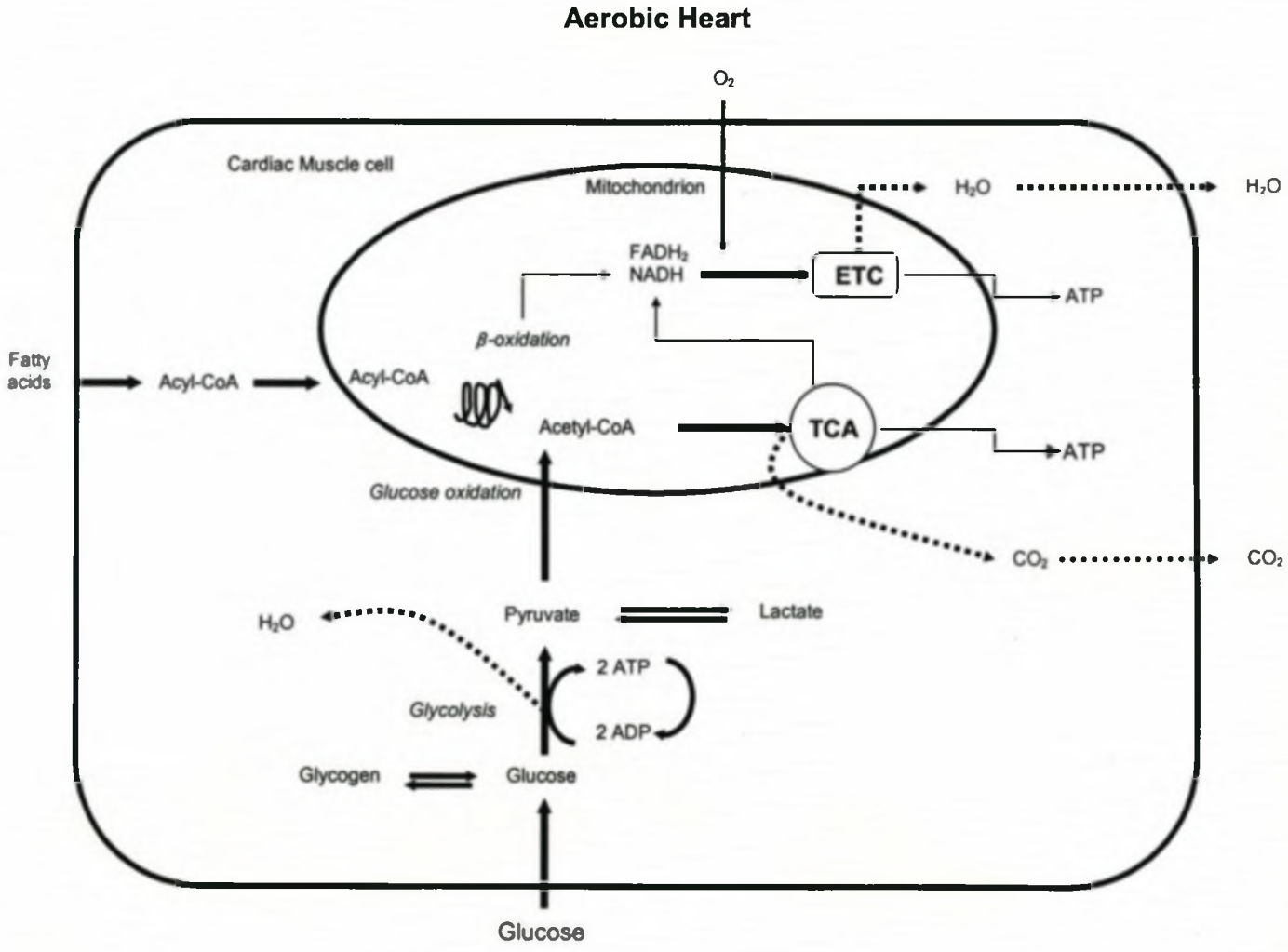
Naturally occurring mutations in many of these enzymes, including MCAD and VLCAD (Kelly & Strauss, 1994), underlie known inborn errors of metabolism which may present early in life with stress-induced hypoglycemia, hepatic dysfunction, cardiomyopathy, and sudden death – a dramatic phenotype illustrating the importance of fatty acid oxidation in normal cardiac energy production.

The process of fatty acid oxidation is termed β-oxidation, since it occurs through the sequential removal of 2-carbon units by oxidation of the β-carbon position of the fatty acyl-CoA molecule. Each round of β-oxidation produces NADH, $FADH_2$ and acetyl-CoA. The acetyl-CoA, the end product of each round of β-oxidation, enters the TCA cycle where it is further oxidized to CO_2 with the concomitant generation of NADH, $FADH_2$ and ATP. The NADH and $FADH_2$ generated during fat oxidation and acetyl-CoA oxidation in the TCA cycle will subsequently enter the respiratory pathway for the production of ATP. Consequently, the oxidation of fatty acids yields more energy per carbon atom than does the oxidation of carbohydrates. However, while fatty acids produce more ATP during complete aerobic oxidation than glucose, this occurs at the expense of a higher rate of oxygen consumption.

2.2.3.1 Energy metabolism in the aerobic, ischaemic and reperfused heart

The high energy demand of the heart is met by utilizing a variety of carbon substrates, including free fatty acids (FFAs), carbohydrates, amino acids and ketone bodies (Kantor *et al.*, 2001). Free fatty acids and carbohydrates are the major substrates from which the heart derives most of its energy (fig 2.20). Under normal, aerobic conditions, 50-70% of the total energy is obtained from fatty acids, while the majority of the rest is obtained from carbohydrates (mainly glucose and lactate). All of the ATP produced from fatty acid oxidation is

Figure 2.20 **Energy metabolism in a normal aerobic heart**
(Sambandam & Lopaschuk, 2003)

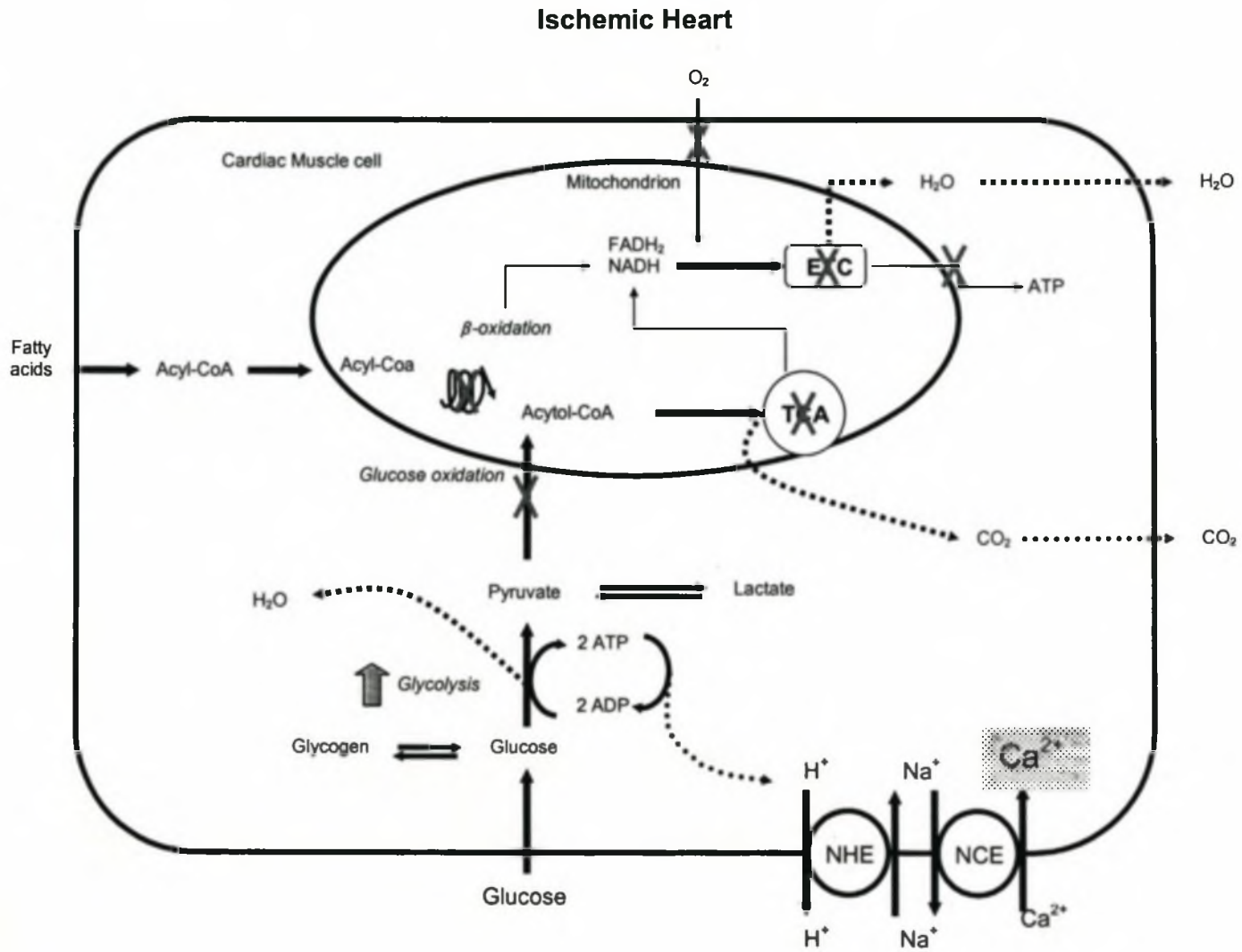


The diagram shows some of the major metabolic pathways, which contribute, to myocardial energy production. Fatty acids are transported into the mitochondria as acyl-CoA which undergoes β-oxidation to release acetyl-CoA. Acetyl-CoA then enters the tricarboxylic acid cycle (TCA) to produce ATP, CO_2 and H_2O. Glucose on the other hand undergoes glycolysis to produce pyruvate. Puruvate is then oxidised via TCA cycle in the mitochondria or converted to lactate extra-mitochondrially. Glucose can also be stored as glycogen in the myocardium.

dependent on the presence of oxygen. In contrast, ATP production from glucose originates from both glycolysis (oxygen-independent) and glucose oxidation (oxygen dependent). During ischaemia, due to restricted oxygen supply to the muscle, both fatty acid and carbohydrate oxidation decreases and ATP production is impaired (fig 2.21) (Lopaschuk, 1997). Glycolysis, a minor source of ATP in the aerobic heart, then becomes a more significant source of energy (Olivier & Opie, 1994). However, pyruvate produced from glycolysis is converted to lactate rather than being completely metabolized to CO_2 and H_2O in the mitochondria. This not only results in the accumulation of lactate but also H^+s, since H^+ produced from hydrolysis of glycolytically derived ATP are not taken up by the mitochondria (with pyruvate) where it eventually is used for H_2O production (fig 2.21). These results in a fall in intracellular pH, the magnitude of which depends on the severity of ischaemia (Kloner & Jennings, 2001; Liu *et al.*, 2001).

During reperfusion of previously ischaemic muscle, there is the possibility of an impaired post-ischaemic functional recovery due to a delayed recovery of metabolism (Schwaiger *et al.*, 1985). However, in the reversibly injured myocardium, fatty acid oxidation rapidly recovers during reperfusion and becomes the dominant source of energy replenishing the ATP pool in the heart (Belke & Lopaschuk, 1997). Glucose oxidation, on the other hand remains suppressed due to the fact that increased fatty acid oxidation inhibits glucose oxidation via the well known Randle cycle (increase in fatty acid oxidation inhibits glucose oxidation in the muscle via inhibiting the rate limiting pyruvate dehydrogenase complex of the glucose oxidation pathway (Kantor *et al.*, 2001). Interestingly, glycolysis is unaffected by these high rates of fatty acid oxidation and remains elevated during reperfusion resulting in an increase in the uncoupling of glycolysis from glucose oxidation and continued H^+ accumulation (Liu *et al.*, 1996). Activation of AMPK during and following ischaemia is an important contributor to these high rates of both glycolysis and fatty acid oxidation.

Figure 2.21 **Energy metabolism in the ischaemic myocardium**
(Sambandam & Lopaschuk, 2003)



Due to a lack of oxygen supply, mitochondrial oxidation of fatty acids and glucose is restricted. However, anaerobic glycolysis persists to meet the energy demand. When pyruvate is not oxidised in the mitochondria, the hydrogen ion generated as a result of hydrolysis of glycolytic ATP accumulates in the cytosol. Accumulation of protons (H^+) then leads to Na^+ influx via Na^+/H^+ exchanger which then leads to Ca^{2+} accumulation via the Na^+/Ca^{2+} exchanger.

Proton accumulation during ischaemia and reperfusion can alter cardiac efficiency by a number of potential mechanisms. It can lead to intracellular Na^+ accumulation via Na^+/H^+ exchange and a subsequent intracellular Ca^{2+} accumulation via Na^+/Ca^{2+} exchange (Liu *et al.*, 1996). Sudden rises in intracellular Ca^{2+} could potentially cause cell death (Liu *et al.*, 1996). Proton accumulation can also result in a decreased efficiency of the contractile proteins, impairment of functional recovery during reperfusion and a reduction in cardiac efficiency (cardiac work/myocardial O_2 consumption). Liu *and* co-workers (1996) have also demonstrated that decreasing H^+ production (by improving uncoupling) and/or inhibiting Na^+/H^+ exchange, improves functional recovery as well as cardiac efficiency during reperfusion. Thus, altered metabolism during ischaemia/reperfusion is an important contributor to post-ischaemic functional impairment of the heart.

2.2.3.2 AMPK control of fatty acid metabolism during ischaemia/reperfusion

AMPK, described as a "cellular fuel gauge", shuts down the energy consuming processes and facilitates energy producing processes during various metabolic stresses (Hardie & Hawley, 2001). Ischaemic stress in the heart leads to an increase in AMPK activity (Kudo *et al.*, 1995, 1996), which modifies two important metabolic pathways during and following ischaemia, namely glycolysis and fatty acid oxidation (for the purpose of this study, the role of AMPK in fatty acid metabolism only will be discussed). AMPK also inhibits creatine kinase and discourages the reverse reaction in which Cr is converted to PCr, and thus makes more ATP available for other critical functions of the cell (Ponticos *et al.*, 1998).

AMPK belongs to a family of serine/threonine kinases which have highly conserved kinase domains (Lefebvre *et al.*, 2001). AMPK was originally identified

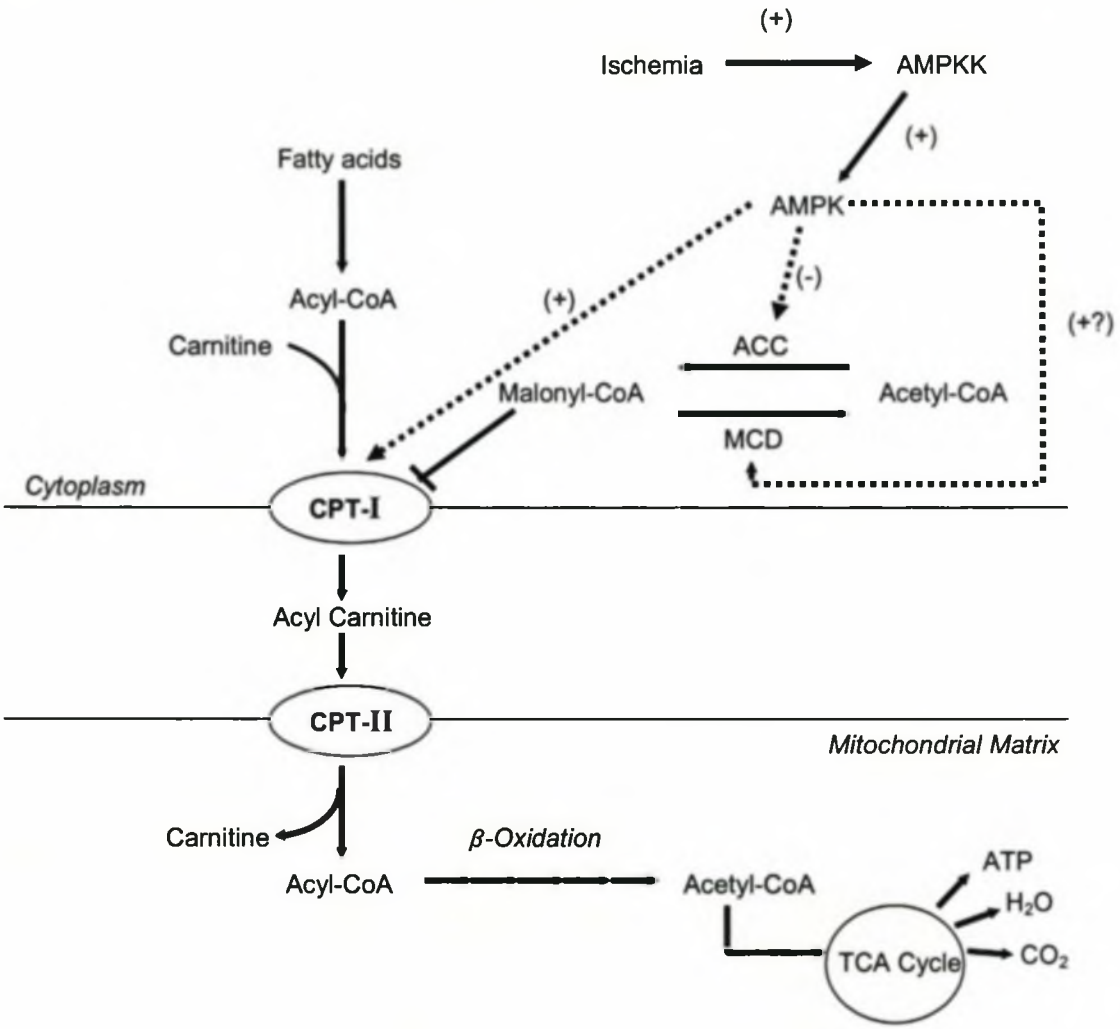
as a HMG-CoA reductase kinase and an acetyl CoA carboxylase kinase (Beg *et al.*, 1978; Keith *et al.*, 1979). However, when it became clear that this kinase phosphorylated a number of proteins and it was activated by 5'AMP, it was renamed as AMPK (Ferrer *et al.*, 1985; Carling *et al.*, 1987).

Under normal conditions, fatty acids are the preferred energy source of the myocardium contributing to 60-80% of the total ATP production (Belke & Lopaschuk, 1997). Although fatty acid oxidation decreases during ischaemia, it rapidly recovers during reperfusion contributing to 90-100% of the ATP production in the heart (Belke & Lopaschuk, 1997). This increased use of fatty acids to support ATP production is due both to an increase in circulating fatty acid levels, and intracellular alterations in the control of fatty acid oxidation, where AMPK appears to have an important role.

In clinical conditions like myocardial infarction, or during and after cardiac surgery, serum fatty acid concentration increases (Opie, 1975; Mueller & Ayres, 1987; Lopaschuk *et al.*, 1994; Fragaso *et al.*, 2002). As a result, cardiac fatty acid supply and oxidation could increase several fold. Indirect evidence suggests that heparin administration decreases the ischaemic threshold in coronary artery disease patients probably by increasing circulating fatty acid levels and thus fatty acid oxidation (Fragaso *et al.*, 2002). Inhibition of fatty acid oxidation protects the heart from detrimental effects of heparin (Fragaso *et al.*, 2002), implicating the detrimental effects of elevated fatty acid oxidation. Experimental evidence further support that over-reliance on fatty acid oxidation is harmful to the functional recovery of the heart during reperfusion following severe ischaemia (Lopaschuk *et al.*, 1988; 1992). Thus, it is important to understand the mechanisms responsible for alterations in fatty acid metabolism during ischaemia/reperfusion.

An important control site of myocardial fatty acid oxidation is at the mitochondrial membrane where fatty acids are transported across as acyl carnitine esters (fig 2.22). This process is facilitated by acyl carnitine translocase and two carnitine

Figure 2.22 **AMPK regulation of free fatty acid oxidation in the myocardium**
(Sambandam & Lopaschuk, 2003)



Activation of AMPK during ischaemia results in a decrease of malonyl-CoA levels either via decreased synthesis [by inhibition of ACC (acetyl-CoA carboxylase)] activity or via increasing degradation of malonyl-CoA [by activation of MCD (malonyl-CoA decarboxylase)]. Lowering of malonyl-CoA levels then removes allosteric inhibition on CPT-I, increase mitochondrial transport of fatty acids and therefore increases β-oxidation.

palmitoyl transferases (CPT-I and CPT-II) (as discussed earlier). Once inside the mitochondria, acyl-CoA undergoes β-oxidation in the matrix producing several acetyl-CoA molecules, which then enter the TCA cycle for complete oxidation. Transport of fatty acids by CPT-I across the mitochondria is the rate- limiting step in fatty acid oxidation (Kantor *et al.*, 2001). Activation of AMPK during ischaemia results in a decrease of malonyl-CoA levels either via decreased synthesis [by inhibition of ACC (acetyl-CoA carboxylase) activity] or via increasing degradation of malonyl-CoA [by activation of MCD (malonyl-CoA decarboxylase)]. Lowering of malonyl-CoA levels then removes allosteric inhibition on CPT-1, increases mitochondrial transport of fatty acids and therefore increases β-oxidation (fig 2.22).

Thus, AMPK could be a potential target for metabolic modulation during ischaemia and reperfusion. It is also proposed that activating AMPK in the heart may be beneficial since it reduces lipid accumulation and therefore the lipotoxicity (Minokoshi *et al.*, 2002). However, it is important to note that activation of AMPK in the heart may also increase fatty acid oxidation and glycolysis which may have deleterious effects especially under the conditions where plasma fatty acids are already elevated and where the heart utilizes exclusively fatty acids as its energy substrate (e.g. in diabetes and myocardial ischaemia).

2.2.4 Fatty acids and apoptosis in the heart

The harmful effects of saturated fatty acids have been reported in several cell types (Buttke, 1984; Zhang *et al.*, 1992; DeVries *et al.*, 1995) including neonatal rat ventricular myocytes (DeVries *et al.*, 1997). The saturated fatty acids palmitate (16:0) and stearate (18:0) have been demonstrated to induce apoptosis, whereas monounsaturated fatty acids, such as oleate (18:1), did not. Both palmitate and stearate are precursors in the *de novo* synthesis pathway of ceramide, a sphingolipid involved in apoptotic signalling.

The role of sphingolipid metabolites, particularly ceramide, in apoptosis signaling pathways is well established (Hannun, 1996; Pena *et al.*, 1997; Obeid & Hannun, 1997; Ariga *et al.*, 1998). A number of stimuli affect ceramide production, including TNF-α, γ-interferon, and ionizing radiation (Kim *et al.*, 1991; Dressier *et al.*, 1992; Haimovitz-Friedman *et al.*, 1994). In the heart, *in vitro* induced ischaemia, led to a significant increase in endogenous ceramide levels (Bielawska *et al.*, 1997). Sphingolipid metabolites have also been implicated in the triggering of apoptosis induced by ischaemia and reperfusion. For example in an ischaemia/reperfusion model, the addition of a cell-permeable ceramide analog, C_2-ceramide, induced apoptotic death of neonatal rat cardiomyocytes.

Cellular ceramide is produced by: (i) cleavage of sphingomyelin with sphingomyelinase, and (ii) *de novo* synthesis (Pena et al., 1997; Hannun & Obeid, 1995; Kolesnick & Fuks, 1995), which involves palmitoyl-CoA and stearyl-CoA, derived from palmitate and stearate, both of which induce apoptosis (de Vries *et al.*, 1997; Paumen *et al.*, 1997). It has been proposed that the accumulation of ceramide associated with ischaemia/reperfusion is caused by sphingomyelin hydrolysis as opposed to *de novo* synthesis, because cardiac ischaemia leads to a rapid loss in ATP from cardiac cells.

Long-chain saturated fatty acids also induce apoptosis in normoxic cells. Paumen and co-workers (1997) demonstrated that induction of apoptosis by palmitate or stearate was associated with *de novo* synthesis of the sphingolipid, ceramide. The same relationship was also observed by Sparagna and co-workers (1999) in cultured rat neonatal cardiomyocytes. Using this model system, they have shown that incubation of myocytes with extracellular palmitate leads to an increase in intracellular ceramide levels. Under the same conditions, incubation with physiologic levels of palmitate leads to an overall increase in fatty acid metabolites, increased oxidation and increased triglyceride synthesis (Sparagna *et al.*, 1999). The temporal sequence of these changes indicates that

the initiation of apoptosis in cardiac myocytes occur while the cells are actively metabolizing palmitate. Thus, one cannot rule out a role for *de novo* ceramide synthesis in cardiac apoptosis.

Inhbition of CPT-1 (the rate-limiting enzyme in the passage of long-chain fatty acids from the cytoplasm into the mitochondria for β-oxidation), which would prevent palmitoyl-CoA from being oxidised, results in an enhancement of palmitate-induced apoptosis and sphingolipid synthesis (Paumen *et al.*, 1997). Thus, the accumulation of palmitoyl-CoA, which occurs when palmitate is present in the cell media (de Vries *et al.*, 1997) or when CPT-1 is inhibited (Paumen *et al.*, 1997), directly influences the production of ceramide and may indeed be the rate-limiting step, leading to cardiac apoptosis.

A direct relationship between saturated fatty acids and other indicators of apoptosis was also found. Sparanga and co-workers (1999) reported that the palmitate-induced elevation of ceramide in neonatal rat cardiac myocytes is associated with a concurrent increase in caspase-3 activity. Mitochondria are intricately linked to both fatty acid oxidation and apoptosis. Not only are heart mitochondria responsible for cardiac fatty acid β-oxidation, which produces most of the ATP for the cell's energy needs, but they also contain CPT-1, which when inhibited, may cause palmitoyl-CoA accumulation and apoptosis (Paumen *et al.*, 1997). Key apoptotic proteins, such as Bcl-2 and cytochrome c are located in the mitochondria. Data from the laboratory of Sparanga (1999) indicated that cardiac myocyte mitochondria lose their membrane potential several hours after exposure to palmitate. During apoptosis, the mitochondria must be able to provide ATP for cleavage and activation of apoptotic proteins, but at a relatively early stage in the apoptotic process, some of them undergo a membrane potential decrease, which is probably associated with the onset of the mitochondrial permeability transition (MPT) (Zamzami *et al.*, 1995; Marchetti *et al.*, 1996). The mitochondrial permeability transition is an event during which large pores open in the mitochondrial inner membrane allowing ions and small

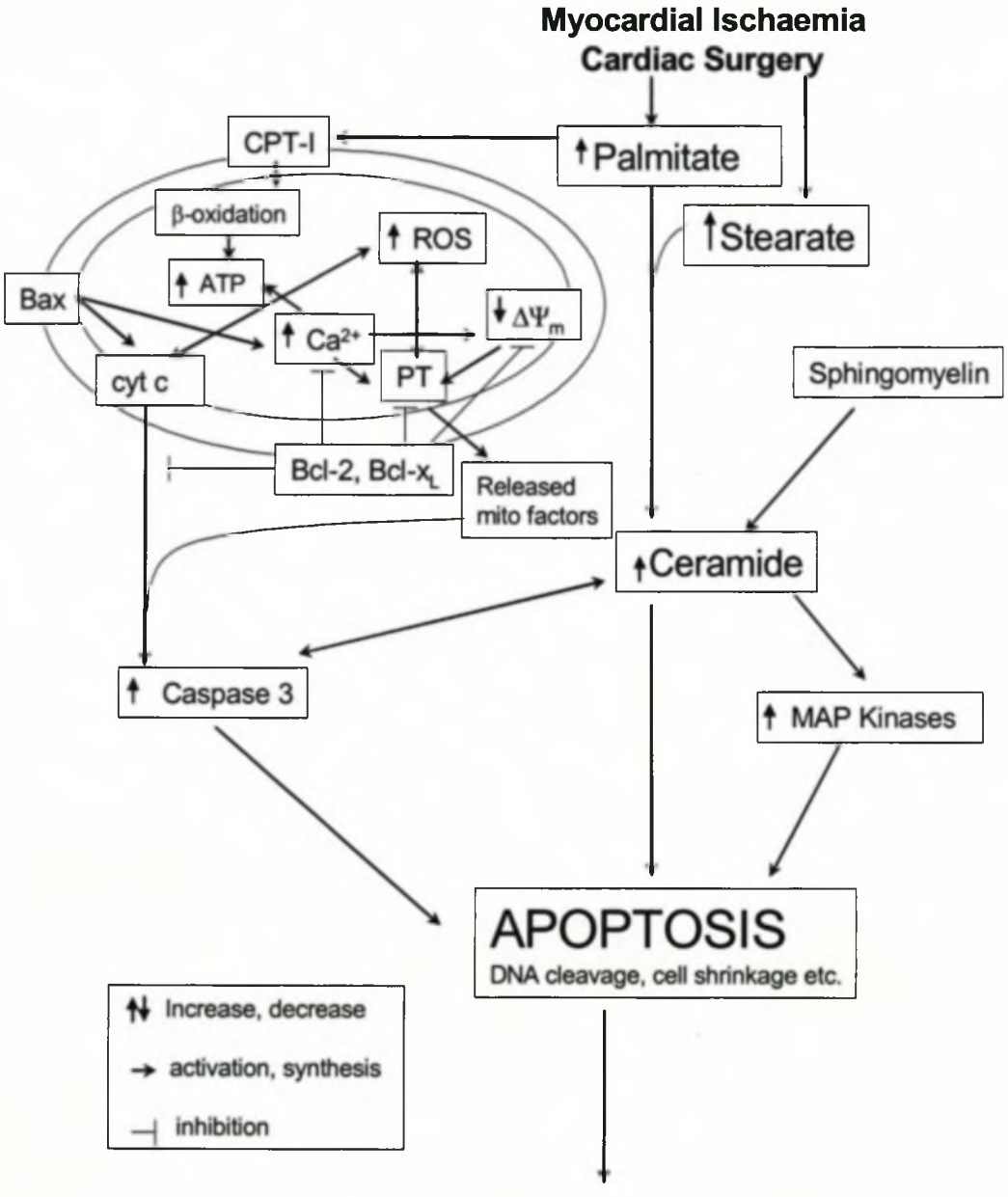
proteins with molecular masses <1500 Da to follow their concentration gradients across the mitochondrial inner membrane (Bernardi, 1996). After undergoing the permeability transition, mitochondria become unable to perform any energy functions linked to their electrochemical gradient, such as the production of ATP. The onset of mitochondrial permeability transition is induced by high levels of mitochondrial matrix free Ca^{2+} in addition to an inducing agent, which in the case of apoptosis, has been hypothesized to be reactive oxygen species (ROS) (Quillet-Mary *et al.*, 1997; Petit *et al.*, 1997) or ceramide (Decaudin *et al.*, 1997). However, Gracia-Ruiz and co-workers (1997) found that ceramide caused the production of ROS, but had no effect on the onset of the permeability transition.

Ischaemia affects mitochondria in several ways, for example, in isolated perfused rabbit hearts, the cytochrome c content is decreased in sub-sarcolemmal, but not interfibrillar mitochondria (Lesnefsky *et al.*, 1997). This could explain how the cell can continue to satisfy the energy requirements associated with apoptosis using one type of mitochondria after a sub-population of mitochondria has undergone permeability transition.

A direct interaction between CPT-I and Bcl-2 has also been suggested (Paumen *et al.*, 1997). This interaction may have some significance in the formation of ceramide, because the activation of CPT-I by Bcl-2 would cause an increase in the rate at which palmitoyl-CoA is oxidized and therefore a decrease in palmitoyl-CoA available to form ceramide. This mechanism would indirectly allow Bcl-2 to prevent apoptosis via control of ceramide concentrations (fig 2.23).

The relationship between fatty acids and the induction of apoptosis in heart tissue deserves further study. The induction of apoptosis by saturated fatty acids may partially explain the link between high levels of circulating fatty acids and heart disease. If fatty acids indeed play an important role *in vivo* in the induction of heart disease, it is important to elucidate the mechanisms of their effects on cells specific to the heart. Mitochondria may play a central role in the relationship

Figure 2.23 Fatty acid-induced apoptosis
(Sparagna & Hickson-Bick, 1999)



PT - mitochondrial permeability transition; $\Delta\Psi_m$ - mitochondrial membrane potential

between fatty acid metabolism and apoptosis in the heart, particularly during ischaemia/reperfusion. The presence of cytochrome c and Bcl-2 and the findings that these proteins interact with themselves as well as with CPT-I, place the mitochondria in the centre of the intricate process of fatty acid induced programmed cell death.

2.2.5 The role of fatty acids in signal transduction

Numerous studies on the molecular basis of fatty acid actions have demonstrated their effects on many steps in cellular signalling. Particular attention has been focused on fatty acid regulation of signals triggered at the level of cell membranes. It is now clear that fatty acids have characteristics generally attributed to modulators and messengers. Fatty acids are good candidates because they can be delivered to cells from extracellular sources and act directly on the cell membrane as first messengers or they are incorporated into membrane phospholipids from which they can be liberated by cellular phospholipases in response to other signals. Once liberated, free fatty acids can propagate the primary message by serving as second messengers or they can modulate signals coming from other pathways such as the binding of steroid hormones to their intracellular receptors.

Many of the enzymes involved in signal transduction are regulated positively or negatively by fatty acids as seen in table 2.1. These include enzymes of the cAMP signalling pathway and those involving PKC, where regulation has been studied in a wide variety of tissues and cells. Fatty acids can also participate in feedback control mechanisms since the phospholipases that are involved in the liberation of fatty acids from membrane phospholipids are themselves modulated by fatty acids. The regulatory action of fatty acids mainly concerns the unsaturated fatty acids rather than the saturated fatty acids. The concentrations of unsaturated fatty acids used in these studies ranged from as low as 1.4 μM to 400 μM. Although it is difficult to determine the local concentration of released

Table 2.1 **Modulatory effects of fatty acids on enzymes and proteins involved in membrane signal transduction (modified from Sumida *et al.*, 1993)**

	Tissues or cells	Fatty acid	References
↑ Phospholipase A_2	human platelets	20:4	Siess *et al.*, 1983
↑ Phospholipase C	human platelets	20:4	Siess *et al.*, 1983
	rat brain	18:1 / 20:4	Irvine *et al.*, 1979
	rat pancreas	20:4	Chaundry *et al.*, 1987
↑ Phospholipase D	various tissues	18:1	Shukla *et al.*, 1991
↑ Adenylate cyclase	adipocytes	18:1	Malgieri *et al.*, 1975
↑ Guanylate cyclase	bovine lung	20:4	Gerzer *et al.*, 1986
↑ G protein	frog heart	20:4	Scherer *et al.*, 1990
↓ PKA	rat brain	PUFAs	Speizer *et al.*, 1991
↑ PKC	human neutrophils	20:4	McPhail *et al.*, 1984
	rat cardiac myocytes	20:4	Mackay & Mochly-Rosen, 2001

18:1 – oleic acid

20:4 – arachidonic acid

PUFAs – polyunsaturated fatty acids

fatty acids at a precise moment in time, intracellular concentrations of 50-100 μM of ARA have been reported after pancreatic islet cell stimulation (Wolf *et al.*, 1986) and in activated platelets (Nishikawa *et al.*, 1988).

In most studies summarised in Table 2.1, fatty acids and not their metabolites, have been shown to be the active regulators, for example GTP binding protein activation in human neutrophils is stimulated by unsaturated fatty acids per se, not by their metabolites, and this activity is correlated with respiratory burst (Abramson *et al.*, 1991). However, some studies have been included which involve metabolites, for example endoperoxides or thromboxane A_2 is probably responsible for the activation of phospholipase C in human platelets (Siess *et al.*, 1983). Metabolites may have opposing activities such as the lipoxygenase metabolites, which stimulate and cyclooxygenase metabolites, which inhibit G-protein in frog heart (Scherer *et al.*, 1990). However, while ARA itself activates guanylate cyclase activity in rat brain, it is inhibited by its hydroperoxide derivatives (Louis *et al.*, 1986).

Fatty acids themselves regulate ion fluxes and Ca^{2+} mobilization directly, much in the same way as they regulate the action of many enzymes (table 2.2). ARA induces Ca^{2+} release from intracellular stores in a manner similar to but independent of inositol 1,4,5-trisphosphate, which is produced via phospholipase C (Chow & Jondal, 1990). When Jurkat cells are exposed to unsaturated fatty acids, they block Ca^{2+} influx through the Ca^{2+} channels (Chow *et al.*, 1990) but activate K^+ channels (Ordway *et al.*, 1991). They appear to interact directly, either with the channel proteins themselves or with some other component of the membrane. These actions of fatty acids have been shown to be due to fatty acids *per se*.

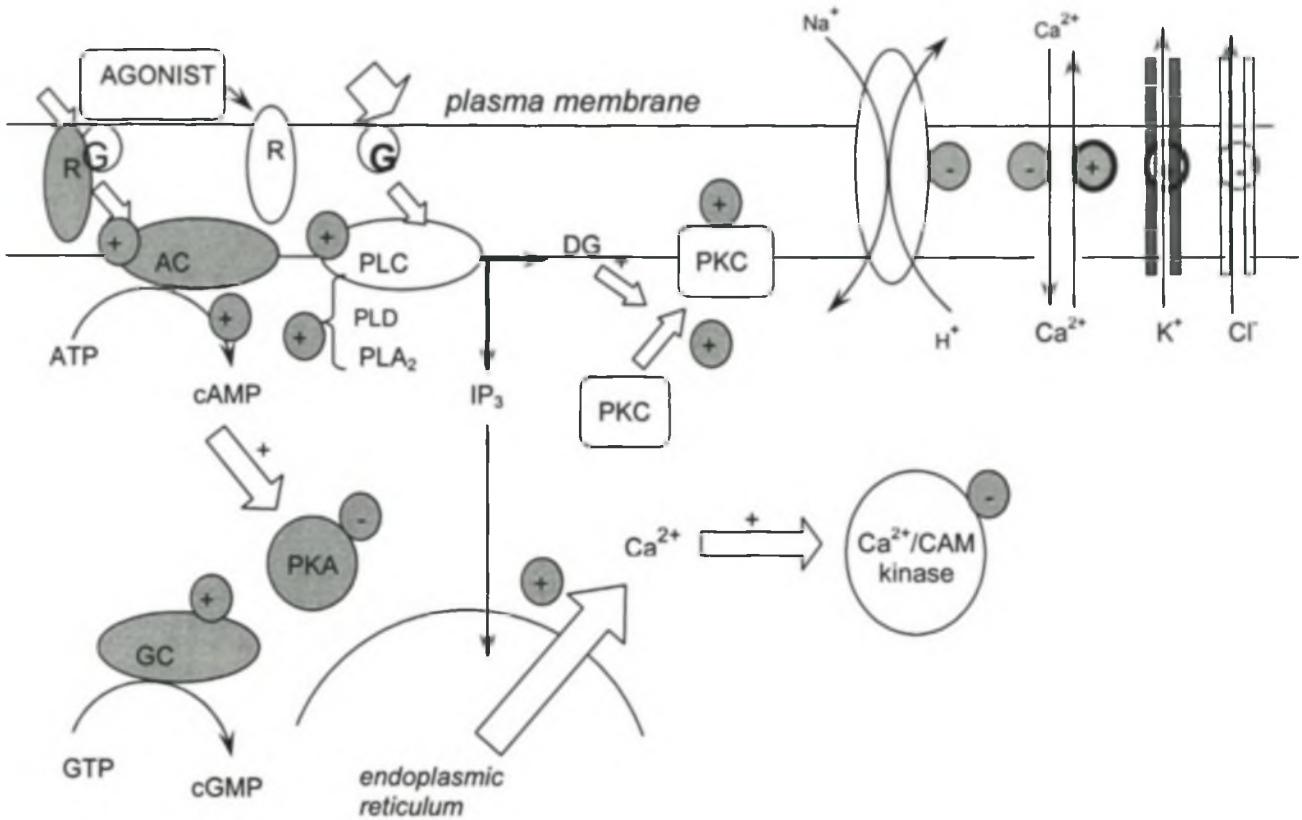
Figure 2.24 summarizes in schematic form various steps in signal transduction where fatty acids have been shown to regulate enzyme and protein activities

Table 2.2 **Effects of fatty acids on ion fluxes and mobilization involved in membrane signal transduction (modified from Sumida *et al.*, 1993)**

	Tissues or cells	Fatty acid	References
Ion channels			
↓ Ca^{2+} channels	Jurkat cells	UFAs	Chow *et al.*, 1990
↑ K^+ channels	muscle	20:4	Ordway *et al.*, 1991
↓ Cl^- channels	fetal trachea	UFAs	Hwang *et al.*, 1990
↑ Na^+ channels	cardiomyocytes	EPA/DHA	Kang & Leaf, 1994
↑ Intracellular Ca^{2+} mobilization	Jurkat cells	PUFAs	Chow *et al.*, 1990
	lymphocytes	20:4	Corado *et al.*, 1990
↑ Ca^{2+} pump	red blood cells	PUFAs	Sarkadi *et al.*, 1982
↑ Na-K-ATPase	rat brain	18:1	Oishi *et al.*, 1990
↑ Na^+/H^+ exchange	cardiac sarcolemma	EPA/DHA	Goel *et al.*, 2002

UFA – unsaturated fatty acid
20:4 – arachidonic acid
PUFA – polyunsaturated fatty acid
18:1 – oleic acid
EPA – eicosapentaenoic acid
DHA – docosahexaenoic acid

Figure 2.24 Role of fatty acids in signal transduction



Fatty acids stimulate (+) or inhibit (-) the activities of enzymes and ion fluxes.
R- receptor; G – G-protein; AC – adenylate cyclase; GC – guanylate cyclase; PKA – protein kinase A; PKC – protein kinase C; PLC – phospholipase C; PLA_2 – phospholipase A_2; PLD – phospholipase D; IP_3 – inositol 1,4,5-trisphosphate; DG – *sn* 1,2-diacylglycerol; Ca^{2+}/CAM kinase – protein kinase II (Ca^{2+} /calmodulin-dependent) (Sumida *et al.*, 1993)

either positively or negatively. In most cases, evidence has been presented for the modulatory action of fatty acids themselves rather than their metabolites.

The activation of phospholipases is often associated with agonist binding to membrane receptors, resulting in the liberation of ARA from the 2-acyl position of the membrane phospholipids. The activation of PKC by ARA is a good example of the role of fatty acids as second messengers in signal transduction. Since the original observation in 1984 by McPhail and co-workers, showing the activation of PKC by ARA and other unsaturated fatty acids, an effect which is not inhibited by cyclooxygenase or lipoxygenase inhibitors, many studies have shown the importance of unsaturated fatty acids in the mechanism of action of PKC (Speizer *et al.*, 1991; Lester *et al.*, 1991; Khan *et al.*, 1992; Mackay & Mochly-Rosen, 2001).

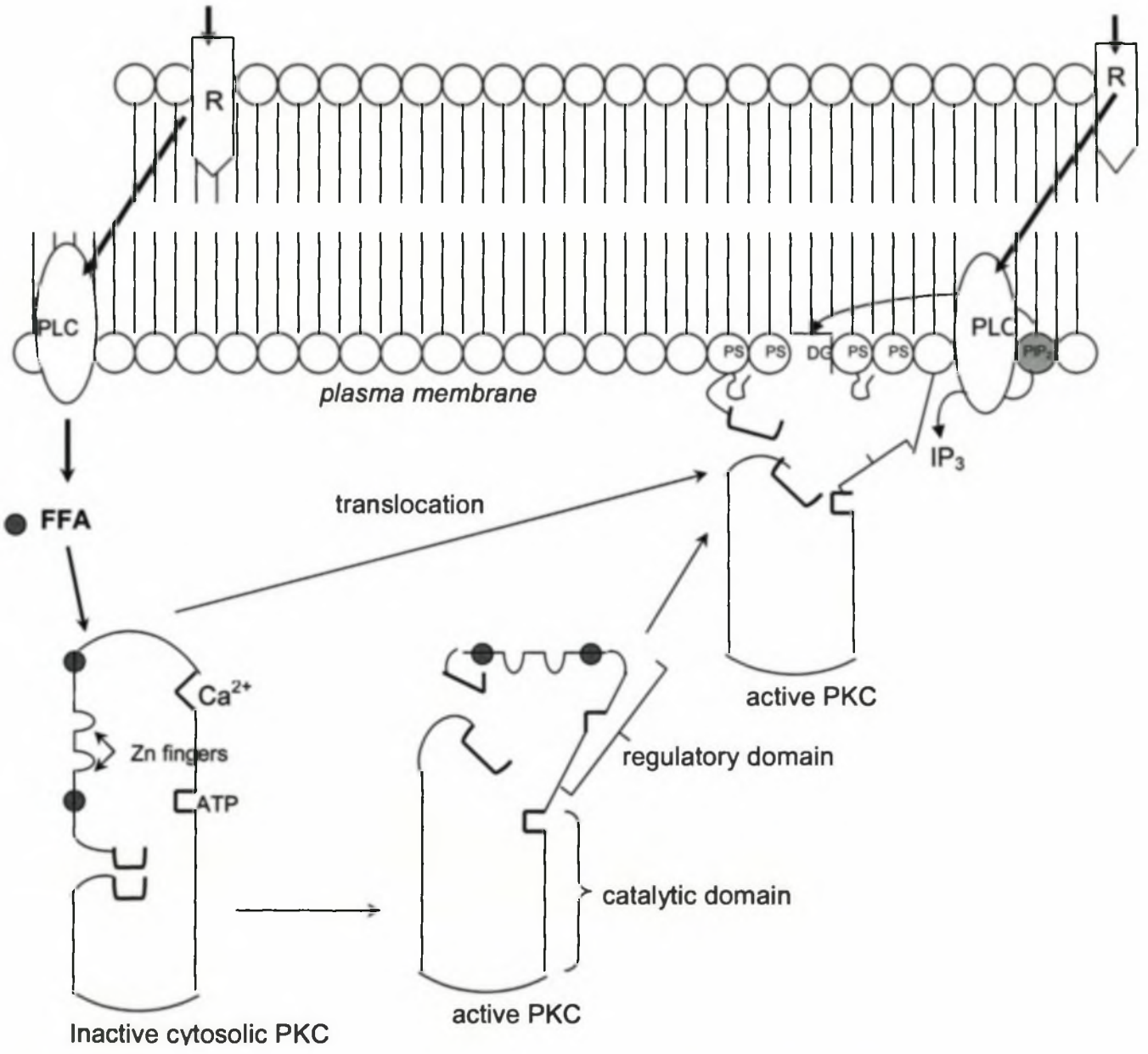
PKC is an ubiquitous, proteolitically activated protein kinase, which usually requires Ca^{2+} and phosphatidylserine (PS) for its activation (Nishizuka, 1986; Kikkawa *et al.*, 1989). Subsequent research has defined a family of at least 10 PKC isozymes related through the carboxy-terminal catalytic domain. These are divided into three subfamilies based on homology in the regulatory domain, which determines the activators required. The classical PKC (cPKC) isozymes (α, βi, βII and γ) are activated by the second messengers Ca^{2+} and diacylglycerol (DAG) in the presence of PS. The novel PKCs (nPKC), δ, ε, η and θPKC, also require PS and DAG for full activation, but are insensitive to Ca^{2+}. Both the cPKCs and the nPKCs are targets for the tumour-promoting phorbol ester PMA, which has been used extensively to study PKC function. The third subfamily, the atypical (aPKCs), λ and ζ, are not activated by PMA, DAG or Ca^{2+} and their regulation is more complex.

The number and levels of PKC isozyme expression vary in different tissues, with developmental stage of the animal studied, and possibly with the species used. This differential localization and sensitivity of isoforms of PKC create a situation

where fatty acids can act as second messengers in synergy with or independent of DAG and Ca^{2+}. Fatty acids activate preferentially α, β and γ isozymes of PKC (El Touny *et al.*, 1990; Khan *et al.*, 1991; 1992). Whether this activation requires Ca^{2+}, DAG or PS depends on the experimental conditions, the specific cell type and the specific isozyme studied. For example, γPKC is a soluble isozyme found in the central nervous system, which is activated by very low (10 μM) concentrations of ARA, independent of Ca^{2+}, PS and DAG (Naor *et al.*, 1988). A direct effect of unsaturated fatty acids on PKC in the cytosol of human platelets under cell-free conditions without DAG or Ca^{2+} generation has also been shown (Khan *et al.*, 1991). Furthermore, interaction of unsaturated fatty acids with cytosolic PKC can also induce its translocation to the membrane where it can then be activated by DAG (Diaz-Guerra *et al.*, 1991). Fatty acids may act in synergy with DAG but do not compete with phorbol esters for the same binding site (El Touny *et al.*, 1990). Indirect evidence points to the interation of fatty acids at the PS regulatory sites (El Touny *et al.*, 1990; Speizer *et al.*, 1991). ARA induces phosphorylation of only a subset of the phosphoproteins which were shown to be produced by phorbol ester activation of PKC in the cytosol of human platelets (Khan *et al.*, 1991). Therefore, PKC isozymes show specific endogenous substrate preferences under physiological conditions in response to ARA and DAG.

Activation of PKC by a fatty acid is presented in Figure 2.25. This activation of soluble PKC by a free unsaturated fatty acid would increase the range of potential protein substrates for PKC phosphorylation. Fatty acid activation might lead to a specific, fatty acid-induced protein phosphorylation, mediating a specific fatty acid response while the activation of membrane-associated PKC would produce other phosphoproteins and responses. Fatty acids and DAG might also act in synergy to sustain PKC activity even when DAG and Ca^{2+} concentrations drop, thereby providing a mechanism of compensation to maintain PKC activity.

Figure 2.25 PKC activation by free fatty acids



Fatty acids increase the activity of PKC and can also induce its translocation.
R – receptor; PL – phospholipases; FFA – free fatty acid; DG – diacylglycerol; PS – phosphatidylserine; PLC – phospholipase C; IP_3 – inositol 1,4,5-trisphosphate; PIP_2 – phosphatidylinositol 4,5-bisphosphate (Sumida *et al.*, 1993)

More and more evidence is accumulating on the molecular mechanisms of the actions of free fatty acids as modulators and/or messengers in signal transduction cascades. These studies clearly show the importance of fatty acids in regulating the transmission of signals from the extracellular environment, via the complex intracellular network of relays, to the nucleus and ultimately modulating the biological response.

2.3 Prevention of coronary heart disease by n-3 long-chain polyunsaturated fatty acids: review of the evidence

Current interest in a possible role of the n-3 long-chain polyunsaturated fatty acids (PUFAs) dates back almost three decades to the epidemiologic studies of Bang and Dyerberg (1976) on the Greenland Inuits. They noted a low mortality rate from coronary heart disease among the Eskimos despite a dietary fat intake comparable to that of the Danes and the Americans. This benefit has been attributed to the relatively high content of two n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the diet of the Inuits.

It was almost ten years after the pioneering work of Bang & Dyerberg (1976), before McLennan and co-workers (1985, 1988, 1993) reported that diets high in saturated fats or olive oil were associated with a high incidence of ventricular fibrillation in rats when their coronary arteries were ligated. A vegetable fat, safflower oil, reduced the incidence of ischaemia-induced ventricular arrhythmias by about 70%, whereas tuna fish oil (n-3 fatty acids) essentially prevented the arrhythmias and death after the experimental infarctions with or without reflow. They also confirmed their findings in marmosets (McLennan *et al.*, 1992).

Billman and co-workers (1994) confirmed the antiarrhythmic effects of n-3 fatty acids in feeding studies in rats by demonstrating prevention of ischaemia-induced 'sudden death' in the conscious, unanaesthetized whole animal. They studied a dog model susceptible to ischaemia-induced ventricular arrhythmias: myocardial infarction was produced by permanent ligature of the left anterior descending coronary artery and a hydraulic cuff placed around the left circumflex artery. After recovery from the surgery, dogs were trained to run on a treadmill to stimulate sympathetic nervous system activity. When a pre-selected exercise level or pulse rate was attained, compression of the left circumflex coronary artery was invariably followed within 2 min by sustained ventricular tachycardia or fibrillation in susceptible animals.

In order to determine unequivocally that the antiarrhythmic effects were caused by the fish oil PUFAs, they administered an intravenous emulsion of a concentrated fish oil preparation (2-5g of 70% n-3 PUFAs, of which 34% was EPA and 25% was DHA, both as their free acids) for 40-60 min just prior to compression of the coronary artery. This infusion prevented the expected fatal ventricular fibrillation. The identical protocol was repeated except that an emulsion of soybean oil rather than fish oil was infused prior to the ischaemic insult: no protection from the ventricular arrhythmias occurred. Eleven out of fourteen dogs tested were protected, whereas no protection was obtained in five out of five dogs infused with the soybean emulsion ($p<0.006$). They have also found in preliminary studies, that both EPA and DHA administered separately as the pure free fatty acids were protective in the canine model of sudden cardiac death. These acute pharmacological interventions complemented the feeding studies in rats (McLennan *et al.*, 1985; 1988; Hock *et al.*, 1990).

To study the mechanisms of this antiarrhythmic effect, Kang & Leaf (1994) have utilized the isolated neonatal rat cardiac myocyte preparation (Thandroyen *et al.*, 1991), which has proven to be a very useful preparation in this regard. The spontaneous, synchronized, rhythmic contractions of syncytia of 10 or more electrically coupled myocytes and the amplitude and rate of contractions can be recorded and measured (Barry *et al.*, 1985). Kang & Leaf (1994) have studied the effects of several agents known to cause fatal ventricular tachyarrhythmias in humans; e.g. high concentration of calcium (5 and 7 μM) or the cardiac glycoside ouabain (0.1mM), added to the fluid perfusing the myocytes. At micromolar concentrations, EPA and DHA will slow the spontaneous beating rate of the myocytes and prevent the arrhythmogenic effects of calcium or ouabain. If added after the arrhythmia is induced, the PUFAs will promptly terminate the arrhythmia. When delipidated bovine serum albumin (BSA, 29 μM, 2mg ml^{-1}) was added to the perfusion without the EPA or DHA, but with continued

arrhythmogenic agents in the medium, the arrhythmia recurred. These simple experiments led to the following important conclusions:

- The free fatty acids of EPA and DHA are antiarrhythmic and act promptly.
- The esterified fatty acids (e.g. the ethyl ester of the triglyceride) are ineffective in preventing the arrhythmias.
- The fact that the arrhythmias recur upon addition of delipidated bovine serum albumin to the perfusion fluid indicates that the active antiarrhythmic form of EPA and DHA is their free acid form.
- Incorporation of these fatty acids into membrane phospholipids is not required for the antiarrhythmic effects of PUFAs.

Albumin has three high-affinity binding sites for fatty acids, and another three or four lower affinity binding sites (Cistola *et al.*, 1987). When delipidated albumin is used, its high affinity binding capability will extract the free fatty acids which are partitioned into the phospholipids of the myocyte membranes, and thus, remove the protective effect of the fatty acids during arrhythmias. Normally, albumin molecules in our serum are loaded with bound fatty acids, which they deliver to the cells of our body for metabolism. Perfusing with the fatty acid-avid, delipidated albumin can reverse the direction of the traffic and extract free fatty acids from the myocytes. Furthermore, Kang & Leaf (1994) have also found that by addition of antioxidants and oxygenase inhibitors, that it is not active oxidised metabolites of EPA or DHA that account for their antiarrhythmic properties. Thus, the findings that addition of free fatty acids can abort the arrhythmias and that the arrhythmias recur when the fatty acids are extracted from the myocyte make it virtually certain that their antiarrhythmic action is a function of the presence of the free acid *per se*.

The current hypothesis regarding the mechanism of action of the n-3 PUFAs to prevent fatal arrhythmias is based on their actions to inhibit the fast, voltage-dependent sodium current (Xiao *et al.*, 1998; 2000) and the L-type calcium currents (Xiao *et al.*, 1997). With a myocardial infarction (MI), a gradient of

depolarization of cardiomyocytes occurs. In the central core of the ischaemic zone, cells rapidly depolarize and die. The depolarization results from the reduction of ATP in the ischaemic cells, which causes a dysfunctional Na^+/K^+-ATPase and the rise of interstitial K^+ concentrations in the ischaemic zone. However, at the periphery of the ischaemic zone, myocytes may be only partially depolarized. They become hyperexcitable because their resting membrane has become more positive, approaching the threshold for generating action potentials (activating fast Na^+ channels). Thus, any additional small depolarizing stimulus may elicit an action potential, which, if it occurs at a vulnerable moment during the cardiac electrical cycle, may initiate an arrhythmia. With nonhomogeneous rates of conduction pathways in the ischaemic tissue, re-entry arrhythmias are likely. In the presence of the n-3 PUFAs, however, a voltage-dependent shift of the steady state inactivation curve to more hyperpolarized potentials occurs. The consequence of this hyperpolarizing shift is that sodium channel availability is decreased, and the potential necessary to return these Na^+ channels in partially depolarized myocytes to a closed but activatable state is physiologically unobtainable. Also, these partially depolarized cells have Na^+ channels, which in milliseconds can slip into "resting inactivation" in response to subthreshold depolarizations without eliciting an action potential (Lawrence *et al.*, 1991; Goldman, 1995) and they do this even faster in the presence of the fish oil fatty acids (Xiao *et al.*, 1995; 1998). Myocytes with normal membrane potentials in the nonischaemic myocardium will not be so drastically affected by the PUFAs and will continue to function normally. Thus, the effect of the n-3 PUFAs on Na^+ channels, inhibition the L-type Ca^{2+} channels (Xiao *et al.*, 2000) and their ability to prevent triggered arrhythmic afterpotential discharges, caused by excessive cytosolic Ca^{2+} fluctuations are the major mechanisms for the antiarrhythmic effects of these PUFAs.

Another mechanism for the antiarrhythmic actions of n-3 PUFAs was proposed by Nair and co-workers (2001). They used a model of isolated cardiac myocytes from adult pig hearts to investigate the effect of ARA, EPA and DHA on inositol

phosphate metabolism and PKC activity. Their results showed that both EPA and DHA were selectively incorporated into the phosphatidylinositol fraction. To study the effect of membrane phospholipid modification on the PLC-mediated inositol lipid cycle, cardiac myocytes were labeled with 4μCi/ml myo-[2-^{3}H]Ins for 48 h and stimulated with epinephrine and phenylephrine. After stimulation, the levels of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 as well as PKC activity in EPA and DHA supplemented myocytes were significantly reduced ($p<0.05$) compared to ARA-supplemented myocytes. From these experiments, it is evident that n-3 PUFA supplementation modulates intracellular signalling also suggesting a possible antiarrhythmic mechanism.

Apart from the antiarrhythmic effects of n-3 PUFAs, Grynberg and co-workers (1995) studied the effect of DHA and EPA incorporation in the phospholipids of rat heart muscle cells on adrenoceptor responsiveness and mechanism. Stimulation of the β-adrenergic receptors (isoproterenol 10^{-7}M) resulted in a positive chronotropic effect, which was significantly higher in the DHA-rich cells. However, this was associated with a decrease in the affinity of the β-receptors for the ligand (dihydroalprenolol) without alteration of the number of β-receptor binding sites and provoked a significant decrease in isoproterenol-stimulated cAMP production (-19%). To further investigate these controversial data, the cardiomyocytes were treated with dibutyryl-cAMP, which elicited a positive chronotropic response significantly higher in the DHA-rich cells. The α-adrenergic stimulation by phenylephrine (3×10^{-6}M) increased the spontaneous rate, but in a similar manner in the DHA- and EPA-enriched cells. Similarly, neither the α-adrenergic receptor binding characteristics nor the production of phosphoinositides was modulated by the membrane DHA content, although the phosphatidylinositol PUFAs were significantly altered. They concluded that increasing the DHA content in the membrane phospholipids had no affect on the α-adrenergic system, but exerted a specific positive influence on the β-adrenergic transduction mechanism, in a manner as yet unknown.

The question now often asked is how do these *in vitro* and animal studies relate to ischaemia-induced lethal ventricular arrhythmias in humans? At present, there have been only a few clinical trials which inadvertently seem relevant to this question. The first study was published in 1989 (Burr *et al.*), before the anti-arrhythmic effects of the n-3 fatty acids were generally appreciated, and thus, no record was kept of the incidence of sudden cardiac death. Nevertheless, in this multifactorial trial, The Death and Reinfarction Trail (DART), 1015 men who have had a recent myocardial infarction were randomly advised to eat fatty fish at least twice per week, and if they could not eat fish, they were given a modest supplement of fish oil. Another matched 1018 men were simply not given the advice to eat fish. At the end of only two years, there was a 29% reduction in mortality in the 1015 men who had received advice to eat oily fish, compared with the 1018 men who had not received such advice.

In 1994, the results of the Mediterranean α-linolenic acid-rich diet in secondary prevention of coronary heart disease were reported (de Logeril *et al.*). In this clinical trial, 302 patients who had just suffered a myocardial infarction were prospectively randomised to the experimental group and another 303 were randomised to be controls. The experimental group reduced their intake of saturated fat, cholesterol and linoleic acid, while increasing that of oleic and α-linolenic acid. After one year, higher concentrations of oleic- α-linolenic acid and eicospentaenoic acid, and reduced concentrations of stearic- linoleic- and arachidonic acid were observed in the plasma lipids of the experimental group. This study was planned to have a five-year follow-up, but the results were so beneficial that the study was terminated earlier for ethical reasons. With a mean follow-up period of 27 months, there was a 70% reduction in all causes of death because of a reduction in cardiovascular deaths (76%). Interestingly, in the experimental group, receiving the α-linolenic acid (18:3n-3), sudden deaths were prevented. It should also be pointed out that these remarkable effects were achieved with no differences in the serum cholesterol levels during the study, or between the experimental and control cohorts.

Clinicians also need to know that dietary n-3 PUFAs (and low-dose n-3 PUFA supplements) may reduce the risk of sudden cardiac death over a period of weeks to months. In a population-based case-control study (Siscovick *et al.*, 1995), recent dietary intake and cell membrane levels of n-3 PUFAs at the time of the event were associated with a lower risk of sudden cardiac death. In the GISSI Prevenzione trial, low-dose n-3 PUFA supplements reduced the risk of sudden cardiac death among post-myocardial infarction patients within four months of initiating treatment (Marchioli *et al.*, 2002).

These n-3 PUFAs have always been an essential component of human diets, only having become reduced in our diets in recent times (Leaf & Weber, 1987). It seems that an important basic level of control of cardiac function by simple dietary choices of the essential PUFAs ingested has been largely overlooked. Much further work, especially carefully planned basic research to further explore the mechanisms as well as human clinical trials, is needed to test whether these natural constituents of a balanced diet will fulfil the need of a safe and effective therapeutic agent.

CHAPTER 3 MATERIALS AND METHODS

3.1 Antibodies and chemicals

Antibodies were purchased from Cell Signalling Technology, collagenase from Worthington Biochemical Corporation, DMEM from Gibco Laboratories. PD98059 (2'-Amino-3'-methoxyflavone), SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole] and Hoechst 33342 from Calbiochem; SP600125 (Anthra[1-9-cd]pyrazol-6(2H)-one) from Tocris, UK; All other chemicals were obtained from Sigma (St. Louis, MO).

3.2 Experimental Animals

Adult male (250-350 g) or 1-2 day old neonatal Wistar rats were used as experimental animals. Rats were allowed free access to food and water. Rats were anaesthetized by injecting sodium pentobarbital (30 mg/adult rat and 3 mg /neonatal rat) intraperitoneally. The animal procedures in this study conformed with the principles outlined in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 85-23, revised 1996. This investigation was also approved by the Ethics committee of the University of Stellenbosch (Faculty of Health Sciences).

3.3 Cell culture preparation

Neonatal cardiac myocytes were isolated and cultured by a modification of the method described by Pinson (1990). Myocytes were dissociated from the ventricles of 1-2 day old Wistar rat hearts using 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in a buffer containing (in mM): NaCl 120, HEPES 19, Na_2HPO_4 1, glucose 6, KCl 5, $MgSO_4$ 0.3 (pH 7.4). The myocytes were purified by passage through a Percoll gradient after digestion. After washing, cells were plated onto

tissue culture dishes precoated with fibronectin at a density of $0.85x10^6$ cells/mm^2. After 48-72 h, the myocyte cultures were confluent and beating spontaneously. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (100U/ml penicillin and 100 μg/ml streptomycin) in a humidified incubator. Confluent, spontaneously contracting myocytes were used for experiments at day 3 or 4 after isolation.

3.4 Simulated ischaemia (SI) and reperfusion

Neonatal ventricular cardiac myocytes were exposed to a balanced salt solution, (composition in mM: NaCl 115, KCl 5, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 2 and HEPES 25) containing 5 mM potassium cyanide (KCN) and 20 mM 2-deoxy-*D*-glucose, for 60 minutes in a 5% CO_2 incubator (37^0C). Cyanide treatment was terminated by rinsing twice with phosphate buffered saline (PBS) [composition in g/l: NaCl 8, KCl 0.2, Na_2HPO_4 1.15, KH_2PO_4 0.2]. The medium was then replaced by maintenance medium (Dulbecco's modified Eagles medium with 100 U/ml penicillin and 100 μg/ml streptomycin) for 30 minutes during the reperfusion period.

3.5 Evaluation of myocyte loss during simulated ischaemia (SI) and reperfusion (R)

To determine whether a gradual loss of myocytes into the supernatant occurred during the SI and reperfusion periods, the incubation medium was collected after 60 minutes of SI as well as after 30 minutes reperfusion and centrifuged. The cells in the respective pellets were suspended in a small volume of buffer, stained by trypan blue (4% in PBS) and counted in a haemocytometer.

3.6 High energy phosphate (HEP) measurement

HEP (adenosine triphosphate, ATP and creatine phosphate, CrP) were measured at indicated time points. Cardiac myocytes (~0.85 x 10^6 cells/mm^2) were treated with 6% ice-cold perchloric acid and centrifuged at 8000 *g* for 5 min at 4°C. The supernatant was neutralized with KOH and filtrated through a 0.45μm (Millipore) filter. Analysis of HEP was done by a reversed phase high-pressure liquid chromatography (HPLC) technique developed by Victor and co-workers (1987). The protein content of the samples was determined in the acid precipitate by the method of Lowry (1951), using bovine serum albumin (BSA) as a standard.

3.7 Preparation of BSA/fatty acid complexes

Fatty acids were prepared according to the method of de Vries and co-workers (1997). Briefly, arachidonic acid or eicosapentaenoic acid was dissolved in 4 ml ethanol to yield a final concentration of 18.75 mM. An equal volume of 10 mM Na_2CO_3 was added. Ethanol was evaporated at 50-60°C under continuous N_2-flow and the fatty acid containing mixture was added dropwise to 10 ml of 10% bovine serum albumin (BSA) in PBS at 40°C. The BSA/fatty acid complexes were subsequently dialyzed four times at 4°C for 4-6 h against 250 ml 0.1 M $[NH_4]HCO_3$, and lyophilized. BSA and BSA/fatty acid complexes were dissolved in DMEM with antibiotics (100U/ml penicillin and 100 μg/ml streptomycin) to yield a final BSA concentration of 1%. After filter sterilization, the media were stored at 4°C until use. The fatty acid/BSA ratio in the medium amounted to 3.3 to 1. For control experiments BSA was prepared similar to the procedure as described above, but in the absence of added fatty acids.

3.8 Experimental protocol for cells treated with fatty acids

DMEM containing either 1% BSA (in control cardiomyocytes) or fatty acids (final concentrations of 20 μM) complexed to BSA was added to the cells 30 minutes before the onset of SI. Following treatment, the medium was removed and replaced with a KCN containing balanced salt solution (composition in mM: NaCl 115, KCl 5, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 2 and HEPES 25) containing 5 mM potassium cyanide (KCN) and 20 mM 2-deoxy-*D*-glucose, for 60 minutes in a 5% CO_2 incubator (37^0C). Cyanide treatment was terminated by rinsing twice with phosphate buffered saline (PBS). During reperfusion, the medium was replaced by maintenance medium (DMEM with 100 U/ml penicillin and 100 μg/ml streptomycin), containing either 1% BSA or fatty acids complexed to BSA, for 30 minutes. (Preliminary studies showed that optimal protection of the myocytes was achieved when fatty acids were present before SI as well as during reperfusion).

3.9 Treatment of cells with SB203580, PD98059, SP600125 and wortmannin

PD98059 (10 μM), a selective inhibitor of MEK1/MEK2, the upstream activator of ERK1/ERK2 (Dudley *et al.*, 1995) or SB203580 (1 μM), an inhibitor of p38-MAPK (Clerk & Sugden, 1998), or SP600125 (10 μM), a JNK inhibitor (Vaishnav *et al.*, 2003), or wortmannin (100 nM), a PI-3-kinase inhibitor (Versteeg *et al.*, 2000) was added to the cells 30 minutes before the onset of SI. Following treatment, the medium was removed and replaced with the KCN containing balanced salt solution (as described above). Inhibitors were present for 30 minutes before SI as well as during the reperfusion period. For controls, cardiomyocytes were incubated with maintenance medium alone containing the appropriate inhibition solvent.

3.10 Treatment of cells with indomethacin, chelerythrine and phosphatase inhibitors

Indomethacin (10 μM), a cyciooxygenase inhibitor, chelerythrine (10 μM), an inhibitor of PKC catalytic activity, okadaic acid (1 μM), an inhibitor of types 1 and 2A serine/threonine phosphatases or orthovanadate (100 μM), a specific inhibitor of tyrosine phosphatases was added to the cells 30 minutes prior to and during fatty acid treatment. Inhibitors were also present during the 30 minutes of reperfusion.

3.11 Analysis of membrane fatty acid composition

To examine the incorporation of supplemented fatty acids into the myocardial phospholipid fraction after a 60 minute incubation period, the cardiomyocytes were rinsed with cold buffer, scraped off and extracted with a 2:1 (vol/vol) mixture of chloroform and methanol which contained 0.01% butylated hydroxytoluene to prevent auto-oxidation of unsaturated fatty acids. The lipid extracts were washed with 0.15 M KCl according to the method of Folch *et al* (1957), and the remaining organic solvents vaporized under nitrogen. Phospholipids were isolated from other lipid classes by thin layer chromatography (Silica gel G plates, 10 x 20 cm; Analtech Inc., Mewark, DE) in hexane:diethyl-ether:acetic acid (80:20:1, v:v:v) (Zail & Pickering, 1979). The band containing phospholipids was removed completely from the plate and transmethylated with boron trifluoride-methanol to yield fatty acid methyl esters (Morrison & Smith, 1964). Individual fatty acid methyl esters were separated on a Varian 3300 gas chromatograph with an SP-2560 capillary column (100 m Supleco Inc., Belfonte, PA). The instrument was programmed for column, injector, and detector temperatures of 175, 225 and 275^{o}C, respectively. Helium was used as a carrier gas at a flow rate of 21 cm per second. Individual peaks were integrated with a programmable Varian 4290

recorder/integrator. The peaks were identified by comparison with authentic standards.

3.12 Determination of cardiomyocyte viability

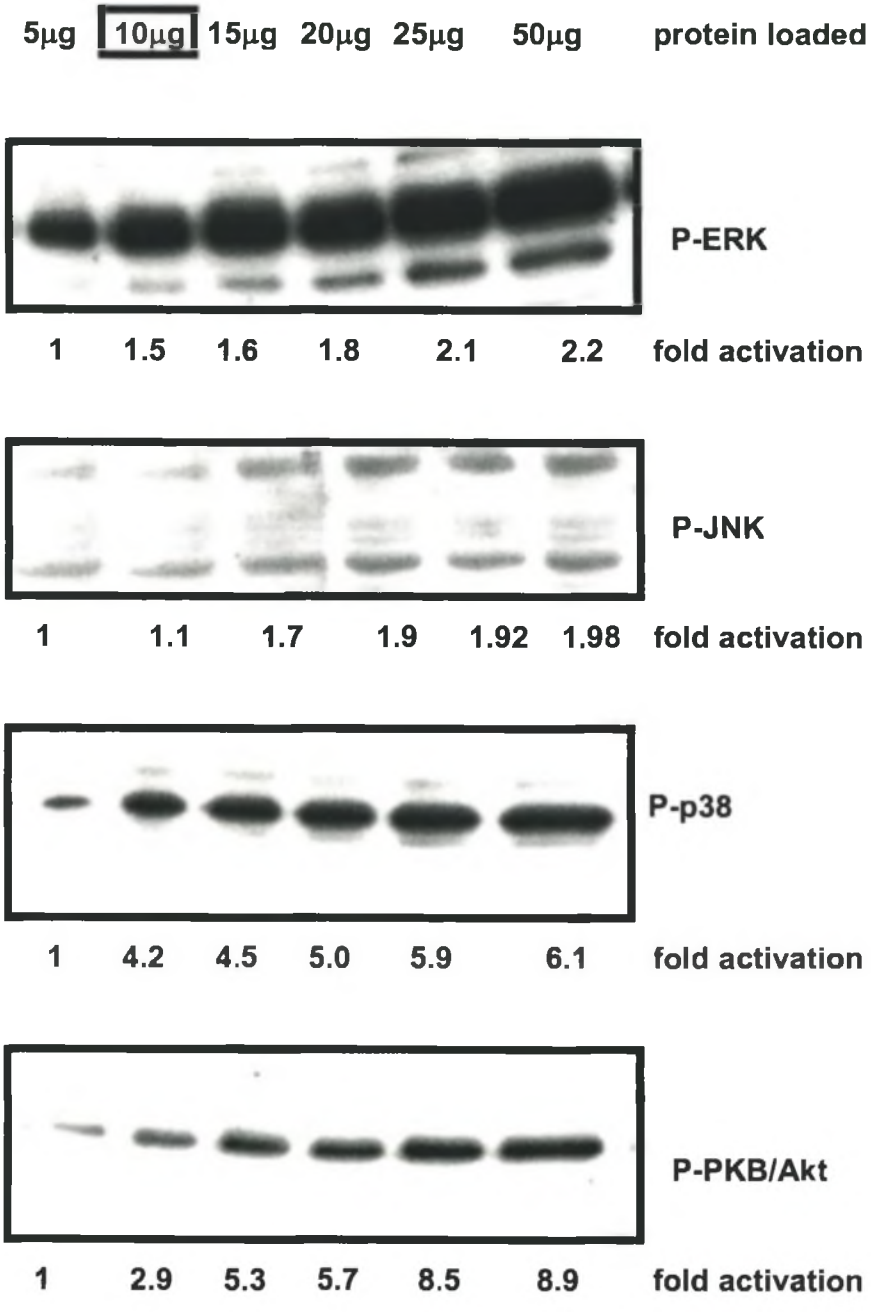
For the assessment of cell viability a modification of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay described by Gomez and co-workers was used (1997). The assay is based upon the principle of reduction of MTT into blue formazan pigments by viable mitochondria in healthy cells. At the end of the experiment, the medium was removed from the petri dishes and the cells washed twice with PBS. MTT (0.01g/ml) was dissolved in PBS, and 500 µl was added to each petri dish. Cells were subsequently incubated for 2 h at 37°C in an atmosphere of 5% CO_2. This time period was found to be optimal for color development associated with formazan product formation. After the incubation period, cells were washed twice with PBS and one ml of HCl-isopropanol-Triton (1% HCl in isopropanol; 0.1% Triton X-100; 50:1) was added to each petri and gently agitated for 5 minutes. This caused the cell membranes to lyse and liberate the formazan pigments. The suspension was then centrifuged at 14 000 rpm for 2 minutes. The optical density (OD) was determined spectrophotometrically at a wavelength of 540 nm and the values expressed as percentages of control values.

3.13 Western-blot analysis

Cardiomyocyte MAPKs, caspase-3 and poly-ADP-ribose-polymerase (PARP) protein were extracted with a lysis buffer containing (in mM): Tris 20, p-nitrophenylphosphate 20, EGTA 1, sodium fluoride (NaF) 50, sodium orthovanadate 0.1, phenylmethyl sulphonyl fluoride (PMSF) 1, dithiothreitol (DTT) 1, aprotinin 10 µg/ml, leupeptin 10 µg/ml. Mitogen-activated protein kinase phosphatase-1 (MKP-1) protein was extracted with a lysis buffer containing (in mM): Hepes 50, EDTA 10, EGTA 10, PMSF 1, Aprotinin (1 ug/ml), Leupeptin (1

ug/ml) and Triton (0.5%). The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 minutes and 10 µg (MAPKs) or 50 µg protein (caspase-3, PARP and MKP-1) were separated by 10% SDS-PAGE. The lysate protein content was determined using the Bradford technique (1976). The separated proteins were transferred to a PVDF membrane (Immobilon^{TM} P, Millipore), which were routinely stained with Ponceau Red for visualization of proteins. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline-0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize phosphospecific ERK p42/p44 (Thr^{202}/Tyr^{204}) and total ERK, p38-MAPK (Thr^{180}/Tyr^{182}) and total p38-MAPK, JNK p54/p46 (Thr^{183}/Tyr^{185}) and total JNK, PKB/Akt ($Ser^{473}$and Thr^{308}) and total PKB/Akt, caspase-3 (p17 fragment pAb) and PARP (p85 fragment pAb). Membranes were subsequently washed with large volumes of TBST (5X5 min) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL^{TM} detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a non-radioactive method (ECL^{TM} Western blotting). Films were densitometrically analysed (UN-SCAN-IT, Silkscience) and phosphorylated protein values were corrected for minor differences in protein loading, if required. Experiments were also performed to ensure that all signals were within the linear range of detection on the autoradiographs under our assay and gel loading conditions (fig 3.1).

Figure 3.1 **Experiments to ensure that signals are within the linear range of detection under our assay- and loading conditions.**



3.14 Morphological assessment and quantification of apoptotic myocytes

Hoechst 33342, a fluorescent nuclear binding dye, which allows clear distinction between apoptotic and normal cells on the basis of nuclear morphology (chromatin condensation and fragmentation), was used for quantification of apoptotic myocytes. Hoechst 33342 (prepared in PBS) was added to the culture medium at a final concentration of 50 μg/ml. Cells were evaluated by fluorescence microscopy according to the following grading system: normal nuclei (blue chromatin with organized structure) and apoptotic cells (bright fluorescent chromatin which is highly condensed or fragmented). For each experimental condition, three separate myocyte populations were prepared. At least 400 cells from three randomly selected fields were counted in each petri and quantified for each experimental condition (total: 1200 cells/petri; >3600 cells/experiment). The apoptotic index [percentage of apoptotic cells] was calculated as number of apoptotic cells/total cells counted X 100. Scoring was done blindly.

3.15 *In vitro* dephosphorylation assay

Myocytes were either incubated with fatty acids (FA) (20 μM) or exposed to 60 minutes SI and 30 minutes reperfusion as described previously. Cells were scraped from the culture dish, transferred to an eppendorf and sonicated for 10 seconds. Phosphatases were extracted with a buffer containing (in mM): Hepes 50, EDTA 10, EGTA 10, PMSF 1, Aprotinin (1 ug/ml), Leupeptin (1 ug/ml) and Triton (0.5%). The samples were centrifuged and the protein content of the supernatant was determined using the Bradford technique (1976). To determine whether the phosphatases were responsible for the inhibition of the kinases, the kinase and phosphatase containing lysates, obtained from myocytes after simulated ischaemia/reperfusion, were mixed (50 μg protein each). Samples not incubated served as controls, while the rest were incubated at 30°C for 45 minutes, with and without the tyrosine phosphatase inhibitor, vanadate (10mM).

The reactions were stopped by addition of sample buffer containing: 250 mM Tris-HCl, 500mM DTT, 10% SDS, 0.5% Bromophenol Blue and 50% Glycerol. Proteins were separated by electrophoresis followed by Western blotting for p38 as described before.

3.16 Perfusion technique of isolated rat heart

The well-characterized retrogradely (Langendorff model) perfused heart was used as experimental model. The hearts were rapidly excised and arrested in ice-cold Krebs-Henseleit bicarbonate buffer, pH 7.4, containing (in mM): NaCl 119, $NaHCO_3$ 24.9, KCl 4.74, KH_2PO_4 1.19, $MgSO_4$ 0.6, Na_2SO_4 0.59, $CaCl_2$ 1.25, glucose 10. The buffer was oxygenated and kept at pH 7.4 by gassing with 95% O_2 / 5% CO_2. Hearts were perfused retrogradely at a constant pressure (100 cm H_2O). A water-filled latex balloon, connected to a pressure transducer was inserted into the left ventricle via the left atrium. Left ventricular end-diastolic pressure was set between 4 and 8 mmHg as baseline. Heart rate and the left ventricular developed pressure (LVDP) were monitored continuously on the computer, and function assessed before and after ischaemia.

3.17 Experimental protocol: functional studies (fig 3.2)

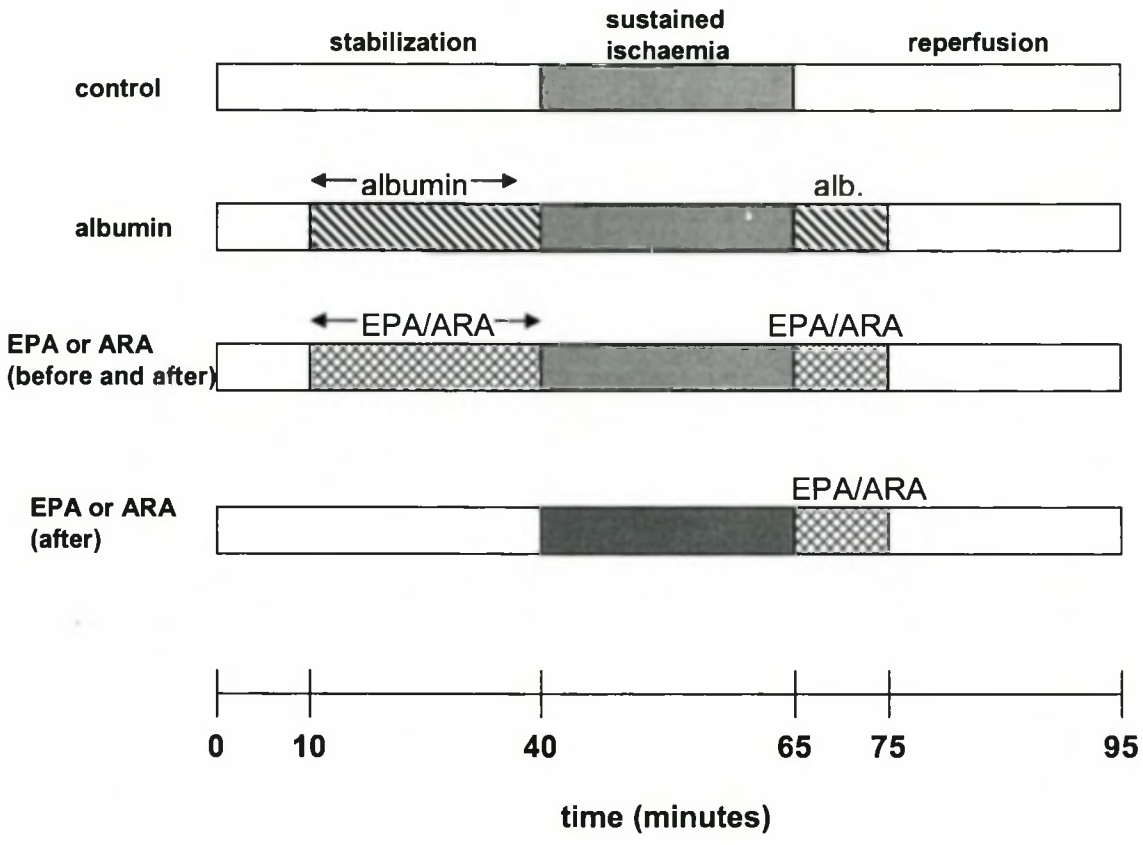
Hearts were perfused retrogradely for 40 minutes (stabilisation) followed by 25 minutes global sustained ischaemia and reperfusion for 30 minutes (control, n=12). In the second protocol, hearts were perfused with albumin as indicated in figure 2. Albumin treated hearts (n=6) were stabilized for 10 minutes without albumin and for 30 minutes in the presence of albumin. This was followed by 25 minutes sustained global ischaemia and 30 minutes reperfusion (10 minutes in the presence of albumin and 30 minutes in the absence of albumin). In the third protocol, EPA (n=7) or ARA (n=6) was added before and after sustained ischaemia as follows: hearts were stabilized for 10 minutes without the fatty acid and 30 minutes in the presence of the fatty acid/albumin. This was followed by 25

minutes of global, sustained ischaemia followed by 30 minutes reperfusion (10 minutes in the presence of the fatty acid/albumin and 20 minutes in the absence of the fatty acid/albumin. In the fourth protocol, the hearts were treated with EPA (n=6) or ARA (n=6) after global, sustained ischaemia only as follows: hearts were stabilized for 40 minutes followed by 25 minutes of global sustained ischaemia and 30 minutes of reperfusion, which included 10 minutes in the presence the fatty acid/albumin and 20 minutes in the absence of the fatty acid/albumin.

3.18 Statistical analysis

All results are expressed as means ± SEM. One-way ANOVA was performed for each group of treatments, using Bonferroni's post-hoc test and p values < 0.05 were regarded as significant. The number of experiments is indicated in the figure legends.

Figure 3.2 Experimental protocol:functional studies



CHAPTER 4 RESULTS AND DISCUSSION: The regulatory roles of MAPKs and PKB/Akt on the development of apoptosis during simulated ischaemia and reperfusion (SI/R) in neonatal cardiomyocytes

4.1 Introduction

During the past few years it has become clear that, in addition to necrosis, apoptosis plays a significant role in myocardial ischaemia/reperfusion injury (Kajstura *et al.*, 1996; Gottlieb & Engler 1999). Anversa and Kajstura (1998) have demonstrated that apoptosis precedes cell necrosis and is in fact the major determinant of infarct size, but this statement has been questioned by others (Schaper *et al.*, 1999 a & b), e.g. Kostin and co-workers recently published results where they concluded that apoptosis, oncosis and autophagy act in parallel to varying degrees in the failing human heart (2003). Shiraishi and co-workers (2001) have underlined the importance of the energy status in neonatal cardiac myocytes, where the presence of intracellular ATP favours a shift from necrosis to apoptosis via caspase activation. However, the precise mechanisms involved in stimulus recognition and progression to apoptosis in response to ischaemic injury or ATP depletion remains largely uncertain. Many studies have suggested that the mitogen activated protein kinases (MAPKs) as well as PKB/Akt may be important regulators of apoptosis in response to myocardial ischaemia/reperfusion, but reports on their precise roles are still conflicting.

Three major MAPKs, namely extracellular signal-regulated protein kinase (ERK), p38 and c-Jun NH_2-terminal protein kinase (JNK) play a pivotal role in the transmission of signals from cell surface receptors to the nucleus (Bogoyevitch *et al.*, 1996; Mansour *et al.*, 1994). In many cell types the ERK cascade appears to mediate specifically cell growth and survival signals. For instance, it has been shown that inhibition of ERK enhances ischaemia-reperfusion induced apoptosis and that sustained activation of this kinase during simulated ischaemia mediates

adaptive cytoprotection in cultured neonatal cardiomyocytes (Yue *et al.*, 2000). On the other hand, the p38 and JNK families appear to be pro-apoptotic in many cell types (Obata *et al.*, 2000; Park *et al.*, 2000), however, their exact roles in regulating cell death are unclear. For example, Hreniuk and co-workers (2001) found that inhibition of JNK46 but not JNK54, significantly reduced reoxygenation-induced apoptosis while Wang *et al* (1998b) reported that activation of JNK by transfection of cultured rat neonatal cardiomyocytes with mitogen activated protein kinase kinase 7 (MKK7), an upstream activator of JNK, induced hypertrophy rather than apoptosis. Despite reports to the contrary (Weinbrenner *et al.*, 1997; Zechner *et al.*, 1998), several investigators support the concept that p38 activation is harmful to the ischaemic heart (Bogoyevitch *et al.*, 1996; Mackay & Mochly-Rosen, 2000). These conflicting results regarding the role of p38 can possibly be attributed to the different isoforms (α and β) expressed in the heart (Saurin *et al.*, 2000), which appear to mediate opposing functions. The p38α-isoform is implicated in apoptosis, whereas p38β is anti-apoptotic in neonatal rat cardiomyocytes (Michel *et al.*, 2001). Further evidence, which links JNK and p38 to apoptosis, is the finding that their signalling pathways are associated with activation of effector caspases, including caspase 3 (Chaudhary *et al.*, 1999).

It has been suggested in several studies that PKB/Akt might be a critical regulator of cell survival. The transfection of a variety of cell types with constitutively active PKB/Akt alleles blocks apoptosis induced by a wide range of apoptotic stimuli, including growth factor withdrawal, ultraviolet irradiation, matrix detatchment, DNA damage and treatment of cells with anti-Fas antibody or transforming growth factor-beta (TGF-β) (Songyang *et al.*, 1997; Dudek *et al.*, 1997; Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Chen *et al.*, 1998; Rohn *et al.*, 1998). Studies of PKB/Akt activation pathways have also shown that PI3-kinase is a mediator of an activator signal for PKB/Akt (Franke *et al.*, 1995; Burgering *et al.*, 1995; Kulik *et al.*, 1997). PI3-kinase is considered one of the intracellular signals responsible for the transmission of anti-apoptotic signals for

controlling cell survival. Overexpression of PI3-kinase in cells has been shown to cause a significant increase in survival of cells exposed to ionising radiation (Krasilnikov *et al.*, 1999; Cataldi *et al.*, 2001). Furthermore, it was also shown by Fujio and co-workers that PKB/Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia/reperfusion injury in the mouse heart (2000).

In the current study, an *in vitro* model of simulated ischaemia/reperfusion (SI/R), induced by KCN and 2-deoxy-*D*-glucose, was used to achieve graded ATP depletion in cultured neonatal cardiomyocytes and to analyse the regulation of cardiomyocyte apoptosis. In addition, the phosphorylation pattern of the MAPKs and PKB/Akt during SI/R was characterized and the significance of their phosphorylation in apoptosis was determined.

4.2 Results

4.2.1 Myocyte loss during SI/R

Myocyte loss during the experimental procedures, should it occur, could severely affect the outcome of the experiments. To establish whether any of the cellular manipulations resulted in a significant detachment of myocytes during the incubation periods, the number of cells in the supernatant (media) was counted after each insult. The number of detached cells was found to be less than 0.01% of the number of cells plated (data not shown). This was further corroborated by the fact that no protein loss [assessed by the Bradford technique (1976)] was detected during SI and reperfusion. Therefore, changes in viability or apoptotic index could not be ascribed to a decrease in cell number.

4.2.2 Cultured rat neonatal cardiomyocytes undergo time-dependent depletion of intracellular ATP and CrP during SI (Figure 4.1A)

Confluent cardiomyocytes subjected to inhibition of glycolysis (with 2-deoxy-*D*-glucose) and oxidative phosphorylation (with KCN) displayed a rapid and

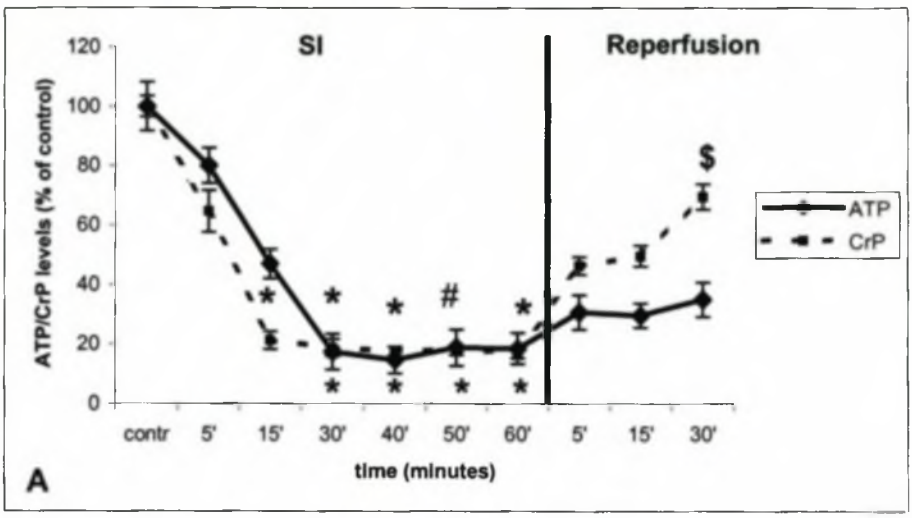


Figure 4.1 A: Intracellular ATP- and CrP levels at various time points during simulated ischaemia (SI) and reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) followed by reperfusion for the indicated time periods. Results are expressed as means ± S.E.M. for four independent experiments (n=4). *$p<0.001$ vs control; $^{\#}p<0.01$ vs control; $^{\$}p<0.01$ vs 60'SI. [Control values before onset of simulated ischaemia: 25 ± 5 nmol ATP/mg protein and 23.1 ± 3.5 nmol CrP/mg protein]

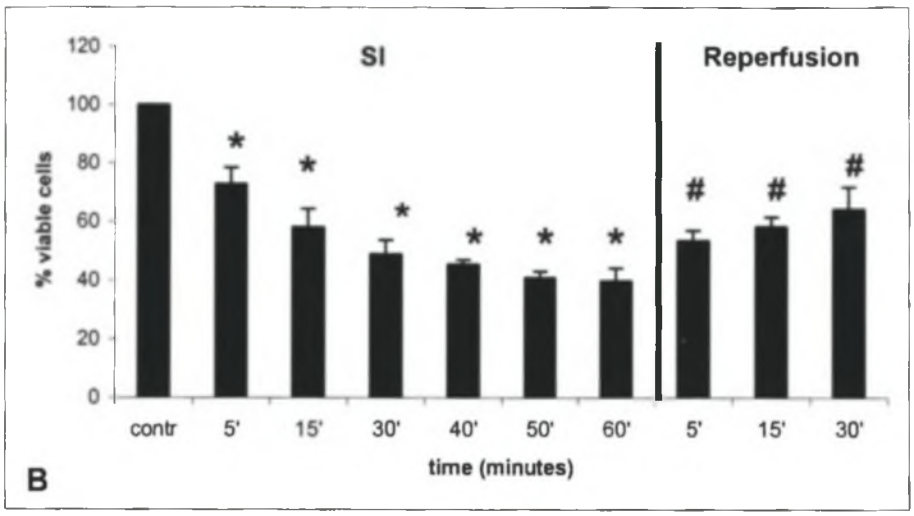


Figure 4.1 B: Cell viability was measured during SI and reperfusion, using the MTT cell viability assay. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) followed by reperfusion for the indicated time periods. Results are expressed as means ± S.E.M. for five independent experiments (n=5). *$p<0.001$ vs control; $^{\#}p<0.001$ vs 60'SI.

profound decrease in intracellular ATP levels to 18 ± 1.4% ($p<0.001$) of controls within 30 minutes of SI. The decrease in CrP was even more rapid compared to ATP: after only 15 minutes SI the intracellular CrP levels were decreased to 21.4 ± 1.4% ($p<0.001$) of controls. A plateaux for both ATP and CrP was reached after 30 minutes SI. After 30 minutes reperfusion, ATP levels increased to 35 ± 1% and CrP to 70 ± 1.2% of their corresponding controls ($p<0.01$, 60 min SI vs 60'SI/30'R for CrP).

4.2.3 Cardiomyocytes undergo time-dependent loss of viability during SI (Figure 4.1B)

The MTT assay showed that a gradual loss of mitochondrial viability in cardiomyocytes occurred during SI reaching 41 ± 1.6% of control after 60 minutes. However, there was a gradual but significant increase in mitochondrial viability during reperfusion, reaching 64 ± 2.8% after 30 minutes. This suggests that in at least 20% of cells the mitochondrial injury was reversible, while the cell membranes were intact.

4.2.4 Caspase-3 activation and PARP cleavage during SI and SI/R (Figure 4.1C)

Caspase-3 activation and PARP cleavage followed more or less the same pattern; both were maximal after 15 minutes SI [2.47 ± 0.23 fold ($p<0.05$) and 2.55 ± 0.29 fold ($p<0.05$) fold increase for caspase-3 and PARP respectively]. Caspase-3 activation appears to be biphasic, reaching a second peak after 60 minutes, while PARP cleavage remained high throughout SI. After 5 minutes of reperfusion, both caspase-3 and PARP returned to almost basal levels with significant increases in activation and cleavage after 15- and 30 minutes reperfusion [Caspase-3: 2.70 ± 0.4 fold ($p<0.05$) and 2.67 ± 0.38 fold ($p<0.05$) for

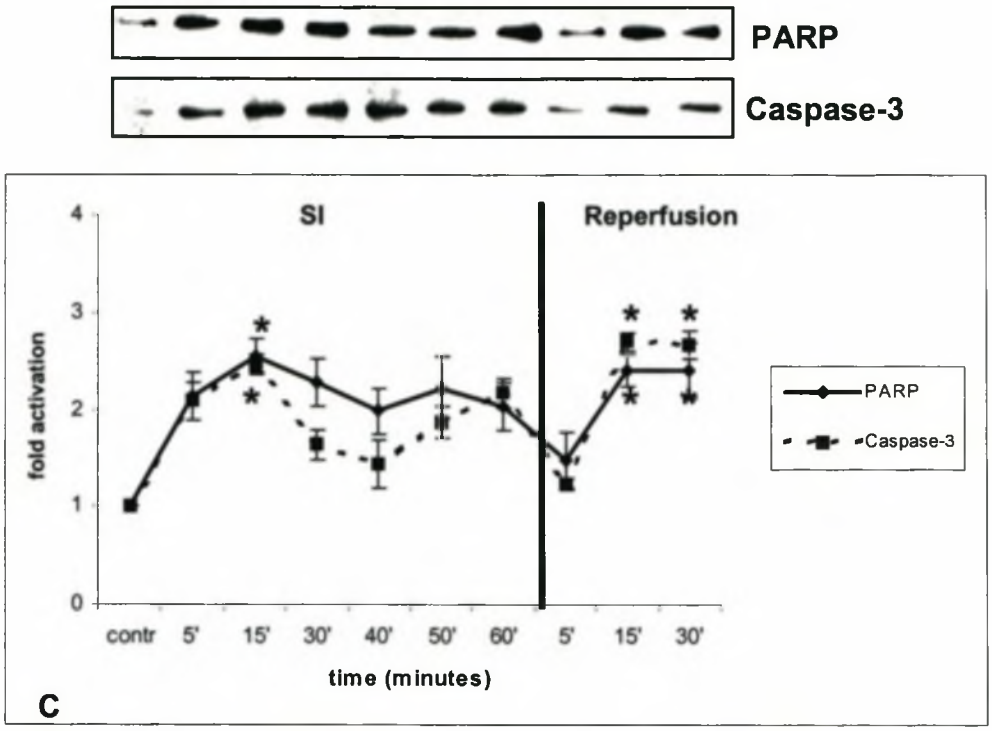


Figure 4.1 C: Time course of PARP cleavage and caspase-3 activation during SI and reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) followed by reperfusion for the indicated time periods. Samples were analysed by Western blotting with antibodies recognizing cleaved PARP and caspase-3. Results were expressed as means ± S.E.M. for three independent experiments (n=3). *$p<0.05$ vs control.

15- and 30 minutes reperfusion respectively; PARP: 2.4 ± 0.32 fold ($p<0.05$) and 2.4 ± 0.31 fold ($p<0.05$) for 15- and 30 minutes reperfusion].

4.2.5 Apoptotic Index during SI and SI/R (Figures 4.1D & E)

Cardiac myocytes, stained with Hoechst 33342 and assessed by fluorescence microscopy, showed condensed chromatin and fragmented nuclei after exposure to SI and reperfusion. During normoxia, 4.6 ± 1.1% cells showed spontaneous cell death (basal), which was maintained during SI. A significant increase in apoptotic cells [22.8 ± 6.58% ($p<0.01$)] was only observed after 60 minutes SI followed by 30 minutes reperfusion.

4.2.6 Phosphorylation of ERK, p38, JNK and PKB/Akt in cardiomyocytes subjected to SI and SI/R (Figures 4.2 and 4.3)

To investigate whether the MAPKs as well as PKB/Akt were phosphorylated during the SI/R protocol, cells were harvested at different times during SI and reperfusion. As shown in figure 4.2A, rapid and transient phosphorylation of p38 occurred after 5 minutes of SI [5.9 ± 0.23 fold ($p<0.001$)], with a gradual decrease in phosphorylation up to 30 minutes where after it slowly increased again. A second peak of phosphorylation was observed at 60 minutes of SI [6.11 ± 0.26 fold ($p<0.001$)]. Five minutes of reperfusion did not induce a further significant increase in phosphorylation [6.83 ± 0.1 fold ($p>0.05$)], compared with 60 minutes SI. p38 phosporylation declined afterwards. No significant changes in phosphorylation of ERK occurred during SI, but upon reperfusion, both the ERK-isoforms were phosphorylated with a peak increase at 5 minutes [6.09 ± 0.37 fold ($p<0.001$) and 1.75 ± 0.06 fold ($p<0.01$) for p44 and p42 respectively]. ERK-p42 remained elevated up to 30 minutes of reperfusion [1.78 ± 0.12 fold ($p< 0.01$)], while p44 phosphorylation gradually decreased from 6.09 ± 0.37 to 3.50 ± 0.22 fold ($p<0.001$). Phosphorylation of both isoforms of JNK (p54/p46) occurred

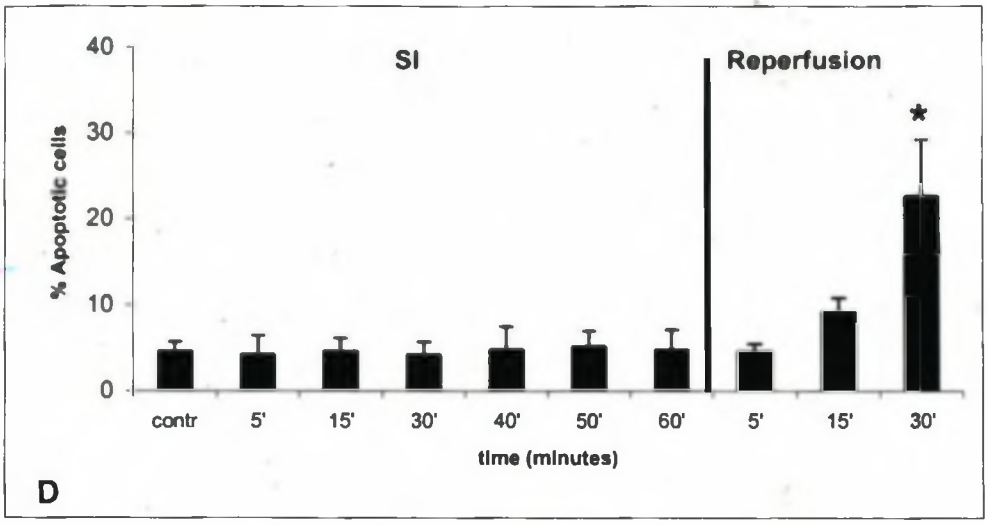


Figure 4.1 D: SI/reperfusion-induced apoptosis in cardiac myocytes. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) followed by reperfusion for the indicated time periods. To quantify apoptotic myocytes, cell monolayers were fixed and stained with Hoechst 33342. The morphological features of apoptosis (cell shrinkage, chromatin condensation) were monitored by fluorescence microscopy. At least 400 cells from three randomly selected fields per dish were counted and each treatment was performed in triplicate. Results were expressed as means ± S.E.M. for three independent experiments (n=3). *$p<0.001$ vs control.

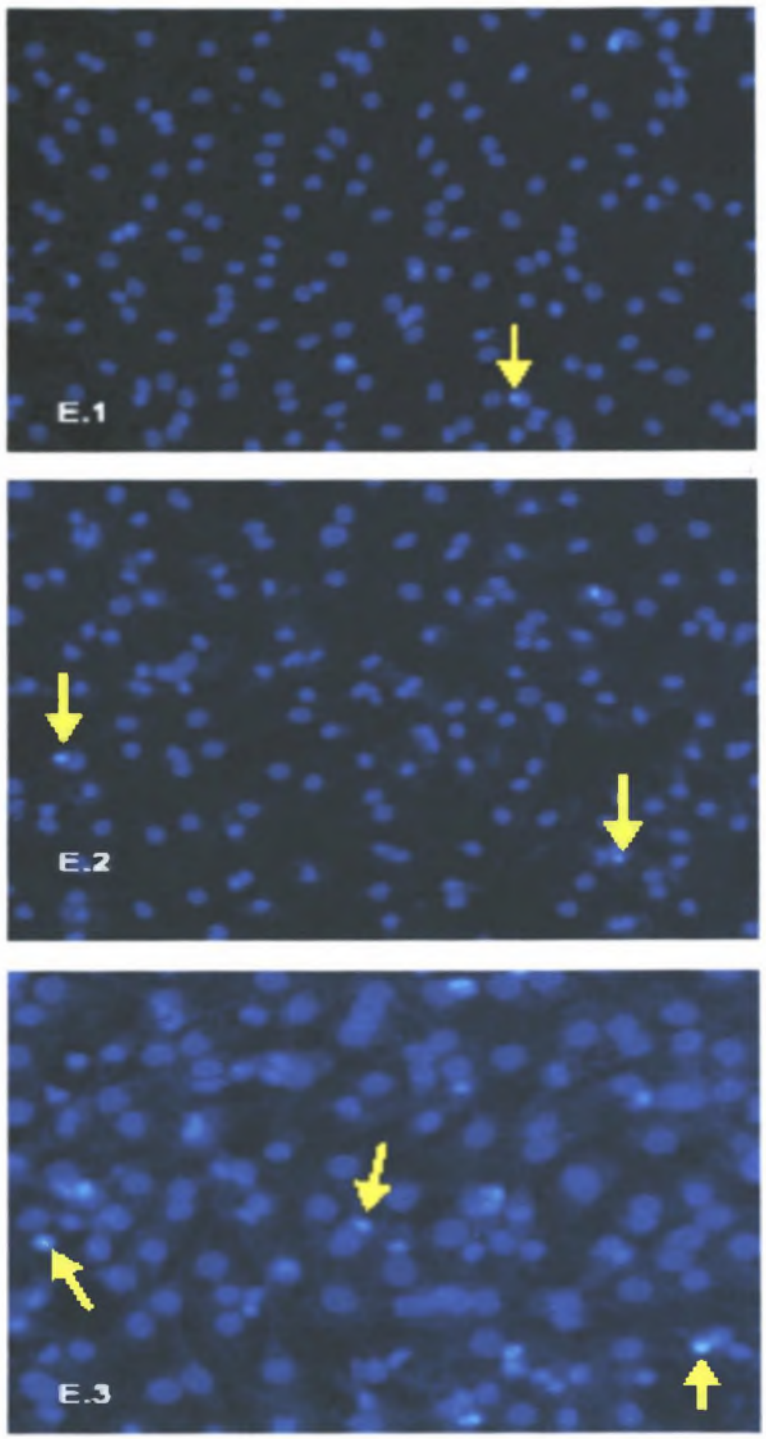


Figure 4.1 E: Simulated Ischaemia / Reperfusion-induced apoptosis in cardiomyocytes (Fluoresence photomicrographs): 1. control; 2. 60'SI; 3. 60'SI/30'R. Arrows indicate apoptotic bodies.

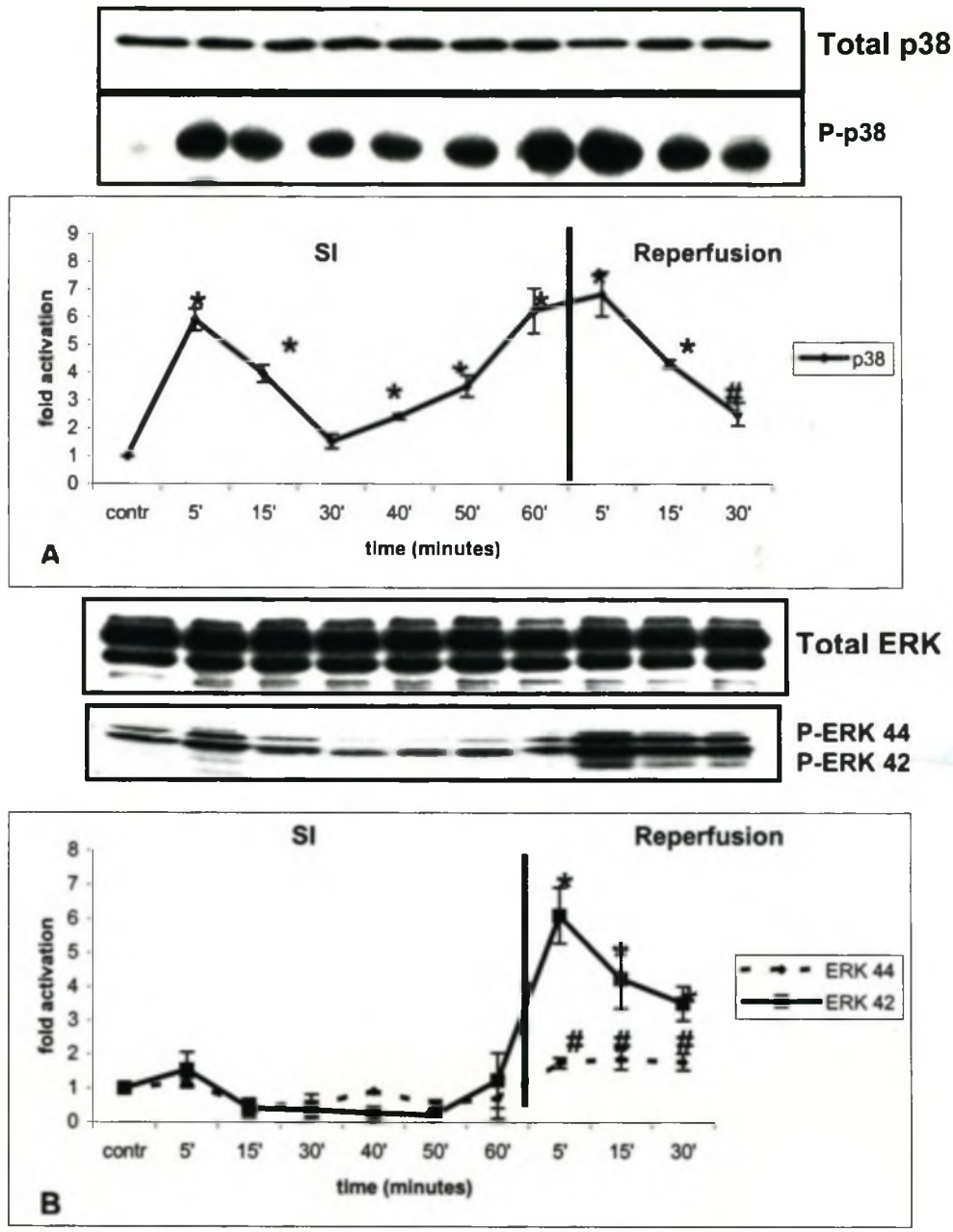


Figure 4.2

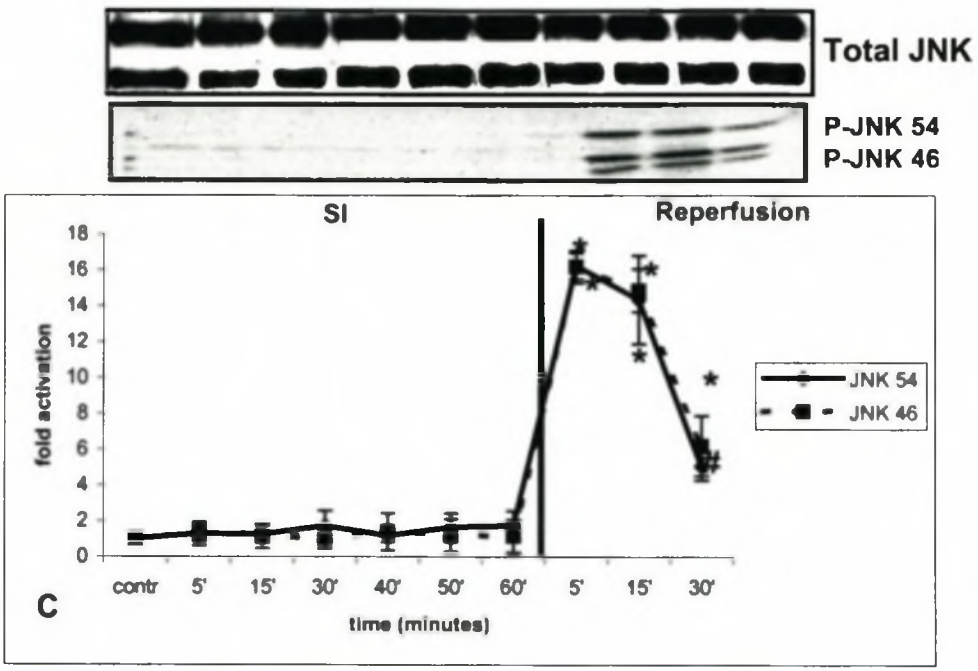


Figure 4.2: Time course of p38, ERK and JNK phosphorylation in cardiomyocytes subjected to simulated ischaemia and reperfusion. Myocytes were exposed to SI (KCN and 2-deoxy-*D*-glucose) followed by reperfusion for the indicated time period. Samples were analysed by Western blotting with antibodies recognizing dual-phosphorylated MAPKs. Results were expressed as means ± S.E.M. for four to seven independent experiments (n=4-7). *$p<0.001$ vs control; #$p<0.01$ vs control.

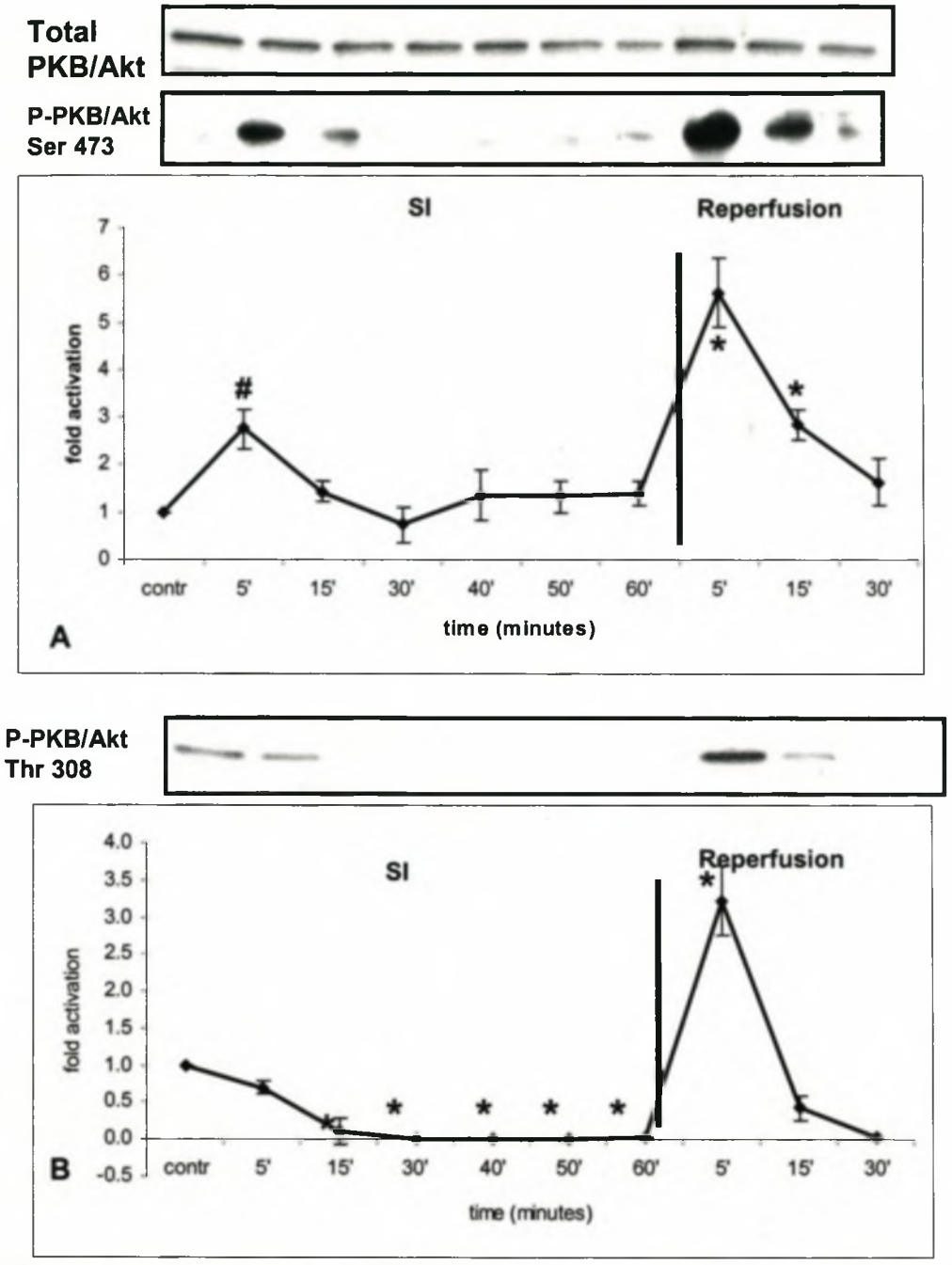


Figure 4.3: Time course of PKB/Akt phosphorylation in cardiomyocytes subjected to simulated ischaemia and reperfusion. Myocytes were exposed to SI (KCN and 2-deoxy-*D*-glucose) followed by reperfusion for the indicated time period. Samples were analysed by Western blotting with antibodies recognizing phosphorylated Ser^{473} and Thr^{308} of PKB/Akt. Results were expressed as means ± S.E.M. for four to seven independent experiments (n=4-7). *$p<0.001$ vs control; $^{\#}p<0.01$ vs control.

during reperfusion only, with maximal phosphorylation at 5 minutes (fig 4.2C) [16.2 ± 0.52 fold ($p<0.001$), declining gradually during further reperfusion.

PKB phosphorylation was determined using antibodies recognizing phospho-Ser^{473} and Thr^{308}. As shown in figure 4.3A, Ser^{473} phosphorylation occurred after 5 minutes SI [2.75 ± 0.23 fold ($p<0.01$)] and returned to basal levels within 15 minutes SI. However, there was no significant increase in Thr^{308} phosphorylation at this specific time-point. Ser^{473} was maximally phosphorylated after 5 minutes of reperfusion [5.63 ± 0.42 fold ($p<0.001$)], returning to basal levels after 30 minutes. Similarly, Thr^{308} phosphorylation also peaked at 5 minutes reperfusion [3.2 ± 0.2 fold ($p<0.001$)] returning to basal levels after 15 minutes.

Please note that the total -p38, -ERK (p44 and p42), -JNK (p54 and p46) as well as -PKB remained unaltered throughout the experimental procedure.

4.2.7 The effects of SB203580, PD98059 and SP600125 on the MAPKs (Figures 4.4 A, B and C)

To evaluate the significance of p38, ERK and JNK activation during SI/R, three specific inhibitors, SB203580, PD98059 and SP600125 respectively were used. Preliminary experiments indicated that, inhibition of the MAPKs was only achieved during reperfusion when the drugs were administered for 30 minutes before the onset of SI as well as during the reperfusion period. Therefore, in all studies, the inhibitors were present before and after SI. Samples were taken at the end of the 60 minutes SI and 30 minutes reperfusion period. In the presence of 1 μM SB203580, p38 phosphorylation was significantly reduced from 3.07 ± 0.59 to 1.03 ± 0.08 fold ($p<0.05$) after SI/R. The MEK1/MEK2 inhibitor, PD98059, at 10 μM, caused a significant decrease in ERK 44 [3.84 ± 0.15 to 2.15 ± 0.26 fold ($p<0.001$)] and ERK 42 phosphorylation [3.62 ±0.07 to 2.7 ± 0.3 fold ($p<0.05$)] during SI/R. SP600125, a specific JNK inhibitor, at 10 μM, caused a significant decrease in JNK 54 [2.16 ± 0.09 to 0.92 ± 0.10 fold ($p<0.001$)] and

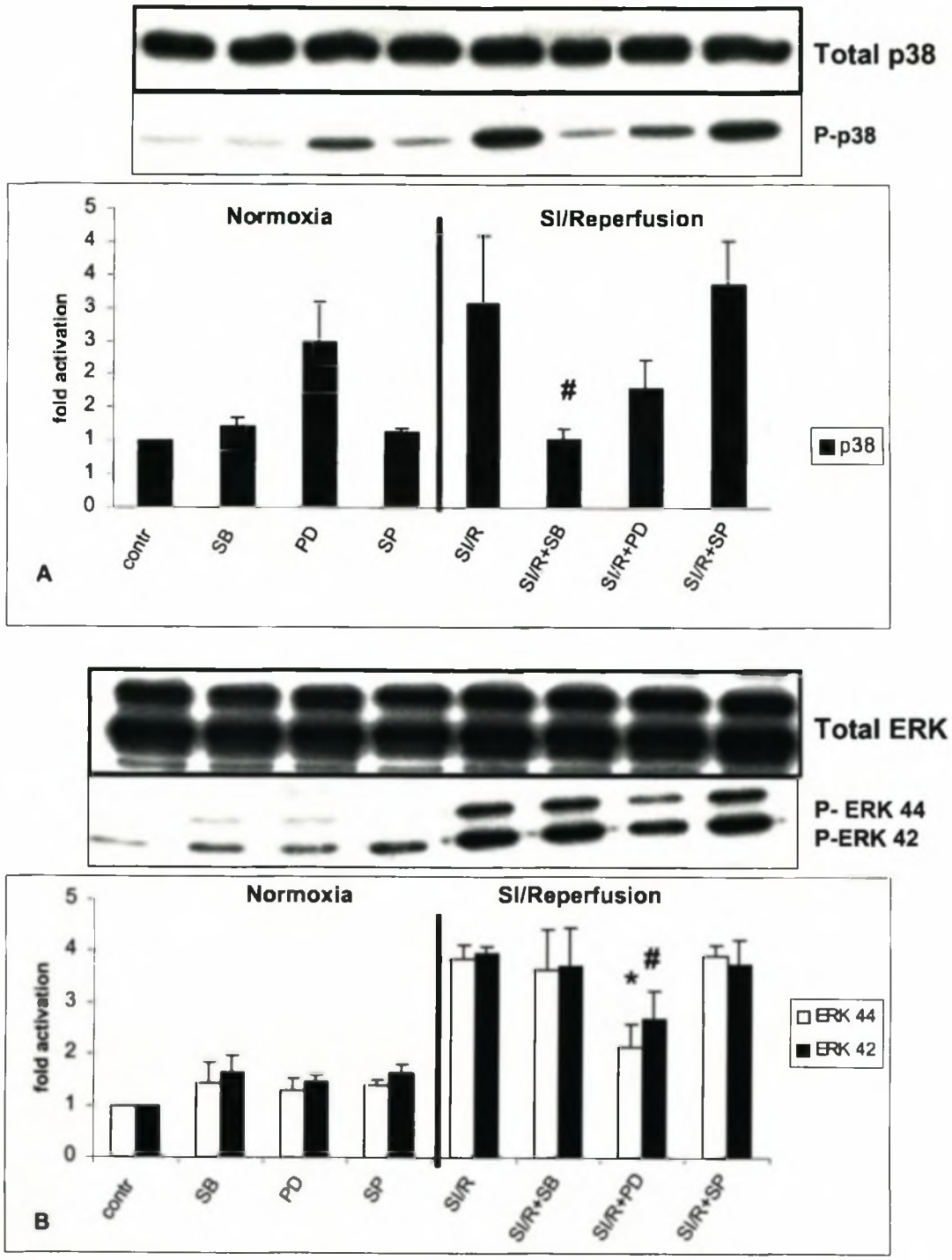
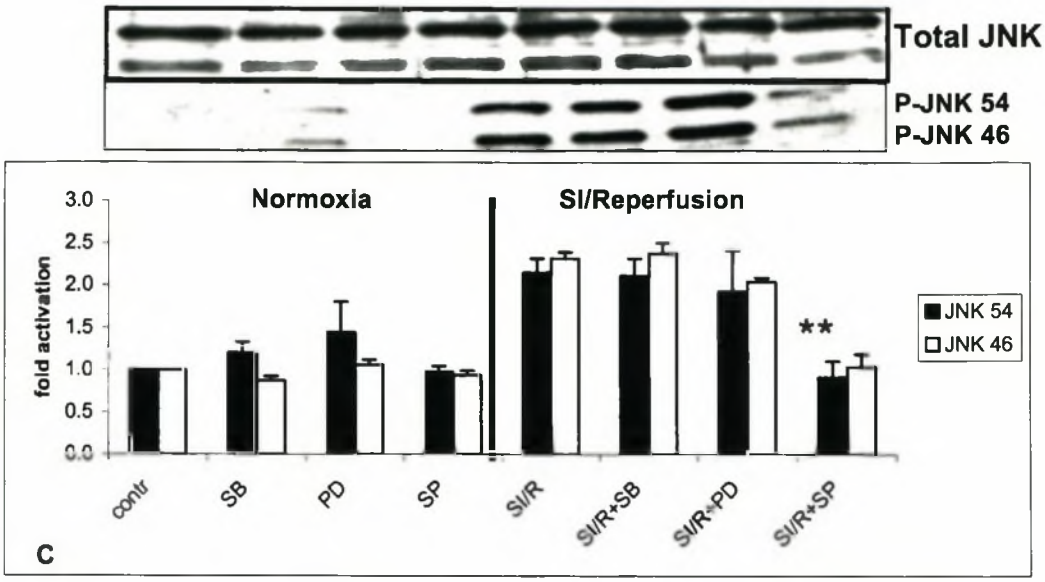


Figure 4.4



Figures 4.4 A, B and C: Effect of SB203580 (1 μM), PD98059 (10 μM) and SP600125 (10 μM) on MAPK phosphorylation during SI and reperfusion. SI and SI/reperfusion were carried out either alone or following treatment with PD98059, SB203580 or SP600125. The drugs were administered 30 minutes before the onset of SI as well as during reperfusion to achieve optimal inhibition. (A) p38: $^{\#}p<0.01$: 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SB. (B) ERK 44: $^{*}p<0.001$: 60'SI/30'reperfusion vs 60'SI/30'reperfusion + PD; ERK 42: $^{*}p<0.05$: 60'SI/30'reperfusion vs 60'SI/30'reperfusion + PD. (C) JNK54 and JNK46 ($^{*}p<0.001$): 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SP. Results were expressed as means ± S.E.M. for three independent experiments (n=3).

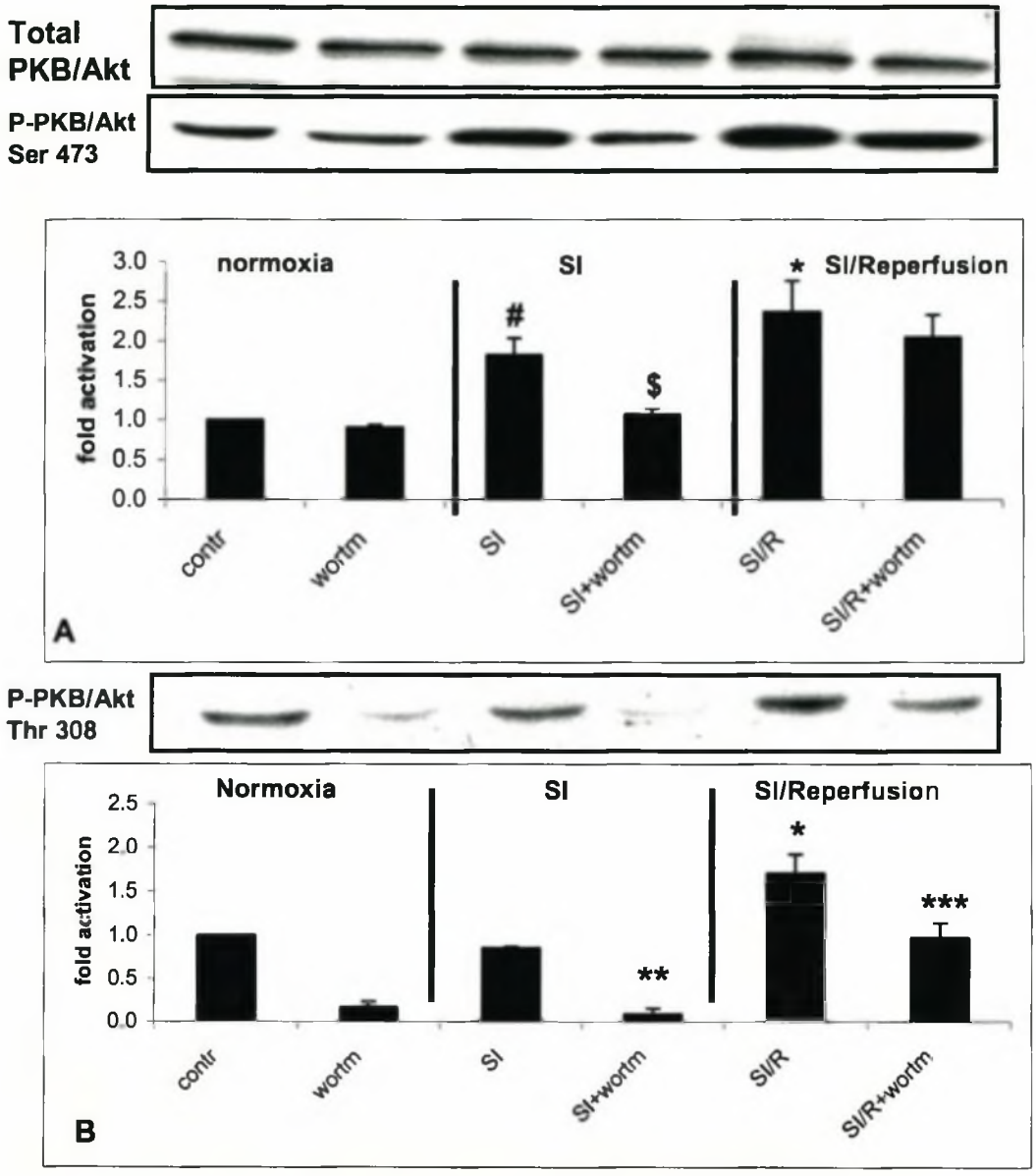
JNK 46 [2.3 ± 0.04 to 1.03 ± 0.08 fold ($p<0.001$)] phosphorylation during SI/R. The inhibitors had no effects on the total kinases.

4.2.8 The effect of wortmannin on PKB/Akt phosphorylation during SI/R (Figures 4.5 A and B).

The significance of PKB/Akt phosphorylation during SI/R in our cell model was evaluated with a PI-3-kinase inhibitor, wortmannin. The inhibitor was administered for 30 minutes before the SI period only. In this study, samples were taken after 5 minutes SI as well as at 5 minutes of reperfusion where phosphorylation has been to the maximum. After five minutes of SI, wortmannin induced a significant inhibition of PKB/Akt Ser^{473} [1.82 ± 0.12 to 1.07 ± 0.05 fold ($p<0.05$)] as well as Thr^{308} [0.85 ± 0.01 to 0.09 ± 0.12 fold ($p<0.001$)] phosphorylation. Wortmannin also caused a significant decrease in PKB/Akt Thr^{308} during reperfusion [1.71 ± 0.12 to 0.96 ± 0.09 fold ($p<0.001$)]. The total PKB/Akt remained unchanged throughout the experiment.

4.2.9 The effect of SB203580, PD98059, SP600125 and wortmannin on cell viability during SI/R (Figure 4.6)

SB203580 (1 μM) significantly increased cell viability during SI/R [63.67 ± 1.85% to 84.33 ± 4.8% ($p<0.05$)]. Although PD98059 (10 μM) and SP600125 (10 μM) reduced cell viability during SI/R this was not significant. Wortmannin (100 nM) caused a small but significant reduction in cell viability during SI/R [69.75 ± 0.48% to 57.25 ± 3.42% ($p<0.05$)]. SB203580, PD98059 and SP600125 were added to the cells 30 minutes before the onset of SI as well as during reperfusion, while wortmannin was added only 30 minutes before the onset of SI.



Figures 4.5 A and B: Effect of wortmannin (100 nM) on PKB/Akt phosphorylation (Ser^{473} and Thr^{308}) during SI and reperfusion. SI and SI/reperfusion were carried out either alone or following treatment with wortmannin. The drug was administered 30 minutes before the onset of the SI period. (A) PKB/Akt Ser^{473}: control vs 5'SI ($^{\#}p<0.01$); control vs 60'SI/5'reperfusion ($^{*}p<0.001$); 5'SI vs 5'SI + wortmannin ($^{\$}p<0.05$). (B) PKB/Akt Thr^{308}: control vs 60'SI/5'reperfusion ($^{*}p<0.001$); 5'SI vs 5'SI + wortmannin ($^{**}p<0.001$); 60'SI/5'reperfusion vs 60'SI/5'reperfusion + wortmannin ($^{***}p<0.001$). Results are expressed as means ± S.E.M. for three independent experiments (n=3).

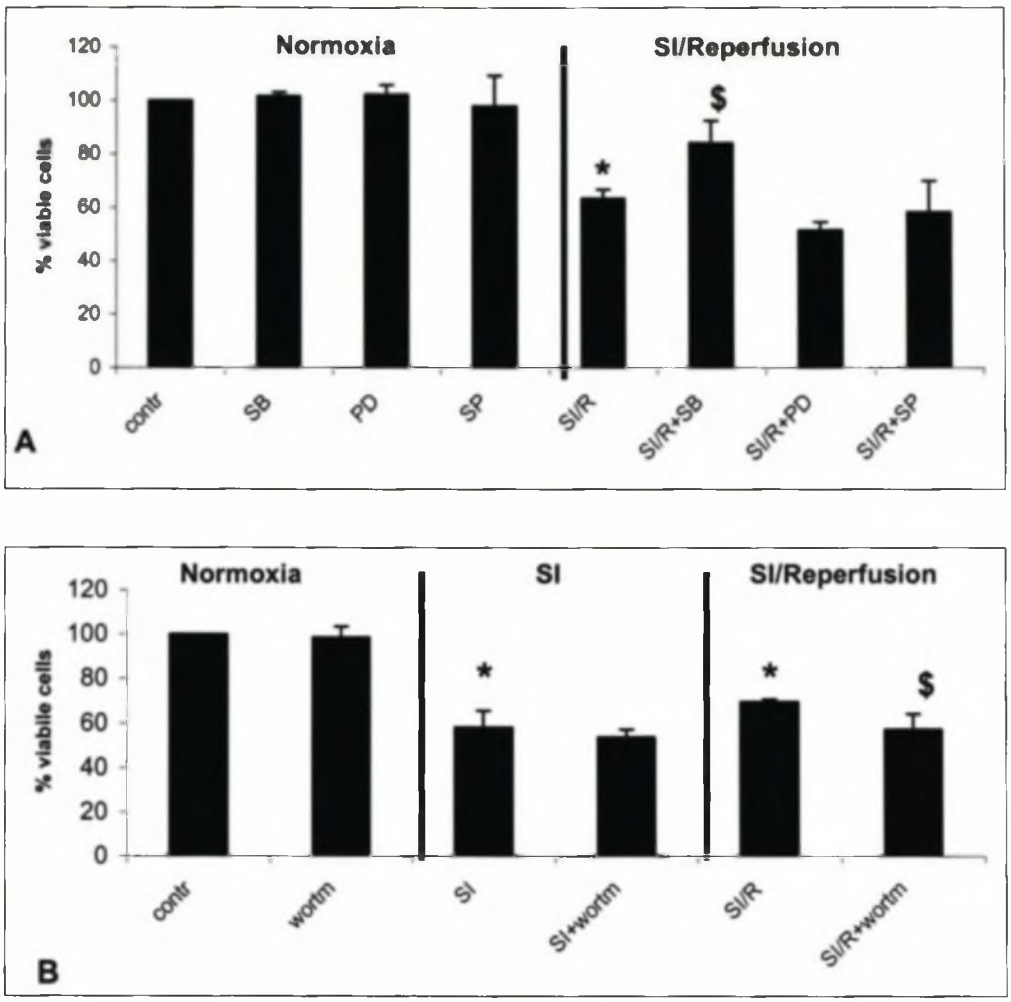


Figure 4.6: Effect of SB203580 (1 μM), PD98059 (10 μM), SP600125 (10 μM) and wortmannin (100 nM) on cell viability using the MTT assay during SI and reperfusion. Results are expressed as means ± S.E.M. for five independent experiments (n=5). **(A):** control vs 60'SI/30'reperfusion (*$p<0.001$); 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SB ($^{\$}p<0.05$) **(B):** control vs 60'SI and control vs 60'SI/30'reperfusion (*$p<0.001$); 60'SI/30'reperfusion vs 60'SI/30'reperfusion + wortmannin ($^{\$}p<0.05$). Results are expressed as means ± S.E.M. for three independent experiments (n=3).

4.2.10 The effect of SB203580, PD98059, SP600125 and wortmannin on caspase-3 activation and PARP cleavage during SI/R (Figures 4.7 A and B)

1 μM SB203580 significantly inhibited caspase-3 activation and PARP cleavage during SI/R [1.66 ± 0.03 fold to 1.06 ±0.07 fold ($p<0.001$) for caspase-3 and 2.17 ± 0.08 fold to 0.80 ± 0.09 fold ($p<0.001$) for PARP]. However, SP600125, significantly increased caspase-3 activation during SI/R [1.66 ± 0.03 fold to 2.56 ± 0.27 fold ($p<0.001$)]. Although 100 nM wortmannin caused a significant increase in PARP cleavage during SI/R [2.26 ± 0.12 fold to 3.43 ± 0.15 fold ($p<0.01$)], caspase-3 remained unaltered during this period.

4.2.11 The effect of SB203580, PD98059, SP600125 and wortmannin on the apoptotic index during SI/R (Figures 4.8 A & B)

As observed before, during normoxia (with and without the inhibitors), 4.6 ± 1.1% cells showed spontaneous cell death (as indicated by the apoptotic index), which was maintained during SI. A significant increase in apoptotic cells [22.8 ± 6.58% ($p<0.01$)] was observed after 60 minutes SI followed by 30 minutes reperfusion when compared to their corresponding controls. Treatment of the cells with SB203580 induced a significant decrease in the number of apoptotic cells during reperfusion [22.6 ± 2.94 % vs 9 ± 0.43% ($p<0.001$)], while SP600125 significantly increased [22.6 ± 2.94% vs 32.75 ± 4.13% ($p<0.05$)] the apoptotic index. Inhibition of ERK with PD98059 and PI3-kinase with wortmannin was without effect on the apoptotic index.

4.3 Discussion

4.3.1 Model

The model used in this study combines the following properties of ischaemia: Inhibition of mitochondrial respiration by cyanide which prevents the oxidation of

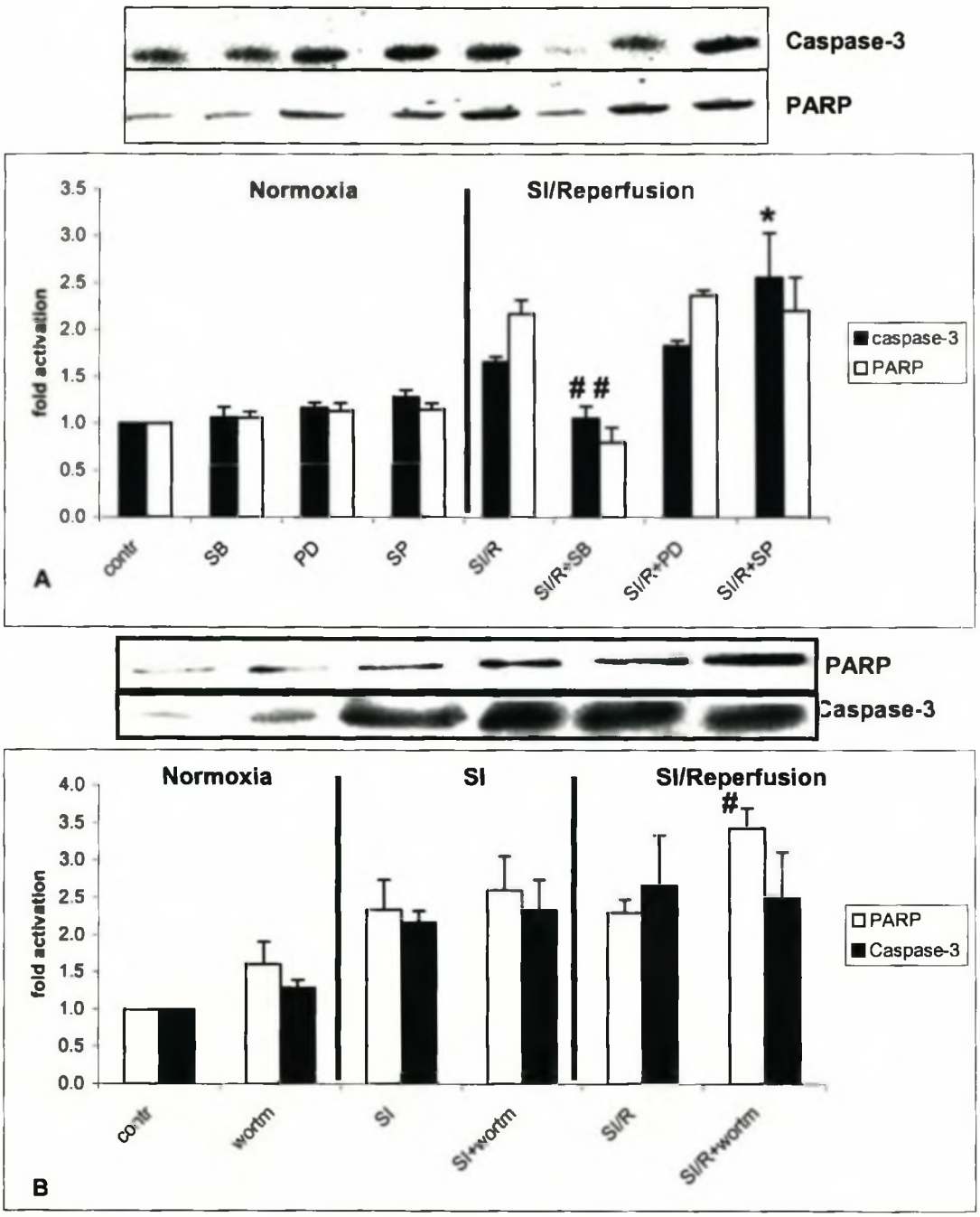


Figure 4.7: Effect of SB203580, PD98059, SP600125 and wortmannin on caspase-3 activation and PARP cleavage during SI and reperfusion. SI/reperfusion was carried out either alone or following pretreatment with PD98059, SB203580 or SP600125. Results were expressed as means ± S.E.M. for three independent experiments (n=3). **(A):** 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SP (*$p<0.001$); 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SB ($^{\#}p<0.05$) **(B):** 60'SI/30'reperfusion vs 60'SI/30'reperfusion + wortmannin ($^{\#}p<0.01$).

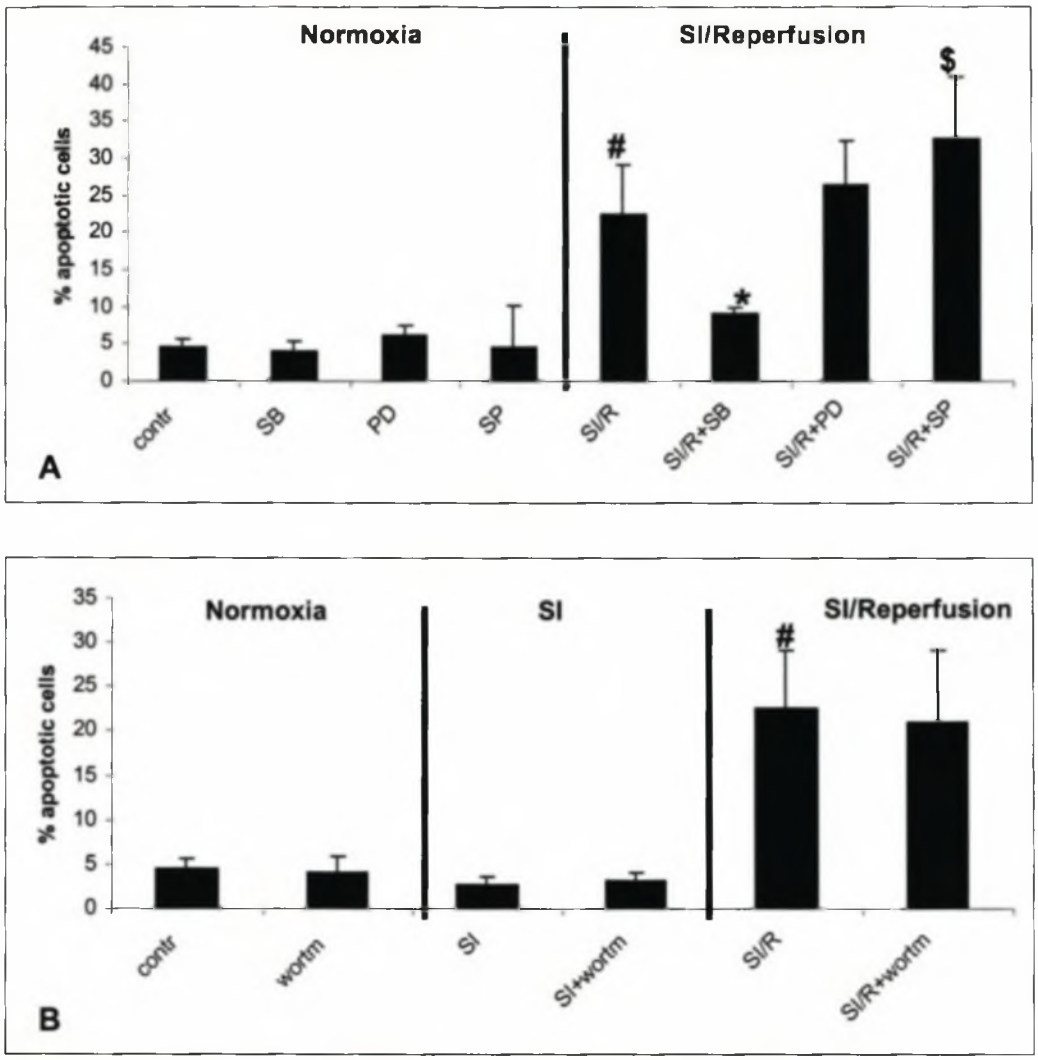


Figure 4.8: The effect of SB203580 (1 μM), PD98059 (10 μM), SP600125 (10 μM) and wortmannin (100 nM) on the apoptotic index during SI and SI/reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) followed by 30 minutes reperfusion. To quantify apoptotic myocytes, cell monolayers were fixed and stained with Hoechst 33342. The morphological features of apoptosis (cell shrinkage, chromatin condensation) were monitored by fluorescence microscopy. At least 400 cells from each of three randomly selected fields per dish were counted, and each treatment was performed in triplicate (~3600 cells/treatment). Results are expressed as means ± S.E.M. for three independent experiments (n=3). **(A):** 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SB203580 ($^{*}p<0.001$); 60'SI/30'reperfusion vs control ($^{\#}p<0.01$); 60'SI/30'reperfusion vs 60'SI/30'reperfusion + SP600125 ($^{\$}p<0.05$) **(B):** 60'SI/30'reperfusion vs control ($^{\#}p<0.01$).

cytochrome a_3, thereby obstructing the electron transport chain and oxidative phosphorylation, while 2-deoxy-*D*-glucose inhibits glycolysis. A major difference from either *in vivo* ischaemia or other models of ischaemia entails a significant decrease in intracellular pH. However, in our model the pH was kept constant at 7.4 due to the use of a buffer. This metabolic inhibition resulted in an immediate time-dependent depletion of intracellular CrP and ATP, reaching a maximum after 30 minutes SI. Although thirty minutes reperfusion caused a significant elevation in CrP, the cells failed to significantly increase ATP levels. Failure of ATP levels to increase significantly during reperfusion has been observed by several other workers; Edoute and co-workers (1983) attributed this failure to depletion of adenine nucleotides and delayed resynthesis in the isolated perfused rat heart model. Despite this severe ischaemic insult induced in the present study, there was no significant loss of detached cells in the medium (supernatant).

The use of isolated cardiac myocytes subjected to simulated ischaemia and reperfusion has some obvious advantages, but also some disadvantages compared to *in vivo* ischaemia. For example, the use of 2-*deoxy*-D-glucose during simulated ischaemia will inhibit glycolysis by trapping phosphate as 2-*deoxy*-D-glucose-phosphate, which is an inhibitor of glycolysis. The 2-*deoxy*-D-glucose-phosphate is retained within the cell during the simulated reperfusion phase of the protocol, and will continue to inhibit glycolysis for a period of time, until the 2-*deoxy*-D-glucose-phosphate is broken down. There is also some evidence that glycolytically-derived ATP may be preferentially used as a substrate for energy-requiring membrane ion transporters, which would impair the myocytes treated with 2-*deoxy*-D-glucose more than myocytes in the isolated heart model. It is also possible that inhibition of glycolysis during the reperfusion phase hampers recovery in this particular model, but this remains to be established.

Furthermore, it must be noted that heterogeneity of cell injury has an impact in models of ischaemia/reperfusion. It could be that a decrease in MTT reduction is due to a generalized decrease in mitochondrial function or it could be due to a small percentage of myocytes with no mitochondrial function, and others with normal or only slightly reduced function. Similarly, an increase in caspase activation could be due to a small amount of caspase activation in all myocytes, or to a large amount of caspase activation in a few myocytes. The problem of heterogeneity of cell injury is inherent to cell models and should be borne in mind in the interpretation of results.

4.3.2 Apoptosis

The stepwise depletion in high-energy phosphates observed in this model of SI prompted us to sequentially evaluate apoptosis and cell viability and to analyse the activation patterns of the MAPKs and the anti-apoptotic kinase, PKB/Akt. Hypoxic injury and intracellular ATP depletion have been shown to induce apoptosis in cultured cardiac myocytes (Shiraishi *et al.*, 2001). Indeed, in our model, ischaemic injury and partial ATP depletion also resulted in the cleavage of PARP to its proteolyzed products, a phenomenon well known to result from caspase-3 activation. Interestingly, both caspase-3 activation and PARP cleavage, albeit low, are evident throughout the SI period. We have demonstrated a significant and specific increase in caspase-3 activation as well as PARP cleavage after 15 minutes of SI in the presence of a 53% reduction in ATP (fig 4.1a & c). Apparently, there is enough residual ATP to maintain ongoing apoptosis which is in accordance with results obtained by Shiraishi and co-workers (2001) who reported that intracellular energy production is required to fuel the apoptotic machinery in ischaemically injured myocytes. The significant increase in DNA fragmentation and chromatin condensation after 30 minutes reperfusion also coincides with a significant rise in CrP (fig.1A), an indicator of resumption of mitochondrial function.

It is of specific interest that in our model, SI-induced caspase-3 activation and PARP cleavage, although associated with decreased mitochondrial viability (as indicated by the MTT assay), preceded any of the visible morphological changes that signify the final stage of apoptosis (see fig.1C & D). It is only in response to 30 minutes reperfusion that the nuclear condensation and fragmentation characteristic of apoptosis can be seen. These results suggest that although the apoptotic cascade was activated early during SI (5-15 minutes), the final execution phase (as indicated by Hoechst staining) was initiated only after reperfusion. The fact that the decrease in viability during SI was not accompanied by an increase in nuclear condensation raises an important point, namely that the MTT assay indicates cells with intact membranes but with compromised mitochondria. However, at this point the mitochondria may possibly still "recover" upon reperfusion, whereas nuclear condensation is irreversible. This may also be an explanation for the fact that there is an increase in cell viability during reperfusion (fig. 1B) – the "recovery of dead cells" reflects mitochondria, which are still able to recover after SI.

4.3.3 MAPKs

We characterized the phosphorylation pattern of the three major MAPK subfamily members during SI/R and found that in response to SI, p38 is activated in a biphasic pattern. Of interest is the observation that p38 was the only MAPK activated significantly during SI, during which time both caspase-3 and PARP cleavage were significantly increased, suggesting a possible causal relationship during this phase of the protocol. The biphasic pattern of p38 activation during simulated ischaemia is in agreement with observations made by Mackay and Mochly-Rosen in cultured neonatal cardiac myocytes (2000).

On the other hand, JNK was phosphorylated in response to 5 minutes reperfusion only and not during SI alone (fig. 2C). This is in contrast with the results obtained by Yue and co-workers (2000) who reported that

cardiomyocytes, exposed to 1% O_2, showed a transient activation of JNK within 5 minutes of hypoxia. These discrepancies may be due to differences in the model of ischaemia used. The ERK pathway, suggested to be required for survival signalling in response to hypoxia/reoxygenation in cardiomyocytes (Abe *et al.*, 2000), was also significantly activated during reperfusion only (fig. 2 C).

The significance of activation of the MAPKs in SI and SI/R-induced apoptosis was further dissected by the use of appropriate inhibitors. PD98059 (an ERK pathway inhibitor) significantly reduced ERK44/ERK42 activation during SI/R, while not affecting p38 and JNK phosphorylation significantly. However, treatment with PD98059 had no significant effect on cell viability or apoptosis (fig. 6 A, 7A & 8A). These observations were contrary to expectations since ERK activation is known to be anti-apoptotic. Among the substrates of ERK, p90 ribosomal S6 kinase (p90RSK) is a ubiquitous and versatile mediator of ERK signal transduction (Frodin & Gammeltoft, 1999) and one of its functions include the phosphorylation of the pro-apoptotic protein BAD at serine 112, which specifically suppresses BAD-mediated apoptosis (Bonni *et al.*, 1999). Yue and co-workers (2000) reported that PD98059 (50 μM) significantly enhanced the activities of both p38 and JNK during hypoxia/reoxygenation, along with an increase in the number of apoptotic myocytes. These discrepancies might be attributed to the hypoxic model used or to the differences in inhibitor concentration (10 μM vs 50 μM) used. Although not significant, PD98059 also caused an inhibition of p38 in our study during SI/R, which might indicate a possible interplay between the MAPK signalling pathways.

SB203580 (an inhibitor of p38) at 1 μM inhibited activation of this kinase during SI/R and reduced caspase-3 and PARP to almost control levels. This inhibition of p38 activation was also associated with a significant improvement in cell viability (fig. 6A) and reduction in the percentage of apoptotic cells (as indicated by Hoechst staining) during SI/R (fig. 8A). These results are consistent with those obtained by Yue and co-workers (2000) who showed that SB203580 rescued

63% myocytes from apoptosis. Despite some results to the contrary (Weinbrenner *et al.*, 1997; Schulz *et al.*, 2002), there is compelling evidence that inhibition of p38 during ischaemia is protective: SB203580 administered before sustained ischaemia decreased infarct size in rat hearts (Minamino *et al.*, 1999, Nakano *et al.*, 2000), while it improves morphology and viability in isolated adult cardiomyocytes (Marais *et al.*, 2001). Furthermore, Schneider and co-workers (2001) also showed by that the inhibition of p38 during sustained ischaemia is protective in the isolated rat heart model. The association between a decrease in p38 activation and a reduction in apoptosis has also been demonstrated in a fibroblast cell line (Han *et al.*, 2001).

SP600125 at a concentration of 10 μM inhibited JNK (both isoforms) phosphorylation (fig. 4A) and significantly increased caspase-3 activation (fig. 7A) as well as the apoptotic index during reperfusion (fig. 8A). These results suggest that activation of JNK phosphorylation promotes survival of neonatal cardiomyocytes during reperfusion. Although several other groups correlated increased JNK activity with apoptosis (He *et al.*, 1999; Hreniuk *et al.*, 2001; Vaishnav *et al.*, 2003) our results are in agreement with the recent study published by Dougherty and co-workers (2002) who reported for the first time that JNK activation in neonatal cardiac myocytes subjected to chronic hypoxia correlated with myocyte survival. These observations were also substantiated by a study on embryonic stem cell derived cardiac myocytes lacking MEKK1, and with significant suppressed JNK activity, which showed sensitization to H_2O_2 treatment (Minamino *et al.*, 1999). Interestingly, Dougherty and co-workers also reported that the activation of JNK during reperfusion is largely independent of p38 (2002).

The targets that determine the effects of JNK and p38 on cell survival or apoptosis are still not clear in cardiomyocytes. It has been shown that p38 can translocate to the mitochondria and phosphorylate Bcl-2 on Ser-87 and Thr-56 which causes cytochrome c release, subsequent activation of downstream

caspases and enhanced apoptosis in B lymphocytes (Torcia *et al.*, 2001). Fiordaliso and co-workers also showed that hyperglycemia can lead to the phosphorylation of p53 at Ser 390 via p38 and this was paralleled by an increase in Bax-mediated myocyte cell death (2001). However, it is not yet known whether p38 targets mitochondria in cardiac myocytes subjected to ischaemia and reperfusion.

4.3.4 PKB/Akt

Several events occur simultaneously during the first 5 minutes of reperfusion, including a decrease in caspase-3 activation and PARP cleavage (fig. 1C), significant stimulation of all the MAPKs as well as PKB/Akt (fig. 2A-C and 3A,B). However, after 15 minutes of reperfusion, caspase-3 activation and PARP cleavage were significantly elevated which coincided with a marked reduction in PKB/Akt phosphorylation (fig 4.1c & 4.3). These observations are in agreement with the suggested pivotal role of PKB/Akt activation in cell survival. Although transient, PKB/Akt phosphorylation appears to be important in preserving cell viability in our model, since inhibition of this phenomenon with wortmannin led to a significant depression in the percentage viable cells and increased PARP cleavage. PKB/Akt phosphorylation also appears to be more important than ERK phosphorylation during reperfusion since inhibition of the latter with PD98059 showed no effect on cell viability or apoptosis.

Full activation of PKB/Akt requires phosphorylation on two regulatory sites, Thr^{308} in the activation loop and Ser^{473} in the hydrophobic domain. However, the contribution of each site towards PKB/Akt activation is not equal, while phosphorylation on Thr^{308} alone is able to increase PKB/Akt activity, phosphorylation on Ser^{473} alone does not significantly stimulate the kinase (Alessi *et al.*, 1996). Furthermore, Hill and co-workers also demonstrated that pretreatment with 1 μM staurosporine abolished insulin-stimulated PKB activation without affecting Ser^{473} phosphorylation (Hill *et al.*, 2001). Therefore, we

examined both phosphorylation sites on PKB in our cell model. Interestingly, wortmannin (PI3-kinase inhibitor) significantly inhibited Thr^{308} but not Ser^{473} phosphorylation during reperfusion, indicating that part of PKB/Akt activation is independent of PI3-kinase in our cell model. PKB is activated downstream of PI3-kinase by the phosphoinositide-dependent protein kinases PDK-1 and PDK-2 (Anderson *et al.*, 1998). PKB in turn phosphorylates a number of downstream targets relevant to cell survival functions, including the pro-apoptotic Bcl-2 family member BAD (del Peso *et al.*, 1997). Phosphorylation of BAD on Ser^{136} by PKB inhibits its pro-apoptotic function, thus promoting cell survival (Datta *et al.*, 1997). Interestingly, BAD is not only a substrate for PKB, but is also phosphorylated by the MAPK kinase MEK (Punn *et al.*, 2000), linking the classical Ras-MAPK pathway to cell survival. Another interesting observation is that PKB inhibition by wortmannin caused a significant increase in PARP cleavage and reduction in cell viability, without a significant effect on either caspase activation or the apoptotic index at 30 minutes reperfusion. This suggests that reperfusion-induced apoptosis occurs partially independent of PI3-kinase. However, it is possible that an increase in the apoptotic index may be seen if the reperfusion period is extended.

4.4 Summary

We have demonstrated that SI and reperfusion in neonatal cardiomyocytes is characterized by apoptosis, particularly during reperfusion. Apoptosis is most likely regulated by interplay between the concomitant changes in the activation pattern of p38, JNK, ERK and PKB. This is substantiated by p38 inhibition, which is associated with a significant improvement of cell viability and attenuation of apoptosis. On the other hand, JNK inhibition caused an increase in caspase-3 activation and apoptosis during SI/R, while PKB inhibition significantly increased PARP cleavage and reduced cell viability. However, ERK inhibition failed to affect apoptosis during SI/R in this particular model. These results indicate that p38 is pro-apoptotic during and following SI/R-induced injury, whereas JNK

activation limits SI/R-induced damage through the inhibition of caspase-3 activation. We have also demonstrated that two distinct apoptotic-regulatory proteins are activated during SI/R. Whereas the monophasic activation of JNK (within 5 minutes of reperfusion) seems to induce protection against subsequent apoptotic events, the biphasic and sustained activation of p38 observed from the onset of SI and throughout reperfusion, strongly induced apoptotic events, thereby overriding the beneficial anti-apoptotic properties of concomitant JNK activation.

CHAPTER 5 RESULTS AND DISCUSSION: Long-chain polyunsaturated fatty acids (PUFAs) attenuate ischaemia/reperfusion-induced apoptosis in neonatal cardiomyocytes via a mitogen activated protein kinase (MAPK) dependent pathway.

5.1 Introduction

Coronary heart disease (CHD) remains one of the leading causes of death in all Western industrialised countries. Although epidemiology and human intervention trials consistently show an inverse relationship between dietary omega-3 fatty acid consumption and mortality from heart disease (Burr *et al.*, 1989; Siscovick *et al.*, 1995; Daviglus *et al.*, 1997; Pepe & McLennan, 2002), the mechanism of the beneficial actions of these fatty acids remains to be elucidated.

Long-chain polyunsaturated fatty acids (PUFAs), taken up by the cell from the extracellular environment or released from cellular triglycerides or membrane phospholipids play an important role in various biological processes in cardiac muscle cells. Not only do they serve as fuel or constituents of membrane phospholipids, but they are also involved in cellular signal transduction. In this regard, fatty acids can act either as second messengers or reversible modulators to amplify, attenuate or deviate a signal at a precise intracellular location (Sumida et al., 1993).

As stated previously, the mitogen-activated protein kinases (MAPKs) are a family of serine-threonine kinases that are activated in response to a variety of extracellular stimuli, (Robinson & Cobb, 1997; Ip & Davis 1998) including during ischaemia and reperfusion of the heart (Bogoyevitch *et al.*, 1996; Knight & Buxton, 1996). The results presented in chapter 4, suggested that activation of the MAPKs, particularly p38, during simulated ischaemia and reperfusion of

neonatal cardiomyocytes, plays a pivotal role in the development of apoptosis in this particular model. Manipulation of the activation state of these kinases by phosphatases may present a possible site of action for the beneficial effects of PUFAs. It is known that a family of dual-specificity phosphatases becomes transcriptionally induced, leading to dephosphorylation and inactivation of specific MAPKs within 30-60 minutes (Haneda *et al.*, 1999). Currently ≈9 dual-specificity phosphatase family members have been described, each of which has a slightly different substrate specificity, tissue distribution, subcellular localization, or inducible expression profile (Haneda *et al.*, 1999). MKP-1 (mitogen-activated protein kinase phosphatase-1) is an important member of this family, which regulates inactivation of p38, JNK and ERK (Chu *et al.*, 1996; Franklin & Kraft, 1997; Li *et al.*, 1999). However, very little or any information is available regarding the effects of long-chain PUFAs on phosphatases in the heart.

Another potential target of long-chain PUFAs might be the serine/threonine kinase PKB/Akt. PKB/Akt contains a pleckstrin homology (PH) domain that is part of a slightly larger portion in the NH_2 terminus, called the Akt homology domain. The phosphoinositide 3-kinase (PI3-K) product phosphatidylinositol-3,4-bisphosphate binds *in vitro* directly to the PH domain and increases enzyme activity (Downward, 1998). PKB/Akt has been shown to be activated by factors that stimulate PI3-K, including thrombin, platelet-derived growth factor, and insulin (Downward, 1998). There is also increasing evidence that the PKB/Akt pathway participates in tissue salvage during ischaemia/reperfusion-induced injury (Brar *et al.*, 2002; Andreucci *et al.*, 2003).

Only a few culture-based studies have investigated the effect of EPA or ARA on cardiac myocytes. Chen and co-workers (2003) have recently showed that EPA inhibits hypoxia/reoxygenation-induced injury by attenuating upregulation of MMP-1 in adult rat cardiac myocytes, while Mackay and Mochly-Rosen (2001) demonstrated that ARA protects neonatal rat cardiac myocytes from ischaemic injury through activation of PKCε. As far as we know, no evidence exists for an

interaction between EPA or ARA and the activation/inhibition of the MAPKs and PKB/Akt in neonatal rat cardiomyocytes during simulated ischaemia (SI) and reperfusion. Therefore, in order to assess the mechanisms of protection of long-chain polyunsaturated fatty acids (PUFAs) in injured/apoptotic heart cells, we treated neonatal cardiomyocytes with EPA and ARA prior to and after simulated ischaemia and determined their effects on cell viability, apoptosis and the activation of the MAPKs and PKB/Akt.

5.2 Results

5.2.1 Fatty acid composition (Table 5.1)

To assess whether in our particular model, fatty acids are incorporated into membrane phospholipids (PL), the fatty acid composition of the phospholipids of the myocytes were determined after a 60 minute incubation period. Results are presented in table 5.1. The cardiomyocytes grown in n-3 culture medium incorporated n-3 fatty acids in their PL which resulted in a phospholipid composition displaying high levels in EPA ($p<0.001$) and C22:5n-3 ($p<0.01$). Conversely, the cardiomyocytes grown in n-6 enriched culture medium were characterised by high levels of ARA ($p<0.001$) and C22:4n-6 ($p<0.01$) in their PL. Interestingly, there was a significant decrease in oleic acid (C18:1n-9) when myocytes were incubated with either ARA or EPA ($p<0.001$).

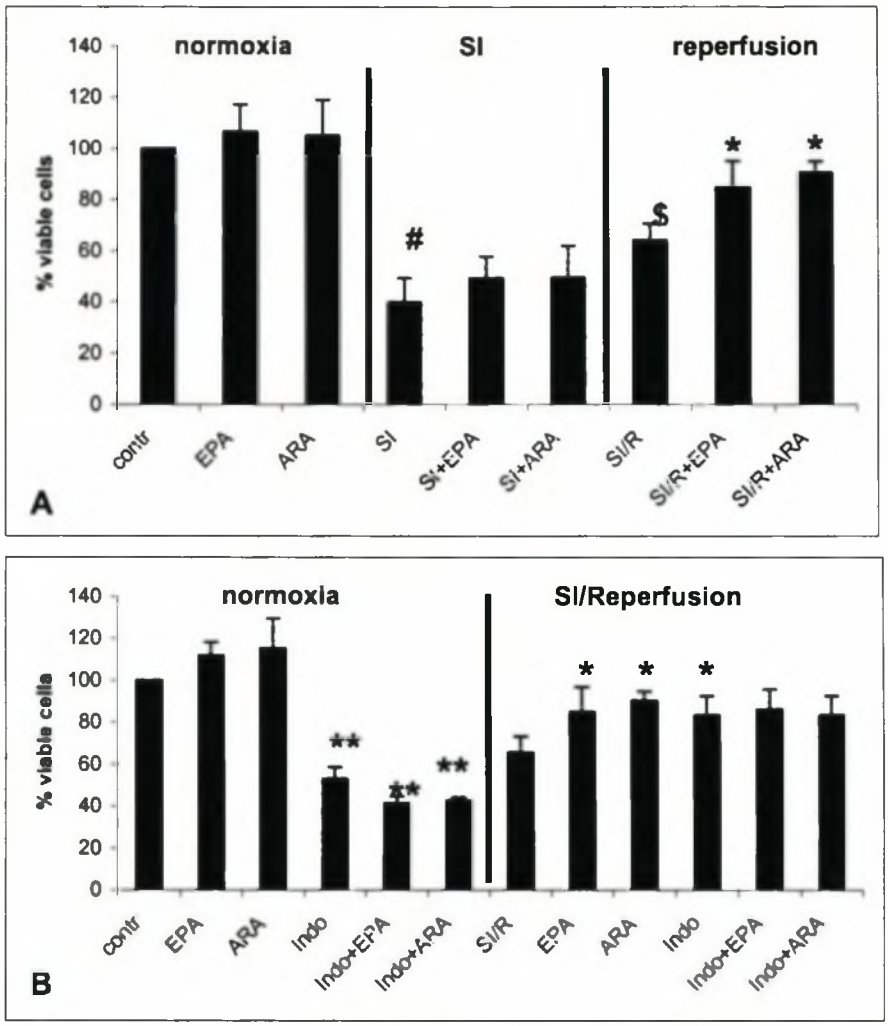
5.2.2 The effect of EPA, ARA and indomethacin on cell viability during simulated ischaemia (SI) and reperfusion (Figures 5.1 A & B)

To establish whether EPA and ARA induced protection in our particular cell model, the MTT cell viability assay was used. In this study, fatty acids were bound to albumin at a ratio of 3.3:1 [albumin has three high-affinity binding sites for fatty acids and another three or four lower affinity binding sites (Cistola *et al.*, 1987)].

Table 5.1. Fatty acid composition of the total phospholipids of control cells, ARA-treated cells and EPA-treated cells

Fatty acid	Control	ARA cells	EPA cells	ANOVA
C16:00	13.10±0.57	12.11±0.73	14.27±1.63	ns
C18:00	14.0±0.31	13.68±0.36	11.78±0.87	ns
C18:1n-9	19.75±1.62	*12.74±1.84	*7.62±0.53	p<0.001
C18:2n-6	3.29±0.22	2.79±0.16	2.34±0.20	ns
C20:4n-6	**10.58±0.43**	***18.86±1.18**	**7.92±0.91**	**p<0.001**
C20:5n-3	**0.85±0.14**	**0.58±0.10**	***8.12±0.72**	**p<0.001**
C22:4n-6	1.83±0.15	*3.39±0.40	1.19±0.17	p<0.01
C22:5n-3	2.28±0.24	2.36±0.39	*5.91±0.76	p<0.01
C22:6n-3	3.28±0.19	2.89±0.53	0.81±0.28	ns

Table 5.1: Fatty acid composition of the phospholipids of rat cardiomyocytes incubated under normoxic conditions for 1 hour in ARA- and EPA (20 μM for both fatty acids) enriched media respectively, in comparison with the phospholipids of cardiomyocytes incubated in standard DMEM medium. Data are expressed as percentage of total fatty acids. Results are expressed as means ± S.E.M. for six independent experiments (n=6). *p<0.001 vs control.



Figures 5.1 A & B: The effect of EPA (20 μM), ARA (20 μM) and Indomethacin (10 μM) on cell viability during SI and reperfusion. Cell viability was measured during SI (simulated ischaemia) and reperfusion, using the MTT cell viability assay. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. Fatty acids were added to the cells for 60 minutes before the onset of SI as well as during reperfusion. Indomethacin was added to the cells for 30 minutes prior to the addition of the fatty acids as well as during fatty acid incubation. **Fig 5.1A:** control SI vs control normoxia ($^{\#}p<0.001$); control SI/R vs control normoxia ($^{\$}p<0.001$); SI/R + EPA and SI/R + ARA vs control SI/R (*p<0.001). **Fig 5.1B:** Indomethacin, EPA+Indomethacin and ARA+Indomethacin vs control normoxia (**p<0.001); Indomethacin, EPA and ARA vs control SI/R. Results are expressed as means ± S.E.M. for six independent experiments (n=6).

Neither EPA nor ARA had an effect on cell viability during SI in our cell model. However, both fatty acids significantly improved cell viability during SI/R [64.13 ± 1.66% to 85.09 ± 3.07% ($p<0.001$) for EPA and to 93.7 ± 0.33% ($p<0.001$) for ARA]. To ensure that eicosanoid biosynthesis was not involved in the fatty acid induced-protection during SI/R, we pre-treated the cells with a specific cyclooxygenase-inhibitor, indomethacin (10 μM). Indomethacin was added to the cells for 30 minutes prior to the fatty acids incubation period as well as during reoxygenation. Interestingly, indomethacin alone significantly reduced cell viability under normoxic conditions, both in the presence and absence of the fatty acids, while having no significant effect on fatty acid-induced protection during SI/R.

5.2.3 The effect of EPA and ARA on caspase-3 activation, PARP cleavage and the apoptotic index during simulated ischaemia and reperfusion (Figures 5.2 A, B and C)

As was also shown in chapter 4, exposure of neonatal cardiomyocytes to 60'SI followed by 30'reperfusion, caused a significant increase in caspase-3 activation and PARP cleavage during SI/R ($p<0.001$ for both). EPA and ARA significantly attenuated caspase-3 activation during SI/R ($p<0.01$ for both). Both fatty acids also significantly inhibited PARP-cleavage during SI [11.97 ± 0.40 fold to 4.66 ± 1.68 fold ($p<0.001$) for EPA and to 4.76 ± 0.34 fold ($p<0.001$) for ARA]. Cardiac myocytes, stained with Hoechst 33342 and assessed by fluorescence microscopy, showed condensed chromatin and fragmented nuclei after exposure to SI and reperfusion. During normoxia (with and without the fatty acids), 4.6% ± 0.50% cells showed spontaneous cell death (basal). The fatty acids had no effect on the percentage of apoptotic cells during exposure to SI. However, the significant increase in apoptotic cells ($p<0.001$) observed after 60 minutes SI followed by 30 minutes reperfusion was markedly reduced by EPA and ARA ($p<0.001$ for both EPA and ARA vs SI/R). Thus, EPA and ARA were equally effective in protecting the neonatal cardiomyocytes from apoptosis.

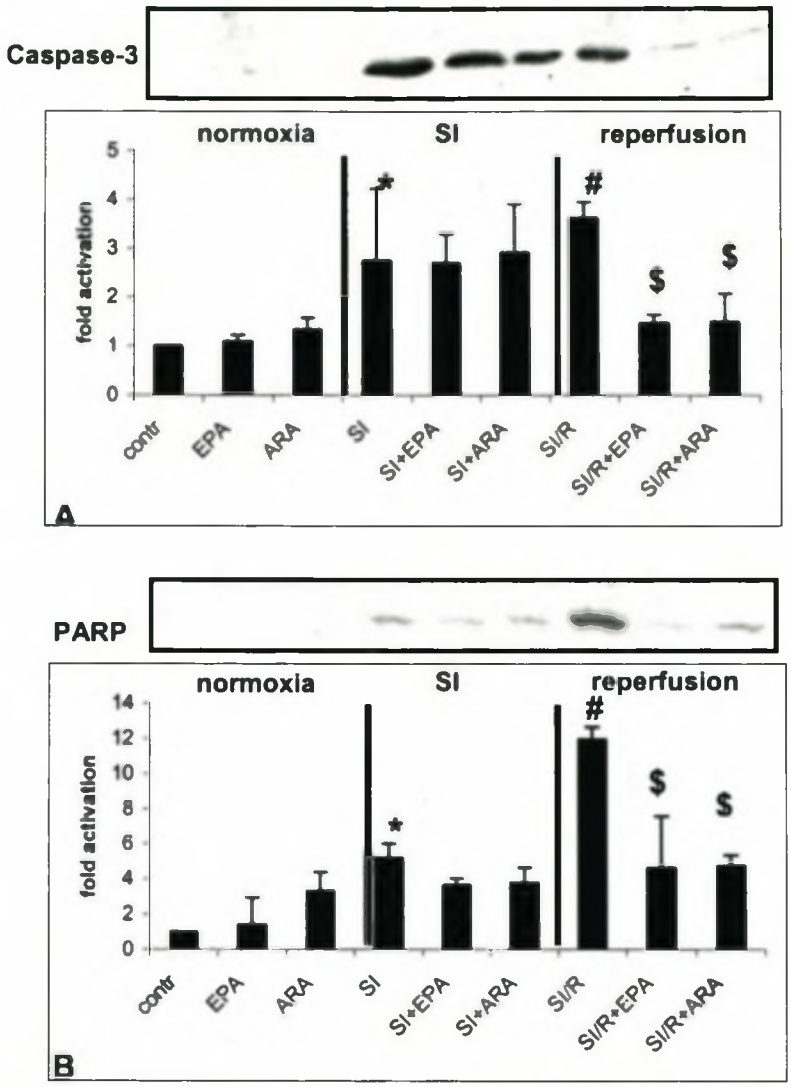


Figure 5.2 A, B: The effect of EPA and ARA on caspase-3 activation and PARP cleavage during SI/Reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. Samples were analysed by Western blotting with antibodies recognizing cleaved PARP and caspase-3. Results are expressed as means ± S.E.M. for three independent experiments (n=3). **Caspase 3:** SI vs control normoxia ($^{*}p<0.05$); SI/R vs control normoxia ($^{\#}p<0.001$); SI/R + EPA and SI/R +ARA vs control SI/R ($^{\$}p<0.01$). **PARP:** SI vs control normoxia ($^{*}p<0.05$); SI/R vs control normoxia ($^{\#}p<0.001$); SI/R + EPA and SI/R + ARA vs control SI/R ($^{\$}p<0.001$).

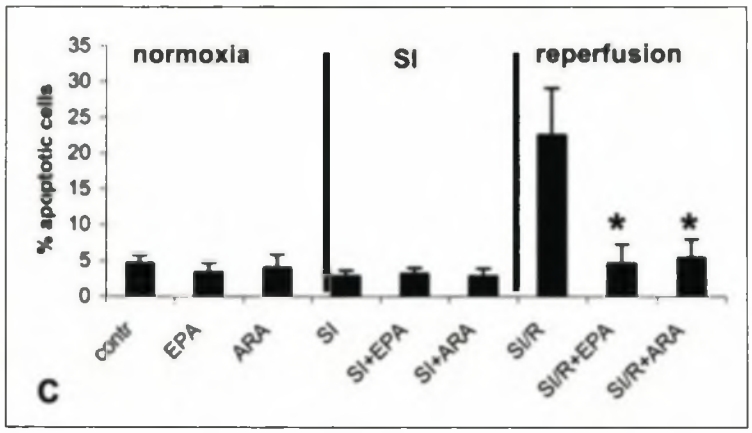


Figure 5.2 C: The effect of EPA and ARA on SI/Reperfusion-induced apoptosis in cardiac myocytes. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. To quantify apoptotic myocytes, cell monolayers were fixed and stained with Hoechst 33342. The morphological features of apoptosis (cell shrinkage, chromatin condensation) were monitored by fluorescence microscopy. At least 400 cells from three randomly selected fields per dish were counted and each treatment was performed in triplicate. Results are expressed as means ± S.E.M. for three independent experiments (n=3). SI/R + EPA and SI/R + ARA vs control SI/R (*$p<0.001$).

5.2.4 The effect of EPA and ARA on the phosphorylation of ERK, p38 and JNK in cardiomyocytes subjected to simulated ischaemia and reperfusion (Figures 5.3 A, B and C)

After having established that both EPA and ARA increased cell viability and reduced apoptosis during exposure of cardiomyocytes to simulated ischaemia and reperfusion, the aim of the next series of experiments was to establish the role of the MAPKs in their protective action. Phosphorylation of ERK 1/2 (p42/p44-MAPK), p38 and JNK (p46/p54-MAPK) was determined by Western blotting using phospho-specific antibodies. As shown before (chapter 4) SI and reperfusion significantly increased ERK and p38 phosphorylation, while JNK was significantly phosphorylated only during reperfusion. As shown in figure 5.3 A, EPA and ARA caused a significant increase in phosphorylation of ERK p42 ($p<0.01$ and $p<0.05$ for EPA and ARA respectively) and ERK p44 ($p<0.01$ and $p<0.05$ for EPA and ARA respectively) during SI/R. Both fatty acids attenuated p38 phosphorylation during both SI ($p<0.001$ and $p<0.01$ for EPA and ARA respectively) and reperfusion ($p<0.05$ for both EPA and ARA). JNK was phosphorylated only during reperfusion and phosphorylation of both JNK p46 and JNK p54 remained unaffected by EPA and ARA during this period. As observed previously, exposure of the cells to SI/R, had no effect on the total amounts of the three kinases investigated.

5.2.5 PKC, an upstream mediator in ARA-induced MAPK phosphorylation (Figure 5.4 A, B)

To establish the possible contribution of PKC to the effects of polyunsaturated fatty acids on MAPK phosphorylation, chelerythrine, an inhibitor of PKC catalytic activity, was used. Chelerythrine (10 μM) was added 30 minutes prior to and during EPA and ARA treatment, before and after SI. Interestingly, chelerythrine on its own, significantly decreased cell viability during SI/R ($p<0.01$), but had no

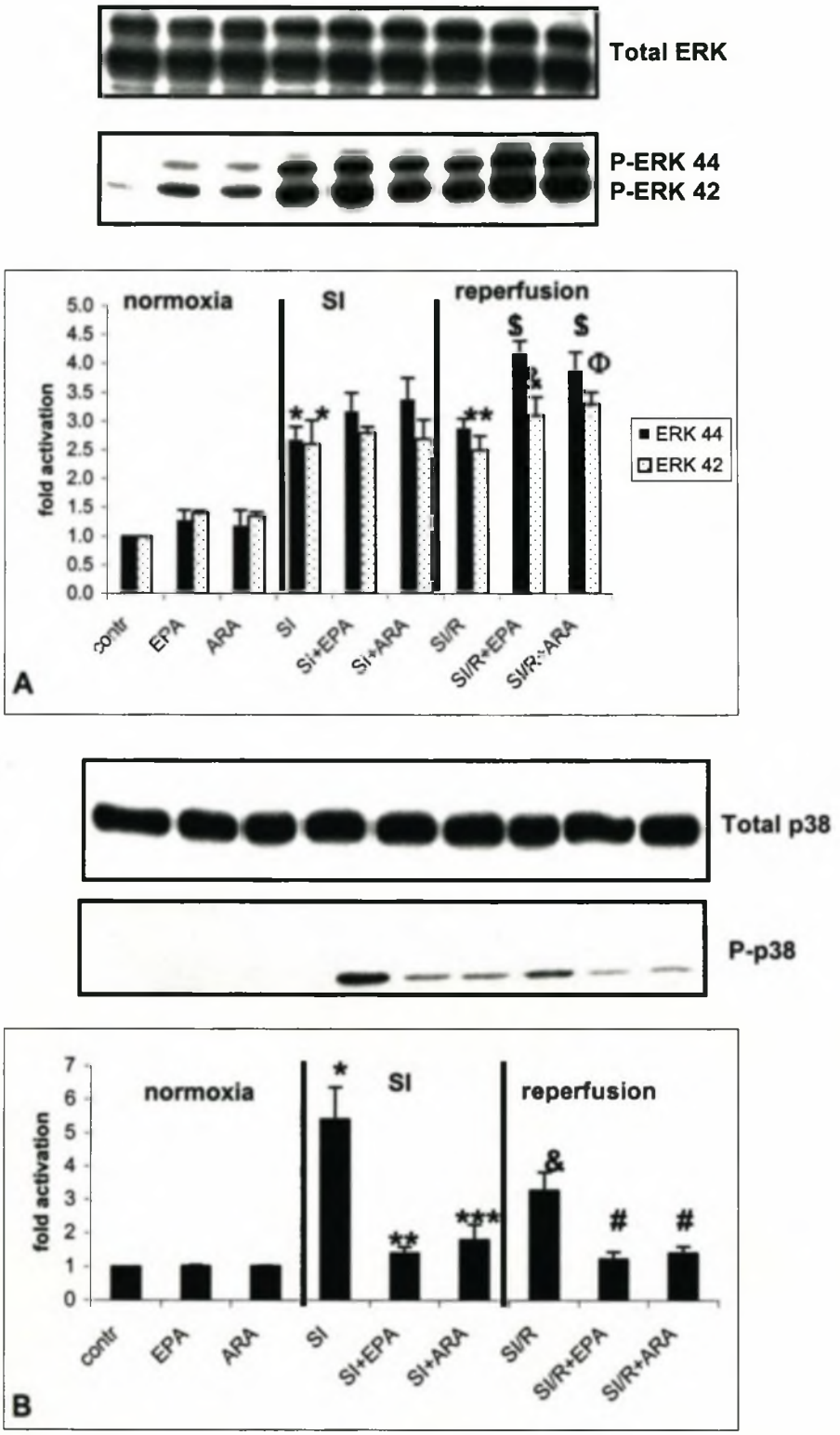


Figure 5.3

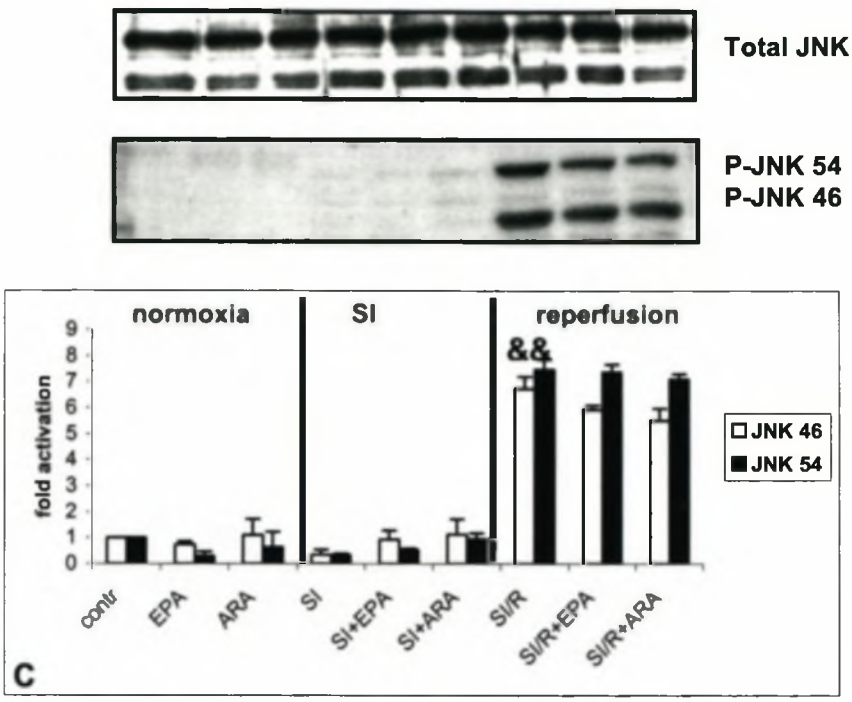


Figure 5.3 A, B and C: The effect of EPA and ARA on p38, ERK and JNK phosphorylation in cardiomyocytes subjected to simulated ischaemia and reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. Samples were analysed by Western blotting with phospho-specific antibodies recognizing dual-phosphorylated MAPKs. Results are expressed as means ± S.E.M. for four to six independent experiments (n=4-6). **ERK 42:** SI and SI/R vs control normoxia (*$p<0.001$); SI/R + EPA vs control SI/R ($^{\&}p<0.01$); SI/R + ARA vs control SI/R ($\Phi p<0.05$). **ERK 44:** *$p<0.001$ vs control normoxia; $^{\#}p<0.001$ vs control normoxia; $^{\$}p<0.001$ vs control SI/R. **p38:** SI vs control normoxia (*$p<0.001$); SI/R vs control normoxia ($^{\&}p<0.05$); SI + EPA vs control SI (**$p<0.001$); SI + ARA vs control SI (***$p<0.01$); SI/R + EPA and SI/R + ARA vs control SI/R ($^{\#}p<0.05$). **JNK 46:** SI/R vs control normoxia ($^{\&}p<0.001$). **JNK 54:** SI/R vs control normoxia ($^{\&}p<0.001$).

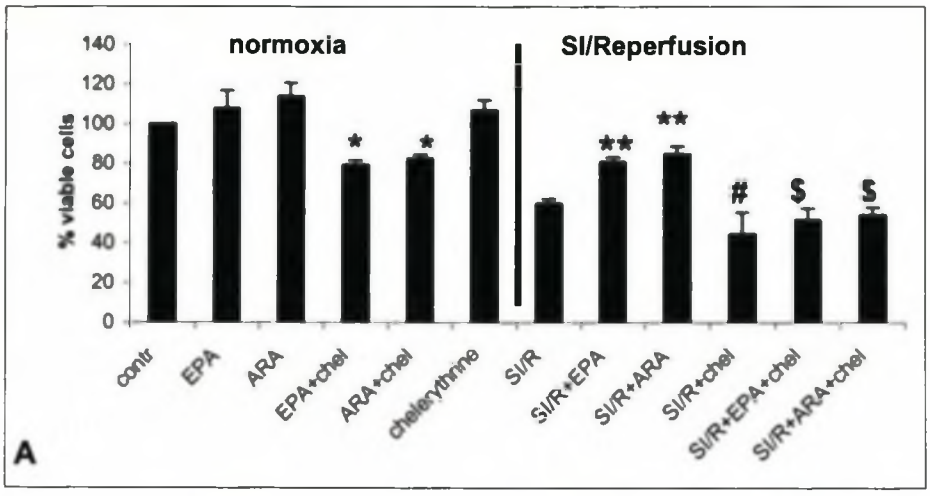


Figure 5.4 A: The effect of PKC inhibition on cell viability using the MTT assay during simulated ischaemia and reperfusion. Chelerythrine (10 μM) was added to the cells for 30 minutes prior to SI as well as during reperfusion. Results are expressed as means ± S.E.M. for three independent experiments (n=3). SI/R + chelerythrine vs control SI/R ($^{\#}p<0.01$); EPA + chelerythrine and ARA + chelerythrine vs control normoxia ($^{*}p<0.001$); SI/R + EPA and SI/R + ARA vs control SI/R ($^{**}p<0.001$); SI/R + EPA + chelerythrine and SI/R +ARA + chelerythrine vs SI/R + EPA and SI/R +ARA respectively ($^{\$}p<0.001$).

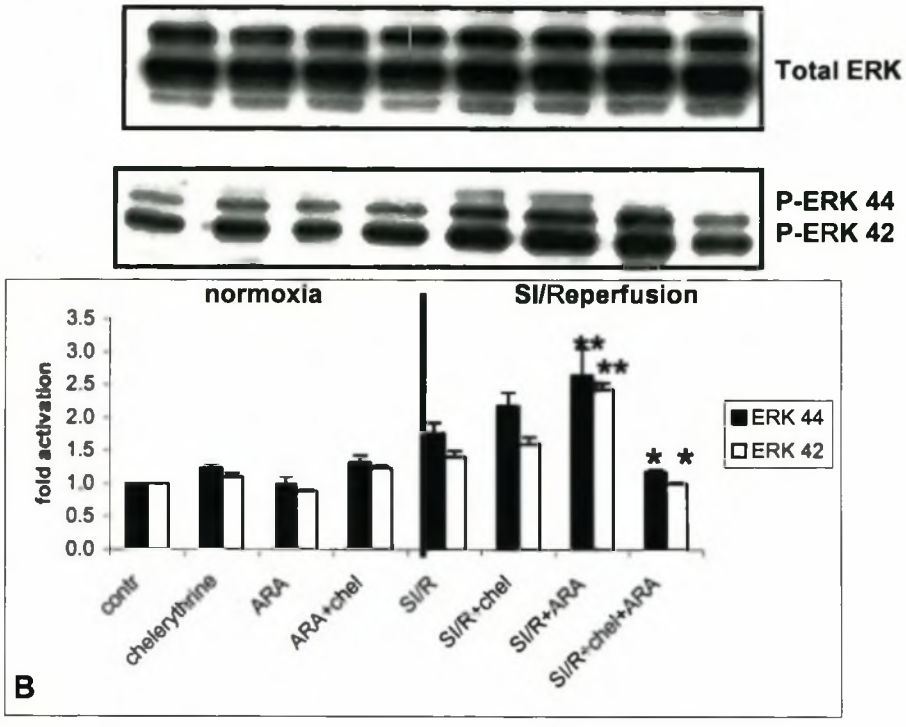


Figure 5.4 B: Effect of PKC inhibition on MAPK phosphorylation during simulated ischaemia and reperfusion. Simulated ischaemia and reperfusion was carried out either alone or following treatment with chelerythrine (10 μM) or/and ARA (20 μM). Results are expressed as means ± S.E.M. for three independent experiments (n=3). SI/R + chelerythrine + ARA vs SI/R + ARA (*$p<0.001$); SI/R + ARA vs control SI/R (**$p<0.001$).

effect during normoxia. Chelerythrine significantly attenuated EPA- and ARA-induced increase in cell viability ($p<0.001$) during normoxia as well as during SI/R as shown in figure 5.4 A. Although chelerythrine significantly inhibited ARA-induced ERK phosphorylation ($p<0.001$ for both ERK-p44 and ERK-p42) (fig 5.4 B), neither p38, nor JNK phosphorylation was affected by PKC inhibition. Similar results were also obtained with EPA (data not shown).

5.2.6 Phosphatases as mediators in ARA-induced MAPK inhibition (Figure 5.5 A, B, C and D)

To determine whether phosphatases were involved in EPA and ARA induced inhibition of p38, we made use of tyrosine- (orthovanadate) and serine-threonine phosphatase inhibitors (okadaic acid). Vanadate (100μM) did not alter cell viability under normoxia, although it significantly improved viability when ARA was added ($p<0.01$). Vanadate significantly inhibited ARA-induced protection during SI/R [84.67% ± 8.51% to 57% ± 4.58% ($p<0.01$)] as shown in figure 5.5 A. Okadaic acid did not have any significant effects under normoxia or during SI/R. As expected, vanadate significantly increased p38 ($p<0.001$) as well as JNK phosphorylation (data not shown) during normoxia. Vanadate also caused a significant increase in p38 phosphorylation when compared with ARA-induced inhibition of p38 (fig 5.5 B) during SI/R [1.49 ± 0.15 fold to 2.72 ± 0.14 fold ($p<0.05$)], but had no significant effect on JNK phosphorylation (data not shown). Both EPA and ARA significantly increased MKP-1 induction during SI/R (fig 5.5 C). Interestingly, vanadate significantly inhibited ARA-induced MKP-1 induction (fig 5.5 D) during SI/R [3.75 ± 0.15 fold to 2.5 ± 0.2 fold ($p<0.05$)], but had no effect under normoxic conditions.

5.2.7 *In vitro* dephosphorylation of p38 (Figure 5.6)

To determine whether MKP-1 upregulation was responsible for attenuation of p38 phosphorylation, we co-incubated the samples where the kinase was

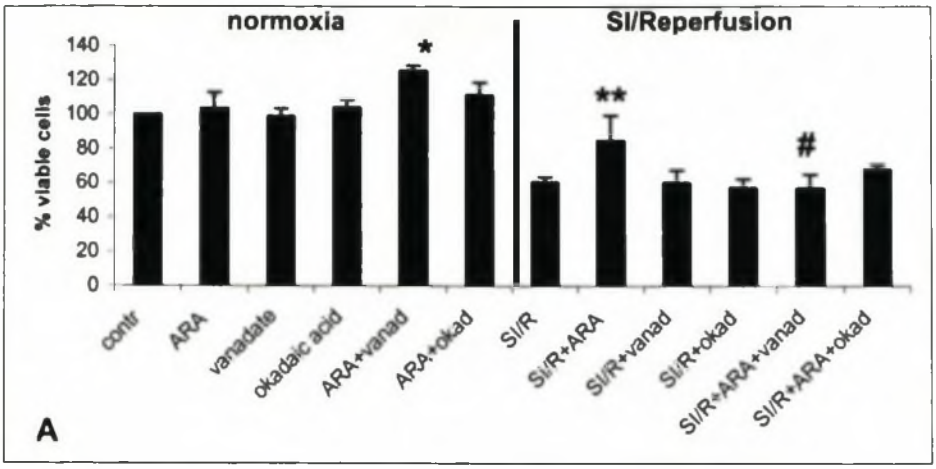


Figure 5.5 A: Effect of okadaic acid and vanadate on cell viability using the MTT assay during SI/Reperfusion. Okadaic acid (1 μM), an inhibitor of types 1 and 2A serine/threonine phosphatases or orthovanadate (100 μM), a specific inhibitor of tyrosine phosphatases was added to the cells 30 minutes prior to and during fatty acid treatment. Inhibitors were also present during 30 minutes of reperfusion. Results are expressed as means ± S.E.M. for three independent experiments (n=3). ARA + vanadate vs control normoxia (*$p<0.01$); SI/R + ARA vs control SI/R (**$p<0.01$); SI/R + ARA + vanadate vs SI/R + ARA ($^{\#}p<0.01$).

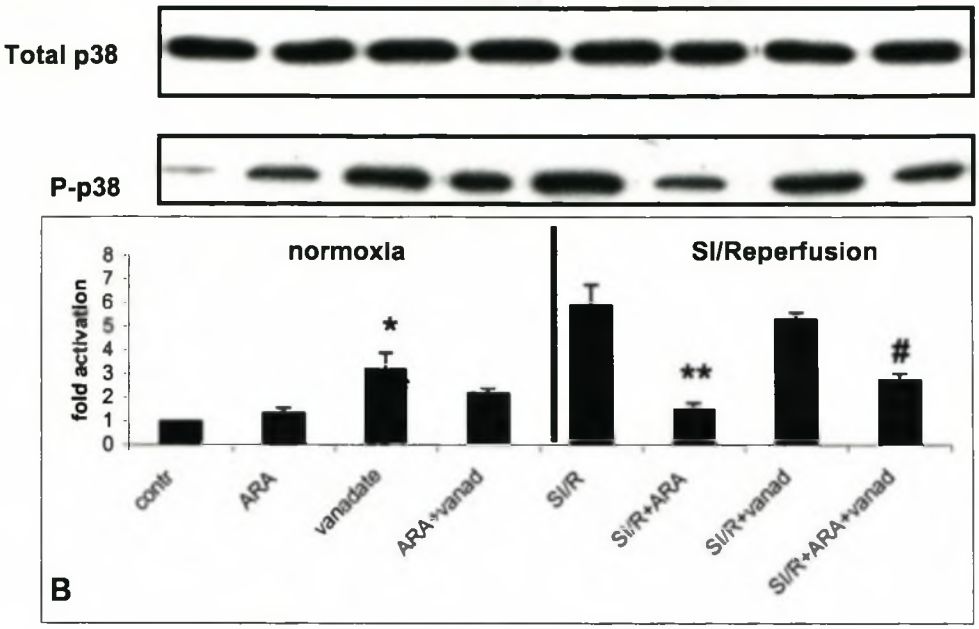


Figure 5.5 B: Effect of vanadate on p38 phosphorylation during SI/R. Simulated ischaemia and reperfusion was carried out either alone or following treatment with vanadate and/or ARA. Orthovanadate (100 μM), a specific inhibitor of tyrosine phosphatases were added to the cells 30 minutes prior to and during fatty acid treatment. Inhibitor was also present during 30 minutes of reperfusion. Results are expressed as means ± S.E.M. for three independent experiments (n=3). vanadate vs control normoxia (*$p<0.001$); SI/R + ARA vs control SI/R (**$p<0.001$); SI/R + ARA + vanadate vs SI/R + ARA ($^{\#}p<0.05$).

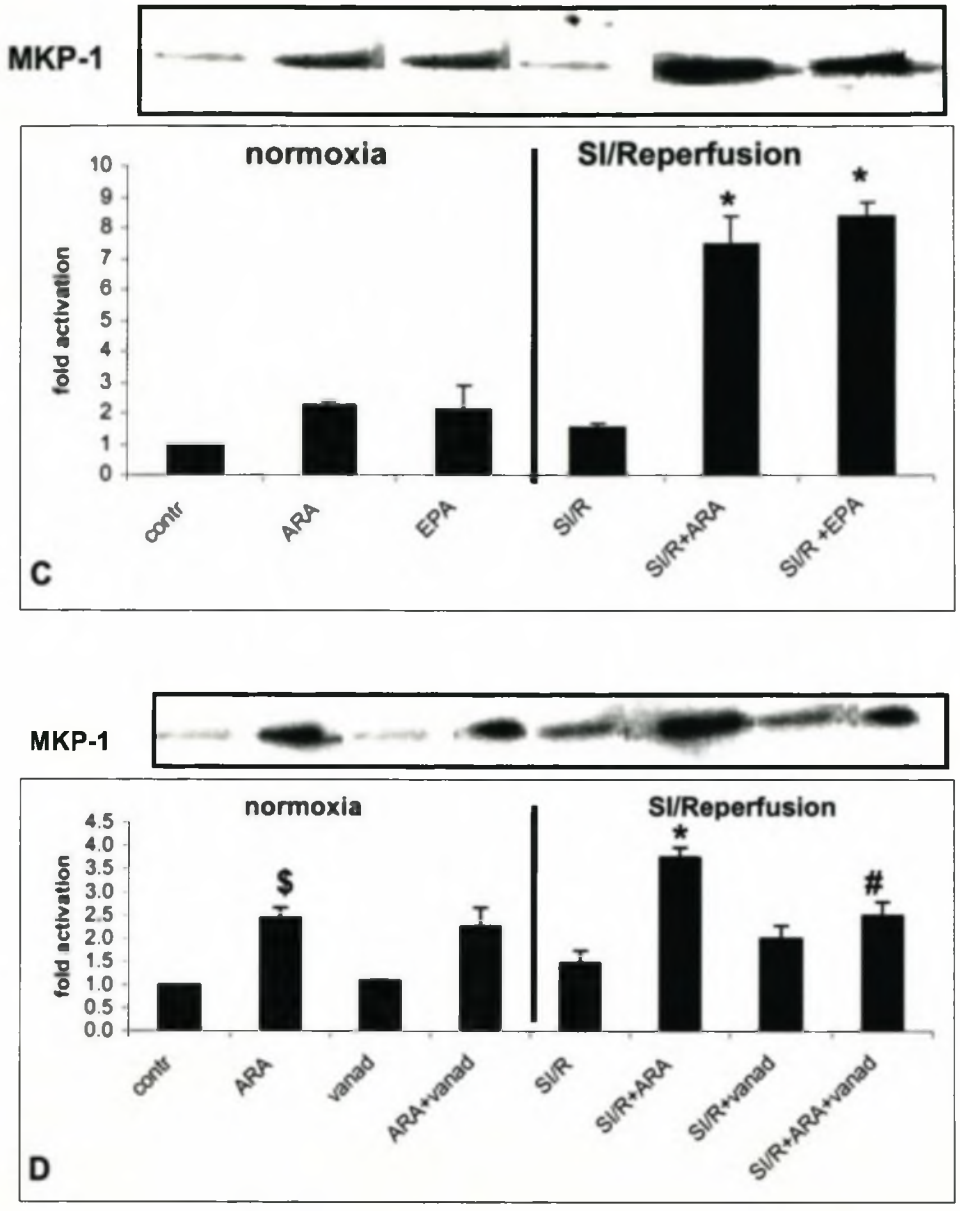


Figure 5.5 C & D: Effect of ARA and vanadate on MKP-1 induction during SI/R. Simulated ischaemia and reperfusion was carried out either alone or following treatment with vanadate and/or ARA or EPA. Orthovanadate (100 μM), a specific inhibitor of tyrosine phosphatases were added to the cells 30 minutes prior to and during fatty acid treatment. Inhibitor was also present during 30 minutes of reperfusion. Results are expressed as means ± S.E.M. for three independent experiments (n=3). SI/R + ARA and SI/R + EPA vs control SI/R (*$p<0.001$); SI/R + ARA + vanadate vs SI/R + ARA ($^{\#}p<0.05$); ARA vs control normoxia ($^{\$}p<0.01$).

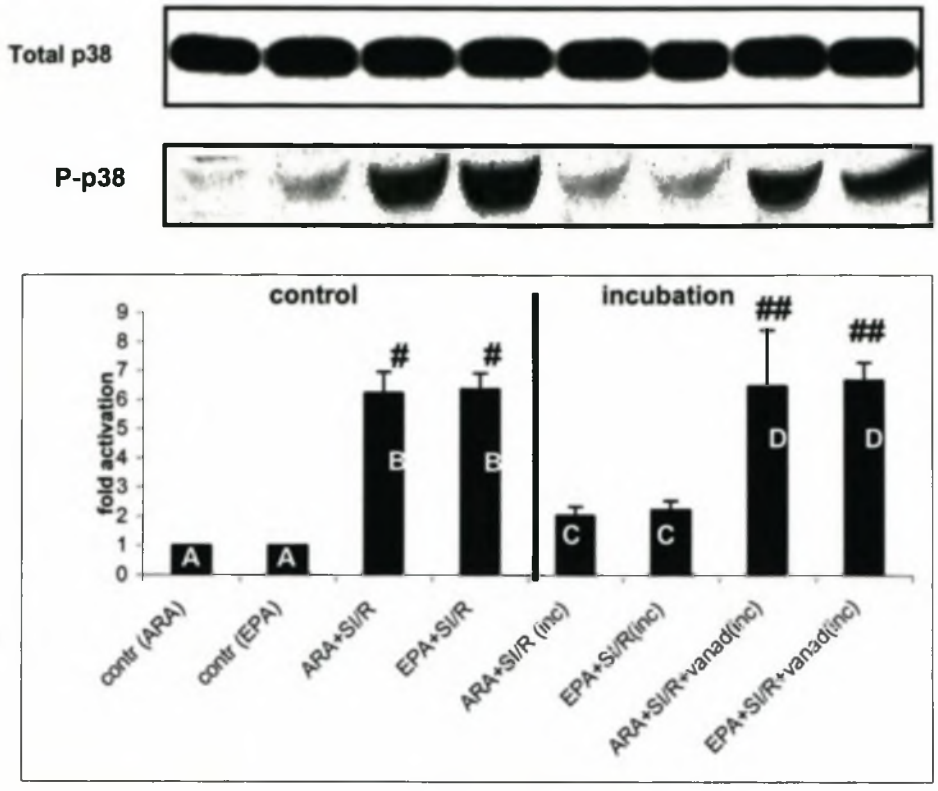


Figure 5.6: *In vitro* dephosphorylation of p38 during SI/R. Myocytes were either treated with fatty acids (20 μM) or exposed to 60 minutes SI followed by 30 minutes of reperfusion. **A:** Fatty acid controls without SI/R. **B:** Fatty acid treated lysates were added to SI/R lysates. **C:** Fatty acid lysates were incubated with SI/R lysates for 45 minutes at 30°C without the phosphatase inhibitor, vanadate. **D:** Fatty acid lysates were incubated with SI/R lysates for 45 minutes at 30°C with the phosphatase inhibitor, vanadate. Results are expressed as means ± S.E.M. for three independent experiments (n=3). ARA + SI/R and EPA + SI/R vs control ($^{\#}p<0.01$); ARA + SI/R (inc.) + vanadate and EPA + SI/R (inc.) + vanadate vs ARA + SI/R (inc.) and EPA + SI/R (inc.) ($^{\#\#}p<0.01$) respectively.

maximally phosphorylated (SI/R) with the sample where the phosphatase was maximally induced (ARA + SI/R). There was a significant decrease in p38 phosphorylation when the SI/R + ARA sample (with 45 minutes incubation) was compared with the control sample (not incubated) [6.24 ± 0.41 fold to 1.9 ± 0.05 fold ($p<0.01$). When vanadate was added during incubation, the effect of the phosphatase on p38 phosphorylation, was abrogated [1.9 ± 0.05 fold to 6.48 ± 1.1 fold ($p<0.01$)]. Similar results were obtained when EPA was used.

5.2.8 The effect of ARA and PI3-K inhibition on PKB/Akt phosphorylation (Figures 5.7 A & B)

To determine whether the PKB/Akt pathway participates in ARA induced protection, wortmannin, an inhibitor of PI 3-K, was used. Phosphorylation of PKB/Akt was determined by Western blotting using antibodies recognising either phospho- Ser^{473} or Thr^{308}. Wortmannin (100nM) was added only for 30 minutes before fatty acid supplementation and not during reperfusion. Wortmannin significantly inhibited PKB/Akt Thr^{308} phosphorylation [0.16 ± 0.03 fold ($p<0.01$)] during normoxia, as well as during SI/R. Interestingly, wortmannin was unable to inhibit PKB/Akt Ser^{473} phosphorylation significantly. ARA caused a significant decrease in PKB/Akt Ser^{473} phosphorylation [2.36 ± 0.22 fold to 0.96 ± 0.12 fold ($p<0.001$)] during SI/R, but Thr^{308} phosphorylation remained unaffected during this period. However, wortmannin significantly inhibited Thr^{308} phosphorylation in ARA-treated cells [1.74 ± 0.12 fold to 0.84 ± 0.06 fold ($p<0.001$)].

5.2.9 The effect of ARA and PI3-K inhibition on cell viability (Figure 5.7 C)

In view of the fact that ARA caused a significant inhibition of PKB/Akt Ser^{473} phosphorylation during SI/R, the effect of ARA and wortmannin was measured on cell viability. Wortmannin caused a significant decrease in cell viability in the SI/R control group ($p<0.05$) as well as in the ARA-treated group [92 ± 2.04 % to 71 ± 2.34 % ($p<0.001$)].

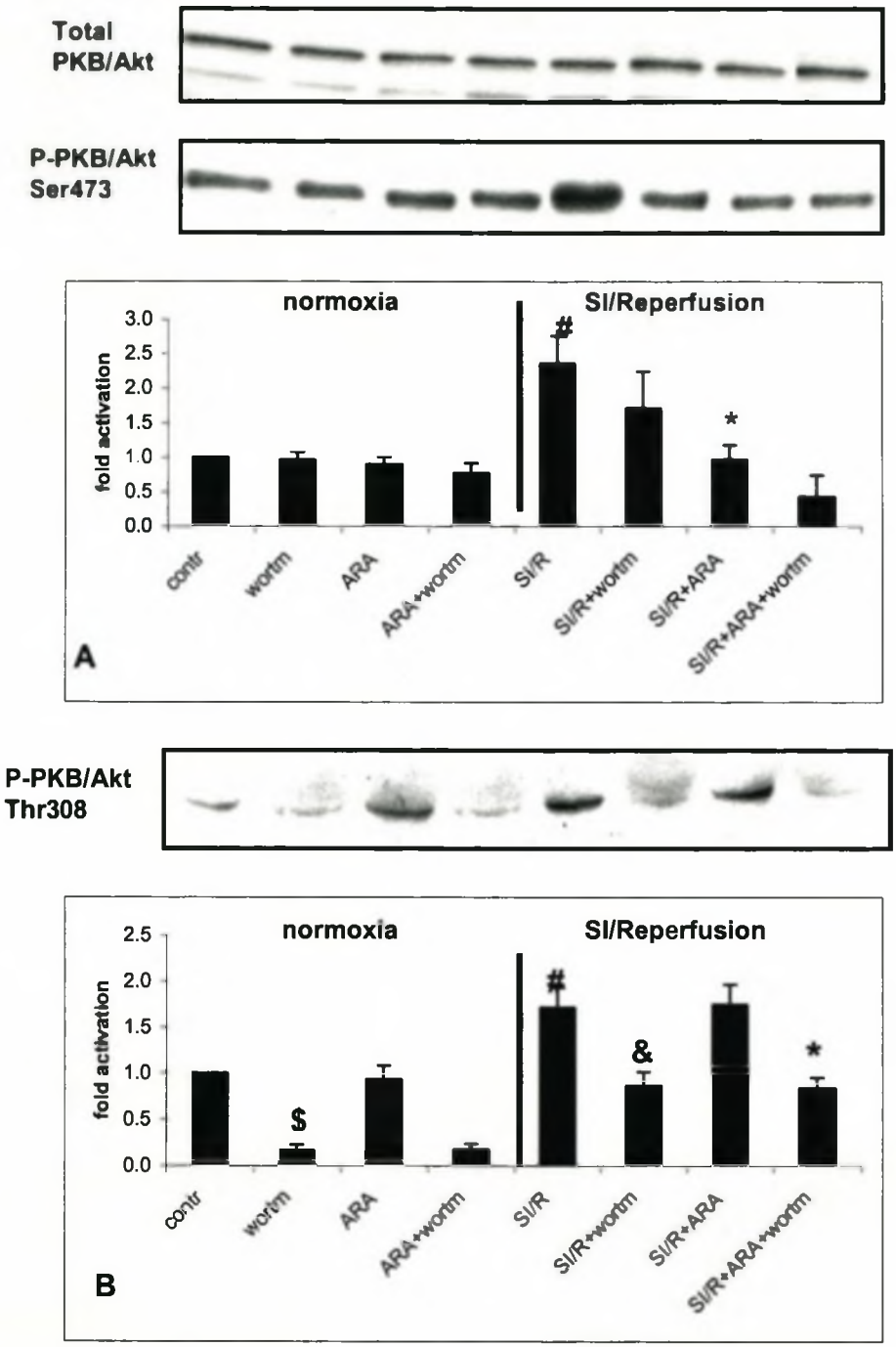


Figure 5.7 A & B: The effect of ARA on PKB/Akt phosphorylation in cardiomyocytes subjected to simulated ischaemia and reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. Wortmannin (100 nM) was added to the cells for 30 minutes prior to SI. Samples were analysed by Western blotting with phospho-specific antibodies recognizing phosphorylated Ser^{473} and Thr^{308} of PKB/Akt. Results are expressed as means ± S.E.M. for four to three independent experiments (n=3). **Ser^{473}:** SI/R vs control normoxia ($^{\#}p<0.001$); SI/R + ARA vs SI/R ($^{*}p<0.001$). **Thr^{308}:** wortmannin vs control normoxia ($^{\$}p<0.01$); SI/R vs control normoxia ($^{\#}p<0.05$); SI/R + wortmannin vs control SI/R ($^{\&}p<0.001$); SI/R + ARA + wortmannin vs SI/R + ARA ($^{*}p<0.001$).

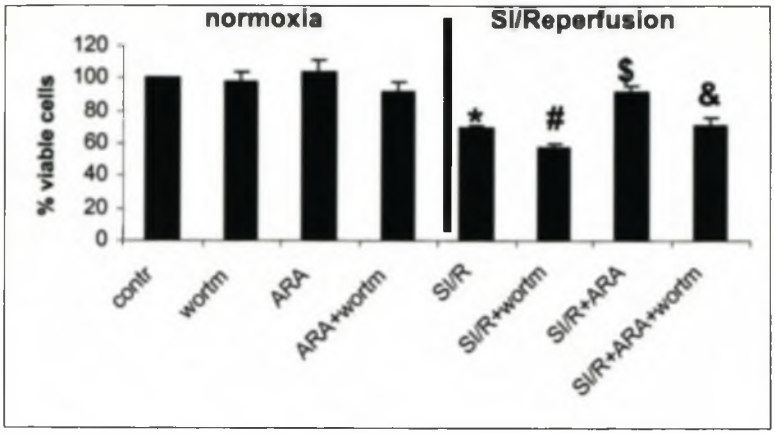


Figure 5.7 C: The effect of ARA and PI3-K inhibition on cell viability using the MTT assay during simulated ischaemia and reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. Wortmannin (100 nM) was added to the cells for 30 minutes prior to SI. Results are expressed as means ± S.E.M. for three independent experiments (n=3). SI/R + wortmannin vs control SI/R ($^{\#}p<0.05$); SI/R vs control normoxia ($^{*}p<0.001$); SI/R + ARA vs control SI/R ($^{\$}p<0.001$); SI/R + ARA + wortmannin vs SI/R + ARA ($^{\&}p<0.001$).

5.2.10 The effect of ARA and PI3-K inhibition on caspase-3 activation and PARP cleavage during SI/R (Figure 5.7 D)

As it was shown in the previous chapter, PI 3-K inhibition caused a significant increase in PARP cleavage during SI/R [2.3 ± 0.1 fold to 2.8 ± 0.12 fold ($p<0.05$)]. ARA significantly inhibited caspase-3 activation as well as PARP cleavage during SI/R [2.3 ± 0.1 fold to 0.73 ± 0.06 fold for PARP and 2.66 ± 0.38 fold to 0.88 ± 0.04 fold for caspase ($p<0.001$ for both PARP and caspase-3)]. Interestingly, when added to the ARA-induced protected group, wortmannin caused a significant increase in caspase activation during SI/R [0.88 ± 0.04 fold to 2.49 ± 0.31 fold ($p<0.01$)].

5.2.11 The effect of ARA and PI3-K inhibition on the apoptotic index (Figure 5.7 E)

SI and reperfusion significantly increased the number of apoptotic cells ($p<0.001$) compared to control cells in the normoxia group. ARA significantly reduced the number of apoptotic cells during SI/R [22.6 ± 2.94% to 5.2 ± 1.02% ($p<0.001$), while the addition of wortmannin to the latter group caused a significant increase in the number of apoptotic cells [5.2 ± 1.02% to 14.8 ± 1.1% ($p<0.05$)].

5.3 Discussion

As also shown in chapter 4, the results presented in this chapter confirmed that the model used is characterized by a reduction in cell viability during SI, increased apoptosis, particularly during reperfusion, activation of ERK, p38 and PKB during both SI and SI/R, while JNK was activated during reperfusion only.

As expected, there was a significant increase in incorporation of the fatty acids in the phospholipids of the cardiomyocytes when they were added under normoxic conditions. There was a significant increase in ARA (C20:4n-6) which was further

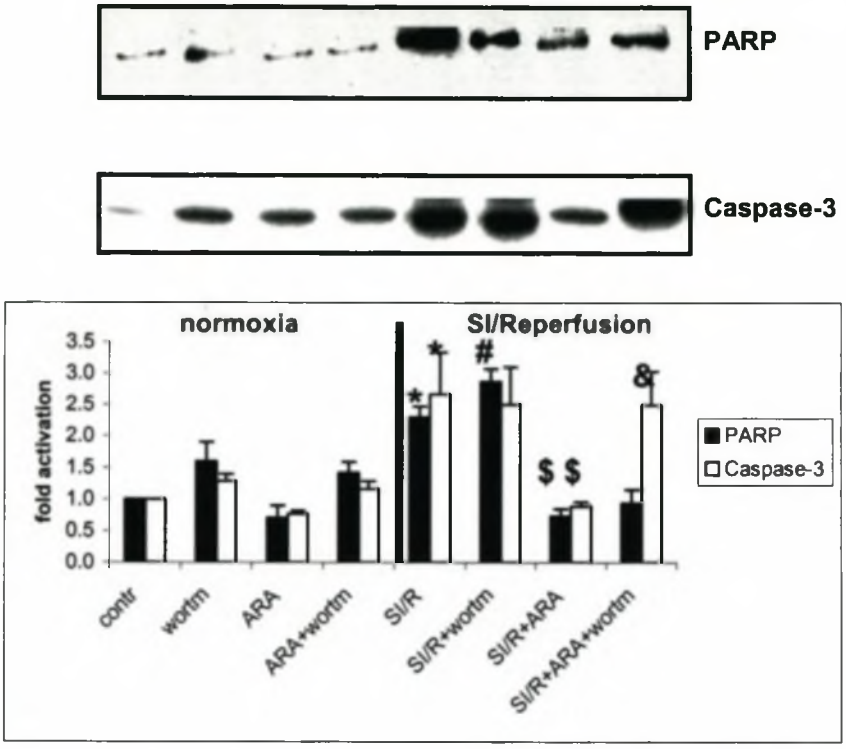


Figure 5.7 D: The effect of ARA and PI3-K inhibition on caspase-3 activation and PARP cleavage during SI/Reperfusion. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. Wortmannin (100 nM) was added to the cells for 30 minutes prior to SI. Samples were analysed by Western blotting with antibodies recognizing cleaved PARP and caspase-3. Results are expressed as means ± S.E.M. for three independent experiments (n=3). **Caspase 3:** SI/R vs control normoxia (*$p<0.001$); SI/R + ARA vs control SI/R ($^{\$}p<0.01$); SI/R + ARA + wormannin vs SI/R + ARA ($^{\&}p<0.01$). **PARP:** SI/R vs control normoxia (*$p<0.001$); SI/R + wortmannin vs control SI/R ($^{\#}p<0.05$); SI/R + ARA vs control SI/R ($^{\$}p<0.001$).

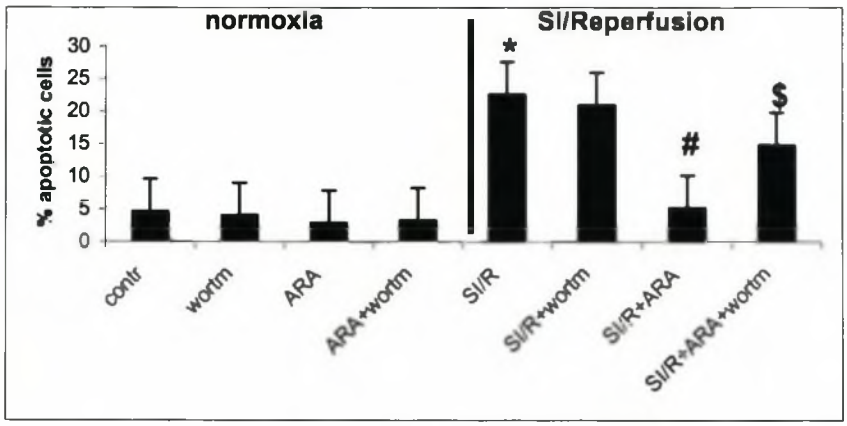


Figure 5.7 E: The effect of ARA and PI3-K inhibition on SI/Reperfusion-induced apoptosis in cardiac myocytes. Myocytes were exposed to simulated ischaemia (KCN and 2-deoxy-*D*-glucose) for 60 minutes followed by 30 minutes reperfusion. To quantify apoptotic myocytes, cell monolayers were fixed and stained with Hoechst 33342. The morphological features of apoptosis (cell shrinkage, chromatin condensation) were monitored by fluorescence microscopy. At least 400 cells from three randomly selected fields per dish were counted and each treatment was performed in triplicate. Results are expressed as means ± S.E.M. for three independent experiments (n=3). SI/R vs control normoxia (*$p<0.001$); SI/R + ARA vs control SI/R ($^{\#}p<0.001$); SI/R + ARA + wortmannin vs SI/R + ARA ($^{\$}p<0.05$).

elongated to C22:4n-6. There was also a significant increase in EPA under these conditions and this fatty acid was further elongated to C22:5n-3. C22:5n-3 was further metabolised via another elongation step, delta-6-desaturation as well as β-oxidation to C22:6n-3. Interestingly, when EPA and ARA were added to the cells, a significant decrease in oleic acid (C18:1n-9) in the phospholipid fractions of the membrane was observed. This might be due to the fact that these fatty acids replaced oleic acid in the sn-2 position of the cell membrane phospholipids.

The results suggest that EPA and ARA, when present as free fatty acids rather than when incorporated into the phospholipid fraction of membranes, are responsible for protecting the neonatal cardiomyocytes from apoptosis. This was evidenced by the fact that although the PUFAs were incorporated into the cell membranes before SI, protection was seen only when the fatty acids were also present during reperfusion (fig 5.1 A). It is therefore possible that EPA and ARA exert their beneficial effect by directly stimulating or modulating a second messenger system, rather than by becoming constituents of membrane phospholipids: if it was acting via membrane phospholipids, it probably would have been beneficial during the SI periods when no free fatty acids were present in the incubation medium.

In this study, it was shown convincingly that the long-chain polyunsaturated fatty acids, EPA and ARA, protect neonatal cardiomyocytes from ischaemia/reperfusion-induced injury as demonstrated by the significant increase in percentage viable cells during SI/R (fig 5.1 A). Although rat heart cells have no detectable lipoxygenase activity and only a very limited cyclooxygenase activity (Hohl & Rōsen, 1987), we have shown through a specific cyclooxygenase-inhibitor, indomethacin, that eicosanoid biosynthesis was not involved in this process during reperfusion. However, it appears that cyclooxygenase activity is essential for maintenance of viability during normoxia (fig 5.1 B).

It was also demonstrated that the increase in cell viability by EPA and ARA was associated with significant inhibition of apoptosis in this model, using different indicators (fig 5.2 A-C). Apoptosis has been consistently observed in cardiac myocytes after ischaemia/reperfusion injury and may represent a direct mechanism by which myocytes are damaged (Yue *et al.*, 2000; Hreniuk *et al.*, 2001). Indeed, SI/R-induced injury resulted in cleavage of PARP to its proteolysed products, a phenomenon that is well known to result from caspase-3 activation. Inhibition of apoptosis may, therefore, offer a unique approach towards amelioration of ischaemic injury.

As discussed previously, the MAPKs are important regulators of apoptosis in response to myocardial ischaemia/reperfusion (Saraste *et al.*, 1997; Yue *et al.*, 2000). Therefore, the effects of EPA and ARA were investigated on the phosphorylation status of the three major MAPK subfamily members that are activated during SI and reperfusion in the neonatal cardiomyocyte. In this study, EPA and ARA alone, failed to stimulate basal MAPK activity. These results partly corroborate the findings of Yang *et al* (Yang *et al.*, 1998), who have also demonstrated that EPA alone failed to stimulated basal MAPK enzyme activity in bovine carotid artery cells. During SI/R, the effects of these fatty acids on the three MAPKs studied, varied markedly: EPA and ARA significantly reduced p38 phosphorylation during SI as well as during reperfusion, while JNK-p54 and JNK-p46 phosphorylation remained unchanged during reperfusion. In contrast, both fatty acids induced a significant increase in ERK-p44 and ERK-p42 phosphorylation during reperfusion (fig 5.3). Although a number of studies have investigated the action of ARA on MAPKs in different cell types (Rao *et al.*, 1994; Hii *et al.*, 1995; Madamanchi *et al.*, 1998), this is the first demonstration that ARA and EPA have profound effects on MAPK phosphorylation in neonatal cardiomyocytes during simulated ischaemia and reperfusion-induced injury.

As discussed above, the effects of these long-chain PUFAs on the activation pattern of the MAPKs differs. Interestingly, chelerythrine, a PKC inhibitor, did not

affect p38 or JNK phosphorylation, but it significantly inhibited ARA-induced ERK phosphorylation demonstrating an upstream role for PKC in this regard (fig 5.4 B). PKC inhibition did not affect viability under normoxic conditions (fig 5.4 A), but it increased susceptibility of the cells to SI/R-induced damage. We also achieved the same reversal of ARA-induced protection by down-regulation of PKC by pre-treating cells with 100μM TPA for 24h (results not shown). These findings are in agreement with those obtained by Mackay and Mochly-Rosen who demonstrated that ARA protects neonatal rat cardiac myocytes from ischaemic injury via PKCε (2001). A number of other studies have also demonstrated the involvement of PKC in activation of the MAPK pathway in different cell types (Clark & Murray, 1995; Chang *et al.*, 1998).

To further investigate the mechanism of action of ARA and EPA on p38 and JNK inhibition during SI/R, it was decided to evaluate the role of the phosphatases in this scenario. Okadaic acid, an inhibitor of type 1 and type 2A serine/threonine phosphatases (Taffs *et al.* 1991) as well as orthovanadate, which is a specific inhibitor of tyrosine phosphatases (including MKP-1) were used (Gordon, 1991; Charles *et al.*, 1993). Although both okadaic acid (results not shown) and vanadate (fig 5.5 B) significantly increased basal levels of p38 phosphorylation, only vanadate could significantly attenuate ARA-induced inhibition of p38 during SI/R. As far as we know, this is the first evidence for a role for a tyrosine phosphatase in ARA-induced inhibition of p38. Although 50 or more tyrosine-specific protein phosphatases (PTPs) have been characterized, little is known about their general importance in terminating MAPK signalling. One subclass of the PTPs, the dual-specificity phosphatases (DSPs), is capable of dephosphorylating both phosphotyrosine and phosphothreonine residues. In this study MKP-1 was targeted, which has been shown to be activated by oxidative stress (Keyse & Emslie, 1992). The results obtained showed that both EPA and ARA (fig 5.5 C) caused a significant increase in MKP-1 induction during SI/R, which suggests that these fatty acids exert their beneficial effects partly through the induction of the DSPs (specifically MKP-1), which in turn can deactivate p38

(Lim *et al.*, 2001; Franklin & Kraft, 1997). This hypothesis was confirmed using an *in vitro* dehosphorylation assay showing that MKP-1 might indeed be responsible for the dephosphorylation of p38 (fig 5.6). Our results are in agreement with those of Metzler and co-workers who reported that ARA induces MKP-1 expression in vascular smooth muscle cells via the activation of tyrosine kinases. In addition, Communal *et al* reported that MKP-associated dephosphorylation of both phosphotyrosine and phosphothreonine residues on JNK1/2 and p38 correlated with their inactivation in the failing human myocardium (Franklin *et al*, 1998).

The involvement of the anti-apoptotic kinase, PKB/Akt, in the cellular response to ARA was also evaluated. It is of great interest that ARA inhibited PKB/Akt Ser^{473} phosphorylation during SI/R, while Thr^{308} remained phosphorylated (fig 5.7 A & B). The results indicated that the continued phosphorylation of PKB/Akt Thr^{308} and the inhibition of the other phosphorylation site (Ser^{473}) of PKB/Akt is associated with ARA-induced protection of neonatal cardiomyocytes, viz an increase in cell viability (fig 5.7 C) as well as a decrease in caspase-3 activation, PARP cleavage and the apoptotic index during SI/R (fig 5.7 D & E). Since we only measured the phosphorylation of PKB/Akt and not its activity, we can only speculate that the sustained phosphorylation of Thr^{308}, while Ser^{473} is inhibited, is adequate for PKB/Akt activation and subsequent inhibition of apoptosis in ARA-induced protected cells. This is substantiated by the findings that phosphorylation on Thr^{308} alone is able to increase PKB activity, while phosphorylation on Ser^{473} alone does not significantly stimulate the kinase (Alessi *et al.*, 1996; Bellacosa *et al.*, 1998)

Studies of PKB/Akt activation pathways have shown that PI3-K is a mediator of an activation signal for PKB/Akt (Kauffmann-Zeh *et al.*, 1997; Kennedy *et al.*, 1997; Franke *et al.*, 1995; Burgering & Coffer, 1995; Kulik *et al.*, 1997). PI3-K is considered one of the intracellular signals responsible for the transmission of anti-apoptotic signals for controlling cell survival and over-expression of PI3-K in

cells has been shown to cause a significant increase in survival of cells exposed to ionising radiation (Kraslinikov *et al.*, 1999; Cataldi *et al.*, 2001). On the other hand, specific inhibitors of PI3-K cause an increase in apoptosis and a decrease in cell survival (Scheid *et al.*, 1995; Kennedy *et al.*, 1997). PKB/Akt may also be activated by a PI3-K independent mechanism, for example, in response to heat shock or increases in intracellular calcium or cyclic adenosine monophosphate (cAMP) (Moule *et al.*, 1997; Konishi *et al.*, 1996; Sable *et al.*, 1997; Filippa *et al.*, 1999). The results obtained in the present study have demonstrated that the protective actions of ARA are PI3-K dependent, since wortmannin significantly increased caspase-3 activation as well as the apoptotic index during SI/R (fig 5.7 D & E). There was also a decrease in cell viability when wortmannin was added to the ARA-treated cells. However, there was no significant increase in PARP cleavage during this period. It is possible that an increase in PARP cleavage might occur if the reperfusion period is extended.

In summary, our results have shown that: i) the increased phosphorylation of ERK by EPA and ARA is beneficial and PKC-dependent, and ii) the reduced phosphorylation of p38 correlates with increased MKP-1 protein levels. Both increased ERK phosphorylation and the inhibition of p38 may contribute to the increased cell viability and decrease in apoptosis induced by either EPA or ARA. Results presented in other studies (Metzler *et al.*, 1998; Communal *et al.*, 2002), as well as our own, indicate that the MAPKs are central regulators of reactive signalling in cardiac myocytes. The ability to directly manipulate MAPK signalling has been shown to protect cardiomyocytes from ischaemia/reperfusion-induced apoptosis/injury. This notion suggests that members of the MAPK signalling cascade would be ideal targets for pharmacological intervention to treat ischaemia/reperfusion injury. Indeed, several investigators have demonstrated that inhibition of p38 with SB203580 protects cardiomyocytes from ischaemia/reperfusion injury and that sustained ERK activation is necessary for this protection (Bogoyevitch *et al.*, 1996; Punn *et al.*, 2000). Thus the results obtained in the present study suggest that these long-chain PUFAs might offer

an alternative, non-pharmacological strategy to protect the heart against ischaemia/reperfusion-induced injury.

CHAPTER 6 RESULTS AND DISCUSSION: The effects of EPA and ARA on functional recovery after global sustained ischaemia in the isolated perfused rat heart.

6.1 Introduction

It is clear form our previous results that ARA and EPA protect neonatal cardiac myocytes from simulated ischaemic and reperfusion injury. Although it is well documented that omega-3 fatty acids are anti-arrhythmic and protect the heart from reperfusion injury (McLennan *et al.*, 1985; 1988; 1992; Kang & Leaf, 2003), there is no data available on the direct effect of these fatty acids on the isolated perfused rat heart model. In addition, in previous studies the omega-3 fatty acids were given as supplements in the diets of rats or *in vitro* to neonatal cardiac myocytes. As far as we know, this is the first study, which describes the effects of EPA as well as ARA when added before and after sustained global ischaemia on functional recovery during reperfusion of the isolated retrogradely perfused rat heart model.

6.2 Results

In order to compare the functional recovery of the hearts in the different groups, the heart rate (beats/min), coronary flow (ml/min) and left ventricular developed pressure (mmHg) were monitored at 10 minute intervals during a 40 minute stabilization phase and again during reperfusion after global sustained ischaemia.

With the exception of EPA, when added before and after ischaemia, all hearts showed a significant ($p<0.05$) reduction in coronary flow, heart rate (HR), left ventricular developed pressure (LVDP) and rate pressure product (RPP = HR x LVDP) during reperfusion after 25 minutes of global ischaemia, when compared to values obtained during the stabilization period.

6.2.1 Coronary flow

Pre-ischaemic coronary flow (CF) averaged 12.6 ± 0.7 ml/min for the control group at the end of the stabilization phase and 8.3 ± 0.6 ml/min at the end of the reperfusion period. When EPA was added before and after ischaemia (EPA b & a), there was a significant increase in CF compared to the control group at the end of the stabilization phase (17.4 ± 0.6 ml/min, $p<0.05$) as well as at the end of the reperfusion period (11.7 ± 0.4 ml/min, $p<0.05$). There were no significant differences in the CF, before as well as during the reperfusion phase, when EPA was added only after sustained ischaemia or when ARA and albumin were compared to the control group.

6.2.2 Left ventricular developed pressure

During the pre-ischaemic phase, the LVDP was similar in all groups (control value: 94 ± 4 mmHg). However, there was a significant increase in LVDP during reperfusion in the groups when ARA or EPA was added before and after global ischaemia [51 ± 5 mmHg for ARA ($p<0.05$) and 80 ± 11.7 mmHg for EPA ($p<0.001$)], when compared with the control untreated hearts or hearts treated with albumin only. Interestingly, there was an almost 80% recovery after global ischaemia in the EPA group (before & after) when compared to pre-ischaemic values. In fact, the significant reduction during reperfusion seen in all the other groups was absent in these hearts. LVDP of hearts having received ARA after global ischaemia only, was significant lower ($p<0.05$) than that of hearts having received the fatty acids before and after global ischaemia and similar to those of untreated controls and albumin-treated hearts.

6.2.3 Rate pressure product (RPP)

The pre-ischaemic values for all groups were similar after 40 minutes of stabilization. However, there was a significant increase in RPP after global ischaemia when ARA (before & after) was compared to the albumin-treated

(5245 ± 1875 to 15200 ± 2275, $p<0.05$) as well as when both EPA groups were compared to the control group [5382 ± 857 to 26580 ± 3330, $p<0.001$ for EPA (before & after) and 14530 ± 2448, $p<0.05$ for EPA (a)]. As was observed for LVDP, RPP of hearts perfused with ARA after global ischaemia only, was significantly reduced and similar to those of untreated controls and albumin-perfused hearts.

6.3 Discussion

The aim of this study was to determine whether the beneficial effects observed upon addition of the long-chain PUFAs to neonatal cardiomyocytes, were also evident in the perfused heart model. It is clear from our results that both EPA and ARA significantly improved functional recovery after global sustained ischaemia, with optimum protection when the fatty acids were present both before and after sustained ischaemia.

The results obtained with EPA are not surprising, since many studies in humans as well as in animal models and cultured myocytes support these findings. For example, mortality by coronary heart disease is reduced as a consequence of dietary long-chain omega-3 PUFA administration (De Lorgeril *et al.*, 1994; Siscovick *et al.*, 1995; Albert *et al.*, 1998; Marchioli *et al.*, 1999). Long-chain PUFAs prevent or retard arrhythmias induced by ischaemia, for example, intravenous infusion of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) into canine myocardium prevented ventricular fibrillation after ischaemia (McLennan *et al.*, 1988; Billman *et al.*, 1994; Leaf *et al.*, 1998; 1999). These anti-arrhythmic effects have been described in a variety of animal species (McLennan *et al.*, 1985; 1992; Hock *et al.*, 1990; Billman *et al.*, 1994) and are thought to function in a similar manner in humans (Burr, 1989; Burr *et al.*, 1989; De Lorgeril *et al.*, 1994; Siscovick *et al.*, 1995). In addition to their anti-arrhythymic effects, these long-chain omega-3 PUFAs also reduce reperfusion injury in the heart. Oskarsson and co-workers (1993) showed that dietary fish oil supplementation significantly

reduced myocardial infarct size in a dog model where the left circumflex artery was occluded for 90 minutes, followed by 6 hours of reperfusion.

The results obtained in this study confirmed the cardioprotective effects of PUFAs observed in nutritional studies and further demonstrated that relatively short term administration of EPA is also effective in the isolated perfused rat heart. However, apart from one study by Mackay and Mochly-Rosen (1999) on isolated neonatal cardiac myocytes, this is the first study to show the protective effect of an omega-6 long-chain PUFA in the isolated rat heart model. We have demonstrated that ARA significantly improved functional recovery when added before and after sustained global ischaemia. Interestingly, although EPA also induced protection when added after global ischaemia only, ARA was without effect when added during this period.

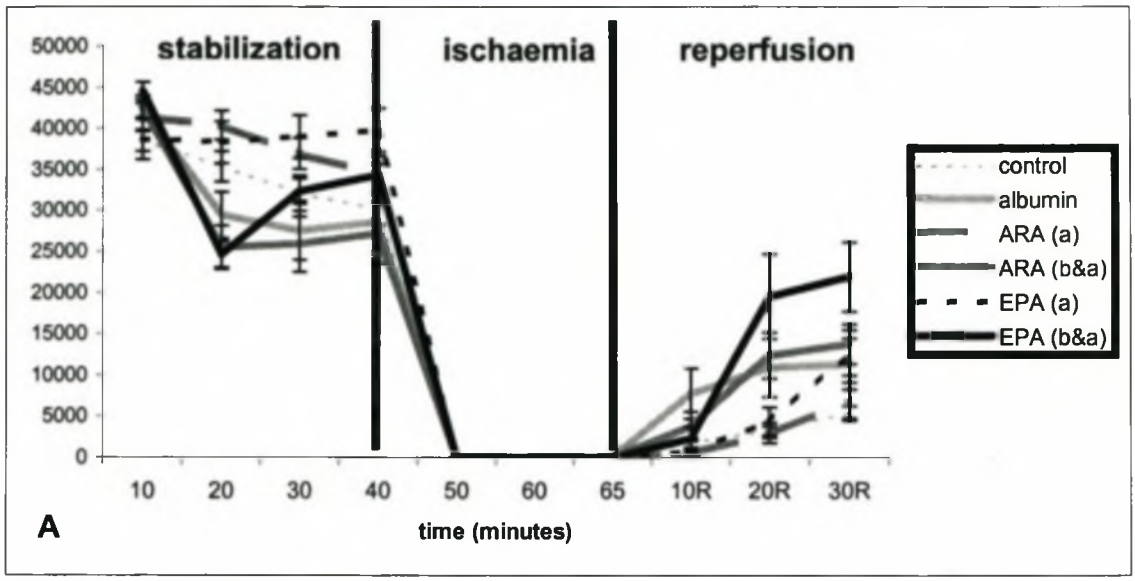
These results are in striking contrast to the well-known harmful effects of long-chain fatty acid oxidation on the ischaemic/reperfused heart, e.g. palmitate in the perfusate greatly increased membrane damage and is toxic to the ischaemic myocardium (De Leiris *et al.*, 1975; Liedtke *et al.*, 1978). It is also well established that the lipid composition of heart membranes is altered during ischaemia, whereas accumulation of nonesterified fatty acids may follow activation of the phospholipases and increased hydrolysis of glycerolipids (Prinzen *et al.*, 1984; Van der Vusse *et al.*, 1997). Disturbed lipid metabolism (Van Bilsen *et al.*, 1991), associated with a defective deacylation-reacylation cycle of phospholipid fatty acids leading to free arachidonic acid accumulation (Das *et al.*, 1986), has been described during the reperfusion phase. Indeed, Das and co-workers (1986) demonstrated a rapid release of free arachidonic acid from the isolated ventricular myocardium within the initial 10 minutes of reperfusion, which was also associated with increased activity of phospholipase A_2. However, it seems that it is not the ARA *per se* which is harmful to the cardiac myocyte, but rather its oxidation products. For example He and co-workers (2001) showed that inhibition of cytochrome P450 monooxygenases which catalyse ARA oxidation to a variety of biologically active eicosanoids, reduced infarct size in a model of Langendorff-perfused rat and rabbit hearts.

The results presented in chapter 5, showed that both ARA and EPA are avidly incorporated into membrane phospholipids of neonatal cardiomyocytes in culture. It is to be expected that this also occurs in the perfused rat heart (de Jonge *et al.*, 1996) and that the membrane phospholipid fatty acid composition is considerably changed during sustained ischaemia and reperfusion (Picard *et al.*, 1998). It is thus possible that the fatty acids (EPA and ARA) presented to the myocytes upon reperfusion will then be available to induce MKP-1 induction and subsequent p38 dephosphorylation. The latter could lead to inhibition of PLA_2 (Degausee *et al.*, 2001) and prevention of reperfusion-induced phospholipid degradation. However, it is clear from the above that the metabolism of long-chain PUFAs by the isolated rat heart needs to be further investigated.

Table 6.1 The effect of albumin, ARA and EPA on functional recovery of hearts subjected to global sustained ischaemia and reperfusion.

	Control (n=12)	Albumin (n=8)	ARA (before & after) (n=6)	ARA (after) (n=6)	EPA (before & after) (n=8)	EPA (after) (n=6)
Coronary flow						
• before	12.6±0.7	13.2±0.78	12.3±1.7	14.1±1.2	$17.4\pm0.6^{*\Upsilon\varepsilon}$	15.2±0.8
• after	8.3 ± 0.6^{o}	8.8 ± 0.6^{o}	7.6 ± 0.8^{o}	6.9 ± 0.4^{o}	$11.7\pm0.4^{o*\Upsilon\varepsilon}$	8.6 ± 0.6^{o}
(ml/min)						
Heart rate						
• before	324±12	326±11	309±24	332±16	344±20	351±9
• after	266 ± 12^{o}	243±19	284±23	276 ± 12^{o}	274 ± 26^{o}	319 ± 15^{o}
(beats/min)						
LVDP						
• before	94±4	98±7	97±9	104±7	101±4	113±5
• after	20 ± 3^{o}	$19.8\pm5.6^{o\delta}$	$51\pm5^{o*}$	$14.3\pm2.8^{o\delta}$	$80\pm11.7^{**\Upsilon\varepsilon}$	44.7 ± 5.7^{o}
(mm Hg)						

* $p<0.05$ vs control; ** $p<0.001$ vs control; $^{\Upsilon}p<0.001$ vs albumin; $^{\delta}p<0.05$ vs ARA (before and after); $^{\varepsilon}p<0.05$ vs EPA after; $^{o}p<0.05$ vs "before vs after"



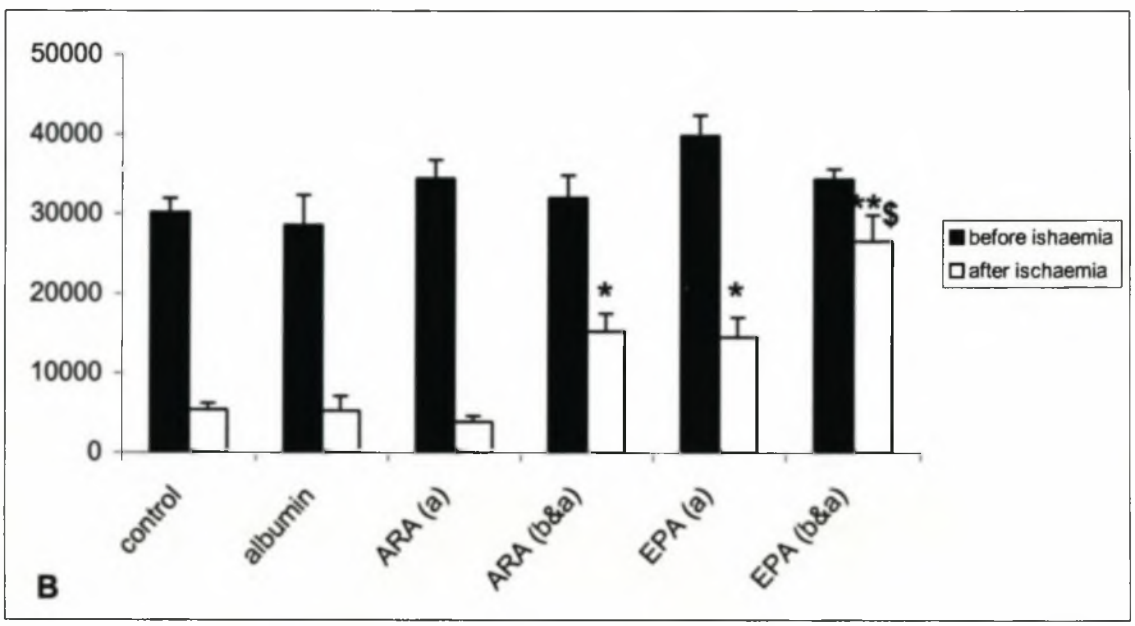


Figure 6.1 A and B: RPP of hearts perfused with standard KH solution containing either albumin, EPA or ARA before and after global ischaemia (b & a) and EPA or ARA just after global ischaemia (a). 20 μM of fatty acid bound to albumin (ratio:3.3:1) was added to the perfusion solution. The rate pressure product was calculated by multiplying the heart rate and the LVDP, (n=6-12); * ARA (b&a) and EPA (a) vs control (after ischaemia) ($p<0.05$); EPA (b&a) vs control (after ischaemia) (** $p<0.001$); EPA (b&a) vs albumin (after ischaemia) ($^{\$}p<0.001$).

CHAPTER 7 FINAL CONCLUSIONS AND FUTURE DIRECTIONS

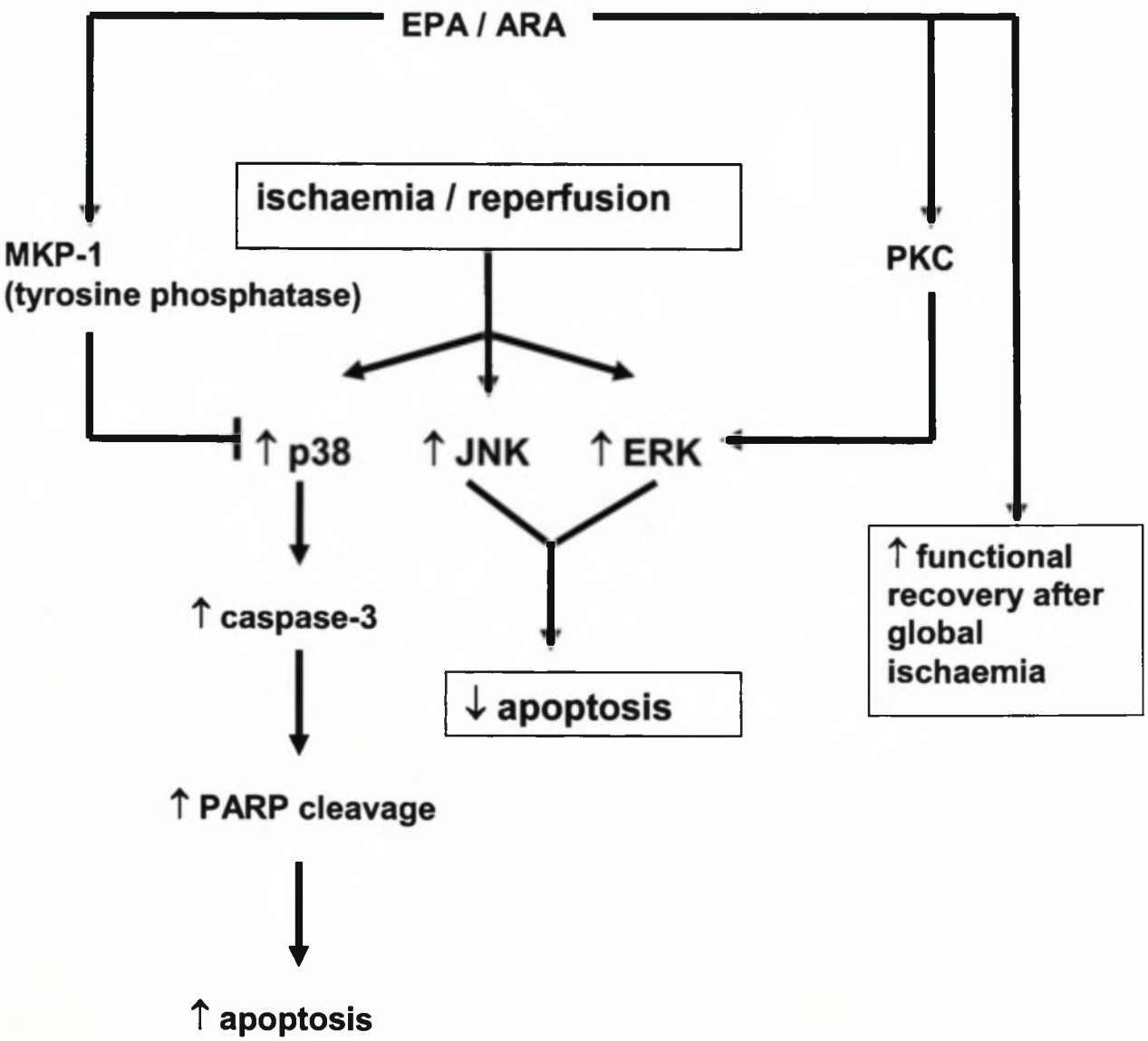
An *in vitro* model of simulated ischaemia/reperfusion (SI/R), induced by KCN and 2-deoxy-*D*-glucose, was used to achieve graded ATP depletion in cultured neonatal cardiomyocytes. It was demonstrated that activation of p38 is associated with development of apoptosis. Myocyte ATP depletion during SI induced p38 activation as well as an upregulation of two molecules that are implicated in the late transduction phases of apoptosis, viz caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage. Reperfusion induced JNK activation and stimulated chromatin condensation and fragmentation, implicating involvement of this MAPK in the final stage of apoptosis. However, our subsequent inhibitor studies clearly indicate that, whereas p38 promotes apoptosis in neonatal cardiomyocytes in response to SI, JNK promotes survival in response to reperfusion. This suggests that p38 and JNK have distinct but different functions during SI/R-induced apoptosis in our cell model.

In order to assess the mechanisms of protection of long-chain polyunsaturated fatty acids (PUFAs) in injured/apoptotic heart cells, we treated neonatal cardiomyocytes with EPA and ARA prior to and after simulated ischaemia. Using this model, we demonstrated for the first time that EPA and ARA protect neonatal cardiomycytes from ischaemia/reperfusion-induced apoptosis through a MAPK-dependent pathway and that these beneficial effects might be exerted through increased induction of a dual-specific phosphatase, MKP-1, which can dephosphorylate p38. We have also shown that ARA inhibits one phosphorylation-site of PKB/Akt (Ser^{473}), while it does not affect Thr^{308}. Also, chelerythrine, a PKC inhibitor, did not affect p38 or JNK phosphorylation, but significantly inhibited ARA-induced ERK phosphorylation, suggesting an upstream role for PKC in this regard during SI/R (fig 7.1).

The beneficial effects of EPA and ARA could also be elicited in the perfused rat heart model subjected to ischaemia/reperfusion. Both fatty acids significantly improved functional recovery when added before and after sustained ischaemia, with EPA being the more effective fatty acid.

The prevention of CHD in the community remains a challenge and only a few drug therapies have demonstrated to reduce the risk of sudden cardiac death. Dietary intake of modest amounts or low-dose n-3 PUFA supplementation in patients with a prior myocardial infarction is low-cost, low-risk interventions. It is time for clinicians, researchers and policymakers to give increased attention to the beneficial effects of these fatty acids and to translate this considerable body of experimental, epidemiological, and clinical evidence into clinical practice.

Figure 7.1 Proposed mechanism for the protective actions of EPA and ARA in the heart



ADDENDUM

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Engelbrecht A-M, Niesler C, Page C, Lochner A. p38 and JNK have distinct regulatory functions on the development of apoptosis during simulated ischaemia and reperfusion in neonatal cardiomyocytes. Basic Res Cardiol 2004; 99: 338-350.

Engelbrecht A-M, Niesler C, Page C, Lochner A. Long-chain polyunsaturated fatty acids protect neonatal cardiomyocytes against apoptosis by upregulation of the phosphatase, MKP-1. J Mol Cell Cardiol, 2004; submitted

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