ROLE OF NUCLEAR FACTOR KAPPA-B IN TNFα-INDUCED CYTOPROTECTION

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Submitted for the Degree of Master of Science in Medicine

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March, 2006

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Declaration

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ACKNOWLEDGEMENTS

This degree would not have been possible without the many people who have influenced and guided me these past few years. First and foremost, I would like to thank Sandrine Lecour, PhD for being the best supervisor I have ever known. Without her motivation, guidance and patience I would have never gotten this far.

Thanks to Professor Opie, PhD, DSc, DPhil for his scientific advice, thoughtful insights and motivation.

I want to thank Lydia Lacerda for teaching me about cell culture and oxygraph techniques and helping me with some troublesome experiments.

Thanks to Naushaad Suleman for his intellectual conversations, not all science.

Anna Wing-Yin Chan for being a true friend and showing me the way here.

To the Hatter for taking a chance on me.

To my parents for being understanding about my absence in the past year and supporting me all the way. This is for them.

To my brother and sister for being there when I needed them most.

To everyone that I've forgotten to acknowledge. Not intentional, just can't remember right now.

And lastly to that radioactive spider that bit Peter Parker.

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ABBREVIATIONS

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2-DG	2-Deoxy-d-Glucose
5-HD	5-Hydroxydecanoate
ACE	angiotensin-converting enzyme
ADP	adenosine diphosphate
ANOVA	analysis of variance
AP-1	Activation protein-1
ATP	adenosine triphosphate
BAD	Bcl-2 antagonist of cell death
CCD	charge coupled device
c-IAP	cellular inhibitor of apoptosis
CTC	Copper tartrate/ carbonate
Cox	cyclooxygenase
DDTC	Diethyldithiocarbamate
DMEM	Dulbecco's Modified Eagle Serum
EMSA	electrophoretic mobility shift assay
Epo	erythropoietin
Erk	extracellular signal-regulated kinase
FCS	fetal calf serum
FLICE	FADD-homologous interleukin-1β-converting enzyme inhibitory protein
H^+	hydrogen ions
HMG-CoA	3-hydroxy-3-methylglutaryl-Co-enzyme A

HS	horse serum
HSP	heat shock protein
I/R	Ischaemia/Reperfusion
IL-1	interleukin-1
IκB	inhibitory kappa B
IKK	IκB kinase
IMD0354	IKK-2 inhibitor V
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
LPS	lipopolysaccharide
LV	left ventricular
MAPK	mitogen –activated protein kinase
MitoK_{ATP}	mitochondrial potassium adenosine triphosphate
Mn-SOD	manganese superoxide dismutase
MPG	N-2-mercaptopropionyl glycine
MRC	Medical Research Council
NaOH	sodium hydroxide
NEMO	NFκB essential modulator
NFκB	nuclear factor kappa B
NH_4	ammonia
NIK	NFκB-inducing kinase
P/S	penicillin/ streptomycin
PBS	phosphate buffered saline
PCD	programmed cell death

PDTC	pyrrolidine dithiocarbamate
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKC/ε /δ	protein kinase C/-epsilon/-delta
PMT	photomultiplier tube
RIP	receptor interacting protein
RISK	Reperfusion Injury Salvage Kinase
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SEM	standard error mean
SI	simulated ischaemia
STAT-3	signal transducer and activator of transcription-3
SWOP	second window of protection
TB	trypan blue
TNFR	TNFα receptor
TNFα	tumour necrosis factor-alpha
TRADD	TNFα receptor associated death domain
TRADD	TNFR-associated death domain
TRAF	TNFα receptor-associated factor
WHO	World Health Organisation
XIAP	X chromosome-linked inhibitor of apoptosis

ABSTRACT

ABSTRACT

Introduction: Although Tumor Necrosis Factor-Alpha (TNFα) is thought to participate in myocardial disease, we have previously reported that this cytokine, in a dose- and time-dependent manner can mimic ischemic preconditioning and thus confer cardioprotection. However, the mechanisms involved in this cytoprotective effect still remain unclear. Nuclear Factor kappa-B (NFκB) is a transcription factor that is activated in response to TNFα exposure. The extent of its role in TNFα-induced cardioprotection as a 'trigger' or 'mediator' or perhaps both, is unknown. Therefore, we investigated the role of NFκB as a possible key regulator in TNFα induced cytoprotection.

Methods: Differentiated C_2C_{12} cells were pretreated with 0.5ng/ml TNFα for 30 minutes with/without an NFκB inhibitor, pyrrolidine derivative of dithiocarbamate (PDTC; 100 μM) or IKK-inhibitor V (IMD0354; 1 μM). After a washout period of an hour, cells were then subjected to a 7 hour simulated ischemia followed by one hour of reperfusion. At the end of the reperfusion period, cell viability was assessed using trypan blue exclusion. To determine whether NFκB acts as a mediator, a similar protocol was applied with the exception that PDTC/IMD0354 was administered during the reperfusion period. Phosphorylation of IκB was measured after 15 minutes of the preconditioning stimulus or post-ischaemic reperfusion. In additional groups, oxygen consumption was evaluated at the end of the simulated ischemia using oximetry.

Results: The cytoprotective effects of TNFα (43.7±8.1% of viable cells in ischemic control vs 70.6±6.1% with TNFα, $p<0.001$, $n\geq9$) was abrogated in C_2C_{12} cells pretreated with either PDTC or IMD0354 (40.6±1.9% and 40.9±2.8% respectively, $p<0.001$ versus TNFα, $n\geq9$). Preconditioning with TNFα increased cytosolic IκB phosphorylation (1.5±0.2 for TNFα versus 1.0±0.0 for untreated, $p<0.01$) but this increase was abolished when TNFα was incubated in the presence of PDTC or IMD0354 (0.8±0.3 and 1.2±0.1 respectively, $p<0.01$ versus TNFα). TNFα improved state 2 respiration but this protective effect was abolished in the presence of PDTC (2.5±0.1 nM/l oxygen/min/million cells for

ischaemic control, 5.7±0.6 TNFα ($p<0.05$) and 3.6±0.3 TNFα+PDTC; n=6). PDTC given after simulated ischemia did not affect the protection afforded by TNFα (70.7±1.7%, $p<0.001$, n≥6). In contrast, IMD0354 abolished cytoprotection (40.9±2.8%, *$p<0.001$ versus TNFα, n≥6). During the reperfusion period, the increase of IκB phosphorylation observed with TNFα was also reduced in the presence of IMD0354 or PDTC (0.91±0.19 and 1.22±0.24, respectively versus 1.78±0.2 for TNFα, *$p<0.01$).

Conclusion: Our data strongly suggest that NFκB is involved both as a trigger and a mediator in the signalling pathway of TNFα mediated cytoprotection.

A. INTRODUCTION

A. Introduction

Cardiovascular disease remains a major cause of morbidity and mortality in the western world and is predicted by the World Health Organisation (WHO) to reach a global scale in the next few years (Murray and Lopez, 1997). In 2005, the South African Medical Research Council (MRC) ranked cardiovascular disease as the leading cause of death in the Western Cape (http://www.mrc.ac.za/bod/).

A myocardial ischaemia refers to a reduced and an inadequate supply of oxygenated blood to the myocardium as a result of occluded coronary flow (Opie, 2004). When the myocardium is subjected to an acute coronary occlusion, the deprivation of oxygenated blood leads to an infarction. An infarction is the resultant dead cells. If the ischaemia is prolonged then progression to total necrosis will commence.

Although myocardial reperfusion has been shown to be detrimental itself, it is the best strategy to avoid further cell death (Jennings and Reimer, 1983). Restoration of coronary blood flow can be achieved either by the administration of a pharmacologic agent (= thrombolytic) to break down the thrombus (blood clot), the use of a device called a stent to physically open the diseased artery (a process known as balloon angioplasty) or by coronary bypass surgery. However, the beneficial effects of reperfusion of the ischaemic myocardium are directly dependant on the time of intervention (Maxwell and Lip, 1997; Park and Lucchesi, 1999). Reperfusion that is not performed within two to three hours of the onset of ischaemia will exacerbate some deleterious effects collectively known as reperfusion injury. This entails the functional impairment of the myocardial contractility (stunning), arrhythmia and the accelerated progression of cell death in severely damaged cardiomyocytes (Opie, 1989 and 2004). Reperfusion is primarily mediated by reactive oxygen species (ROS) and calcium overloading (Bolli and Marbàn, 1999). Since it was possible for

reperfusion to cause irreversible injury if not performed timeously, an alternative therapeutic means was required to limit the size of the infarction.

1. Concept of Preconditioning

Ischaemia causes severe cell death in the myocardium and dead myocytes are not replenished by division of surviving myocytes. Reperfusion therapy is insufficient to reduce infarct size to appreciable extent that promotes viability of the myocardium and is not always successful. In 1986, Murry et al. published a seminal paper in which they described a phenomenon whereby four cycles of five minutes of ischaemia with intermittent reperfusion prior to a subsequent and prolonged ischaemia attenuated infarct size by 75% in the canine myocardium. Initially, they found that brief periods of ischaemia reduced the rate of adenosine triphosphate (ATP) depletion during subsequent ischaemic episodes (Reimer et al., 1986). Intermittent reperfusion also served to prevent the cumulative effects of ischaemic injury by washing out potentially harmful catabolites such as lactate, hydrogen ions (H^+) and ammonia (NH_4). The reduction in ATP depletion was also associated with the limitation in infarct size. Given these findings and that the procedure could be reproduced successfully, this cardioprotective phenomenon was termed 'ischaemic preconditioning' (IPC, Murry et al., 1986).

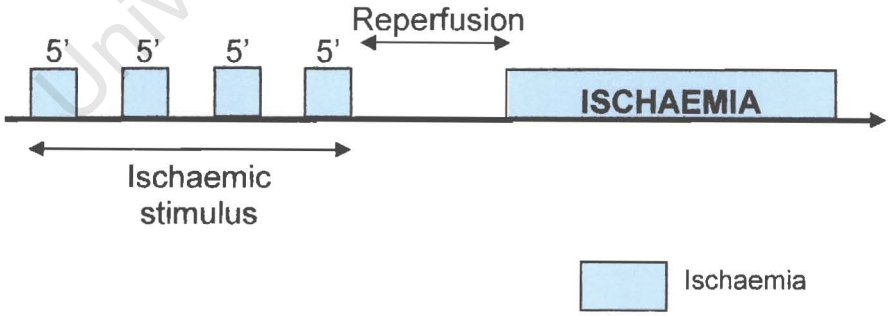


Figure 1: **Schematic representation of the ischaemic preconditioning protocol as demonstrated by Murry et al (1986).**

1.1 Classic vs. delayed preconditioning

Preconditioning the myocardium results in a significant reduction in infarct size compared with the non-preconditioned myocardium that is subjected to a sustained coronary occlusion. Cardioprotection is initially transient and the preconditioned state lasts for only 1-2 hours (Murry et al., 1991). However, within 24 hours of the preconditioning stimulus, a late phase of protection is evident that is less robust but more prolonged, lasting up to 72 hours after the preconditioning stimulus (Baxter et al., 1997). The initial phase of protection is referred to as 'classic' or 'early' phase and the latter phase as the second window of protection (SWOP) or delayed preconditioning (figure 2).

Classic preconditioning involves the activation of G protein coupled receptors and the opening of ATP dependent potassium channels in the sarcolemma and mitochondria (See review, Yellon and Downey, 2003). Inhibition studies with genistein and lavendostin have demonstrated a role for protein tyrosine kinase in delayed preconditioning (Imagawa et al., 1997; Dawn et al., 1999). A role for protein kinase C (PKC) has also been demonstrated in the SWOP (Yamashita et al., 1994). PKC is not unique to SWOP and is also involved in the early preconditioning (Mitchell et al., 1995; Ytrehus et al., 1994). There are steps in the signal transduction cascade that are common to both classic preconditioning and SWOP but due to the prolonged time course with which SWOP occurs allows for possible *de novo* protein synthesis and post translational protein modification.

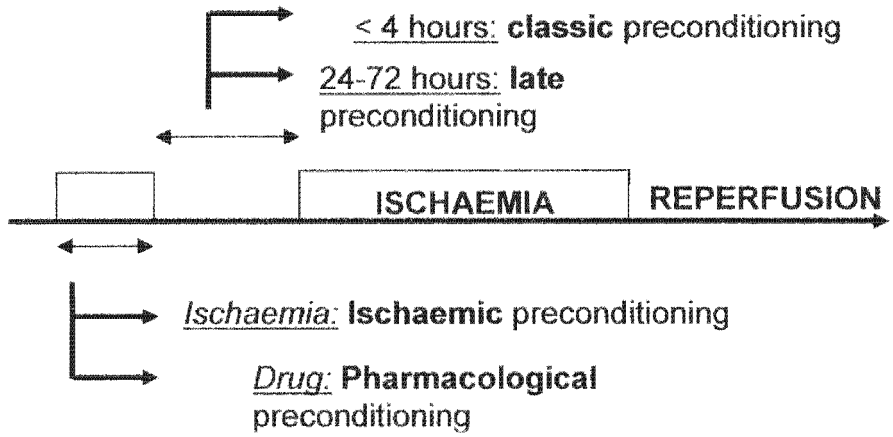


Figure 2: **Schematic representation of classic and delayed preconditioning.**

1.2 Pharmacological Preconditioning

It is now possible to mimic ischaemic preconditioning by pharmacological means. In this process, the myocardium is treated with a drug instead of being subjected to an ischaemic preconditioning stimulus.

Adenosine is important to the preconditioning process (discussed later in the thesis). Exogenous administration of adenosine receptor agonists can precondition the heart (Liu et al., 1991). In addition, pretreatment with dipyridamole, an inhibitor of adenosine's cellular uptake, an increase of extracellular adenosine concentration was observed which significantly reduces the infarct size in rabbits exposed to an index ischaemic period (Miura et al., 1992).

Bradykinin itself can also mimic ischaemic preconditioning (Goto et al., 1995). Administration of angiotensin-converting enzyme (ACE) inhibitors has led to infarct size reductions in animal studies (Schwarz et al., 1997; Miki et al., 1996). Similarly, ACE inhibitors are administered to prevent the breakdown of

bradykinin and thereby increase its endogenous concentration (See review, Baxter and Ebrahim, 2002).

Opioid receptor agonists such as morphine have been used to precondition the rat myocardium *in vivo* (Schultz et al., 1996). In this study, three intermittent drug infusions were given prior to 30 minutes occlusion and 2 hours reperfusion. The resultant infarction reduction size was comparable to ischaemic preconditioning. Nalaxone, an opioid receptor antagonist completely blocked the cardioprotective effect of morphine (Schultz et al., 1996).

Tumour necrosis factor-alpha (TNFα) is released during ischaemia and was originally thought to contribute to cell death. However, TNFα administered in a low dose has been successfully shown to mimic ischaemic preconditioning (Lecour et al., 2002).

It has been shown in rat cardiomyocytes and the isolated mouse and rabbit models that an acute exposure of ethanol prior to the ischaemic event can mimic ischaemic preconditioning (Krenz et al., 2001). Preconditioning with ethanol leads to the activation of protein kinase C isozymes, protein kinase C epsilon (PKCε) (Miyamae et al., 1998) and protein kinase C delta (PKCδ) (Inagaki et al., 2003; Inagaki et al., 2003b). PKCε mediates protection against ischaemic injury whilst PKCδ is involved in reperfusion injury. Though, recently it has been discovered that PKCδ mediates PKCε activation in ethanol-induced cardioprotection (Inagaki and Mochly-Rosen, 2005).

Recently, 3-hydroxy-3-methylglutaryl (HMG)-Co-enzyme A (CoA) reductase inhibitors or statins, have been investigated to protect the myocardium from ischaemia/ reperfusion injury. Statins are usually used to halt the progression of atherosclerosis but can also mimic the effects of ischaemic preconditioning (Jones and Lefer, 2001).

Erythropoietin (Epo) is a glycoprotein that is synthesized in renal peritubular interstitial cells in response to reduced oxygen delivery. This cytokine is able to confer cardioprotection against ischaemia injury in several models such as isolated cardiomyocytes and the isolated perfused heart (Calvillo et al., 2003; Tramontano et al., 2003; Cai and Semenza, 2004; Shi et al., 2004). Immediate resistance is observed after treatment with Epo which suggests that induction of new genes is not required to facilitate cardioprotection (Calvillo et al., 2003).

Ultimately, it is expected that pharmacological preconditioning becomes practical for human use. However, the problem with preconditioning in human requires that the heart is preconditioned prior to the ischaemic insult. Therefore, it is imperative that drug treatment strategies should be focused on the period where ischaemia has already begun.

1.3 Remote Preconditioning

In 1993, Kloner and colleagues demonstrated a new form of preconditioning. By preconditioning one region in the dog heart, they were able to protect a remote region against I/R injury (Przyklenk et al., 1993). Since then, remote preconditioning has been repeated in other organs such as the short bowel (Gho et al., 1996), kidney (Pell et al., 1998) and hind limb (Birnbaum et al., 1997). Early remote ischaemic preconditioning of a non-cardiac tissue has been shown to mimic the myocardial infarct size reduction observed with classical preconditioning in animal models such as rats (Gho et al., 1996), rabbits (Tang et al., 1999) and pigs (Kharbanda et al., 2002). The mechanisms of remote preconditioning are not well defined although it is suggested that it may be some neuronal involvement (Gho et al., 1996). Nakano et al., tried to reproduce the initial experiment by Przyklenk et al. in the rabbit heart but were unsuccessful. They concluded from this study that remote preconditioning may not apply to all species (Nakano et al., 2002).

1.4 Other forms of preconditioning

Brief ischaemia results in the dilation or stretch of the myocardium. Ovize et al., induced myocardial stretch by acute volume overload and were successfully able to precondition the canine myocardium (Ovize et al., 1994). This study was repeated in the rabbit heart and found to share a common mechanism with ischaemic preconditioning via the protein kinase C (Gysembergh et al., 1998). A brief period of rapid right ventricular pacing prior to a myocardial ischaemia has also been shown to limit infarct size (Koning et al., 1996). Subjecting male Wistar rats to transient hyperthermia is able to protect the myocardium immediately after ischaemia and 24 to 72 hours after the heat stress (Yamashita et al., 1998). This protection is mediated by the activation of manganese superoxide dismutase (Mn-SOD) which acts as a free radical scavenger (Yamashita et al., 2000). Interestingly, during the late phase of ischaemic preconditioning, heat shock protein 72 (HSP72) is also involved in mediating protection (Marber et al., 1993).

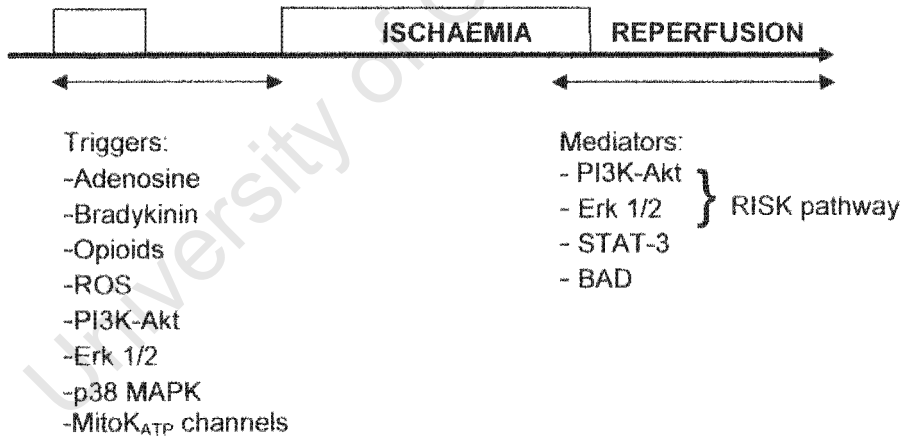
1.5 Concept of Postconditioning

Since it is impossible to predict the myocardial infarction, the procedure of preconditioning the myocardium cannot be applied clinically in this context. In 2003, Vinten-Johansen and colleagues demonstrated a novel strategy whereby the canine myocardium underwent three cycles of intermittent occlusion and reperfusion following one hour of left anterior descending artery occlusion. The resultant infarct size was comparable to that of ischaemic preconditioning and this phenomenon was termed as ischaemic postconditioning (Zhao et al., 2003). Similar to ischaemic preconditioning, the mechanism of postconditioning also involves the recruitment of the survival kinase pathway of PI3K-Akt and Erk1/2 (Hausenloy et al., 2005; discussed later in thesis). Recently, the human

myocardium has been successfully postconditioned (Staat et al., 2005, Loukogeorgakis et al., 2006). It is hoped that future study should focus on the administration of pharmacological agents to mimic postconditioning in clinical practice (Yellon and Opie, 2006).

1.6 Triggers vs. Mediators

The mechanisms by which preconditioning confers cardioprotection against ischaemic injury involves a series of signals that can be classified as either triggers or mediators subject to the sustained ischaemia (See review, Yellon and Downey, 2003). Signalling molecules that are upregulated between the preconditioning stimulus and the sustained ischaemia are considered as *triggers*. A *mediator* would be any molecule that exerts their activity steps during and after the sustained ischaemia. The final step in the signalling pathway that causes the protection against the sustained ischaemia is referred as the end



effector.

Figure 3: **Some triggers and mediators in ischaemic preconditioning. ROS= reactive oxygen species, PI3K-Akt= phosphatidylinositol 3-kinase (PI3K)-Akt, Erk= extracellular signal-regulated kinase, MAPK= mitogen –activated protein kinase, MitoK$_{ATP}$= mitochondrial potassium adenosine triphosphate, STAT-3= signal transducer and activator of transcription-3, BAD= Bcl-2 antagonist of cell death.**

1.6.1 Triggers

The adenosine receptor was the first signal transduction element to be identified in the preconditioned myocardium (Liu et al., 1991). Adenosine is a byproduct from the breakdown of adenosine triphosphate (ATP). During the ischaemic preconditioning stimulus, there is an increase in adenosine concentration within the myocardium. The blockade of adenosine A_1 receptors almost completely abolishes the protective effects of ischaemic preconditioning in the rabbit heart and the transient exposure of an A_1 receptor agonist induces cardioprotection. This discovery revealed that the early phase of IPC could be receptor mediated.

Likewise, a role for bradykinin in ischaemic preconditioning has also been described (Schulz et al., 1998). Blockers of the bradykinin receptor in the rabbit heart abolished ischaemic preconditioning that was afforded by a single cycle of ischaemia/ reperfusion (Goto et al., 1995). Interestingly, multiple cycles of ischaemia/ reperfusion did not require the activation of bradykinin to achieve the preconditioning effect.

The involvement of opioids as a trigger has also been investigated. The administration of opioids receptor antagonist nalaxone completely inhibited the preconditioning effect in rats (Schultz et al., 1995) and to a lesser degree in the rabbit heart. Similarly to the bradykinin study, only one cycle of preconditioning was blocked with nalaxone. It was suggested that opioids act together with adenosine and bradykinin to contribute to the initiation of ischaemic preconditioning.

Ischaemic preconditioning is also a receptor-independent mediated event. Reactive oxygen species (ROS) are released during the preconditioning ischaemia (Baines et al., 1997). The inhibition of ROS during the preconditioning stimulus with N-2-mercaptopropionyl glycine (MPG), an

antioxidant, blocked the infarct size reduction achieved by ischaemic preconditioning in rabbits. ROS are generally considered to be harmful and perpetuate ischaemia/ reperfusion injury but can also be beneficial as they can act as a trigger to initiate protection.

Protein kinase C (PKC) is also involved in ischaemic preconditioning (Mitchell et al., 1995). This intracellular enzyme is activated by the upstream signaling molecule, phosphatidylinositol-3-kinase (PI3K) (Tong et al., 2000). PI3K activates the serine/ threonine kinase Akt by phosphorylation. Akt in turn, phosphorylates and inhibits the proapoptotic kinase glycogen synthase kinase-3 (GSK-3) (Tong et al., 2002).

Mitogen-activated protein kinase (MAPK) kinases (MKKs) are located downstream of PKC. The extracellular signal-regulated kinases (ERK 1/2), the p46 and p54 c-Jun NH_2-terminal kinases (JNK)/ stress-activated protein kinases (SAPK), and p38 MAPK are the primary MAPKs found in the myocardium that are implicated in the signaling mechanism of ischaemic preconditioning (Maulik et al., 1996).

The opening of mitochondrial K_{ATP} (mitoK_{ATP}) channels has been linked to cardioprotection (Gross and Auchampach, 1992). These potassium channels are aptly named due to their normal inhibition at physiological levels of ATP. Pain et al. (2000) and Wang et al. (2002) used rabbit hearts to determine whether mitoK_{ATP} channels are involved as a trigger or mediator in ischaemic preconditioning. Using 5-Hydroxydecanoate (5-HD), a putative mitochondrial ATP channel inhibitor, they have shown that 5-HD could only block the protection when administered during the preconditioning stimulus and thus supporting a role as a trigger.

1.6.2 Mediators

Yellon and colleagues have investigated the role of PI3K-Akt and ERK1/2 at the onset of the reperfusion phase that follows the sustained ischaemia. The activation of these prosurvival kinases at the time of reperfusion that subsequently leads to myocardial protection against lethal ischaemia/ reperfusion injury has been termed the Reperfusion Injury Salvage Kinase (RISK) pathway (Hausenloy et al., 2005a and b).

Ischaemic preconditioning of the isolated rat perfused heart leads to the phosphorylation of Akt and ERK 1/2 at the time of reperfusion (Hausenloy et al., 2005a). Inhibition of Akt or ERK 1/2 with PI3K inhibitor, LY294002 or MEK 1/2 inhibitor, PD98059, respectively, for the first 15 minutes of reperfusion abrogates the protective effects of IPC. Similar results have been confirmed in the rabbit heart model (Solenkova et al., 2006).

Activation of the RISK pathway leads to the phosphorylation of the proapoptotic protein Bcl-1 antagonist of cell death (BAD) (Datta et al., 1997). However, phosphorylation of BAD by survival factors results in BAD binding to 14-3-3 regulatory proteins which sequester it from the mitochondria. In this way, apoptosis is prevented (Zha et al., 1996). BAD may also act independently of the RISK pathway. Indeed, BAD has recently been shown to mediate protection at reperfusion that is concomitant with STAT-3 activation. Inhibition of STAT-3 with AG490 at reperfusion abolishes myocardial protection and BAD phosphorylation (Lecour et al., 2005). The STAT-3 pathway appears as an alternative protective pathway at the time of reperfusion, independent of the activation of the RISK pathway.

2. TNFα

Tumour necrosis factor (TNF) is a pro-inflammatory cytokine. Cytokines are small proteins that are primarily involved in heat responses to disease or infection. (Dinarello, 2000).

There are two known isoforms of TNF, TNFα and TNFβ. TNFα is smaller and more abundant than TNFβ. It is produced predominantly by macrophages. TNFβ was first described as 'lymphotoxin' (Ruddle and Waksman, 1967). It is a larger molecule that is less potent and abundant and produced by T-cells. Only TNFα will be discussed further in this thesis.

There are two types of TNFα receptors that have been characterised, namely TNFα receptor 1 (TNFR1, or p55) and TNFα receptor 2 (TNFR2, p75) (Baxter et al., 1999). These receptors are either membrane bound or can also exist as truncated soluble forms (Baker and Reddy, 1995). Both receptor subtypes are present in rat and human cardiomyocytes (Torre-Amione et al., 1995; Krown et al., 1996). TNFR1 is the main subtype found in the heart and also the most studied. The activity and TNFα signalling effects of TNFR2 is less well elucidated.

More recently, a mitochondrial binding protein for TNFα has been discovered. This suggests the presence of a pathway capable of delivering TNFα directly from the cell surface to the mitochondria independently of the activation of the cell surface TNFα receptors (Ledgerwood et al., 1998; Busquets et al., 2003).

2.1 TNFα in the heart

TNFα is implicated as a mediator in cardiovascular disease, including acute myocardial infarction (Maury and Teppo, 1989), atherosclerosis (Barath et al., 1990), chronic heart failure (Levine et al., 1990; Fichtlscherer et al., 2001) and ischaemia/ reperfusion (Squadrito et al., 1993; Gurevitch et al., 1996). Macrophages are present in the heart and are a rich source for this pleiotrophic cytokine among others (Gurevitch et al., 1996). TNFα can also be produced by endotoxin-induced cardiomyocytes (Kapadia et al., 1995).

When TNFα is expressed at a sufficiently high concentration, it is able to mimic the heart failure phenotype that includes progressive left ventricular (LV) dysfunction, pulmonary edema, LV remodelling and cardiomyopathy (Bozkurt et al., 1998; Kubota et al., 1997). Cytokines do not elicit heart failure but rather the overexpression of the cytokine cascades contributes to its progression (Seta et al., 1996). TNFα is able to induce cardiomyocyte apoptosis via signalling through the TNFα receptor associated death domain (TRADD) (Vanderabeele et al., 1995).

2.2 TNFα and Ischaemia/ Reperfusion

Controversies exist as to whether reperfusion of the ischaemic myocardium is required to rescue the tissue or trigger an inflammatory process that leads to severe lethal cell injury (Hansen, 1995; Hearse and Bolli, 1992). Kurrelmeyer et al. demonstrated a cytoprotective role for endogenous TNFα signalling against cardiomyocyte apoptosis after acute ischaemic injury (Kurrelmeyer et al., 2000). They observed an increase in infarct size in mice lacking both TNFα receptors compared to wild-type littermate controls. TNFα has also been shown to be protective against hypoxic injury in cultured cardiomyocytes (Nakano et al.,

1998). In contrast, TNFα knockout mice have been shown to be protected against ischaemia/ reperfusion injury (Maekawa et al., 2002). This left coronary occlusion study demonstrated a significant reduction in infarct size in TNFα knockout mice hearts as compared to wild-type mice. A reason for this discrepancy is difficult to explain. The involvement of second messenger systems and divergent pathways of TNFα has not been completely elucidated. It is possible that TNFα may be protective in cardiomyocytes during ischaemia without reperfusion but harmful during reperfusion (Maekawa et al., 2002). The deleterious and beneficial role of TNFα might depend on its concentration produced by the myocardium during ischaemia with and without reperfusion (See review, Sack et al., 2000).

2.3 TNFα and Ischaemic preconditioning

TNFα can be activated by toll-like receptors in the myocardium in response to ischaemia and reperfusion injury (Kupatt et al., 1999; Meldrum et al., 1998). Toll-like receptors are pattern-recognition receptors that bind extracellular pattern-recognition molecules (such as lipopolysaccharides) and initiate the subsequent innate immunity cytokine cascade (See review Smith et al., 2002).

Cardioprotection induced by ischaemic preconditioning has been demonstrated to produce reduced TNFα levels during ischaemia-reperfusion *in vivo* in rabbits (Belosjorow et al., 1999) Ischaemic preconditioning in an isolated perfused mouse model resulted in upregulation of TNFα (Smith et al., 2002b). This study suggested that cardiac TNFα production is a requirement for ischaemic preconditioning-induced cardioprotection since TNFα knockout mice could not be preconditioned in the isolated perfused mouse heart. In addition, administration of TNFα can mimic ischaemic preconditioning in a dose and time dependant manner and thus lead to cardioprotection in the isolated rat heart model (Lecour et al., 2002).

The mechanisms involved in TNFα induced cardioprotection still remain poorly understood. It is also possible that the protective signals mediated by TNFα and ischaemic preconditioning may be activated via similar but divergent pathways. Free radicals are involved as a trigger of ischaemic preconditioning (Das et al., 1999). Since TNFα are known to activate free radicals (Suematsu et al., 2003), Lecour et al., were able to demonstrate a similar role for free radicals in TNFα preconditioning (2005). Sphingolipids are involved in the signalling cascade in TNFα-mediated preconditioning but not in ischaemic preconditioning (Lecour et al., 2002). Activation of p38-MAPK is involved in ischaemic preconditioning (Nakano et al., 2000). However, a preconditioning dose of TNFα did not activate p38-MAPK in the murine heart (Tanno et al., 2003). At higher non-cardioprotective concentrations, TNFα induced p38-MAPK but was not protective (Tanno et al., 2003).

It has been established that ischaemic preconditioning confers cardioprotection via the activation of prosurvival kinases Akt and Erk 1/2 (RISK pathway; Hausenloy et al., 2005b). IPC also requires the recruitment of STAT-3 and BAD, possible downstream targets of Akt and Erk 1/2 (Hattori et al., 2001; Xuan et al., 2001; Hausenloy et al., 2005a and b). Recently it was shown that STAT-3 can be activated in TNFα preconditioning independent of the RISK pathway (Lecour et al., 2005). It was also suggested in this paper that BAD may be phosphorylated by the activation of both pathways. Therefore the exact mechanism of TNFα mediated cardioprotection is currently unknown and needs to be clearly defined.

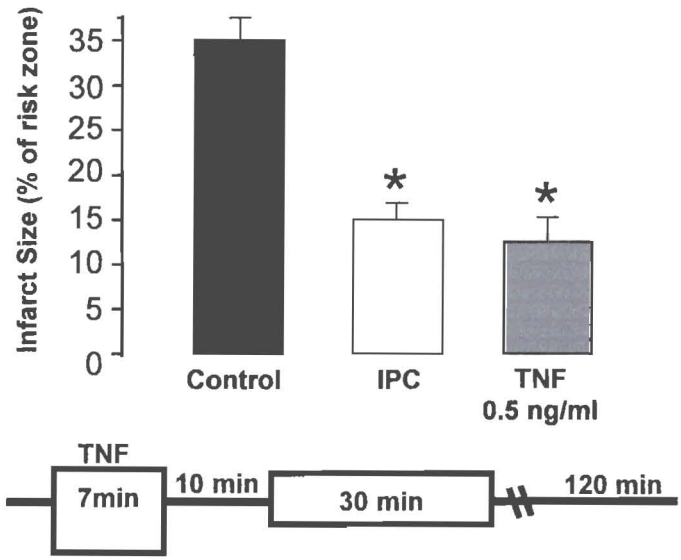


Figure 4: **TNFα can mimic ischaemic preconditioning and thus confer cardioprotection in the isolated rat heart model.** Hearts preconditioned with 0.5ng/ml TNFα prior to the sustained ischaemia showed a significant reduction in infarct size. $p<0.05$ vs Control IPC= ischaemic preconditioning; TNF= Tumour necrosis factor-alpha (Lecour et al., 2002)

3. Nuclear factor kappa B (NFκB)

In 1986, Sen and Baltimore discovered a transcription factor known as nuclear factor kappa B (NFκB) that was initially thought to exclusively bind the kappa light chain immunoglobin enhancer in β cells. However, they subsequently identified NFκB in other cell types in an inactive cytoplasmic form (Sen and Baltimore, 1986a and 1986b). NFκB is primarily involved in the regulation of the immune system as well as inflammatory and stress responses. Due to its involvement in many diseases, NFκB has been intensively investigated in the past two decades. NFκB can function to prevent programmed cell death (PCD) through the induction of target genes for antiapoptotic proteins such as FLICE (FADD [Fas-associated death domain]-homologous interleukin-1β-converting enzyme [ICE]) inhibitory protein (FLIP), Bcl-X_L, cellular inhibitor of apoptosis (c-IAP) and X chromosome-linked inhibitor of apoptosis (XIAP) (Luo et al., 2005).

3.1 Activation of NFκB

NFκB is composed of a complex of two variable subunits belonging to the Rel/ NFκB family. These proteins contain a Rel homology domain which is involved in the binding to DNA, dimerization and interaction with other proteins (See review, Jones et al., 2003). The first subunit, comprising of p65, c-rel, v-rel, or rel B, contains a nuclear localization domain and one or more transcriptional activation domains. The second unit has only a transcriptional activation domain, NFκB1 (p105 and p50) or NFkB2 (p100 and p52). NFκB consists of a dimer of these subunit proteins with the most prevalent being p65/p50 (Dawn et al., 2001). Homodimers of p50/p50 do exist and are thought to be involved with the repression of gene expression (Perkins, 1997; Haudek et al., 2001).

Figure 5 shows a schematic representation of NFκB activation. Inactive NFκB resides in the cytoplasm bound to inhibitory IκB proteins (Baeuerle, 1991). NFκB activation can be initiated through receptor-mediated events such as ligand binding (eg. TNFα, interleukin-1 [IL-1] or lipopolysaccharide [LPS]) or non-receptor-mediated pathways such as oxidative stress or UV radiation (Caamaño and Hunter, 2002). This triggers the phosphorylation and activation of the IκB kinases (IKKs) by upstream protein kinases (such as NIK, MEKK-1 or Akt). The IKK complex consists of three components, namely IKKα, IKKβ and NEMO/ IKKγ. The IKK complex in turn, phosphorylates IκB at its amino terminus leading to its disassociation from NFκB and subsequent proteosomal degradation by ubiquination (Karin and Ben-Neriah, 2000). NFκB, now in its active form, translocates to the nucleus where it binds to specific recognition sequences and regulates transcription. As a negative feedback loop, NFκB will induce IκB gene transcription to regulate its activity (Brown et al., 1993).

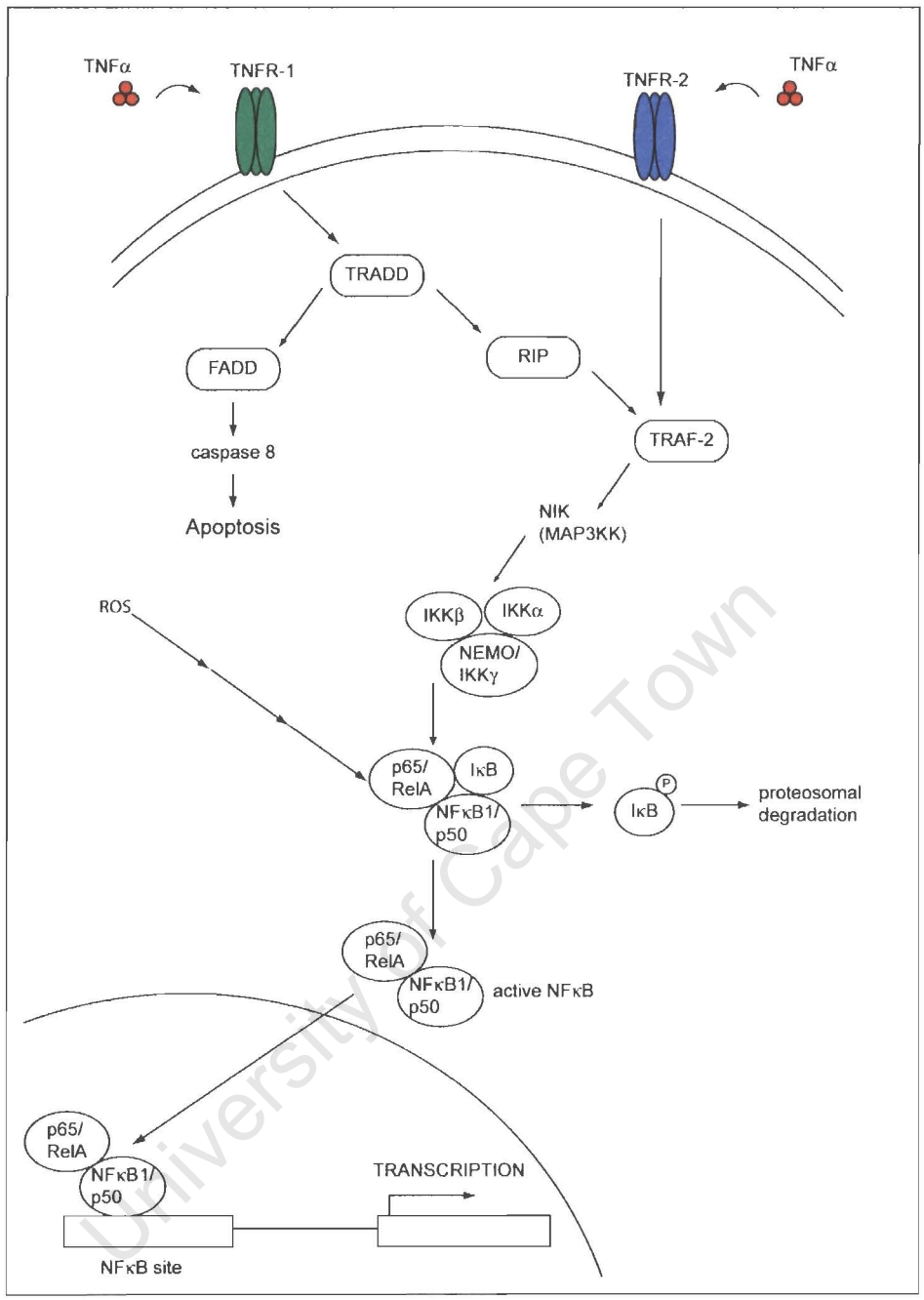


Figure 5: **Schematic representation showing the activation of NFκB and some of the associated factors involved.** Details are discussed thoughout the text. FADD= Fas-associated death domain, IKK= IκB kinase complex, MAP3KK= mitogen-activated protein kinase kinase, NEMO= NFκB essential modulator, NFκB= nuclear factor kappa-B, RIP= receptor-interacting protein, ROS= reactive oxygen species, TNFα= tumour necrosis factor-alpha, TNFR= TNF receptor, TRADD, TNF receptor associated death domain, TRAF-2= TNF receptor associated factor-2. Adapted from Sack et al., 2000.

3.2 NFκB and Ischaemia/ Reperfusion

The induction of NFκB in the isolated myocardium has been demonstrated shortly after the initiation of ischaemia and at the time of reperfusion in all species including humans (Li et al., 2001; Canty et al., 1999; Maulik et al., 1998) where it is involved in the regulation of many genes for cardiomyocyte function, cell death/ survival and stress responses (Balligand et al., 1994; Li et al., 1999). NFκB activation following an *in vivo* myocardial infarction is biphasic peaking after 15 min and 3h reperfusion. This may be explained by a primary activation of NFκB by reactive oxygen intermediates and a secondary activation by proinflammatory cytokines produced by the first activation (Chandrasekar et al., 1997). Recently, Tillmanns et al have developed a new and novel technique where they were able to detect *in vivo* non-invasive NFκB activity after myocardial infarction by using a light sensitive CCD camera in a mouse model (Tillmanns et al., 2006). They found an increase of NFκB activity following myocardial infarction with a maximum at three days.

A detrimental role of NFκB during reperfusion is suggested indirectly by functional studies of the product of genes that NFκB regulates during the reperfusion phase such as the leukocyte cell adhesion molecules, cytokines and chemokines (Gumina et al., 1996; Cain et al., 1999; Li et al., 1999). In a direct study, a double-stranded decoy oligonucleotide with a high affinity for NFκB was transfected intracoronarily into rats before coronary artery ligation. This synthetic DNA bound the transcription factor and blocked activation of NFκB during reperfusion, resulting in a reduced myocardial infarction (Morishita et al., 1997). A homogeneous distribution of decoy is difficult to obtain and there are other transcription factors that bind or overlap with NFκB DNA binding elements. Therefore, these results may not be very specific.

Brown et al. used specific transgenic mice to analyse the involvement of Ser32/Ser36 and Tyr42 of IκB isoform IκBα. Subjecting mice that repress only

Ser32/Ser36 or all three residues to ischaemia/reperfusion injury completely abrogated NFκB activation and reduced infarct size by 70%. This demonstrated that the activation of NFκB during I/R injury was more dependent on the phosphorylation of Ser32/Ser36 than Tyr42 residues on the IκB protein and that NFκB contributes to infarct development after I/R (Brown et al., 2005).

The deleterious role of NFκB in ischaemia/reperfusion has also been studied using pharmacological NFκB inhibitors. Diethyldithiocarbamate (DDTC) and pyrrolidine dithiocarbamate (PDTC) are well-known antioxidants that inhibit NFκB (Schreck et al., 1992) and reduce I/R injury (Irem et al., 1991). However, both of these pharmacological agents are non-specific and are also known as free radical scavengers. Thus, the attenuated NFκB activity may be an indirect effect of reduced ROS levels (Jones et al., 2003). Recently, the use of IKK-2 inhibitor V (IMD0354), a highly specific inhibitor of IKKβ, which phosphorylates IκBα at Ser32/Ser36 was able to successfully block NFκB activation after myocardial I/R and reduce infarct size by 59% (Onai et al., 2004).

3.3 NFκB and Ischaemic preconditioning

NFκB is activated during the preconditioning stimulus and during the sustained ischaemia and reperfusion. The cytoprotective effects of ischaemic preconditioning have been linked to the activation of NFκB by classic and delayed ischaemic or pharmacological preconditioning (Maulik et al., 1998; Morgan et al., 1999; Xuan et al., 1999). It is easy to understand the involvement of NFκB in mediating protection during delayed preconditioning because there is time for *de novo* cardioprotective gene transcription and translation. The beneficial role of NFκB in classic preconditioning is more difficult to understand because there is a short timeframe involved for NFκB to initiate transcription (see review Valen, 2004).

Das and colleagues demonstrated that NFκB was essential to tyrosine kinase signaling of p38 MAP kinase regulation during adaptation to ischaemia in isolated rat perfused hearts. The beneficial effects of ischaemic preconditioning were abrogated with pretreatment of NFκB inhibitor, SN50 peptide before the ischaemic stress adaptation (Maulik et al., 1998). Similarly, Morgan et al. determined that NFκB activity was increased during ischaemic preconditioning stimulus and attenuated after I/R injury following IPC in an *in vivo* rabbit model (1999). Inhibition of NFκB during the ischaemic preconditioning stimulus also abolished cytoprotection against I/R injury. It was suggested that NFκB might induce myocardial protection by the downregulation of the inflammatory response during reperfusion.

Das et al. also suggested a role for oxygen free radicals as a second messenger during the ischaemic preconditioning stimulus in the myocardium. Free radical signaling leads to phosphorylation of tyrosine kinase resulting in the activation of p38 MAP kinase and MAPKAP kinase 2 and ultimately, NFκB. This process was independent of protein kinase C (PKC) which would suggest separate signaling pathways in ischaemic preconditioning, one involving PKC and the other one involving NFκB (Das et al., 1999).

Delayed preconditioning with adenosine A1 receptor stimulation has been shown to confer protection in rabbits via upregulation of NFκB-regulated manganese superoxide dismutase (Dana et al., 2000). Stimulation of the adenosine A3 receptor in the heart also protects against I/R injury via activation of NFκB (Zhao and Kukreja, 2002).

No previous studies have explained the role of NFκB at the time of reperfusion in an ischaemic or pharmacological preconditioning model. Heat shock proteins have been shown to modulate the activity of NFκB DNA binding activity and might be involved in reducing NFκB activation at reperfusion by downregulation of the inflammatory response (Carter, 1997).

3.4 TNFα and NFκB

TNFα is a potent activator of NFκB. TNFα binds to its type I receptor and triggers a series of signaling molecules such as TNFα receptor-associated factor (TRAF)-2, TRAF-5, TNFR-associated death domain (TRADD) and receptor interacting protein (RIP) to assemble on the intracellular domain of the receptor. The IKK complex is subsequently activated by NFκB-inducing kinase (NIK) leading to the phosphorylation of IκB (Sakurai et al., 2003). TNFα can also initiate cardioprotective programs against ischaemia/reperfusion injury via Activation protein-1 (AP-1) through TNFR-1 (see review, Sack et al., 2000).

TNFα may also bind to its type II receptor which results in the accumulation of TRAFs and cellular inhibitor of apoptosis (cIAP) proteins and lead to NFκB activation (Darnay and Aggarwal, 1997). However, only activation of TNFR-1 and not TNFR-2 receptor complex can progress to programmed cell death. The induction of ROS by TNFα may contribute to NFκB activation (Schreck et al., 1992).

Since TNFα is a critical mediator of heart failure, the cardiac NFκB levels of transgenic mice that continuously overexpressed TNFα was analysed against healthy wild-type mice that were acutely exposed to TNFα. Although the abundance of NFκB p50-p65 homodimers located in the nucleus did not differ between the two groups, p50 homodimers was only detected in the transgenic mice. The p50 subunits contain a nuclear translocation domain but lack a transactivation domain which implies that these homodimers may act to downregulate the NFκB binding activity by blocking transcription and prevent progressive heart failure in the transgenic mice (Haudek et al., 2001). Whether this regulatory mechanism occurs in humans is unknown.

The beneficial effects of TNFα may also result from the activation of NFκB. TNFR-1/2 double knockout mice displayed an increased number of apoptotic cells and larger infarcts after coronary occlusion with no reperfusion (Kurrelmeyer et al., 2000). It is possible that TNFR1 and TNFR2 might confer cytoprotective signaling through TRAF-2 which has demonstrated to mediate NFκB activation (Rothe et al., 1995). TNFα has also been shown to protect cultured cardiomyocytes against hypoxic injury (Nakano et al., 1998).

In contrast, TNFα has been shown to exacerbate myocardial I/R injury by the activation of NFκB in TNFα knockout mice (Maekawa et al., 2002). It is difficult to explain the differences between the results but perhaps NFκB dependent gene expression programs may differ between the scenarios which ultimately determine the pro cell death or pro cell survival actions of NFκB.

4. Summary

With the increasing rates of heart attacks in the world, major therapeutic and preventative strategies are required. Ischaemic preconditioning represents a means to achieve this, whereby a few short bursts of ischaemia with intermittent reperfusion can significantly attenuate infarct size. However, applying ischaemic preconditioning to the myocardium in a clinical setting is difficult. Therefore, it is important to elucidate the signalling pathways involved in mediating the protective effects of preconditioning which may provide targets for therapeutic drug design and better treatments. TNFα is activated during ischaemic preconditioning and can be beneficial in a dose and time dependent manner. Nuclear factor kappa-B is a transcription factor that has been found to be upregulated during ischaemic preconditioning and concomitantly activated with anti-apoptotic factors. NFκB has also been shown as being pro cell death. It is likely that different signalling pathways are activated depending upon the specific stimuli that leads to either cell death or survival. It is possible that a preconditioning dose of TNFα may activate NFκB which initiates the transcription of pro survival factors.

B. OBJECTIVES AND HYPOTHESIS

B. Objectives and Hypothesis

The role of nuclear factor kappa-B in TNFα-mediated preconditioning

Tumour necrosis factor alpha (TNFα) is a pleiotrophic cytokine that has been associated with cardiovascular diseases such as heart failure and atherosclerosis. Recently, we have shown that TNFα can mimic ischaemic preconditioning in a dose and time manner and thus confer cardioprotection. However, the mechanisms involved in this cardioprotective effect still remain unclear. Nuclear factor kappa-B (NFκB) is a transcription factor that is known to be activated in response to a TNFα stimulus. **Using a cell culture model (differentiated C_2C_{12} cells), we hypothesised that TNFα induced preconditioning requires the activation of NFκB.**

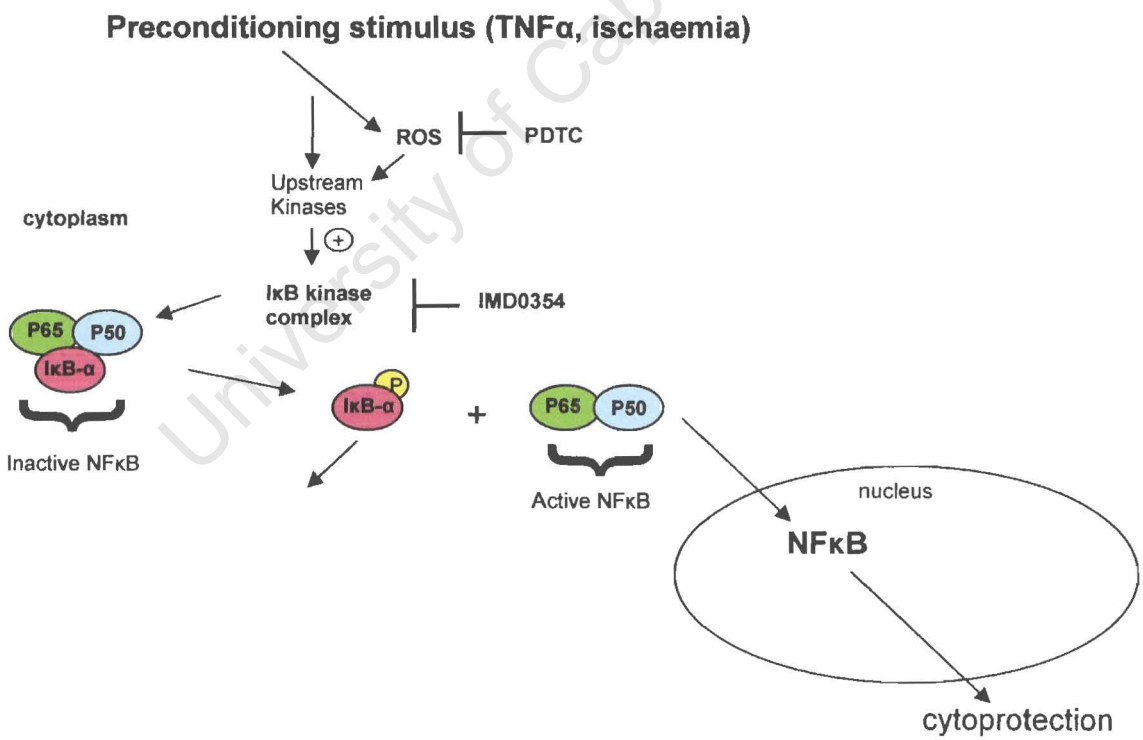


Figure 6. **Proposed TNFα/NFκB signalling pathway leading to cytoprotection.** The sites of inhibition of PDTC and IMD0354 are indicated in the diagram. IMD0354= IKK-2 inhibitor V, NFκB= nuclear factor kappa-B, PDTC= ammonium pyrrolidone derivative dithiocarbamate, ROS= reactive oxygen species, TNFα= tumour necrosis factor alpha

C. MATERIALS AND METHODS

Materials and Methods

1. Cell culture

The protective effect of classical preconditioning can be induced in the skeletal muscle cell in a similiar manner to the heart (Pang et al., 1995). For this study, C_2C_{12} myoblasts derived from mouse skeletal muscle cells were obtained from the European Collection of Cell Cultures (Centre for Applied Microbiology and Research, UK). These myoblasts can be differentiated into multinucleated myotubes that are phenotypically similar to cardiomyocytes and are able to propagate rapidly in tissue culture flasks that do not require prior coating for cell adherence. These characteristics make them suitable for a cardiac study model.

Cells were stored in cryovials containing 1 x 10^6 cells/ ml in liquid nitrogen at -196°C. When required, a cryovial was thawed for 30-40 seconds in a 37°C water bath. The contents were sterilely transferred to a 75 cm^3 tissue culture flask containing Dulbecco's Modified Eagle Serum (DMEM) with 4.5 g/L glucose, 0.110 g/L sodium pyruvate and L-glutamine supplemented with 10% fetal calf serum (FCS) and 1% (wt/vol) penicillin/ streptomycin (P/S); (All purchased from Highveld Biological, RSA). The myoblasts were grown in 5% CO_2 trypsinised with 0.25 % (w/v) Trypsin (Highveld Biological, RSA) supplemented with 0.2 % EDTA (w/v); (Sigma, Germany) made up in phosphate buffered saline (PBS). The trypsinisation process was allowed for 3 minutes at 37°C before it was neutralized with twice the volume of 10 % FCS DMEM. The cells, now in suspension, were transferred to 50 ml polypropylene conical tubes and centrifuged for 5 minutes at 1000 rpm. Cells were counted in a Neubauer haemocytometer before centrifugation. 25 cm^3 Tissue culture flasks were seeded with sufficient cells to allow 80 % confluency in two-three days. At this point, differentiation was initiated with DMEM supplemented with 1% horse serum (HS); (Sigma, Germany) and 1 % P/S. Differentiation was maintained

with fresh 1 % HS DMEM every two days till day 8. Experiments were performed using differentiated myotubes between day 8 and 10.

2. Preconditioning Protocol

During physiological ischaemia, the pH becomes mildly acidic, potassium concentration increases and metabolic activity is perturbed. Myocardial ischaemia was simulated in the C_2C_{12} study model under these conditions. The protocol was adapted from Esumi et al (1991). The simulated ischaemia (SI) buffer (137 mM NaCl, 12 mM KCl, 0.5 mM MgCl, 0.9 mM $CaCl_2$, 20 mM Hepes and 20 mM 2-Deoxy-d-Glucose [2-DG]) was adjusted to a pH of 6.4. The inclusion of 2-DG served to inhibit glycolysis and thereby disrupting metabolic activity. To simulate ischaemia, low oxygen consumption was also required. Hence, a multigas incubator (SANYO Electric Co., Ltd, Japan) supplying 5 % CO_2, 94 % N_2 and 1 % O_2 was employed to house the 25 cm^3 tissue culture flasks in a hypoxic environment. The preconditioning protocol involved the following groups which are schematically represented in Figure 7:

- Group 1: the normoxic control group represented untreated cells that were maintained in their normal environment in the standard 5 % CO_2 incubator for the period of the experiment.

- Group 2: the SI control group consisted of cells that were incubated for 7 hours in the hypoxic incubator followed by incubation with 1 % HS DMEM for 1 hour in the normoxic incubator.

- Group 3: cells were treated with TNFα (recombinant murine TNFα, Peprotech, USA, 0.5 ng/ ml in DMEM with 1 % HS) for 30 minutes in the normoxic incubator. Cells were then reincubated with fresh 1 % HS DMEM for 60 minutes in the normoxic incubator before incubation with SI buffer for 7 hours in the

hypoxic buffer. Finally, cells were incubated with 1 % HS DMEM for one hour in the normoxic incubator.

- Group 4: In the IPC group, cells were incubated in the hypoxic incubator with SI buffer, pH 6.2 without 2-DG to avoid inhibition of glycolysis during the preconditioning stimulus. After 30 minutes, cells were supplemented with fresh 1 % HS DMEM prior to normoxic incubation for one hour. This was followed by incubation with SI buffer for 7 hours in the hypoxic incubator. Cells were then incubated with 1 % HS DMEM for one hour in the normoxic incubator.

- Groups 5-8: to explore the potential involvement of NFκB as a trigger, two NFκB inhibitors, IKK-2 inhibitor V (1 μM, Onai et al., 2004); (IMD0354); (Calbiochem, Germany) or ammonium pyrrolidone derivative dithiocarbamate (PDTC); (100 μM, Schreck et al, 1992); (Sigma-Aldrich, USA) were added prior to the addition of TNFα (0.5 ng/ ml) in 1 % HS DMEM and incubated in the normoxic incubator. Similiarly, these inhibitors were added to the SI buffer (no 2-DG, pH 6.2) during the ischaemic preconditioning stimulus for 30 minutes in the hypoxic incubator. Following reincubation for 60 minutes with 1 % HS DMEM in the normoxic incubator, cells were incubated for 7 hours in the hypoxic incubator. Cells were then incubated in 1 % HS DMEM for one hour in the normoxic incubator.

- Groups 11-14: to investigate the role of NFκB as a mediator, IMD0354 or PDTC were added at the beginning of the reperfusion period after 7 h simulated ischaemia for the duration of one hour.

- Groups 9, 10, 15 and 16: For inhibitor control groups, IMD0354 or PDTC were either added for 30 minutes in 1 % HS DMEM followed by two washes, 1 % HS DMEM for 1 hour in the normoxic incubator or after the 7 h SI for 1 hour in the normoxic incubator.

In all the groups, cells were washed twice with phosphate buffered saline (PBS; pH 7.4) prior to the change of medium.

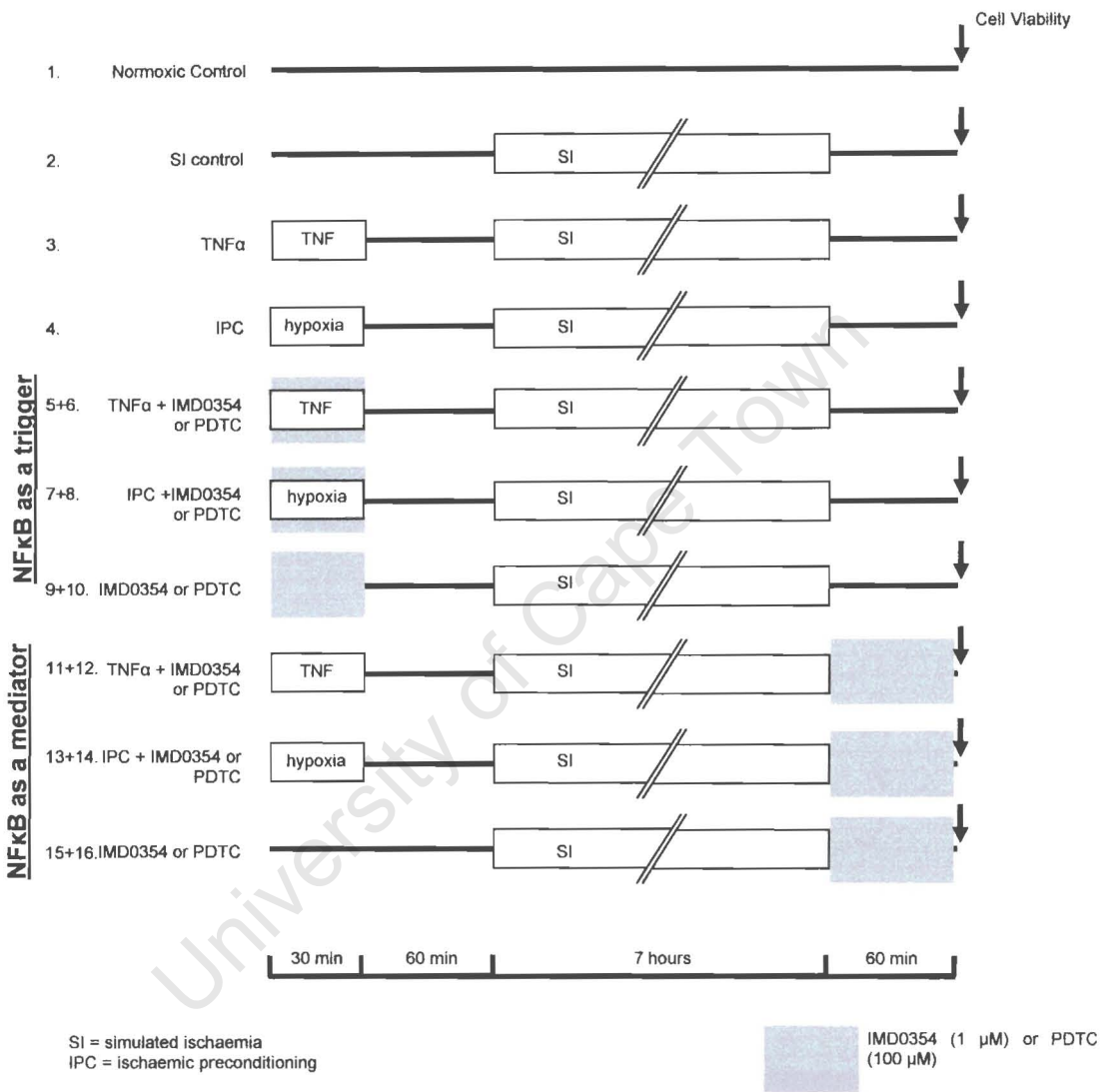


Figure 7: **Schematic representation of preconditioning protocols** – investigation of NFκB as a trigger and mediator. IMD0354= IKK-2 inhibitor V, IPC= ischaemic preconditioning, SI= simulated ischaemia, PDTC= ammonium pyrrolidone derivative dithiocarbamate, TNF= tumour necrosis factor alpha

3. Cell viability

3.1 Propidium Iodide

Flow cytometry was employed to assess the cell viability (Boise et al., 1993, FACS Calibur, Becton-Dickinson, USA). This was achieved by measuring the uptake of propidium iodide (PI); (Sigma, Germany). The principle of the technique involves the penetration of PI into a cell and its binding to DNA. A fluorescence signal is emitted that is measured. PI does not transverse the cell membrane of an intact cell and thus the technique is useful for identifying dead cells. In flow cytometry, measurements are made as the individual cells pass in a fluid stream through one or more laser beams. The scattered and emitted fluorescent light is converted to electrical signals by photomultiplier tubes (PMT) and data is outputted on a computer.

Following the preconditioning protocol, cells were trypsinised and resuspended in 1 x 10^6 cells/ ml in PBS. PI (1.5 µM) was then added and cells were incubated at 37°C for 5 minutes in the standard 5 % CO_2 incubator. The PI fluorescence was measured in a population of $1x10^4$ cells and analysed using CellQuest software.

3.2 Trypan Blue

Cell viability was also evaluated using the trypan blue exclusion method (Kitakaze et al., 1997). Cells were trypsinised and resuspended in PBS. A small volume of cell suspension was mixed in a 1:1 ratio with trypan blue (4 µM); (Sigma, Germany). The trypan blue seeps through the disrupted membrane of dead or dying cells and in the process, stains them in blue. The mixture is pipetted onto a Neubauer haemocytometer and the counting is assessed on a

light microscope. The ratio of blue stained cells to grey, unstained live cells is calculated and expressed as a percentage.

4. Respiratory Parameters

Oxygen consumption during cellular respiration by the mitochondria fuels the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). ATP provides the driving force for many enzymatic functions in the cell. The metabolism of endogenous substrates as a result of respiration in whole cells is termed State 2 respiration. Ischaemic and pharmacological preconditioning have previously been shown to increase cellular respiration prior to a sustained ischaemia (Minners et al., 2001). This study was performed to confirm a protective role for NFκB in ischaemic and TNFα-mediated preconditioning. An increase in cellular respiration would suggest the induction of the preconditioning effect. PDTC, an NFκB inhibitor was used to test the transcription factor's involvement in triggering cytoprotection. Respiration studies were conducted immediately after the simulated index ischaemia as shown in Figure 8.

State 2 respiration was measured using an Oxytherm respirometer equipped with a Clark-type electrode and a Peltier temperature control unit (Oxytherm, Hansatech, Norfolk, UK). Calibration was set with PBS at a temperature of 37°C. The temperature did not fluctuate significantly and remained consistent throughout the experiment. Calibration was verified by the addition of 1ml PBS into the chamber followed by a baseline reading. Cell viability was assessed with the propidium iodide method. The percentage viability was used to calculate a factor from which the respiration values from viable cells could be determined.

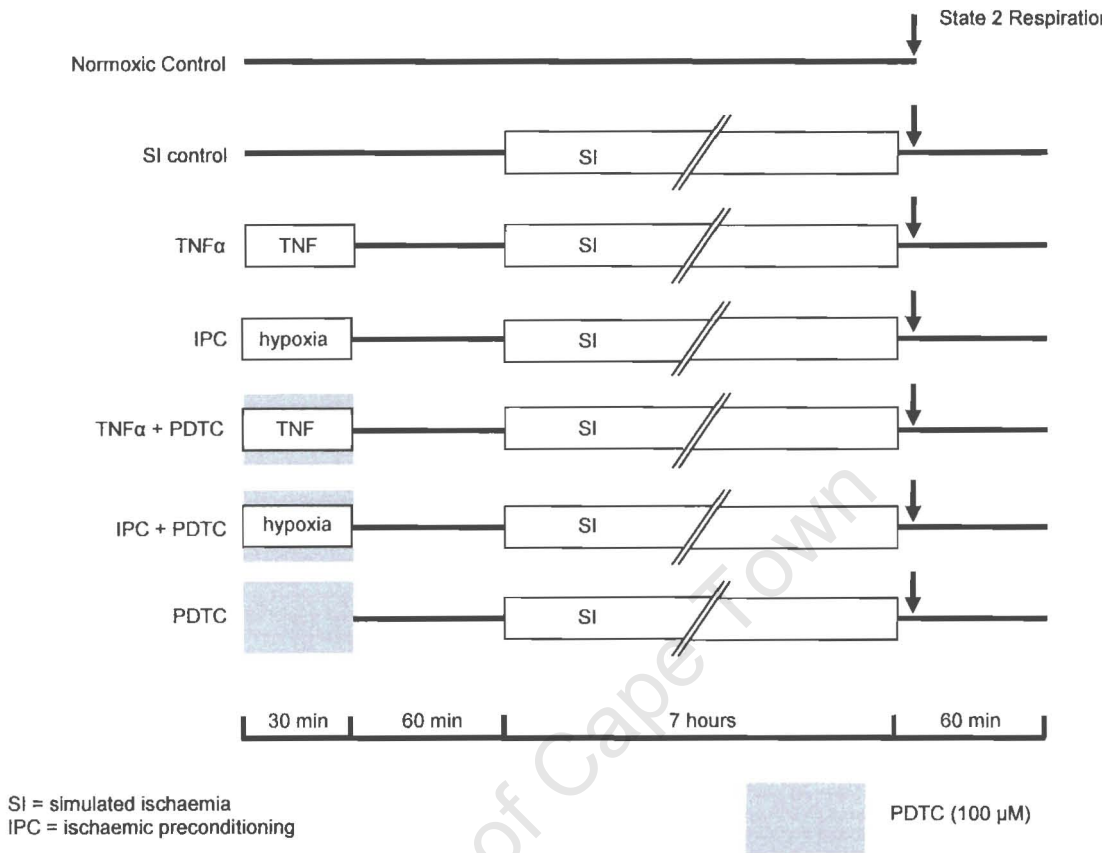


Figure 8: **Schematic representation of state 2 respiration protocol.** IPC= ischaemic preconditioning, SI= simulated ischaemia, PDTC= ammonium pyrrolidone derivative dithiocarbamate, TNF= tumour necrosis factor alpha

5. Protein Isolation

To investigate the role of NFκB as a trigger, either IMD0354 or PDTC were administered during the ischaemic or TNFα preconditioning stimulus. Samples were collected after 15 minutes (figure 9) as this timepoint was observed as the peak of IκB phosphorylation (data not shown). To explore the involvement of NFκB as a mediator, IMD0354 or PDTC were given immediately after the 7 h simulated ischaemia. Following 15 minutes of reperfusion, samples were

extracted for IκB phosphorylation analysis (figure 9, peak of IκB phosphorylation – data not shown). After treatment the cells were washed quickly with phosphate-buffered saline (pH 7.4) and then transferred to the respective eppendorf in the presence of 500 µl solution lysis buffer containing 10 % Nonidel P-40, 4 M NaCl, 1 M Hepes (pH 7.9), 500 mM EDTA and complete EDTA-free protease inhibitor cocktail (Roche Systems, USA). Samples were centrifuged at 3000 rpm for 30 seconds and the supernatant was transferred to a new tube. These tubes were spun for 5 minutes at 5000 rpm. The subsequent supernatant fraction contained the cytosolic proteins. Protein concentrations were determined using the Protein Lowry method (Lowry et al., 1951). Extracts were stored at -80°C.

6. Bio-plex Array Analysis for Phosphoprotein Testing

Bio-plex phosphoprotein assays are bead-based singleplex or multiplex assays that detect phosphorylation of proteins in cell and tissue sample lysates (www.bio-rad.com/bio-plex/). The lysates are coupled to internally dyed beads and incubated with the specific biotinylated detection antibody in a 96-well plate. Streptavidin-phycoerythrin (streptavidin-PE) is then added to bind the detection antibodies. Data is acquired using a dual laser, flow-based microplate reader system and outputted as fluorescence intensity on the Bio-plex Manager™ software.

A singleplex assay was performed to test for phosphorylated IκB proteins from cell lysates. The Bio-Plex phosphorylation assay and testing reagent kits were purchased from Bio-Rad Laboratories Inc, USA. The desired number of wells in a 96-well filter plate was washed followed by addition of 50 µl of coupled beads (diluted 1:1 with wash buffer). The wells were vacuumed filtered and washed twice. The vortexed lysates containing the cytosolic proteins (50 µl) were then added to each respective well and incubated on a mini-plate shaker for 4 hours

in the dark. After being vacuumed filtered and washed thrice, the biotin-labeled p-IκB detection antibodies were added to the wells and incubated for 30 minutes. The wells were vacuumed filtered again followed by three wash steps. Strepavidin-phycoerythrin (1 x streptavidin-PE) was added to each well and incubated for 10 minutes. Samples were vacuumed filtered then rinsed with resuspension buffer 3 times. 125 µl of resuspension buffer added to each well. After 30 seconds incubation, samples were analyzed using the Bio-Plex suspension array system.

7. Lowry Protein Determination

Protein concentrations were determined using the Lowry method (Lowry et al., 1951). Protein samples were added to 1 ml of distilled water in a 1:200 volume ratio. 1ml of Solution A containing a composite of three solutions in equal volumes; CTC reagent, 10% sodium dodecyl sulfate (SDS) and 50 µM sodium hydroxide (NaOH) was added to each tube. CTC reagent contained 2% (w/v) sodium carbonate (Na_2CO_3) mixed in a ratio 100:1:1 (v:v:v) with 1% (w/v) copper sulphate ($CuSO_4.5H_2O$) and 1% (w/v) di-potassium tartrate hemihydrate ($C_4H_4K_2O_6.0.5H_2O$). After 10 minutes at room temperature, 0.02 N Folin phenol reagent was added to each tube. Following 30 minutes of incubation, the absorbance was measured at 750nm.

8. Statistical Analysis

Data are expressed as mean ± SEM. Statistical significance between multiple groups was determined by one-way analysis of variance (ANOVA) followed by the Student Newman-Keul post hoc test (Graph Pad Instat), A value of $P < 0.05$ was considered significant.

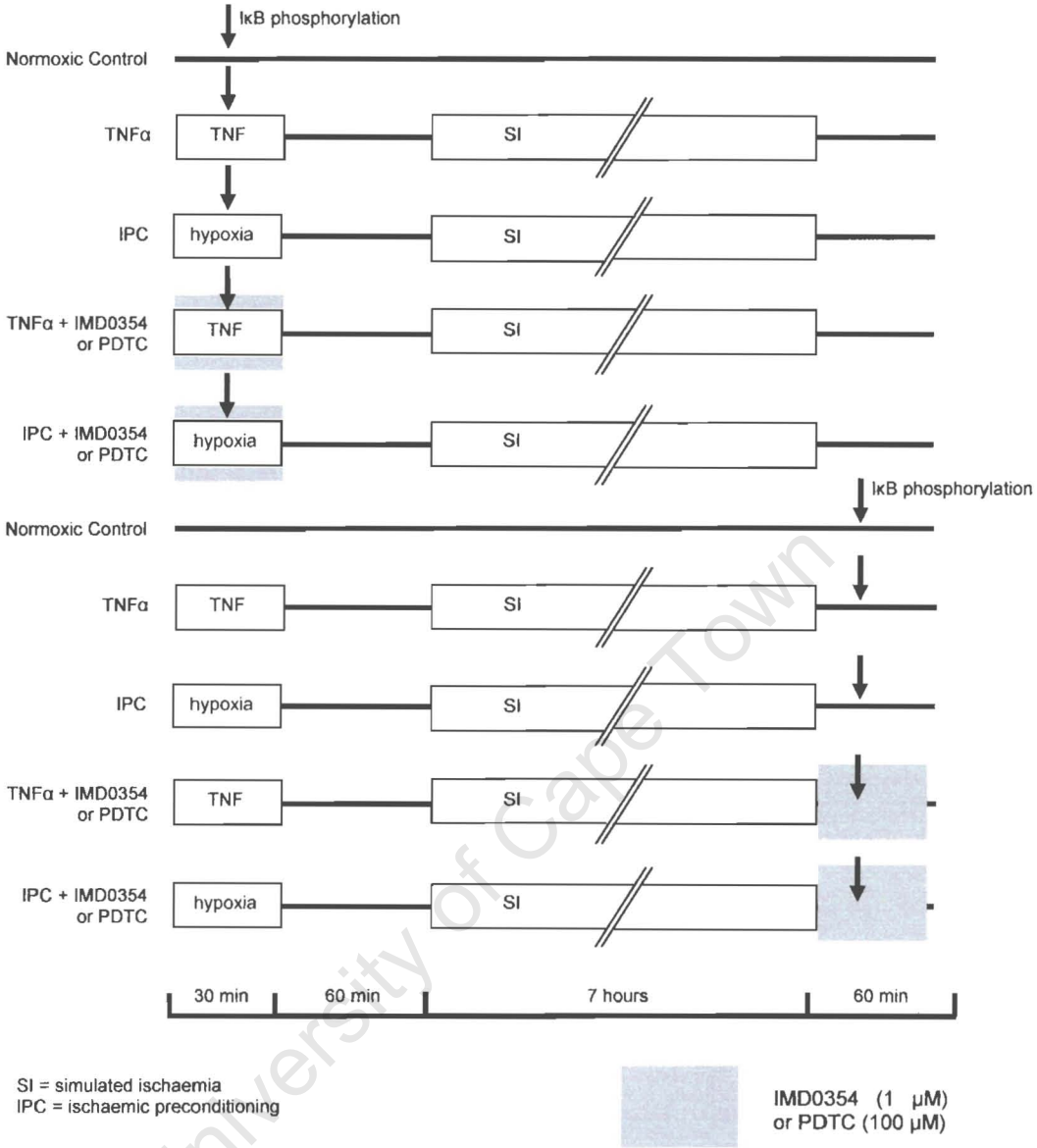


Figure 9: **Schematic representation of the timepoints used to analyze IκB phosphorylation.** IMD0354= IKK-2 inhibitor V, IPC= ischaemic preconditioning, SI= simulated ischaemia, PDTC= ammonium pyrrolidone derivative dithiocarbamate, TNF= tumour necrosis factor alpha

D. RESULTS

D. Results

1. TNFα can mimic ischaemic preconditioning in C_2C_{12} cells

1.1 Cell viability

Cell viability was assessed with Trypan Blue (TB) exclusion test (figure 10) and confirmed with the propidium iodide (PI) method (figure 11). As shown in figure 10, untreated cells (N) that were maintained under normoxic conditions during the full protocol had a cell viability of 84.3±1.3% (TB test) and 82.8±3.02% (PI test). Seven hours of simulated ischaemia followed by one hour of reoxygenation (IC) dramatically reduced the cell viability to 43.7±8.1% and 30.7±11.8% for TB and PI tests, respectively. Preconditioning the cells with 30 minutes of simulated ischaemia prior to the index ischemia (IPC) significantly improved the cell survival to 66.3±1.2% (TB) and 72.0±12.01% (PI) (*p< 0.001 vs IC group). Similarly, cells preconditioned with TNFα (0.5ng/ml) prior to the index simulated ischaemia restored the cell viability to 70.6±6.1% (TB test) and 69.0±3.6% (PI test) (*p< 0.001 versus IC group).

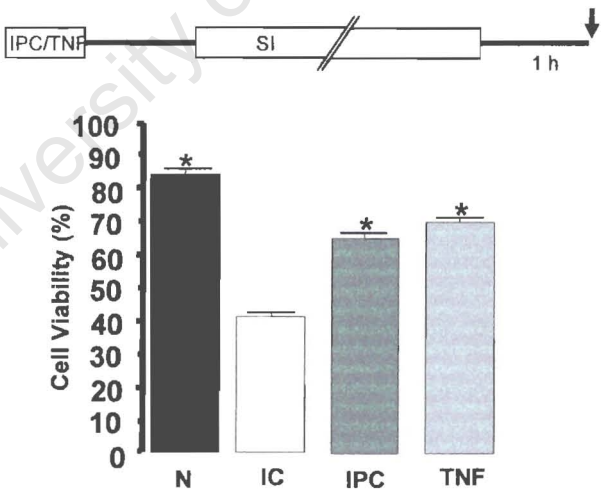


Figure 10: **Cell viability in C_2C_{12} myotubes measured with the Trypan Blue method.** Cell viability (expressed as a percentage) was reduced in cells subjected to 7h simulated ischaemia and 1h of reperfusion (IC). Ischaemic preconditioning (IPC) or TNFα (TNF, 0.5ng/ml) restored the cell viability. *p< 0.001 versus IC group. n≥ 9 per group.

As cell viability results did not differ between the trypan blue technique and the propidium iodide, further cell viability results presented in this thesis will only be given using the trypan blue technique.

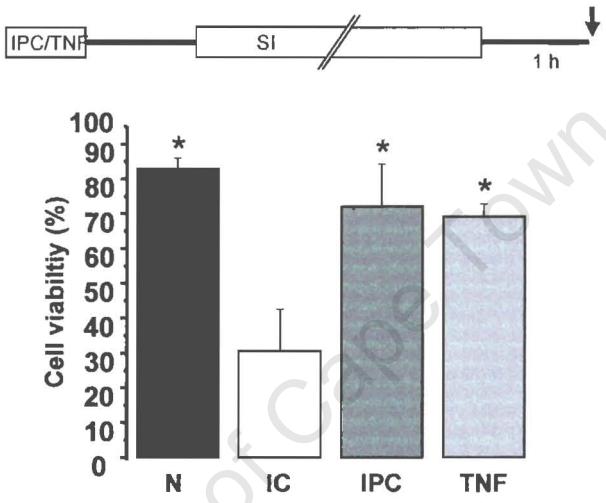


Figure 11: **Cell viability in C_2C_{12} myotubes measured with the propidium iodide method.** Cell viability (expressed as a percentage) was reduced in cells subjected to 7h simulated ischaemia and 1h of reperfusion (IC). Ischaemic preconditioning (IPC) or TNFα (TNF, 0.5ng/ml) restored the cell viability. *p< 0.01 versus IC group.

1.2 Respiratory parameters

Cellular oxygen consumption (state 2 respiration) in C_2C_{12} myotubes was measured immediately after the index simulated ischaemia. Untreated cells (N) presented a state 2 respiration of 6.3±0.4 nM of oxygen/million of viable cells/min. Following seven hours of simulated ischaemia, state 2 respiration was reduced to 2.5±0.1. Both ischaemic preconditioning (IPC) and TNFα preconditioning significantly improved the state 2 respiration (5.8±0.6 for IPC and 5.7±0.6 for TNFα, *p<0.05 versus IC).

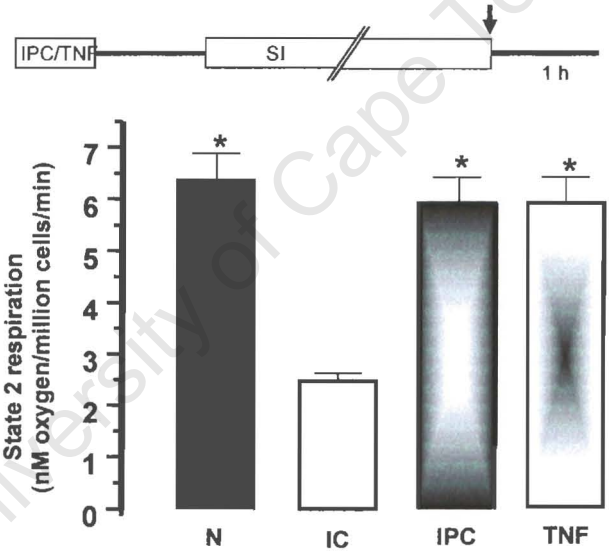


Figure 12: **State 2 respiration in C_2C_{12} myotubes measured after the simulated ischaemic insult.** Both ischaemic preconditioning (IPC) and TNFα (TNF, 0.5ng/ml) improved state 2 respiration (expressed in nM of oxygen/million viable cells/min). N=normoxic; IC=ischemic control. *p< 0.05 versus IC group. n≥ 6 per group

2. Role of NFκB as a trigger in preconditioning

2.1. Ischaemic preconditioning

2.1.1 Cell viability

To investigate the role of NFκB as a **trigger** in ischaemic preconditioning, we used two classic NFκB inhibitors, PDTC (100 μM) and IMD0354 (1 μM) that were given **during the preconditioning stimulus** insult (figure 13). As previously described (section 1.1, p.43), ischaemic preconditioning improved cell viability (66.3±1.2% versus 43.7±8.1% for IC group, *p<0.001). When used on their own, both IMD0354 and PDTC had no effect on the cell viability. In contrast, administration of either IMD0354 or PDTC during the preconditioning stimulus both abolished the protective effect of IPC (34.8±2.4% and 39.4±0.9% respectively, $p<0.001 versus IPC).

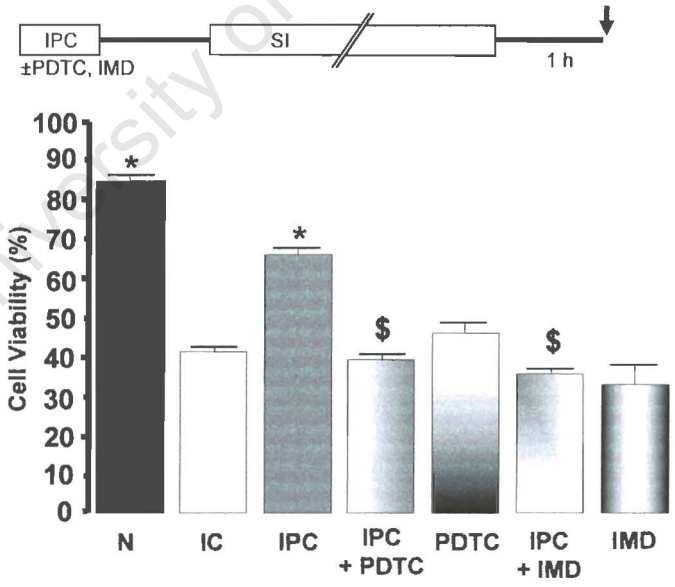


Figure 13: **Effect of NFκB inhibitors given during the preconditioning stimulus on cell viability in $C_{2C}C_{12}$ myotubes subjected to ischaemic preconditioning.** Cell viability was assessed using trypan blue technique. Addition of PDTC (100 μM) or IMD0354 (IMD, 1 μM) during the preconditioning stimulus abolished the protective effect of IPC. *p< 0.001 versus ischaemic control (IC) group; $p<0.001 versus IPC. n≥9 per group.

2.1.2 Respiratory Parameters

As previously described in section 1.2, p.45, ischaemic preconditioning improved state 2 respiration in C_2C_{12} myotubes subjected to 7h simulated ischaemia. When used on its own, PDTC (100 μM) did not affect the state 2 respiration. Addition of PDTC during the preconditioning stimulus abolished the protective effect of IPC on state 2 respiration (IPC +PDTC: 2.1±0.1, $^{\$}p<$ 0.05 versus IPC).

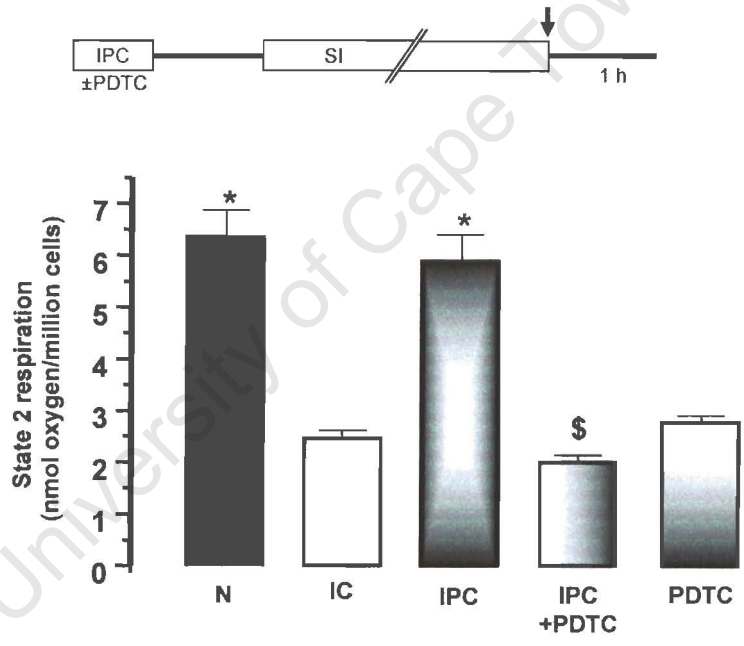


Figure 14: **Effect of NFκB inhibitor given during the ischaemic preconditioning stimulus on the oxygen consumption in C_2C_{12} myotubes.** The addition of PDTC (100 μM) abolishes the increase of state-2 respiration induced by ischaemic preconditioning (IPC). *p<0.05 versus ischaemic control (IC) group; $^{\$}p<0.05$ versus IPC. n≥ 6

2.1.3 Phosphoprotein assay

The phosphorylation of IκB is an indication of NFκB activation. Phosphorylation of IκB was measured after 15 min during the 30 min ischaemic preconditioning stimulus. IPC increased IκB phosphorylation compared to the untreated cells (in arbitrary units [AU] 1.89±0.03 for IPC versus 1.00±0.00 for N, *p< 0.001). The phosphorylation of IκB was reduced in the presence of IMD0354 (0.81±0.12 for IPC+IMD, $p<0.01 versus IPC) and to a lesser extent with PDTC (1.39±0.11, versus IPC, *p<0.01).

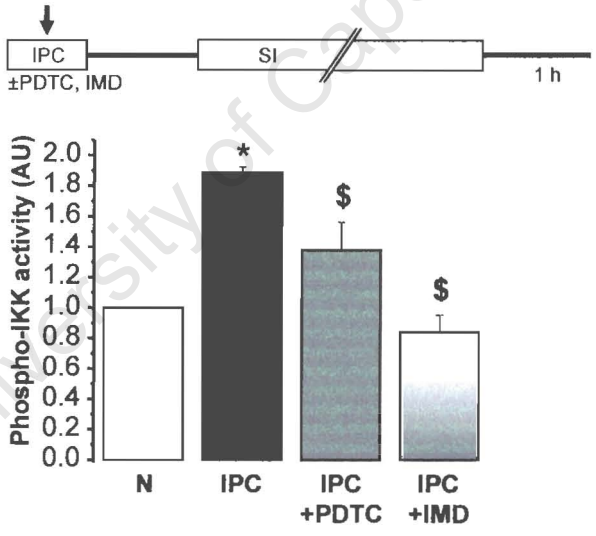


Figure 15: Effect of NFκB inhibitors on the phosphorylation of IκB during the preconditioning stimulus in C_2C_{12} myotubes subjected to ischaemic preconditioning.

The addition of PDTC (100 μM) or IMD0354 (1 μM) reduced the phosphorylation of IκB observed during the ischaemic preconditioning stimulus. *p< 0.01 vs N, $p< 0.01 vs IPC. n≥3 per group

2.2 TNFα-induced preconditioning

2.2.1 Cell viability

As described in chapter 1.1, TNFα preconditioning improved cell viability (70.6±6.1% versus 43.7±8.1% for IC group, *p<0.001). According to figure 16, the administration of either IMD0354 or PDTC during the TNFα preconditioning stimulus insult abolished the cytoprotective effect of TNFα (40.9±2.8% and 33.6±1.2% respectively, $p<0.001 versus TNFα).

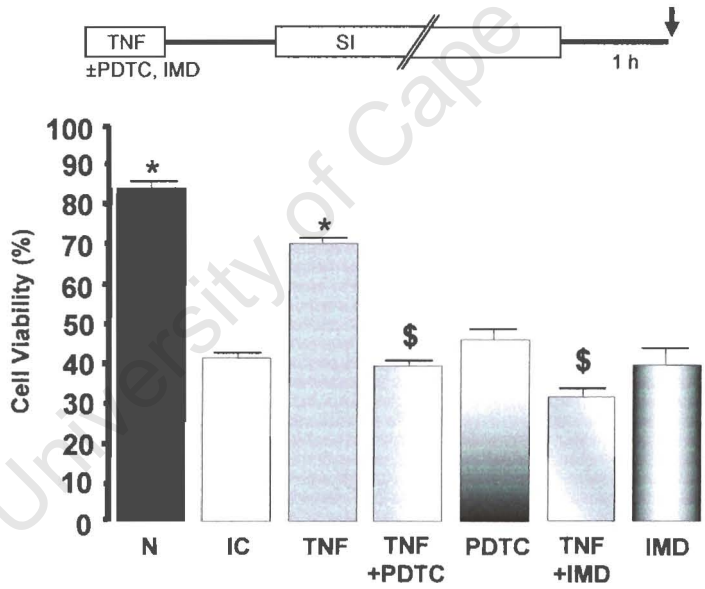


Figure 16: **Effect of NFκB inhibitors given during the preconditioning stimulus on cell viability in $C_{2C}C_{12}$ myotubes subjected to TNFα mediated preconditioning.** Cell viability was assessed using trypan blue technique. Addition of PDTC (100 μM) or IMD0354 (IMD, 1μM) during the preconditioning stimulus abolished the protective effect of TNFα. *p< 0.001 versus ischaemic control (IC) group; $p<0.001 versus TNFα. n≥9 per group

2.2.2 Respiratory parameters

As previously described (section 1.2), cells preconditioned with TNFα improved state 2 respiration. Administration of PDTC during the TNFα preconditioning stimulus reduced the oxygen consumption (TNF+PDTC: 3.6±0.3, *p<0.05 vs TNFα).

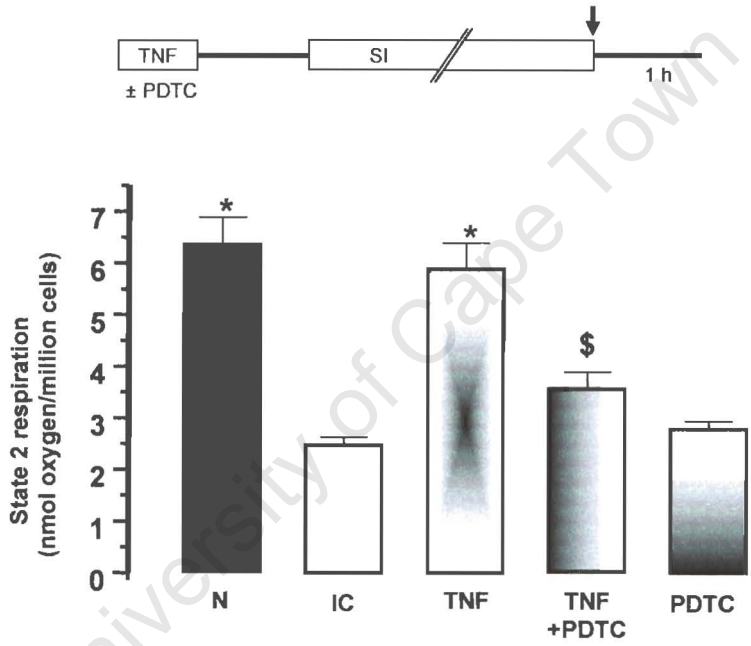


Figure 17: **Effect of NFκB inhibitor given during the TNFα preconditioning stimulus on the oxygen consumption in C_2C_{12} myotubes.** PDTC (100 μM) abolishes the increase of state-2 respiration induced by TNFα preconditioning (TNF). *p< 0.05 versus ischaemic control (IC) group; $p<0.05 versus TNFα. n≥ 6

2.2.3 Phosphoprotein assay

IκB phosphorylation was measured after 15 min during the 30 min TNFα preconditioning stimulus. As shown in figure 18, preconditioning with TNFα increased the phosphorylation of IκB (1.5±0.2 for TNFα versus 1.0±0.0 for N, $p<0.01$) and was abolished when TNFα was incubated in the presence of PDTC or IMD0354 (0.8±0.3 and 1.2±0.1 respectively versus TNFα, $p< 0.01$).

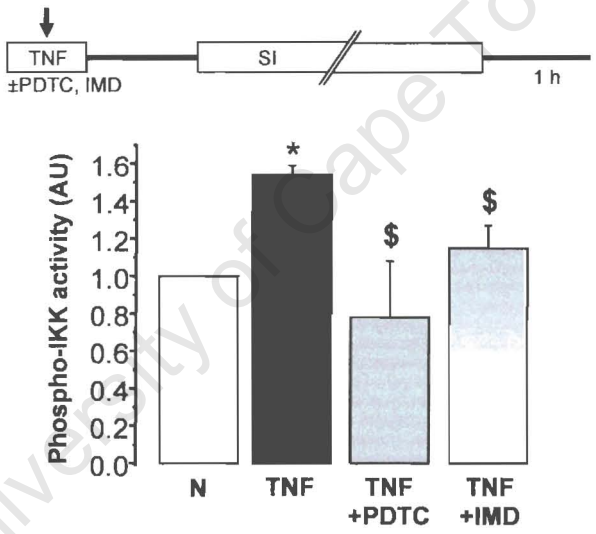


Figure 18: **Effect of NFκB inhibitors on the phosphorylation of IκB during the preconditioning stimulus in C_2C_{12} myotubes subjected to TNFα mediated preconditioning.** The addition of PDTC (100 μM) or IMD0354 (1 μM) reduced the phosphorylation of IκB observed during the TNFα preconditioning stimulus. *$p< 0.01$ vs N, $^{\$}p< 0.01$ vs TNFα. n≥3 per group

3. Role of NFκB as a mediator in preconditioning

3.1 Ischaemic preconditioning

3.1.1 Cell viability

We were also interested as to whether NFκB might be involved as a **mediator** in ischaemic preconditioning. The NFκB inhibitors, PDTC (100 μM) and IMD0354 (1 μM) were given **at the time of reperfusion** (figure 19). Ischaemic preconditioning improved cell viability as previously described in section 1.1, p.43. The NFκB inhibitors had no effect on cell viability when used on their own. PDTC did not attenuate the protective effect induced with ischaemic preconditioning (73.2±2.4% versus 43.7±8.1% for IC group, *p<0.001). In contrast, IMD0354 reduced cell survival when given at reperfusion (37.7±0.9% versus IPC group, p<0.001).

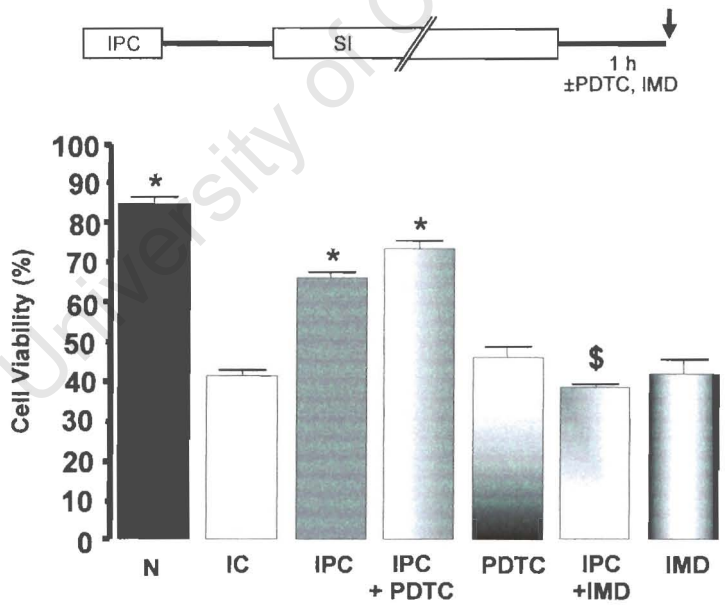


Figure 19: **Effect of NFκB inhibitors given at the time of reperfusion on cell viability in C_2CC_{12} myotubes subjected to ischaemic preconditioning.** Only the addition of IMD0354 (1 μM) removed the protective effects at the time of reperfusion. *p< 0.001 vs N, $^{\$}$p< 0.001 vs IPC. n≥9 per group

3.1.2 Phosphoprotein assay

As an indication of NFκB activation at the time of reperfusion, the phosphorylation of IκB was measured following 15 min of reperfusion. IPC increased IκB phosphorylation compared to the untreated cells (1.31±0.02 for IPC versus 1.00±0.00 for N, *p<0.01). PDTC, given at reperfusion did not significantly affect the phosphorylation of IκB during IPC (1.27±0.04 for IPC+PDTC versus IPC). In contrast, IMD0354 reduced IκB phosphorylation (0.78±0.22, versus IPC, $^{\$}$p<0.01).

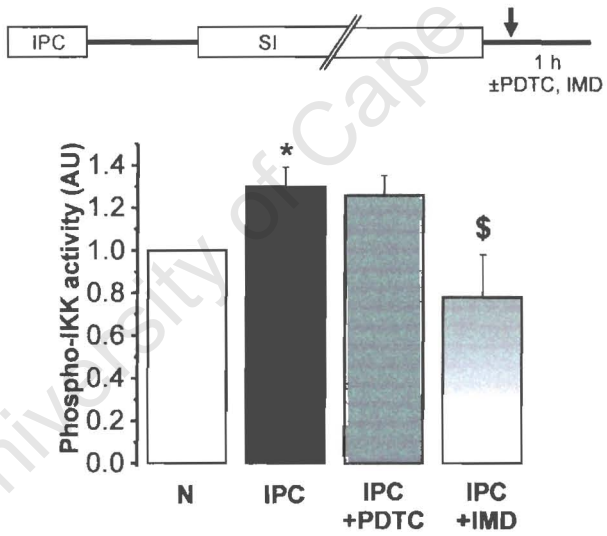


***Figure 20. Effect of* NFκB inhibitors on IκB phosphorylation given at the time of reperfusion in C_2C_{12} myotubes subjected to ischaemic preconditioning.** IMD0354 (1 μM) significantly attenuates the increase in IκB phosphorylation shown with IPC ($^{\$}$p<0.01 vs IPC). PDTC (100 μM) does not have any noticeable effect when given at the time of reperfusion. n≥3 per group

3.2 TNFα preconditioning

3.2.1 Cell viability

As previously described (section 1.1, p.43), TNFα preconditioning improved cell viability (70.6±6.1% versus 43.7±8.1% for IC group, *p<0.001). In figure 21, PDTC did not reduce the high cell viability induced with TNFα (70.7±1.7%, ns vs TNFα) whereas IMD0354 had a more noticeable effect and lowered cell survival to levels observed in the simulated ischaemic control (40.9±2.8%, $p<0.001 versus TNFα). On their own, both NFκB inhibitors had no effect on the cell viability.

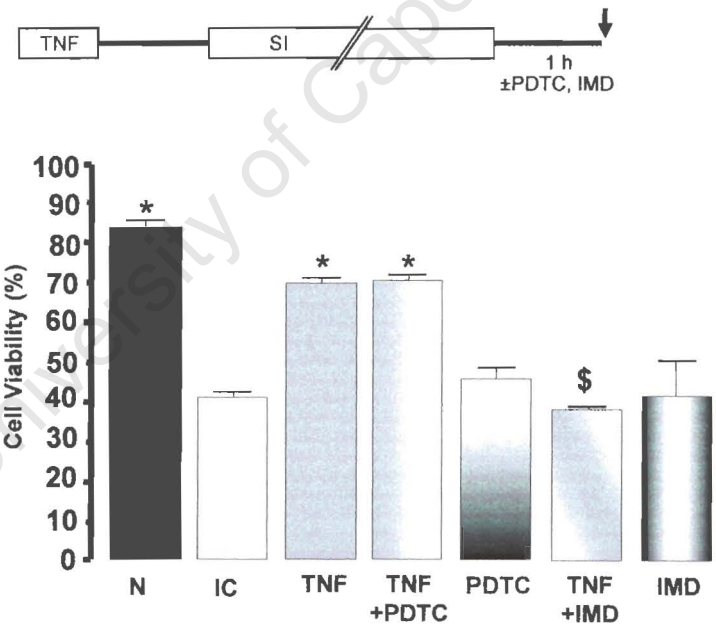


Figure 21: **Effect of NFκB inhibitors given at the time of reperfusion on cell viability in C_2C_{12} myotubes subjected to TNFα mediated preconditioning.** The addition of PDTC (100 μM) did not abolish the protective effect of TNFα preconditioning (TNF). However, IMD0354 reduced cell viability. *p< 0.001 versus IC. $P< 0.001 versus TNFα. n≥9 per group

3.2.2 Phosphoprotein assay

NFκB activation by IκB phosphorylation during the mediator phase was examined in TNFα preconditioning. TNFα preconditioning increased the phosphorylation of IκB compared to untreated cells (previously described in section 2.2.3, p.51). However, this phosphorylation of IκB was reduced in the presence of IMD0354 (0.91±0.19 versus TNFα, $^{\$}p<0.01$) and to a lesser extent with PDTC (1.22±0.24 for TNF+PDTC versus 1.78±0.2 for TNFα, $^{\$}p<0.01$).

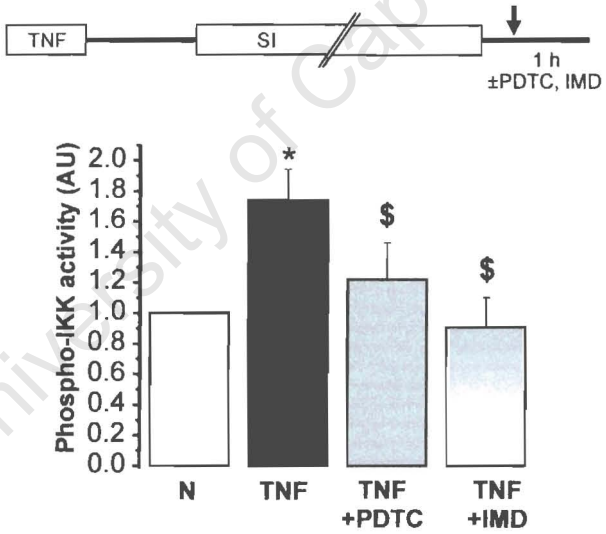


Figure 22: **Effect of NFκB inhibitors on the phosphorylation of IκB at the time of reperfusion in C_2C_{12} myotubes subjected to TNFα preconditioning.** The addition of PDTC (100 μM) or IMD0354 (1 μM) reduced the phosphorylation of IκB induced by TNFα preconditioning. $^{\$}p< 0.01$ vs TNFα. $^{\$}p<0.01$ vs N. n≥3 per group

E. DISCUSSION

E. Discussion

The cytokine TNFα is known to mimic ischaemic preconditioning and thus confer cytoprotection in different models such as the isolated rat heart model (Lecour et al, 2002), the isolated cardiomyocytes (Lecour et al, 2005) or the C_2C_{12} myotubes (Lecour et al, 2005). However, the mechanisms involved in this cytoprotective effect still remain unclear. The combination of our findings in the present thesis suggests that the transcription factor NFκB is involved as a trigger and a mediator in TNFα-induced preconditioning. Using C_2C_{12} myotubes, we have found that IκB was activated in both ischaemic and TNFα induced preconditioning. In addition, inhibition of NFκB during the preconditioning stimulus or at the time of reperfusion abolished the protective effect of both TNFα and ischaemic preconditioning.

TNFα mimics ischaemic preconditioning in C_2C_{12} myotubes.

Using an isolated rat heart model, Lecour et al (Lecour, 2002) previously reported that TNFα can mimic ischaemic preconditioning in a dose and time-dependent manner and that the dose of 0.5ng/ml did confer the best protection. Similarly, James Meiring (Meiring, MSc thesis, 2001) established that the dose of 0.5ng/ml given for half an hour in C_2C_{12} provided maximal protection in C_2C_{12} myotubes.

In the present study, we used C_2C_{12} myotubes to delineate the mechanisms involved in TNFα induced cytoprotection. We have confirmed that the dose of 0.5ng/ml was protective as it improved the cell viability by 60%.

To determine the cell viability, we have used both trypan blue exclusion and the propidium iodide technique. Our results obtained with the propidium iodide in the ischaemic preconditioning protocol are conformed to those previously described in the literature (Minners et al, 2001). As we obtained similar results with the trypan blue technique, we can conclude that the trypan blue technique is a validated method to measure cell viability.

An increase in cellular oxygen consumption was determined in C_2C_{12} cells that were preconditioned with TNFα which was comparable to observations with ischaemic preconditioning. This allowed us to proceed with the study and test the influence of NFκB inhibitors in both ischaemic and TNFα preconditioning in our cell culture model.

NFκB acts as a trigger and mediator in ischaemic preconditioning.

It has already been reported that NFκB activation during the preconditioning stimulus is essential to mediate the cytoprotective effects of ischaemic preconditioning in the isolated rat perfused heart (Maulik et al, 1998) or in an *in vivo* rabbit model (Morgan et al, 1999). Here, we delineated the role of NFκB in ischaemic preconditioning using a cell culture model. Two well known NFκB inhibitors, PDTC and IMD0354 were used in our investigations. NFκB inhibitors given during the preconditioning stimulus abolished the protective effect of ischaemic preconditioning, confirming data previously reported in the literature (Maulik et al, 1998; Morgan et al, 1999). In addition, we reported for the first time to our knowledge that NFκB activation is also required at the time of reperfusion and therefore acts as a mediator in ischaemic preconditioning. It has been postulated that the activation of NFκB in ischaemic preconditioning may be mediated via p38 mitogen activated protein kinases (Maulik et al., 1998), nitric oxide (Zhao and Kukreja, 2002) or heat shock proteins (Marber et al., 1993). As we have recently shown that TNFα is activated in ischaemic preconditioning (Deuchar et al, 2005), it is likely that NFκB activation in ischaemic preconditioning may be mediated via TNFα.

NFκB acts as a trigger and mediator in TNFα-mediated preconditioning

The administration of NFκB inhibitors, PDTC and IMD0354 during the preconditioning stimulus abrogated the protective effect of TNFα-mediated cardioprotection. Similar results were obtained when these inhibitors were given at the time of reperfusion. These findings suggest a role for NFκB as a trigger and mediator in TNFα-mediated preconditioning. NFκB has been

shown to activate various cytoprotective proteins in myocardial adaptation to ischaemia such as the Bcl-2, Bcl-X_L, BAD, cellular inhibitor of apoptosis (c-IAP) and X chromosome-linked inhibitor of apoptosis (XIAP), inducible nitric oxide synthase (iNOS), cyclooxygenase (Cox) -2 and the endogenous antioxidants manganese superoxide dismutase and hemeoxygenase-1 (Wang et al., 1998; Xuan et al., 1999; Bolli et al., 2002; Luo et al., 2005). In our experimental conditions, it is possible that NFκB might confer protection through induction of these factors but this remains to be investigated. However, the timeframe in which NFκB is able to evoke protection in classic preconditioning, is insufficient for *de novo* transcription of cardioprotective genes (for review, see Valen 2004). It is also possible to consider that NFκB may have a protective effect by down-regulating the inflammatory response during the reperfusion period.

Protective or harmful role of NFκB

It has been shown that activation of NFκB by ischaemia-reperfusion is detrimental to the myocardium (Chandrasekar et al., 1997; Shimizu et al., 1998; Li et al., 1999). In contrast, in a preconditioning model with ischaemia or TNFα, we have shown that NFκB activation is required and protective. It is likely that the excess levels of NFκB during I/R are attenuated by both TNFα and ischaemic preconditioning and that the reduced production of NFκB is protective. This requirement for the transcription factor is supported by inhibition of NFκB during mediator phase which abrogates the protective effect of IPC and TNFα-mediated preconditioning. To prove this hypothesis, we will need to compare the levels of NFκB activation (directly or by IκB phosphorylation studies) at reperfusion between the ischaemic control and IPC groups.

Specificity of the inhibitors

Interestingly, our study showed discrepancies in the results during the mediator phase between IMD0354 and PDTC. While IMD0354 abolished the protective effect of both TNFα and ischaemic preconditioning, PDTC failed to

do this. The specificity of the inhibitors may explain these conflicting results. IMD0354 specifically inhibits the phosphorylation of IKK complex that is require for the phosphorylation of IκB. Inhibition of this action prevents the activation of NFκB (Onai et al., 2004). In contrast, PDTC is known as a ROS scavenger and therefore is less specific (Schreck et al., 1992; Irem et al., 1991). The lack of inhibition of NFκB at reperfusion by PDTC during both ischaemic and TNFα-mediated preconditioning may also suggest the concentration of the inhibitor PDTC (100 μM) used in this study is not sufficient to inhibit NFκB.

NFκB inhibitor, PDTC affect the oxygen consumption

For a long time, the mitochondria have been proposed as the end effector in ischaemic preconditioning. More specifically, activation of the mitochondrial K_{ATP} (mitoK_{ATP}) channel is thought to be the likely target (Gross and Auchampach, 1992). The mechanism by which the mitoK_{ATP} channel exerts ischaemic tolerance is not well understood. Previously it has been shown that both ischaemic and pharmacological preconditioning can induce mitochondrial uncoupling in C_2C_{12} cells (Minners et al., 2001). Mitochondrial uncoupling is a result of perturbation in ATP synthesis where the H^+ gradient dissipate across the inner mitochondrial membrane. This followed by an increase in oxygen consumption that is independent of ATP production. The respiratory parameters obtained in our study were used to measure the oxygen consumption of the cells. We were able to confirm an improvement in cellular respiration with both TNFα and ischaemic preconditioning. The inhibition of NFκB by PDTC removed this protective effect, suggesting that NFκB exerts its effect above the level of the mitochondria.

Limitation of the Study

The C_2C_{12} cell culture model used in our study is derived from the murine skeletal muscle. In a serum depraved medium, the myoblasts differentiate into myotubes which exhibit behavioural characteristics of cardiomyocytes. The advantage of C_2C_{12} cells is that they propagate rapidly and it is easy to

obtain large quantities of cells to work with. However, any cell culture study is an isolated model and our investigations does not take into account the intracoronary activation of leukocytes which may affect the levels of TNFα and produce a negative harming effect in the setting of myocardial ischaemia. Therefore, this study should be confirmed in an *in vivo* animal model.

We measured IκB phosphorylation to examine the activation of NFκB. This is an indirect technique that may not reflect the reality as it is possible that there may be some protein deterioration. During this study, we have explored the p65 and p50 unit using western blot analysis technique but this method was not sensitive enough for our model to study the activity of NFκB. A more direct measurement of NFκB by means of an electrophoretic mobility shift assay (EMSA) would be required to confirm the data. Recently, Tillmanns et al (Tillmanns et al., 2006) have developed a non-invasive *in vivo* molecular imaging of NFκB activation of the heart. The implementation of this novel technique will undoubtedly provide a much needed insight into the role of NFκB in TNFα-mediated preconditioning.

Unfortunately, during the course of this study, we have encountered dramatic problems with the cell culture of the C_2C_{12}. The phenotypical characteristics of our cell line appeared to have changed as it became impossible to differentiate the cells. After four months of intensive investigations, we have discovered that the problem was related to the bad quality of the medium used in our study. Using the same medium issue from a different supplier restored the differentiation process of the cells. Consequently, the respiratory parameters measuring the effect of IMD0354 during the trigger and mediator phase in both ischaemic and TNFα-mediated preconditioning could not be accomplished in time for the thesis and some of the experiments will also require repetition to increase their statistical 'n' value.

F. Conclusion

Our study demonstrates a role for NFκB in ischaemic and pharmacological preconditioning with TNFα as a trigger and mediator of cytoprotection in C_2C_{12} myotubes. This was confirmed by inhibiting NFκB either during the preconditioning stimulus or at the time of reperfusion following the sustained ischaemia. We have also shown that NFκB exerts it protective effects above the level of the mitochondria in the preconditioning signalling pathway.

Future direction

As mentioned earlier, this cell culture model is an indication of what would be expected in cardiomyocytes and the findings of this study need to be confirmed in an *in vivo* animal model. The use of EMSAs should also be employed as a direct measure of NFκB activation. Implementation of a non-invasive *in vivo* molecular imaging of NFκB activation during both ischaemic and TNFα mediated preconditioning would be desirable. The possible downstream factors of NFκB should also be examined between both types of preconditioning. It would be interesting to explore the mechanisms involved and determine if there is a further overlap or divergence between the two cytoprotective signaling cascades.

F. CONCLUSION

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G. PUBLICATIONS

G. Publications

Conference Outputs and Meetings

Somers S., L. Lacerda, L.H. Opie and S. Lecour. "NFκB triggers TNFα induced cytoprotection in C_2C_{12} cells." Abstracts from the 25th Annual ISHR European Section Meeting, Tromso, Norway, June 21-25, 2005. Journal of Molecular Cardiology, Volume 38, Issue 6 (June 2005). Pages 997-1094.

Somers S., L. Lacerda, L.H. Opie and S. Lecour. Tumour Necrosis Factor-alpha (TNFα) confers cytoprotection: A role for Nuclear Factor kappa-B (NFκB). 33rd Meeting of the Physiological Society of South Africa, Cape Town, South Africa. September 7-9, 2005. *Poster presentation*

Somers S., L. Lacerda, L.H. Opie and S. Lecour. Tumour Necrosis Factor-alpha (TNFα) confers cytoprotection: A role for Nuclear Factor kappa-B (NFκB). AstraZeneca Research Day, Cape Town, South Africa. October, 2005. *Oral presentation*

Publication

Lecour, S., N. Suleman, G.A. Deuchar, S. Somers, B. Huisamen and L.H. Opie (2005). "Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase)." Circulation **112**(25): 3911-8.

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