The Role of Signal Transducer and Activator of Transcription-3 (STAT-3) in Ischaemic and Pharmacological Preconditioning

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Declaration

I, Naushaad Suleman, hereby declare that the work on which this thesis is based is my original work (except where acknowledgement indicates otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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"It takes heart to do heart research"

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Abbreviations

5-HD -	5-hydroxydecanoate
ACE -	Angiotensin converting enzyme
ANT -	Adenine nucloetide transfer
ATP -	Adenosine triphosphate
Bad -	Bcl-2 associated death agonist
Bcl-2 -	B-cell lymphoma-2
cAMP -	Cyclic adenosine monophosphate
COX -	Cyclooxygenase
DAG -	1,2-diacylglycerol
ERK -	Extracellular signal-regulated kinases
F -	Floxed
FADD -	Fas-associated death domain
FGF -	Fibroblast growth factor
G Proteins -	Guanyl nucleotide binding proteins
Gi -	G inhibitory
Gs -	G stimulatory
GSK 3 -	Glycogen synthase kinase 3
HPC -	Hypoxia-induced preconditioning
HSP 27 -	Heat shock protein 27
I/R -	Ischaemia/reperfusion
IGF-1 -	Insulin-like growth factor-1
IP3 -	Inositol 1,4,5-triphosphate

IPC -	Ischaemic preconditioning
Jak -	Janus kinase
JNK -	c-Jun NH_2-terminal kinases
KATP -	Potassium ATP sensitive channel
KO -	Knockout
LIF -	Leukemia inhibitory factor
MAPK -	Mitogen activated protein kinases
MAPKAPK2 -	MAPK-activated protein kinase 2
MKK -	MAPK kinase
MKKK -	MAPK kinase kinase
MLC-2V -	Myosin light chain-2V
mPTP -	Mitochondrial permeability transition pore
mTOR -	Mamalian target of rapamycin
N -	Normal
NFκB -	Nuclear factor κB
NOE -	n-oleoylthanolamine
PCR -	Polymerase chain reaction
PI2 -	Phosphatidylinositol 4,5 bisphosphate
PI3K -	Phosphatidylinositol 3 kinase
PKB -	Protein kinase B
PKC -	Protein kinase C
PLC -	Phospholipase C
PLD -	Phospholipase D
RACK -	Receptor for activated C kinases
RIP -	Receptor interacting protein

RISK -	Reperfusion injury salvaging kinases
ROS -	Reactive oxygen speceis
RTK -	Receptor tyrosine kinases
RXR -	Retinoic acid receptor
SH-2 -	Src homology domain 2
STAT -	Signal transducer and activator of transcription
SWOP -	Second window of protection
T -	Transgene
TACE -	TNFα converting enzyme
TGF b1 -	Transforming growth factor-b1
TNFR1 -	TNFα receptor 1
TNFR2 -	TNFα receptor 2
TNFα -	Tumour necrosis factor alpha
TRADD -	TNF receptor associated death domain
TRAF -	TNF receptor associated factor
TTC -	2,3,5-triphenyltetrazolium chloride
VDAC -	Voltage dependent anion channel
WT	Wildtype

Abstract

Background: Ischemic preconditioning is a protective mechanism whereby repetitive transient ischaemia confer protection against the subsequent prolonged lethal ischaemia that is observed during myocardial infarction. This protective phenomenon that lessens myocardial infarction can be mimicked by the cytokine tumor necrosis factor alpha (TNFα) but the signalling pathways involved still remain unclear. We hypothesized that the signal transducer and activator of transcription 3 (STAT-3) plays a critical role in both ischemic and TNFα preconditioning.

Methods: To explore this hypothesis, we first generated cardiac specific STAT-3 deficient mice using Cre-LoxP technology. Cardiomyocytes, isolated from wildtype (WT) or cardiac specific STAT-3 knockout mice (KO), were subjected to 26 hours of simulated ischaemia followed by 2 hours of reperfusion. Myocytes were preconditioned either with 30min of hypoxia or TNFα (0.5ng/ml). An inhibitor of STAT-3 (AG490; 100nM) or an inhibitor of PI3K/Akt (wortmannin, 100nM) was given during the preconditioning stimulus or at the time of reperfusion. Cell viability was assessed via cell morphology and trypan blue exclusion. Similarly, isolated langendorff perfused hearts (mice or rats) were subjected to a regional ischaemia/reperfusion insult and were preconditioned with either short periods of ischaemia or exposure with low dose of TNFα (0.5ng/ml). Infarct size was measured using triphenyltetrazolium chloride technique. Western blots analysis were performed to evaluate the activation of STAT-3 and Akt during the preconditioning stimulus or at the time of reperfusion.

Results: In isolated cardiomyocytes and in an isolated mouse hearts, we demonstrated that, in contrast to their littermate controls, the STAT-3 deficient

mice failed to be preconditioned with TNFα or an ischemic stimulus. In addition, both ischemic and pharmacological preconditioning were associated with an activation of STAT-3 during the preconditioning stimulus and at the time of reperfusion in the wildtype mice and further investigations conducted with STAT-3 inhibitors demonstrated that this activation was required for preconditioning to confer cardioprotection. In ischaemic preconditioning, our data suggest the possibility of a cross-talk between Akt activation and STAT-3 activation. In contrast, we showed that the cardioprotection afforded by TNFα is independent of activation of the classic prosurvival kinase Akt, therefore suggesting that STAT-3 can protect via a novel and Akt-independent pathway.

Conclusion: In this thesis, we have delineated a novel cardioprotective pathway against myocardial infarction. This pathway involves the activation of STAT-3 and it may have potential therapeutic significance in the development of future drug to promote cell survival in the heart.

A. Introduction

The heart is a highly specialized pump with the functional role of transporting blood throughout our body and maintaining a healthy circulatory system. Under physiological circumstances, the heart pumps 7 500 litres worth of blood a day that equates to 136 875 000 litres over the course of a healthy 50 years (http://kidshealth.org/kid/body/heart_noSW.html). The efficiency of this pump would tend to diminish as we get older and the negative accumulative effects associated with an unhealthy lifestyle, will result in premature death due to cardiovascular disease.

Cardiovascular disease is the end point of an accumulation of "risk factors". These factors, which increase an individual's risk of entering a diseased state of the myocardium (heart muscle), include smoking, high cholesterol levels, an unhealthy diet which can lead to obesity culminating in type II diabetes, elevated blood pressure and lack of sufficient physical activity (http://www.americanheart.org). According to the World Health Organisation, coronary heart disease accounted for 13% of all deaths worldwide in 2002 (http://www.who.int/en/). By 2020, heart disease and stroke are predicted to become the leading cause of both death and disability worldwide, with the number of fatalities projected to increase to 24 millions a year by 2030 (http://www.who.int/en/). The statistics in South Africa show a daunting 6.3 million people suffering from hypertension (chronic long term elevation of blood pressure), 4.5 million with high blood cholesterol level, 1.5 million with Type II diabetes with and one individual suffering from a heart attack every eight minutes (www.heartfoundation.co.za).

The above-mentioned risk factors progressively lead to the narrowing of the heart arteries. As a result, the flow of oxygenated blood to the cardiomyocytes (muscle cells of the heart) is reduced resulting in an ischaemia. An ischaemia is defined as the insufficient amount of oxygenated blood flowing to the myocardium to sustain its oxygen and nutrient requirements (http://www.americanheart.org; Opie 2004). Ischaemic heart disease is an outcome of coronary artery disease and ultimately ending in heart attack.

The majority of cell death during prolonged coronary occlusion occurs *via* necrosis (cell death resulting from irreversible damage to the cell) (Harper *et al.* 2003).In order to salvage the myocardium, the occluded artery can be re-opened thus allowing the reperfusion of the heart. However, if not controlled correctly, reperfusion itself contributes towards cellular damage and myocyte death in an apoptosis (programmed cell death which can be reversed) dependent manner. This is termed reperfusion injury (see review by Yellon *et al* (Yellon *et al.* 2003)).

In this setting of ischaemia/reperfusion injury, it is imperative to understand the biochemical functioning of the heart and how it can intrinsically protect itself, in the perspective of developing new and novel therapies against ischaemic injury.

1. Cardiac preconditioning – A biological mechanism of cardioprotection

In 1986, while performing experiments on adenosine triphosphate (ATP) levels in the failing canine heart, Murry *et al* came across a biological mechanism of instinctive cardioprotection whereby the ischaemic myocardium was protected from the maximal damage afforded by sub-lethal ischaemia/reperfusion (Murry *et*

al. 1986). They observed that 4 cycles of 5 minutes ischaemia/reperfusion prior to a 40 minute ischaemia lead to a significant improvement of the *via*ble cardiac tissue and a reduction in the infarct size. This experiment demonstrated that brief episodes of ischaemia and reperfusion, preconditioned the heart against sub-lethal ischaemia. This type of cardioprotection was then described as ischaemic preconditioning.

Preconditioning-like protection can also be induced by other means of chemical and physical stimuli including hypoxia (Bruer *et al.* 1997; Milano *et al.* 2002; Uchiyama *et al.* 2004), thermal stress (Heads *et al.* 1996), stretch (Iliodromitis *et al.* 2002) and pharmacological agents including adenosine (Auchampach *et al.* 1993), bradykinin (Goto *et al.* 1995) and opioids (Cohen *et al.* 2001). Preconditioning protects the myocardium by reducing the infarct size (Jonassen *et al.* 2001; Lochner *et al.* 2003a) and/or improving the contractile function of the heart (Liu *et al.* 2002; Lochner *et al.* 2003b). Although first observed in the heart, the phenomenon of ischaemic preconditioning has successfully been demonstrated to occur in multiple tissue types, including the skeletal muscle (Kohin *et al.* 2001), the brain (Kis *et al.* 2003), the liver (Ates *et al.* 2002; Koti *et al.* 2002) and the kidneys (Kosieradzki *et al.* 2003) of a wide variety of mammals. These widespread observations of induced protection due to ischaemic preconditioning suggest that it may be an evolutionary conserved cell survival program enabling many tissue types to exhibit a heightened tolerance to ischaemic injury.

1.1 The "first" and "second" windows of protection

Further studies revealed that the protection observed in ischaemic preconditioning is the first of two "windows" of protection from sub-lethal ischaemia occurring in the myocardium. The first window of protection or Classical Preconditioning occurs up to 3 hours following a preconditioning stimulus (Figure 1). The second window of protection (SWOP) or delayed protection, was observed to occur 24-72 hours following the ischaemic preconditioning stimulus (Yellon *et al.* 1995; Baxter *et al.* 1997; Ebrahim *et al.* 2001) and is protective to a lesser extent when compared to the first window (Figure 1). In this thesis, only the classical method of protection will be discussed further.

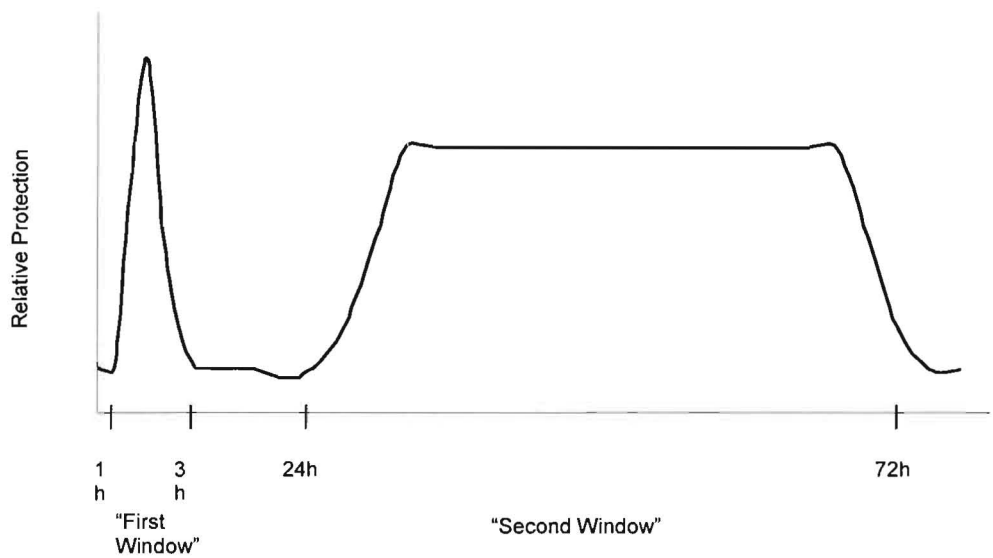

Figure 1: The "first" and "second" windows of cardioprotection (modified from Yellon *et al* (Yellon *et al.* 2003)).

1.2 The trigger and mediator phases of cardiac preconditioning

In all biological systems, a cascade of signal events is crucial to attain a desired outcome. Preconditioning the heart requires the initiation of one or possibly more signals, which promote the activation or the transcription/translation of proteins that are mandatory to protect the myocardium from sustained ischaemia. Over the past few decades, a large amount of work has been done to delineate these signalling pathways but the mechanism of protection has not yet been completely elucidated.

The action of signalling molecules involved in cardiac preconditioning can be divided into 2 phases (Figure 2):

1. **The trigger phase:** This period occurs between the moment the heart is subjected to a preconditioning stimulus and the beginning of the index ischaemia. Preconditioning triggers initiate a change in the physiology of the heart rendering it more resistant to the forthcoming ischaemia/reperfusion injury (Yellon *et al.* 2003).
2. **The mediator phase:** This phase begins at the start of index ischaemia and ends with the termination of reperfusion. The signalling molecules here form part of a signalling cascade which terminates with the activation of end effectors.

The end effectors of preconditioning are currently unknown. Exacerbating this situation is that the mechanism of damage to the myocardium has not yet been

identified. Some signalling molecules are common to both the trigger and the mediator phases which may suggest a convergence at a point, in the signalling pathway leading up to cardioprotection.

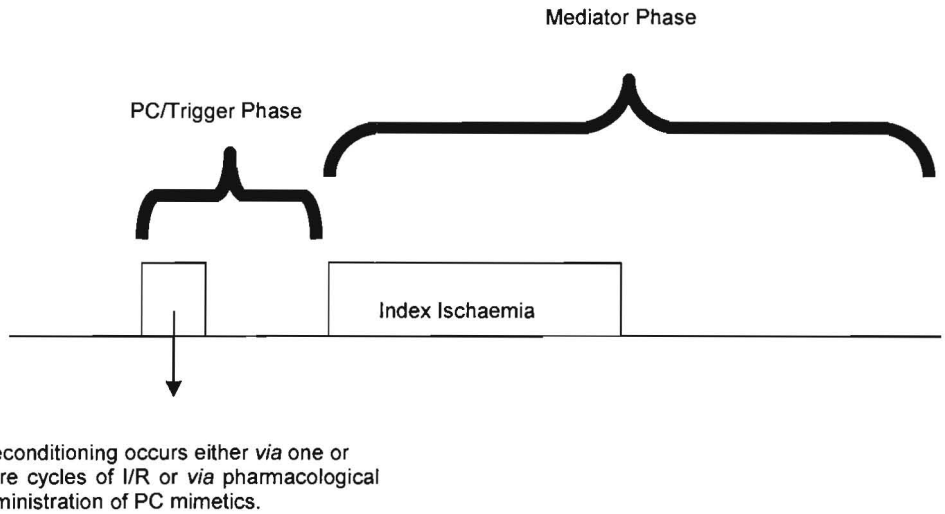

Figure 2: The preconditioning/trigger and mediator phases of cardioprotection. Cardiac PC can be divided into a PC phase that occurs prior to index ischaemia and a mediator phase that occurs from the index ischaemia onwards. I/R- ischaemia/reperfusion, PC -Preconditioning.

a) Preconditioning with ischaemia

The mechanism by which ischaemic preconditioning protects the heart is incompletely delineated. By subjecting the heart to transient bursts of ischaemia/reperfusion, signalling molecules such as adenosine, bradykinin, norepinephrine and opioids are released. These molecules bind to the receptors located at the cell surface membranes and initiate the preconditioning cascade. Using different animal species, experimental models, protocols and endpoints, the literature reports some controversial findings. Ischaemic preconditioning is commonly studied in the isolated working rat heart model or in the retrograde

perfusion model (Langendorff). The severity of ischaemia can vary between regional ischaemia (ligation of the left coronary artery) or global ischaemia and the endpoint can be functional recovery of infarct size. Lochner et al have compared these different models and they concluded that infarct size analysis was a more reliable endpoint than functional recovery (Lochner *et al.* 2003b).

The number and duration of ischaemia/reperfusion cycles employed to induce cardioprotection have an additive effect culminating in protection. Using two conventional models of ischaemia preconditioning in the isolated rat heart, Awan et al observed varying degrees of protection (Awan *et al.* 1999). The first model with one cycle of 10 minutes ischaemia/reperfusion improved functional recovery by 157% while 4 cycles of 5 minutes ischaemia/reperfusion improved functional recovery by 440%.

Similarly, inhibitors of cardiac preconditioning may prevent cardioprotection from a single ischaemia/reperfusion cycle only. Hence, Goto et al demonstrated that inhibition of bradykinin could prevent cardioprotection from one short cycle of ischaemia/reperfusion but not from several cycles (Goto *et al.* 1995). It was suggested that a preconditioning threshold had to be reached for cardioprotection to occur. The number of ischaemia/reperfusion cycles had to be increased to overcome the action of the inhibitor. In support of this data, Morris *et al* (Morris *et al.* 1997) demonstrated that individual administration of either a "sub-threshold" ischaemic stimulus or insufficient concentration of an angiotensin converting enzyme (ACE) inhibitor failed to precondition the myocardium. However, by concommitent addition of the two stimuli lead to cardioprotection (Morris *et al.* 1997).

Ischaemic preconditioning can be mimicked by means of pharmacological agents. Exogenous administration of adenosine (Cain *et al.* 1998), acetylcholine (Mullane *et al.* 1995), bradykinin (Goto *et al.* 1995), endothelin (Erikson *et al.* 1996), opioids (Patel *et al.* 2002), cytokines (Smith *et al.* 2002b) and β adrenergic agonists (Lochner *et al.* 2003b) have all been shown to confer protection in a broad spectrum of animals. The relative concentration and time of administration of these preconditioning agents may vary between species (see review (Yellon *et al.* 2003)). Pharmacological preconditioning can be a useful tool for the understanding of intracellular mechanisms involved in this complex phenomenon.

b) G_I-protein coupled receptors

Receptors of triggering agents of cardioprotection, such as adenosine, bradykinin and noradrenalin are coupled to heterotrimeric guanyl nucleotide binding proteins (G proteins) (Thornton *et al.* 1993; Schultz *et al.* 1998). These proteins mediate the preconditioning signal from activated receptors to downstream targets that transmit the cardioprotective signal intracellularly. G-proteins are located within the lipid bilayer of cells and they can move freely, thus allowing a multitude of G-proteins to be activated by a single occupied receptor complex (Lubert 1988). Two sub-types of G-proteins have been identified. The G stimulatory protein (G_s), which is cholera toxin sensitive, and the G inhibitory (G_i), which is pertusis toxin sensitive. Both are constituted of three subunits, G_α (45 kD), G_β (35kD) and G_γ (7 kD). In the inactive state, Guanosine Diphosphate (GDP) is bound to the G_α subunit thereby facilitating the interaction of the three subunits. In the presence of an occupied receptor, GDP is exchanged for guanosine triphosphate (GTP) at the G_α subunit (Figure 3). This results in a conformational change to the G-protein

separating the G_{α}-GTP bound subunit from the $G_{\beta\gamma}$ subunits, thus activating this signal transducer. The activation is reversed when the bound GTP is hydrolysed to GDP by the G_{α} subunit allowing re-association with the $G_{\beta\gamma}$ subunits (Lubert 1988).

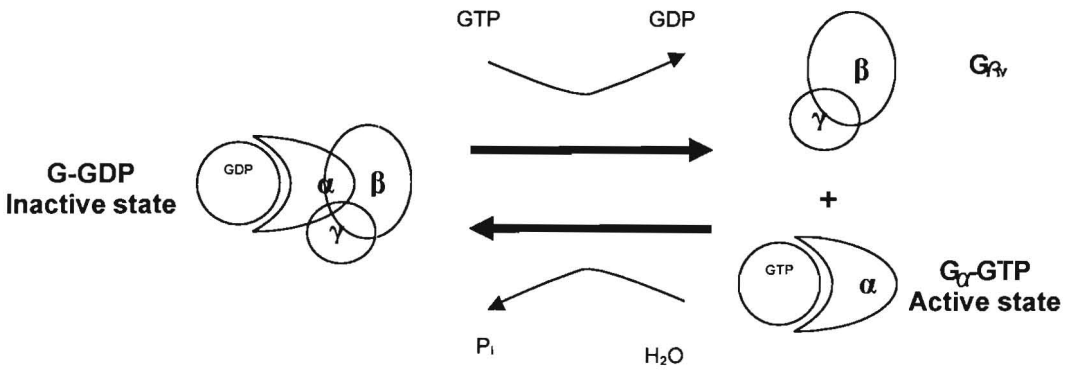

Figure 3: **G-protein activation.** G-proteins consist of 3 subunits, Gα, Gβ and a Gγ subunit. When inactive, GDP binds the complex together. In response to an extracellular signal, GDP is exchanged for GTP at the Gα-subunit causing the G-protein complex to become active after disassociation. The signal is switched off when GTP is hydrolysed to GDP. GDP- Guanosine diphosphate; GTP – Guanosine triphosphate. Adapted from Grisham (Grisham 1997).

Stimulation of the G_s-protein complex will activate adenylate cyclase resulting in the catalytic formation of the second messenger cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). An excessive concentration of intracellular cAMP is detrimental to cardiac function (Hammarberg *et al.* 2004). Once G_i-protein is activated, the $G_{\beta\gamma}$ subunits are released and they bind to the $G_{s\alpha}$-GTP bound subunit preventing it from inducing cAMP *via* adenyl cyclase activation. The role of the G_i-protein in cardioprotection has been intensively studied and proven to be controversial. Adenosine accumulates rapidly in the ischaemic myocardium and signals *via* these G_i-proteins to activate the stress responsive kinases (Hammarberg *et al.* 2004). Inhibition with pertusis toxin has

been demonstrated to block the effects of preconditioning when administered prior to the ischaemic preconditioning stimulus in rabbits (Thornton *et al.* 1993). In addition, stimulation of the G_i-protein coupled muscarinic (M2) receptor with carbachol, induced preconditioning-like protection from an ischaemic insult (Thornton *et al.* 1993). In contrast, studies in the rat myocardium have generated controversial data with evidence both for (Parratt *et al.* 1993) and against (Cave *et al.* 1993; Liu *et al.* 1993) the involvement of G_i-proteins in cardioprotection. The activation of many G_i-protein coupled receptors has been shown to be able to induce preconditioning-like protection from ischaemia including bradykinin and adenosine receptors (Fryer *et al.* 2002).

G_i-protein activation is followed by the activation of phospholipase C (PLC) and phospholipase D (PLD) (Guillon *et al.* 1992). PLC degrades the membrane phospholipid phosphatadylinositol 4,5 bisphosphate (PI_2) to inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Lubert 1988; Yellon *et al.* 1998). DAG in-turn activates protein kinase C (PKC) facilitating its translocation and induction of cardioprotection (Figure 4). PLD degrades other membrane lipids such as phosphatidylcholine, which is metabolised into DAG *via* a phosphohydrolase. Usually, the production of DAG is short lived, peaking within 30s. In contrast, PLD-mediated formation of DAG takes longer, facilitating the prolonged activation of PKC (Cohen *et al.* 1996).

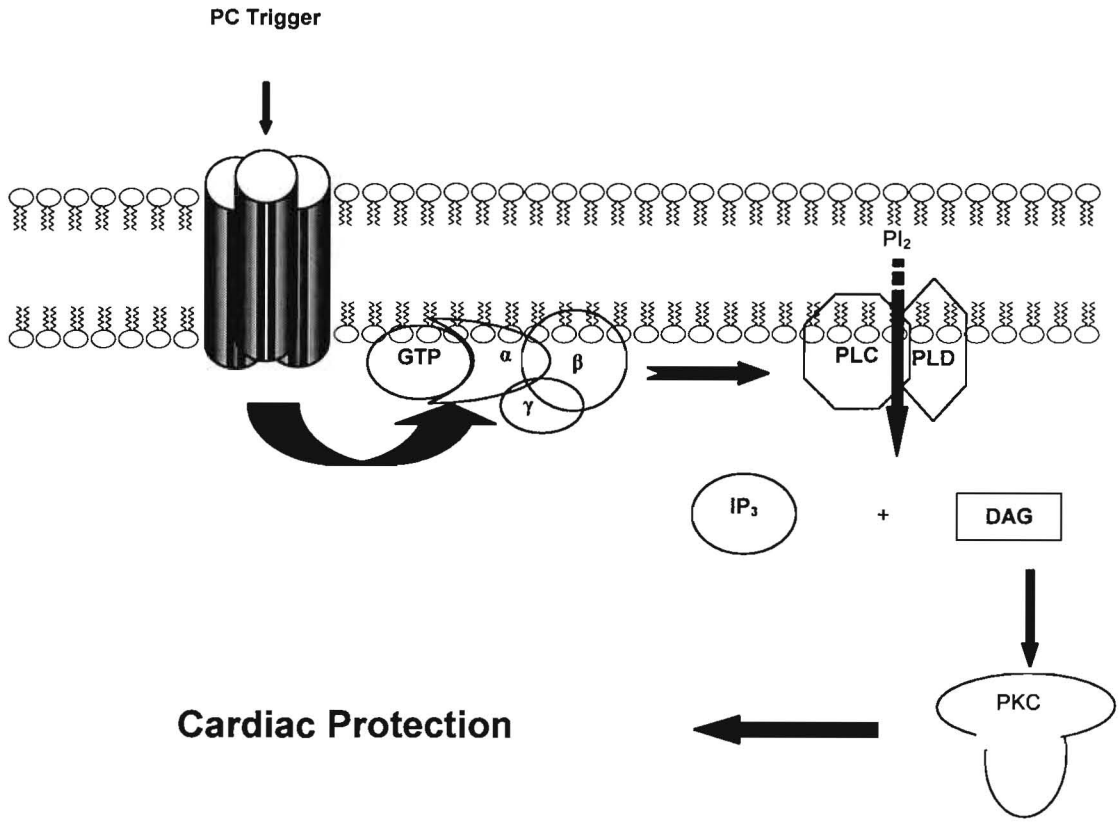

Figure 4: Signalling cascade in cardiac preconditioning: Activation of protein kinase C (PKC). G-protein coupled receptors are activated in response to a PC stimulus culminating in the activation of PKC *via* DAG formation by both PLC and PLD. DAG- Diacylglycerol, GTP- Guanosine triphosphate, IP_3- Inositol 1,4,5-triphosphate, PLC and PLD- Phospholipase C and D, PC- Preconditioning, PI_2- Phosphatadylinositol 4,5 bisphosphate. Adapted from (Grisham 1997).

c) Protein kinase C (PKC)

PKC is an important mediator of cardiac preconditioning (Fryer *et al.* 1999). It is a serine-threonine kinase that functions as an intracellular signalling peptide for many growth factors, such as angiotensin, endothelin and vascular endothelial growth factor (Naruse *et al.* 2000). PKC can also be activated in response to stress-related factors such as ischaemia, hypoxia and oxidants (Pain *et al.* 2000). Binding of DAG to the regulatory subunit of PKC causes a conformational change in PKC allowing it to interact with its "internal receptors" known as

receptor for activated C kinase, RACK, within the cells. RACKs are bound to various intracellular structures/organelles and form docking sites for activated PKC, functionally bringing this peptide into close proximity of its target substrate proteins. To date, 12 isoforms of PKC have been identified (Naruse *et al.* 2000) and they can be divided into three groups:

1. **Conventional PKC's** – which are calcium and DAG dependent for their activation. These include the α, β_1, β_2 and γ isoforms;
2. **Novel PKC's** – which are calcium independent but DAG dependent. These include the δ, ε, η and θ isoforms;
3. **Atypical PKC's** – which are calcium and DAG independent regarding activation. These include the ζ, and ι/λ isoforms of PKC.

Activation and subsequent translocation of PKC, has been shown to be necessary for the protective effects of ischaemic preconditioning (Ping *et al.* 1997). Inhibition of PKC with staurosporin, polymyxin B or chelerythrine, administered prior to and/or during ischaemia, abrogates the protection associated with preconditioning in most animal models (Speechly-Dick *et al.* 1994; Pain *et al.* 2000; Yellon *et al.* 2003). Supporting these findings, direct activation of PKC, either with the DAG analogue 1,2-dioctanoyl-sn-glycerol (Speechly-Dick *et al.* 1994) or the PKC agonist phorbol myristate acetate (Gray *et al.* 1997) both induce preconditioning-like protection in isolated rat hearts and isolated myocytes, respectively. In addition, inhibition of cellular microtubular activity with colchicine, which prevents translocation of PKC, also abolishes protection

afforded by ischaemic preconditioning, supporting the concept that PKC translocation is essential for protection (Liu *et al.* 1994).

Of the above-mentioned isoforms, PKCε and PKCα play active roles in cardioprotection. Following an ischaemic stimulus, PKCα is activated in the dog heart (Naruse *et al.* 2000) while PKCε is activated in the mouse, rat, rabbit and pig heart (Gray *et al.* 1997; Ping *et al.* 1997; Liu *et al.* 1999). In addition, Mochly-Rosen's group has been able to induce cardioprotection in an *in vitro* and *in vivo* settings of coronary artery ligation in mice and pigs using a specific PKCε agonist (Liu *et al.* 1999).

d) Mitogen activated protein kinases (MAPK)

Intracellular signal transduction is also mediated by tyrosine kinases. These proteins induce gain or loss of function by phosphorylating target proteins on tyrosine residues. Tyrosine kinases were first proposed to have a protective role by Maulik *et al* (Maulik *et al.* 1996). Administration of genistein, a broad-spectrum tyrosine kinase inhibitor, blocked protection from ischaemic preconditioning. Interestingly, PKC activation was impaired suggesting that tyrosine activation occurred upstream of PKC. The role of tyrosine kinases in preconditioning was confirmed by Baines *et al* in the rabbit heart (Baines *et al.* 1998). However, they suggested that the tyrosine kinase activation occurred distal to or even possibly in parallel to PKC activation. Since then, several studies have suggested that the tyrosine kinases act in parallel with PKC (Vahlhaus *et al.* 1998; Fryer *et al.* 1999) and that they belong to the MAPK family.

MAPK are a family of intracellular tyrosine kinases which are activated in response to external stimuli and cellular stresses such as heat shock, stretch and ischaemia (Michel *et al.* 2001; Steenbergen 2002). MAPK activation is the final step of a tightly regulated signalling cascade which is initiated *via* the activation of a MAPK kinase kinase (MKKK). These in turn activate a MAPK kinase (MKK), which finally activate the MAPK (Widmann *et al.* 1999; Michel *et al.* 2001; Steenbergen 2002) (Figure 5).

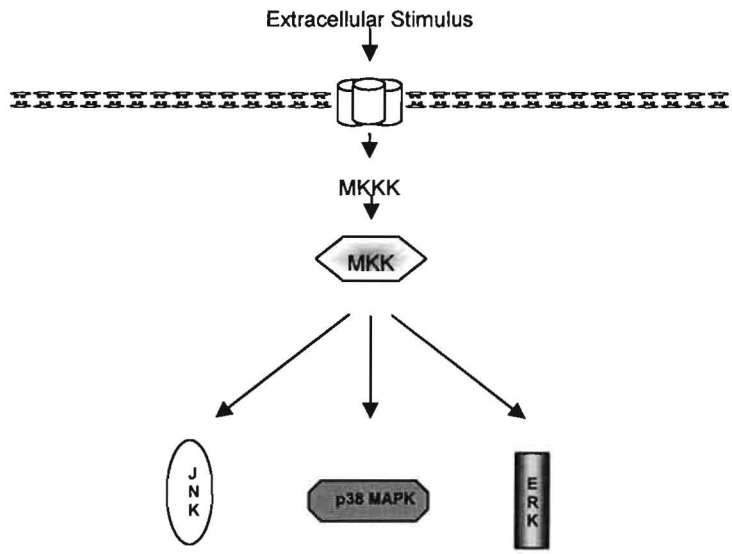

Figure 5: General MAPK signalling cascade. MAPKKK are activated in response to external stimuli. They in turn phosphorylate and activate the MAPKK which then activate specific MAPK. ERK- Extracellular signal-regulated kinase, JNK- cJun NH_2-terminal kinase, MKKK- MAPK kinase kinase, MKK- MAPK kinase, MAPK- Mitogen activated protein kinase.

The MAPK activate transcription factors, both in the cytosol and nucleus, as well as other kinases and structural or functional proteins. To date 14 MKKK, 7 MKK and 12 MAPK have been identified in mammalian cells (Widmann *et al.* 1999;

Michel *et al.* 2001; Steenbergen 2002). Three of the MAPK sub-families are thought to have a role in cardiac preconditioning:

1. **p38 MAPK family** - p38 α–MAPK, p38 β-MAPK, p38 γ-MAPK and p38 δ-MAPK.
2. **cJun NH_2-terminal kinase (JNK) family** – JNK1, JNK2 and JNK3.
3. **Extracellular signal-regulated kinase (ERK) family** – ERK1 and ERK2.

i) p38 MAPK

The role of p38 MAPK as a mediator of cardiac preconditioning is controversial. Early studies in preconditioning noted that p38 MAPK was phosphorylated following an ischaemic preconditioning stimulus and during the index ischaemia in rats (Maulik *et al.* 1998; Yellon *et al.* 2003) and rabbits (Weinbrenner *et al.* 1997). In addition, direct activation of p38 MAPK with anisomysin, a dual activator of both p38 MAPK and JNK, has been demonstrated to induce cardioprotection from ischaemia/reperfusion injury and this protection was abolished using the p38 MAPK inhibitor SB 203580 (Lochner *et al.* 2003a). p38 MAPK is thought to induce protection *via* MAPK-activated protein kinase 2 (MAPKAP2) (Figure 6) which will activate heat shock protein 27 (Hsp27) (Maulik *et al.* 1998). Hsp27 promotes actin assembly which increases the tolerance of the cytoskeleton to stress. Ischaemic preconditioning, adenosine and anisomycin have all been reported to increase MAPKAP2 activation and subsequent protection from ischaemia *via* p38 MAPK (Maulik *et al.* 1996). In contrast, other studies did not observe an activation of p38 MAPK in pigs (Yellon

et al. 2003) and rats (Fryer *et al.* 2001a) following an ischaemic preconditioning stimulus. In direct contradiction, attenuation of p38 MAPK activity was associated with improved recovery (Marais *et al.* 2001). Similarly, SB 203580, has been demonstrated to induce cardioprotection in rats and pigs (Steenbergen 2002) and to inhibit p38 MAPK, but not JNK 1/2 in a dose dependent manner (Clerk *et al.* 1998).

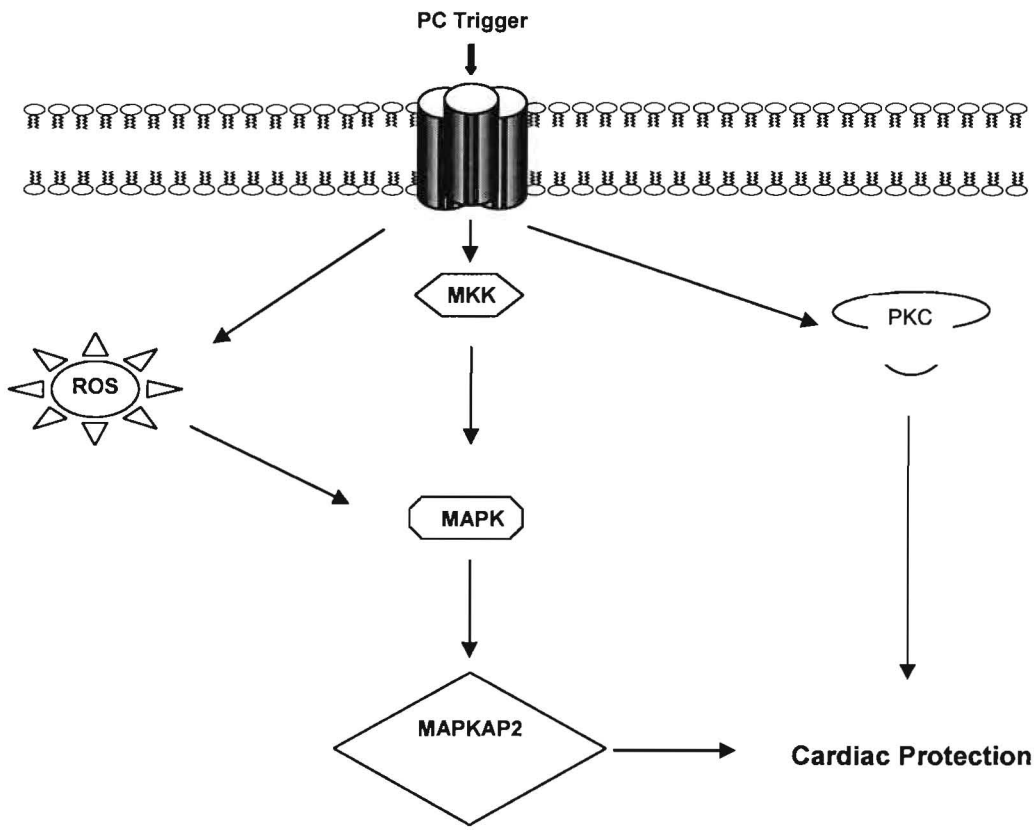

Figure 6: Signalling cascade in cardiac preconditioning: Activation of the MAPK family of tyrosine kinases. The MAPK are activated following a PC trigger. This occurs *via* the intracellular signal mediators ROS, PKC and through the MAPK signalling cascade. The MAPK activate MAPKAP2 inducing cardioprotection. MAPK- Mitogen activated protein kinases MKK- MAPK kinase, PKC- Protein kinase C, MAPKAP2- MAPK-activated protein kinase 2, PC- Preconditioning, ROS- Reactive oxygen species.

Despite the large number of studies undertaken into the role of p38 MAPK in cardioprotection, substantial ambiguity remains. The contradictive findings may be due to the diverse animal models used, the variations in the biochemical methods used to determine p38 MAPK activation and the reported variable effects of p38 MAPK activators and inhibitors in the heart. Furthermore, the period during which p38 MAPK is activated may play a vital role in determining whether cardioprotection occurs. Using an isolated rat heart model, Lochner *et al* have demonstrated that p38 MAPK activation is required to trigger cardioprotection but its attenuation during ischaemia/reperfusion is needed for a reduction in infarct size (Lochner *et al.* 2003). In addition, the various isoforms of p38 MAP, p38 MAPKα or β, may have opposing effects. For example, in neonatal rat cardiomyocytes, p38 α-MAPK is apoptotic while p38 β-MAPK is anti-apoptotic (Michel *et al.* 2001).

ii) cJun NH_2-terminal kinase (JNK)

JNK is a stress responsive kinase of which 3 isoforms, JNK1, JNK2 and JNK3, are known to exist. JNK1 and JNK2 are predominantly expressed in the heart and are involved in cardioprotection. JNK is activated by cellular stresses such as heat, osmotic shock, cytokines and ischaemia/reperfusion (Armstrong 2004). Studies have shown that JNK is activated following an ischaemic preconditioning or pharmacological preconditioning stimulus (Sato *et al.* 2000). However, JNK phosphorylation levels decrease at reperfusion following an ischaemic preconditioning stimulus (Sato *et al.* 2000), suggesting that JNK may not attenuate reperfusion injury. Pharmacological preconditioning with anisomycin and the opioids receptor agonist TAN-67 both precondition by activating JNK

(Fryer *et al.* 2001a). The induced cardioprotection could be abrogated with curcumin, a selective JNK inhibitor (Fryer *et al.* 2001a). JNK has also been implicated as a downstream signalling intermediate in PKC mediated cardioprotection. Subsequently, up-regulation of PKCε *via* transfection studies increases JNK expression (Ping *et al.* 1999).

Recently, Shao *et al* provided new evidence that JNK prevents apoptosis from hypoxia/reoxygenation injury contributing to the activation of the pro-survival kinase Akt during reoxygenation (Shao *et al.* 2006). Furthermore, they revealed a unique JNK phosphorylation site on Akt that they suggest is required for the re-activation of Akt at reoxygenation for cardioprotection to occur.

iii) Extracellular signal-regulated kinase (ERK)

The role of ERK1 and ERK2 (ERK 1/2) has been extensively studied as a trigger and mediator of cardioprotection. ERK 1/2 is phosphorylated and activated in the preconditioning period following an ischaemic (Kim *et al.* 1999; Fryer *et al.* 2001b) or pharmacological preconditioning stimulus. Administration of the opioid receptor agonist, TAN-67 ((Fryer *et al.* 2001b) also see review by Hausenloy *et al* (Hausenloy *et al.* 2004b)), Transforming Growth Factor-β1 (TGF-β1) (Baxter *et al.* 2001) and cardiotropin-1 (Brar *et al.* 2001) increase ERK 1/2 phosphorylation levels prior to index ischaemia. These phosphorylation levels decrease during the index ischaemia with ERK1/2 activation once again enhanced at the time of reperfusion (see review (Hausenloy *et al.* 2004b)). Inhibition of ERK 1/2 activation during the preconditioning phase with PD 98059, results in a loss of cardioprotection (Armstrong 2004). ERK 1/2 is also activated in a PKC dependent

manner in response to PKC activating phorbol esters linking ERK 1/2 as a downstream mediator of PKC induced cardioprotection. Similar to p38 MAPK, ERK 1/2 activates the cardioprotective Hsp27 *via* MAPKAP2 improving the tolerance of the cytoskeleton to stress (Kim *et al.* 1999) (Figure 6).

More recently, ERK 1/2 has been implicated, with Akt, as a major component of the so-called "Reperfusion Injury Salvaging Kinases" (RISK) pathway (Hausenloy *et al.* 2004b). The RISK pathway stipulates that activation of the pro-survival kinases, ERK 1/2 and Akt, during early reperfusion attenuates reperfusion induced cell death *via* the upregulation of anti-apoptotic mechanisms (Hausenloy *et al.* 2004b). Supporting this, ERK 1/2 phosphorylation levels have been shown to be increased during early reperfusion following an ischaemic or pharmacological preconditioning stimulus (Mocanu *et al.* 2002). In addition, direct activation of ERK 1/2 by staging the reperfusion (staged reperfusion) with pharmacological agents such as Insulin-like Growth Factor-1 (IGF-1) (Parrizas *et al.* 1997), TGF-β1 (Baxter *et al.* 2001), cardiotropin-1 (Brar *et al.* 2001), adenosine (Hammarberg *et al.* 2004), Fibroblast Growth Factor (FGF) (Buehler *et al.* 2002) or urocotin (Buehler *et al.* 2002) can induce cardioprotection (see review Hausenloy *et al* (Hausenloy *et al.* 2004b)). Consequently, inhibition of ERK 1/2 activation during early reperfusion, with the inhibitor PD 98059, abrogates cardioprotection following ischaemic or pharmacological preconditioning (see review Hausenloy *et al* (Hausenloy *et al.* 2004b)).

e) Phosphatidylinositol-3-kinase (PI3K)/Akt pathway

The PI3K/Akt pathway is a survival-enhancing pathway, which attenuates apoptosis, regulates glycogen synthesis, and glucose transport in the myocardium (Murphy *et al.* 2003). Akt (also known as protein kinase B (PKB)) is a serine/threonine kinase that is activated upon the production of phosphatidylinositol-3-phosphate by PI3K (Murphy *et al.* 2003). Akt has been shown to mediate its function as a pro-survival kinase *via* the phosphorylation and subsequent inactivation of the pro-apoptotic kinases, bcl-2 associated death agonist (BAD), glycogen synthase kinase-3β (GSK-3β) and activation of anti-apoptotic transcription factors such as mammalian target of rapamycin (mTOR) and p70s6 kinase (Jonassen *et al.* 2001) (Figure 7). This kinase is of interest in this study as it has been conclusively shown to play a central role in the development of acute cellular adaptation to ischaemic and hypoxic stress as well as being critical as both a trigger and mediator in ischaemic preconditioning. Several studies have documented Akt activation following ischaemic or pharmacological preconditioning stimuli such as acetylcholine, cardiotropin-1 and bradykinin (Mocanu *et al.* 2002; Hausenloy *et al.* 2004b). In all circumstances, inhibition of this Akt activation, during the trigger phase, with the selective PI3K inhibitor wortmannin, resulted in a loss of the protection associated with ischaemic and pharmacological preconditioning (see review Hausenloy *et al* (Hausenloy *et al.* 2004b)).

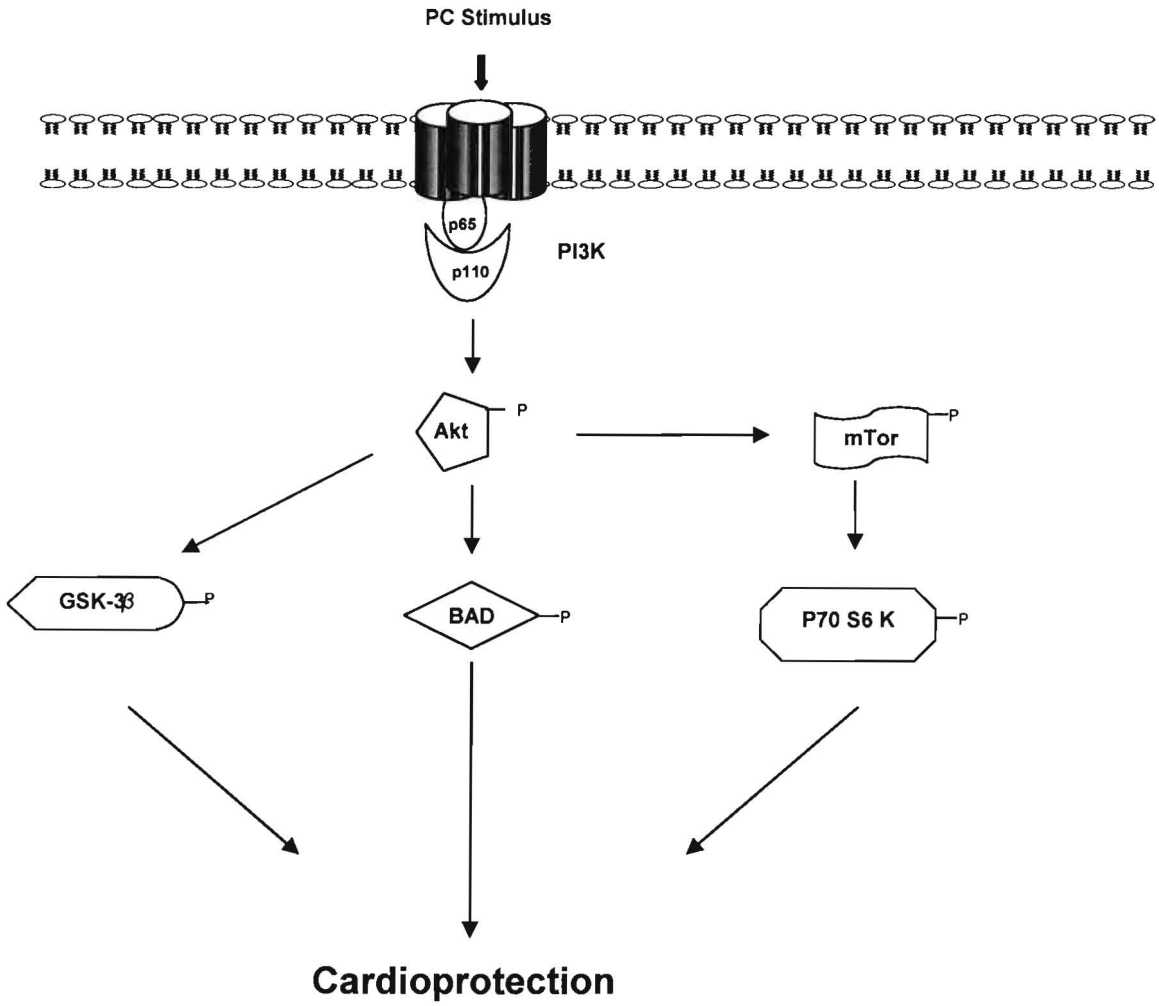

Figure 7: **Signalling cascade in cardiac preconditioning: Activation of PI3K/Akt.** Akt is phosphorylated and activated following a preconditioning (PC) stimulus. Akt phosphorylates numerous downstream peptides leading to cardiac protection *via* the inhibition of apoptosis. GSK-3β- Glycogen synthase kinase-3β, PI3K- Phosphatidylinositol-3-kinase, mTOR- mammalian target of rapamycin.

As previously mentioned, Akt is a major signalling molecule of the "RISK" pathway. Similar to ERK, Akt phosphorylation levels have been shown to increase during early reperfusion following an ischaemic or pharmacological preconditioning stimulus (Hausenloy *et al.* 2002). In addition, staged reperfusion *via* pharmacological activation of Akt at the onset of reperfusion rescues the myocardium from reperfusion injury. Hence, in studies conducted by Jonassen *et*

al insulin, given at the onset of reperfusion, induced protection following ischaemia in isolated rat hearts and in isolated cells following hypoxia (Jonassen *et al.* 2000). This protection was attributed to the activation of the PI3K/Akt pathway. Interestingly, if insulin was administered following 15 minutes of reperfusion, no protection ensued. These results clearly indicate that pharmacological activation of Akt during early reperfusion (within the first 15 minutes of reperfusion) mediate a cardioprotective-signalling cascade (Jonassen *et al.* 2001).

f) K_{ATP} channels

K_{ATP} channels are ATP sensitive potassium conducting channels. They are normally closed or inhibited at physiological ATP levels and they open as ATP levels drop (Trapp *et al.* 1997). K_{ATP} channels play a key role in preconditioning, with activation resulting in cardioprotection (Forbes *et al.* 2001) and inhibition negating induced protection (Auchampach *et al.* 1992). Although a large number of studies have elucidated the regulation and manner in which K_{ATP} channels are activated, the true physiological determinants of this channel are not known (Flagg *et al.* 2005).

Two distinct populations of K_{ATP} channels exist within the cardiomyocytes, the sarcolemmal and the mitochondrial K_{ATP} pools (Kukreja *et al.* 1990; Kim *et al.* 1994). Each displays distinctive properties and can be activated or inhibited using specific pharmacological compounds. Even though such pharmacological tools exist, a large amount of controversy exists as to which channel is important for cardioprotection following ischaemic preconditioning (Schultz 2001).

i) The Sarcolemmal K_{ATP} channel

The sarcolemmal K_{ATP} channel opening occurs following hypoxia or ischaemia (Sakmann *et al.* 1983). This was hypothesised to protect the myocardium and decrease infarct size by preventing excessive calcium from entering the myocytes and thereby reducing calcium overload during ischaemia and early reperfusion (Gross *et al.* 1999). Since, several studies have proven roles for and against the role of the sarcolemal K_{ATP} channel in cardioprotection (Gross *et al.* 1999). However, the mitochondrial K_{ATP} channel is favoured as to predominantly playing a protective role in cardioprotection. Specific activation of the mitochondrial K_{ATP} channel, by diazoxide can confer preconditioning-like protection against lethal ischemia in the hearts of rabbits, rats and mice (Garlid *et al.* 1997; Pain *et al.* 2000). Also, 5-hydroxy-decanoate (5HD), a mitochondrial K_{ATP} blocker, abrogates cardioprotection by ischaemic preconditioning (Garlid *et al.* 1997) in the above species. In contrast the sarcolemmal K_{ATP} inhibitor, HMR 1883, could not duplicate these results (Toyoda *et al.* 2000).

ii) The Mitochondrial K_{ATP} Channel

The mitochondrial K_{ATP} channel has a role during both the trigger and the mediator phases of cardioprotection. Opening of the mitochondrial K_{ATP} channel with diazoxide administration is followed by a burst of free radical production, triggering the preconditioning effect (Figure 8) (Pain *et al.* 2000). Using free radical scavengers, n-acetylcysteine or n-2-mercaptopropionyl glycine, the diazoxide induced protection is blocked (Forbes *et al.* 2001). Pharmacological preconditioning with acetylcholine, bradykinin, norepinephrine or opiods is also

associated with a burst of free radicals all of which can be inhibited by blocking the mitochondrial K_{ATP} channel (Cohen *et al.* 2001).

To determine the source of the free radicals within the mitochondria, the presence of myxothiozol, which blocks the flow of electrons from site III in the electron transport chain, also inhibited free radical production in preconditioning (Ludwig *et al.* 2004). This not only implicates the mitochondrial K_{ATP} channel but specifically suggests that the free radicals come from the electron transport chain (Ludwig *et al.* 2004).

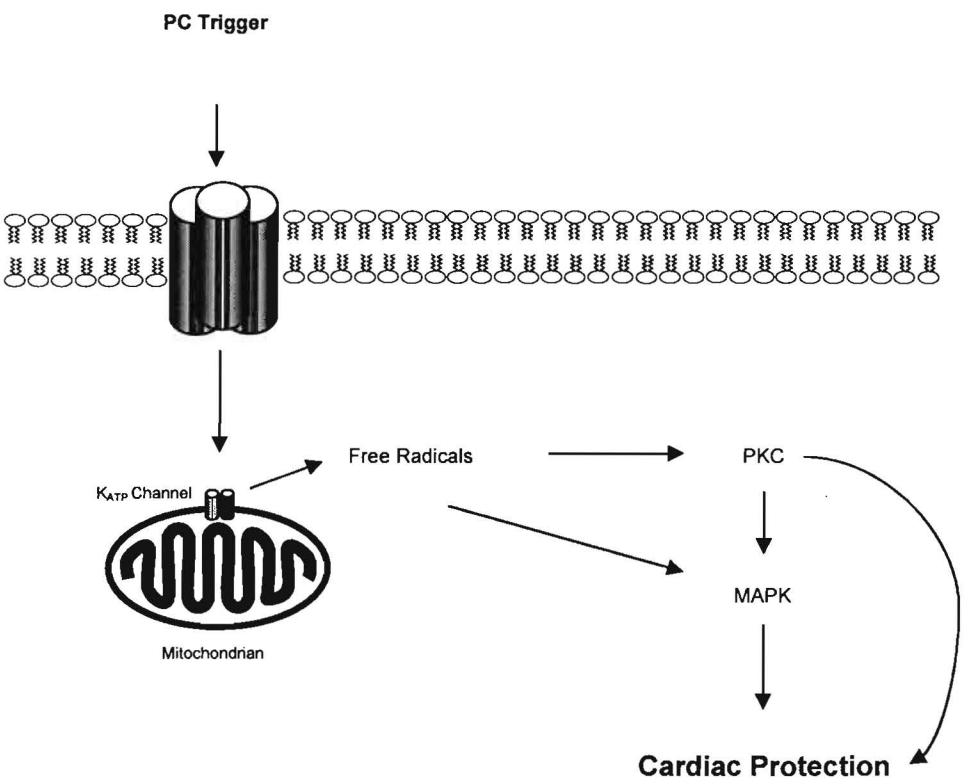

Figure 8: Signalling cascade in cardiac preconditioning: Activation of the mitochondria. PC stimuli such as diazoxide or ischaemia trigger the release of free radicals from the mitochondrial K_{ATP} channel. These free radicals act as signalling intermediates initiating the activation of the MAPK cascade as well as PKC leading to cardiac protection. K_{ATP}- Potassium adenosine triphosphate, MAPK- Mitogen activaed protein kinase, PKC- Protein kinase C.

The mitochondrial K_{ATP} channel has also been implicated as a mediator of cardiac preconditioning. Pharmacological activation of both PKC (Hopper *et al.* 2000) and MAPK (Yue *et al.* 2002), which induces cardioprotection, can be inhibited with 5HD indicating that the mitochondrial K_{ATP} channel may also be downstream to these preconditioning mediators (Hopper *et al.* 2000).

In addition to its role as both a trigger and a mediator of cardioprotection, the mitochondrial K_{ATP} channel may also function as an end effector of preconditioning. In support of this theory, falling ATP levels during ischemia re-open this channel, which has been demonstrated to be essential for cardioprotection (Griffiths 2000). It is unclear as to the reason that the opening of the mitochondrial K_{ATP} channels should be protective as this causes mitochondrial swelling and also slight uncoupling which are both undesirable during ischaemia (Yellon *et al.* 2003).

g) Mitochondrial permeability transition pore (mPTP)

Under normal physiological conditions, the mitochondrial inner membrane is impermeable to most molecules. However, during stressful circumstances a non-specific pore, known as the mitochondrial permeability transition pore, can open in the inner mitochondrial membrane allowing any molecule less that 1.5kDa to enter the inner mitochondria (Hausenloy *et al.* 2004a) (Figure 9). This causes an increase in the osmotic pressure of the mitochondria resulting in a subsequent increase of the inner matrix volume and the rupture of the outer mitochondrial membrane (Halestrap *et al.* 2004). These series of events result in apoptosis *via* the release of cytochrome c into the cytosol (Hausenloy *et al.* 2002). Opening of the

mPTP occurs under conditions of oxidative stress and ATP depletion. Consequently it is closed during ischaemia and it re-opens for the first few minutes of reperfusion when these conditions are present (Figure 9) (Griffiths *et al.* 1995).

The core components of this pore are as follows:

1. **Adenine nucleotide transporter (ANT)** – which is a transporter of adenine nucleotides both into and out of the mitochondria.
2. **Voltage dependent anion channel (VDAC)** – this protein, also known as porin, is found in abundance in the outer mitochondrial membrane and usually forms a channel allowing the passage of small metabolites.

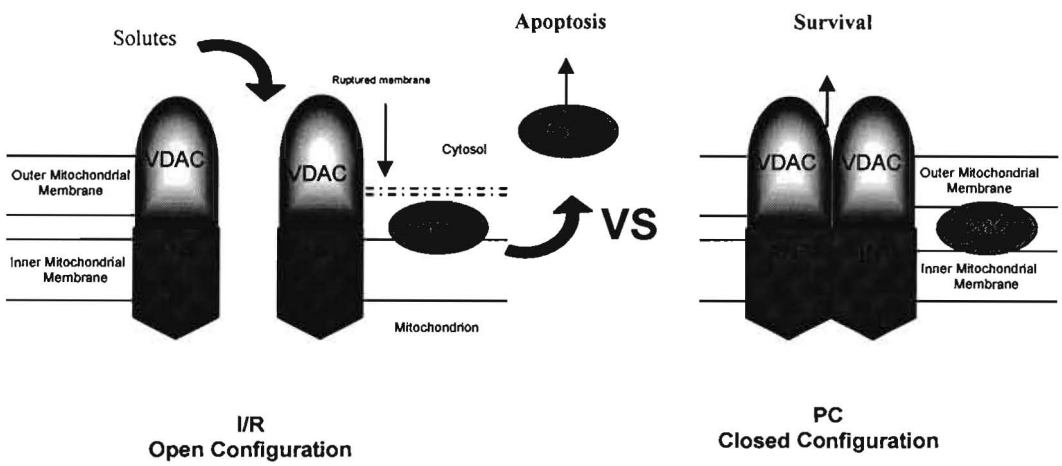

Figure 9: Induction of apoptosis or survival by the mitochondrial permeability transition pore (mPTP). I/R results in the formation of the mPTP. This allows solutes entrance to the mitochondria resulting in the disruption of the outer mitochondrial membrane. Subsequently cytochrome C is released into the cytosol initiating apoptosis. In the setting of cardiac PC, the mPTP remains in a closed configuration preventing cytochrome C release. ANT- Adenine nucleotide transporter, cyt C- Cytochrome C, PC- Preconditioning, VDAC- Voltage dependent anion channel, VS- versus.

Cardiac preconditioning with ischaemia and diazoxide, the mitochondrial K_{ATP} channel opener, have been shown to keep the mPTP in the closed configuration during early reperfusion (Halestrap *et al.* 2004). Opening the mPTP with atractyloside, a specific pore opener, can reverse the protective effects of ischaemic preconditioning and diazoxide-induced cardioprotection when given at the onset of reperfusion (Hausenloy *et al.* 2002). In addition, prevention of the pore formation with the cyclosporin A induces preconditioning-like protection (Griffiths 2000).

h) Reactive oxygen species (ROS)

Free radicals are molecules which have an odd number of electrons making them unstable and highly reactive. ROS are a type of free radicals where the un-paired electron occurs on oxygen. ROS are formed *in vivo* as by-products of cellular enzymatic reactions and as undesirable by-products of oxidative phosphorylation within mitochondria (Kulisz *et al.* 2002). When produced in excess, ROS cause intracellular damage by oxidation of proteins, lipids and nucleic acids (Guaiquil *et al.* 2004). This can result in loss or gain of function, which can be detrimental to the cell (Duranteau *et al.* 1998; Guaiquil *et al.* 2004). However, in moderate concentrations, ROS play an important role as regulatory mediators of cellular signalling processes (Heads *et al.* 1996) and recently, it was demonstrated that ROS can induce preconditioning-like protection (Forbes *et al.* 2001). Under physiological circumstances, the ROS are produced in small amounts which are targeted and removed by endogenous anti-oxidants such as vitamin C and manganese superoxide dismutase (MnSOD) (Guaiquil *et al.* 2004). However,

when ROS are produced in excess, the physiological anti-oxidants in the heart are quickly depleted resulting in an accumulation of intracellular ROS.

Excessive ROS production is implicated as a leading cause of the damage in ischaemia/reperfusion injury. ROS are produced at the onset of ischaemia followed by a second large burst at the onset of reperfusion (Maupoil *et al.* 1988; Guaiquil *et al.* 2004). By limiting the excess of ROS produced, it is possible to reduce myocardial damage. To test this hypothesis, *Guaiquil et al* preloaded isolated cardiomyocytes with the anti-oxidant vitamin C to decrease the ROS production observed in their tissue culture model of ischaemia/reperfusion injury (Guaiquil *et al.* 2004). By lowering the amount of ROS produced, they successfully increased the survival rate of isolated cardiomyocytes following a hypoxic insult (Guaiquil *et al.* 2004). In addition, the results were reproduced *in vivo* using an experimental model of myocardial infarction.

Paradoxically, ROS production is required to precondition the heart in ischaemic and pharmacological preconditioning. By triggering the transient production of free radicals, (using the Mitochondrial K_{ATP} channel opener diazoxide), the intracellular signalling cascade of cardiac preconditioning could be initiated (Forbes *et al.* 2001). Furthermore, inhibition of ROS production has been shown to avert protection from preconditioning in the rabbit, rat, mouse and chick cardiomyocytes(Pain *et al.* 2000; Cohen *et al.* 2001; Forbes *et al.* 2001; Kulisz *et al.* 2002; Sauer *et al.* 2004). Previously, Downey's group used the free radical scavenger N-2-mercaptopropionyl glycine to prevent pharmacological preconditioning with acetylcholine, bradykinin, opioids and phenylepherine

(Cohen *et al.* 2001). Moreover, they observed that adenosine-induced preconditioning was not inhibited by N-2-mercaptopropionyl glycine, suggesting not all means of inducing cardioprotection was dependent on ROS signalling. In addition to this, ROS generation has been shown to activate the so-called survival kinases, which are important for myocardial protection, in the heart (Kulisz *et al.* 2002; Pesse *et al.* 2005).

2. Tumour necrosis factor alpha (TNFα)

TNFα is a pleiotropic cytokine that was first discovered and described in 1985 by Old as a protein that could induce necrosis of certain mouse tumours (Old 1985). Two isoforms of TNF exist, TNFα and TNFβ sharing 30% of homology. TNFα is first expressed as a 26kDa transmembrane protein that is cleaved, by TNFα converting enzyme (TACE), to release a 17kDa soluble form allowing for more widespread effects of this cytokine (Figure 10) (MacEwan 2002).

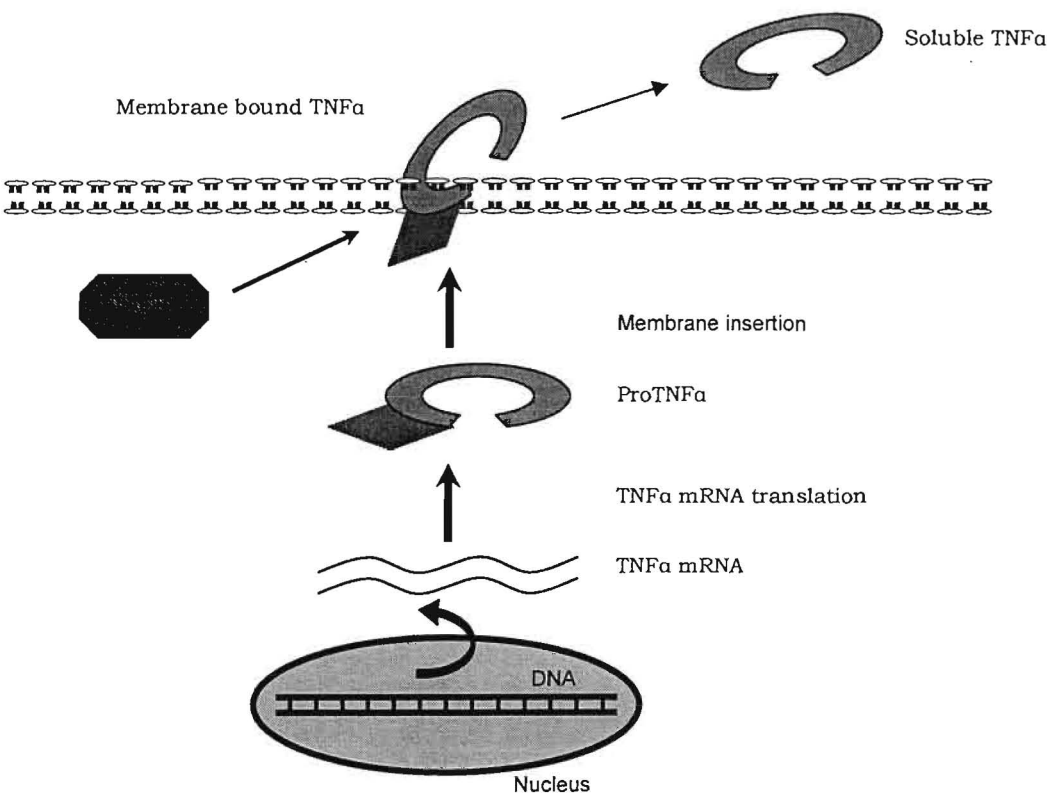

Figure 10: The production and release of TNFα. TNFα is first synthesized as a transmembrane proTNFα allowing TNFα to exist in its membrane bound form. ProTNFα is cleaved by TACE releasing the soluble form of TNFα. mRNA- messenger ribonucleic acid, TACE- TNFα converting enzyme, TNFα- Tumour necrosis factor alpha. Adapted from (Meldrum 1998).

2.1 TNFα in the heart

TNFα is largely produced in macrophages but recent studies have shown it to be expressed in cardiomyocytes (Benigni *et al.* 1996). TNFα expression in the heart does not occur in a constitutive manner and its synthesis is controlled at a translational level (Sack *et al.* 2000). The role of TNFα in the myocardium can be described as a double-edged sword, with both adaptive and maladaptive roles proposed for this protein. These contradictory avenues of thought are reconciled by the view first proposed by Mann *et al* (Mann 1996), and further expanded by Sack *et al* (Sack 2002). They proposed that a low acute dose of TNFα would be protective while a high or sustained dose of TNFα would be maladaptive (Figure 11).

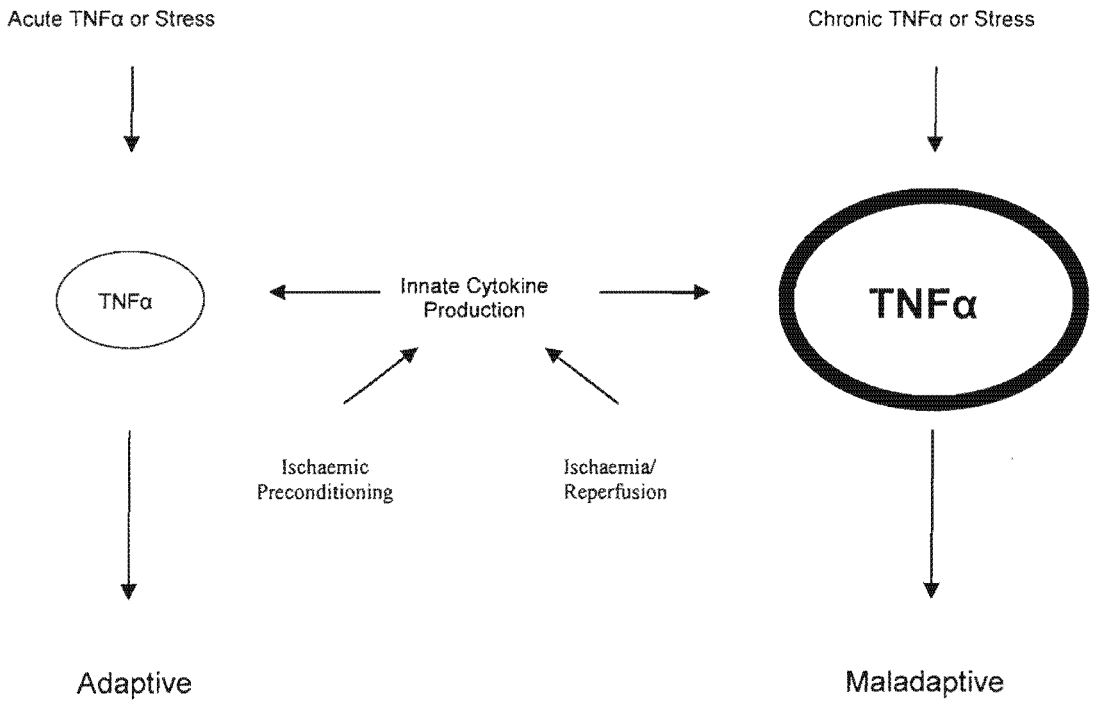

Figure 11: Adaptive vs maladaptive roles of TNFα. A low level of TNFα production, or administration, due to either an acute stress or ischaemic preconditioning gives an adaptive response. In contrast, a large production or administration of TNFα results in a maladaptive response. Adapted from Sack *et al* (Sack *et al.* 2000).

2.2 TNFα induced signalling

TNFα signalling has been implicated as a mediator of diverse physiological and pathophysiological events including inflammation, cell survival, growth, differentiation and apoptosis (see review by Mann (Mann 1996)). It achieves its effects after binding to either one of its cognitive receptors (Meldrum 1998), the 55kD TNFα receptor 1 (TNFR1) or the 75kD TNFα receptor 2 (TNFR2). Both receptors are expressed in the myocardium (Torre-Amione *et al.* 1995) and they can exist either as membrane-bound or truncated soluble forms (see review (MacEwan 2002)). They occur as single transmembrane glycoproteins sharing only 28% homology, mostly in their extracellular regions. When activated by TNFα, both receptors form homotrimers thereby facilitating signal transduction (Figure 12).

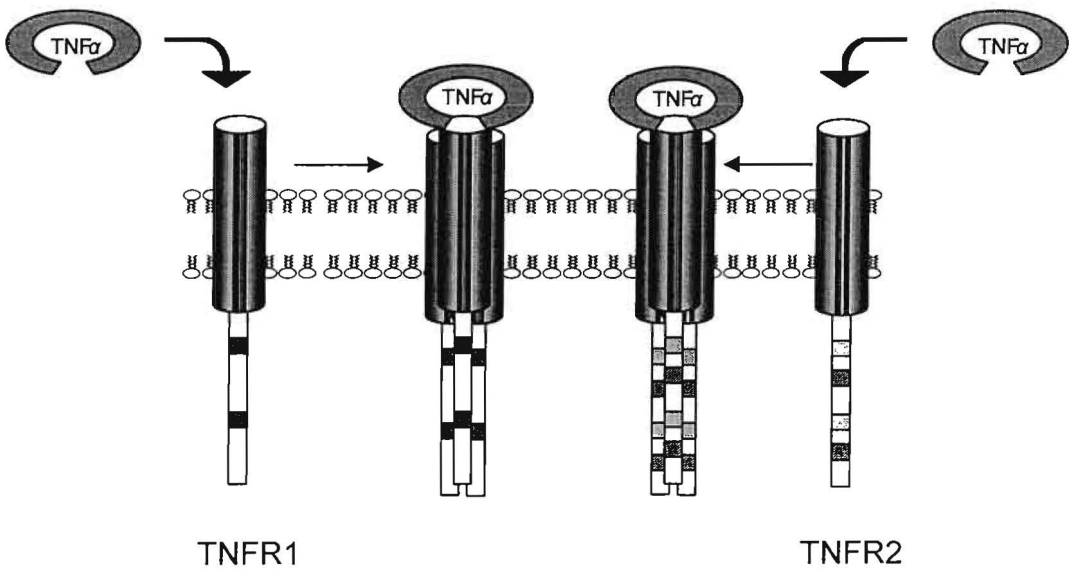

Figure 12: TNFα triggered receptor activation. Binding of TNFα to its receptor promotes receptor trimerisation and subsequent activation of the receptors. TNFR- Tumour necrosis factor receptor.

The cytoplasmic domains of the two receptors are, in contrast, very distinct thus allowing the receptors to activate separate intracellular signalling pathways (Grell 1995). These domains lack kinase ability and therefore bind adapter molecules, which mediate their effects.

Recent studies have also proposed the existence of a mitochondrial TNFα - binding protein as well as an intracellular TNFα trafficking pathway which allows TNFα to be transported from the cell surface directly to the mitochondria (Busquets *et al.* 2003).

2.3 TNFα receptor 1 (TNFR1)

Of the two receptors, TNFR1 has been more extensively studied. The signalling pathways coupled to TNFR1 include both apoptotic and protective signalling (see review Sack (Sack 2002)). Apoptosis is induced by the adapter protein, fas-associated death domain (FADD) protein which recruits and activates the apoptosis initiator caspase-8 (Harper *et al.* 2003). FADD recruitment to the receptor occurs indirectly through another adaptor protein, TNF receptor-associated death domain (TRADD) protein (Figure 13). TRADD can also bind to two other adaptor molecules named receptor-interacting protein (RIP) and TNF-receptor associated factor-2 (TRAF-2) which may mediate the protective effect of TNFα *via* nuclear factor κB (NFκB) and JNK activation (see reviews by Meldrum and Sack (Meldrum 1998; Sack 2002)), respectively. Recently, the intracellular domain of the TNFR1 was shown to bind TRADD, RIP and TRAF-2 but not FADD, even though the activation of FADD is necessary for TNFα induced apoptosis. TNFα receptor binding also leads to DAG formation *via* PLC (Schutze

et al. 1992). This, in turn, activates the spingolipid signalling pathway and PKC (Wiegmann *et al.* 1994).

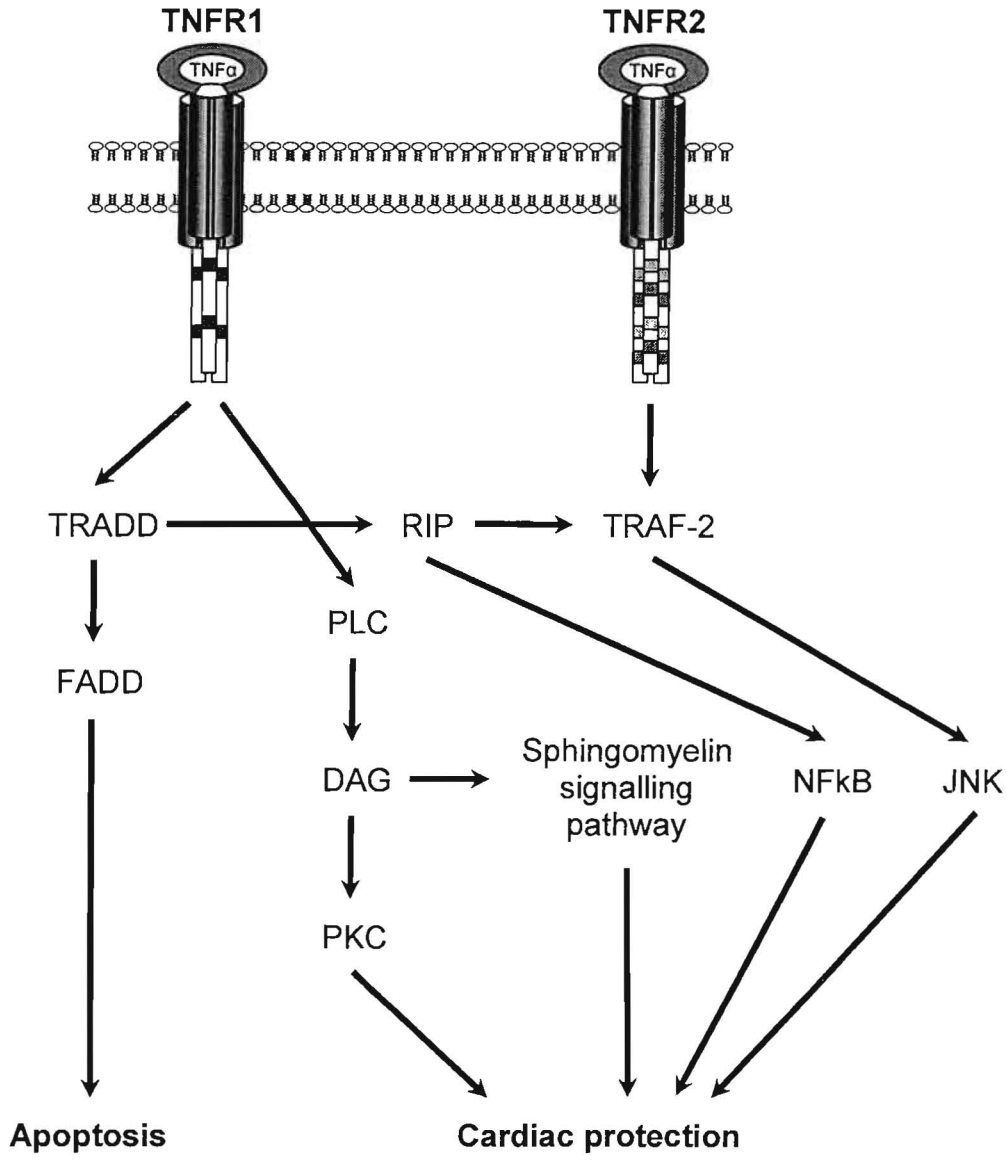

Figure 13: TNFα triggered signaling pathways. The binding of TNFα to TNFR1 leads to apoptosis *via* the recruitment of the adapter protein FADD which initiates apoptosis. TNFR1 activation also leads to cardiac protection *via* the activation of PKC, NFκB, JNK and the sphingomyelin signalling pathway. TNFR2 activation recruits the adapter protein TRAF-2 which leads to cardiac protection *via* the activation of NFκB and JNK, TNFR. FADD- Fas-associated death domain, JNK- cJun NH_2-terminal kinase, PKC- Protein Kinase C, NFκB- Nuclear factor kappa B, TRAF-2- TNF receptor activated factor-2, TNFR- TNFα Receptor. Adapter from MacEwan (MacEwan 2002),

2.4 TNFα receptor 2 (TNFR2)

In contrast to TNFR1, the signalling pathways that are coupled to TNFR2 are poorly understood. Information is limited but TNFR2 has been shown to recruit TRAF-2 in preference to TRADD suggesting that this receptor may initiate the protective effect of TNFα (MacEwan 2002) (Figure 13). Supporting this proposal, genetic ablation of TNFR2 was observed to result in excessive heart failure possibly due to the loss of this protective signalling pathway (Kurrelmeyer *et al.* 2000; Higuchi *et al.* 2004). In addition, recent evidence described by Wang *et al* (Wang *et al.* 2006), show an improvement in post-ischaemic recovery of myocardial function in TNFR1 knockout mice when compared to their male wild-type counter parts.

2.5 The maladaptive role of TNFα in the heart

Earlier studies have highlighted the pathogenic role of TNFα in the development of myocardial disease. A direct perfusion of TNFα to the myocardium produces hypertension by coronary constriction (Edmunds *et al.* 1998). Abnormally high levels of TNFα are also detected in cardiac injury with a direct correlation between serum TNFα levels and the severity and progression of heart failure (Levine *et al.* 1990). In the setting of ischaemia/reperfusion injury, TNFα levels are increased at the onset of reperfusion (Cain *et al.* 1998). Adenosine mediated cardiac preconditioning prevents this upregulation (Meldrum *et al.* 1997; Cain *et al.* 1998).

The maladaptive effect of TNFα in the heart is emphasised in transgenic mice designed to over-express TNFα in a cardiac restricted pattern. These mice

developed myocarditis while their wild-type counterparts remained relatively healthy (Kubota *et al.* 1997). In addition, the necessity of TNFα signalling for protection from ischaemic damage is observed in the TNFα , TNFR1 or TNFR2 knockout (KO) mice (Kurrelmeyer *et al.* 2000). In TNFR1 and TNFR2 KO mice, ventricular infarct size assessed following ischaemia/reperfusion was larger than their wild-type counter parts (Kurrelmeyer *et al.* 2000). Moreover, genetic ablation of TNFα results in a loss of the cardioprotection due to ischaemic preconditioning (Smith *et al.* 2002b). Taken together, these results suggest that endogenous TNFα signalling gives rise to a cytoprotective signal.

2.6. The protective role of TNFα

Transient low doses of TNFα administration pharmacologically preconditions the myocardium in a dose dependent manner (Lecour *et al.* 2002) in rats (Lecour *et al.* 2002), rabbits (Nelson *et al.* 1995) and mice (Smith *et al.* 2002b) (Figure 14). However, this protective effect is lost when TNFα is administered at a higher dose (Lecour *et al.* 2002).

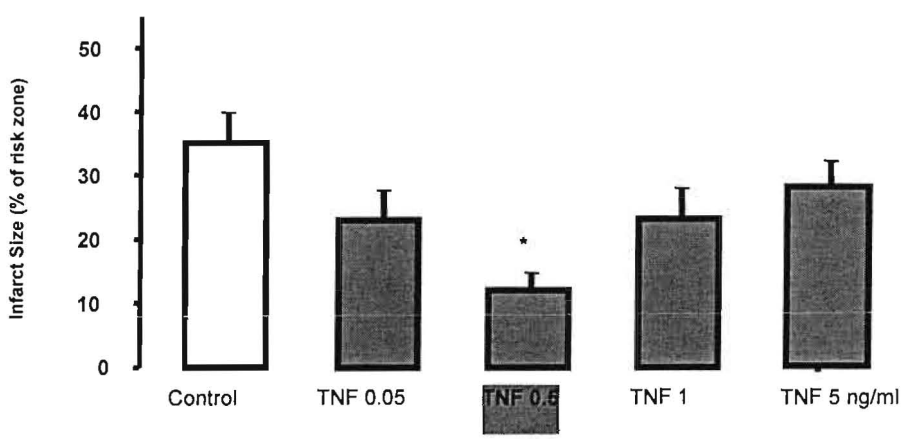

Figure 14: Dose response of TNFα-induced cardiac protection. Exogenous administration of 0.5ng/ml TNFα (7 minutes followed by a 10 minute washout period) induces a reduction in infarct size in an isolated rat heart model of I/R injury. This protective effect is lost with higher doses. * P < 0.05 vs control. Adapted from Lecour *et al* (Lecour *et al.* 2002).

In addition, TNFα is released at low levels from the myocardium in response to an ischaemic preconditioning stimulus (Kurrelmeyer *et al.* 2000). This transient release of TNFα may function as a trigger of preconditioning. Furthermore, anti-bodies against TNFα prevent the induced protective effect of preconditioning (Smith *et al.* 2002a).

This cardioprotective phenomenon may occur in part *via* sphingolipid signalling (Lecour *et al.* 2002) and ROS generation (Figure 13) (Lecour *et al.* 2005). Both n-oleoylethanolamine (NOE), an inhibitor of the sphingolipid pathway, or the free radical scavenger n-2-mercaptopropionyl glycine attenuated the protection due to TNFα mediated cardioprotection. TNFα administration also leads to PKC activation (Figure 13) (Lecour *et al.* 2002; MacEwan 2002) and it stimulates the release of the protective heat shock proteins, HSP 72 and HSP 27 (MacEwan 2002).

The intracellular signal transduction pathway of TNFα mediated protection still requires a substantial amount of work to delineate this protective pathway. In recent studies, TNFα-triggered protection was found to be independent of p38 MAPK activation (Tanno *et al.* 2003). One potential signalling cascade to be investigated is the PI3K/Akt signalling pathway that has been extensively demonstrated to be essential in ischaemic and pharmacological preconditioning (see review Hausenloy et al (Hausenloy *et al.* 2004b)). Another potential signalling protein in TNFα induced cardioprotection is the transcription factor

signal transducer and activator of transcription-3 (STAT-3), which forms part of the janus kinase (JAK)–STAT signalling pathway.

3. The janus kinase- signal transducer and activator of transcription (JAK-STAT) signalling pathway

The JAK-STAT pathway is a novel signal transduction pathway, which, in response to a diverse number of extracellular signals, activates a variety of genes in numerous tissue types (Darnell *et al.* 1994). The pathway has been linked to a wide range of biological events including embryonic development, apoptosis, organogenesis, innate and adaptive immunity and cell cycle regulation (see review (Imada *et al.* 2000)). In the heart, this pathway has been implicated in cardiac hypertrophy (Kunisada *et al.* 1996), apoptosis (Sheng *et al.* 1997), inflammation (McWhinney *et al.* 1997) as well as the early (Hattori *et al.* 2001) and the late phases of preconditioning (Bolli *et al.* 2001; Xuan *et al.* 2001; Xuan *et al.* 2005).

3.1 Janus kinase family (JAK)

JAK proteins are a family of tyrosine kinases composed of four members, janus kinase 1 (JAK1), janus kinase 2 (JAK2), janus kinase 3 (JAK3) and tyrosine kinase 2 (TYK2). They range in size from 120 kD – 140 kD and they are characterised by a functional kinase domain as well as a pseudokinase domain which may serve as a docking site for STAT proteins (see review Imada *et al* (Imada *et al.* 2000)). JAK members are constitutively associated with the cytoplasmic domains of cytokine receptors. When the receptor is activated, it forms a dimer allowing the cytoplasmic tail of the receptor and the associated JAK proteins to phosphorylate. This phosphorylation creates a docking site for a STAT protein (Figure 15). Once docked, the STAT protein is activated by phosphorylation from the relevant JAK. Different JAK proteins can activate the

same STAT protein as specificity within the pathway is due to the interaction of the relevant STAT molecule with the receptor rather than due to the JAK which is localised at the receptor (see review by Imada *et al* (Imada *et al.* 2000)).

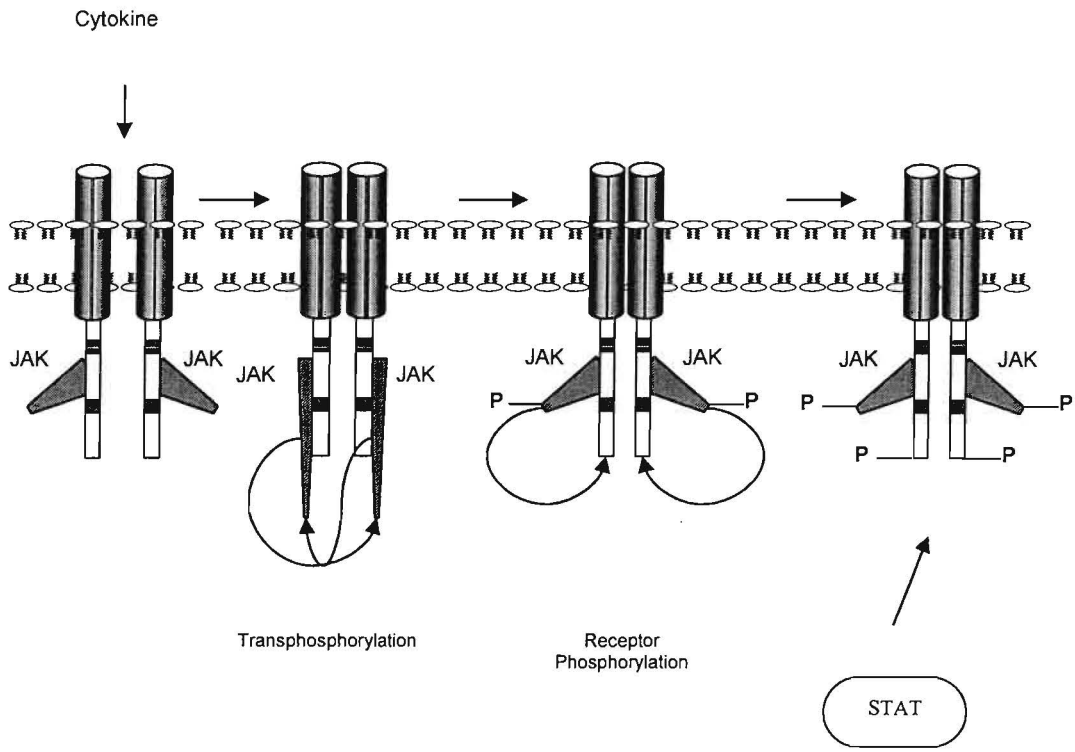

Figure 15: JAK receptor dependent activation. Receptor dimerisation occurs in response to ligand binding. This brings the associated JAK proteins in close proximity allowing them to phosphorylate one another. The phosphorylated JAK protein also phosphorylates the cytoplasmic domains of their respective receptors creating docking sites for STAT proteins. Adapted from (Darnell 1997).

3.2 STAT proteins

STAT proteins were discovered while investigating the gene regulation of interferon type 1 (Darnell *et al.* 1994). They are a family of cytoplasmic signalling proteins that initiate gene translation. Seven STAT proteins are known to occur, STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT6 (Copeland *et al.* 1995), each mediating various functions (Table 1). They contain

between 700 and 800 amino acids in length and possess several conserved domains which have been found to be critical to STAT functional abilities (Figure 16).

STAT Isoforms	Main Function
STAT-1	Impaired Interferon (IFN) α,β and γ signalling.
STAT-2	Impaired IFN α,β and γ signalling as well as reduced expression of STAT-1 in some cells.
STAT-4	Impaired Interleukin-12 (IL-12) signalling as well as an increased susceptibility to pathogens.
STAT-5a	Defective mammary gland development.
STAT-5b	Defective sexual dimorphic growth.
STAT-6	Impaired T-Helper cell-2 differentiation coupled with a susceptibility to infection.

Table 1: STAT isoforms and their main function. (See review by Darnell, (Darnell 1997))

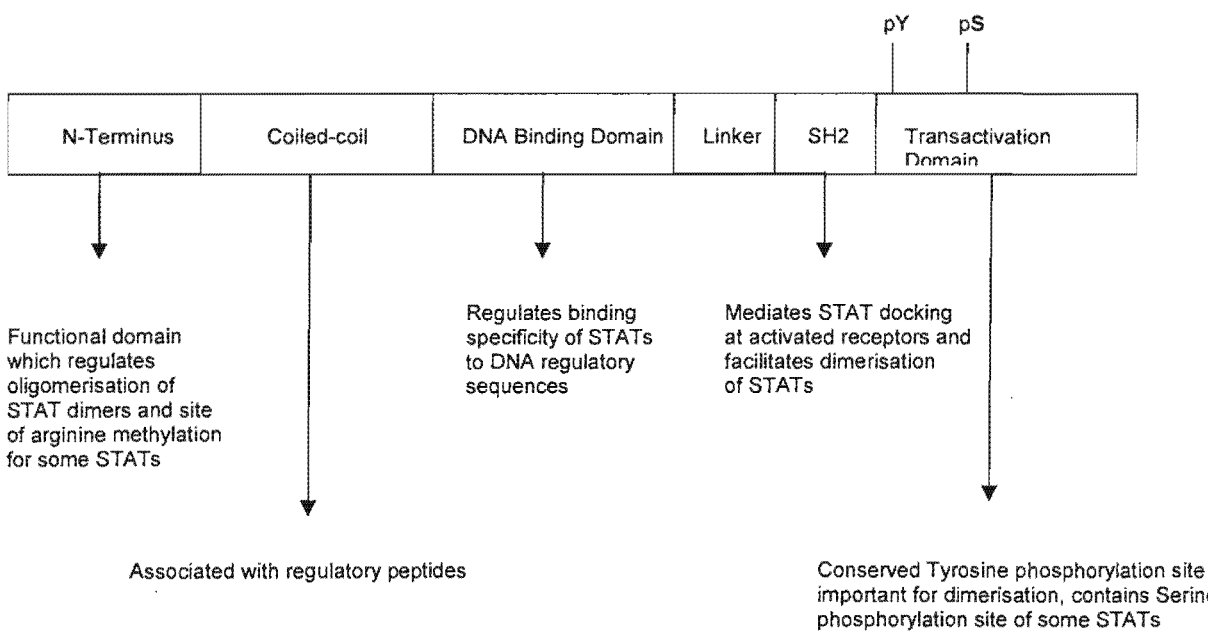

Figure 16: Structural domains and functions of STAT proteins.

3.3 STAT regulation

The regulation of STAT signalling allows for the appropriate physiological response to stimulation. STAT proteins are activated by phosphorylation of a conserved tyrosine residue. Latent cytoplasmic STAT proteins are recruited to the cellular membrane *via* a conserved src homology 2 (SH2) domain, which binds to a phosphorylated receptor (see review (Imada *et al.* 2000)). Here, the JAK associated with the activated receptor, phosphorylates then releases the STAT molecule. Tyrosine phosphorylated STAT proteins form either homodimers or heterodimers that translocate to the nucleus initiating transcription of target genes (Figure 17) (Darnell 1997). The dimerisation of STAT proteins is an integral part of their function as nuclear translocation and DNA binding only occurs in the presence of tyrosine phosphorylated STAT dimers (Figure 17)(Darnell 1997; Imada *et al.* 2000)) . In addition to tyrosine phosphorylation, STAT dimers can be further phosphorylated on a conserved serine residue *via* a PKCε-ERK1/2 dependent manner (Xuan *et al.* 2005).

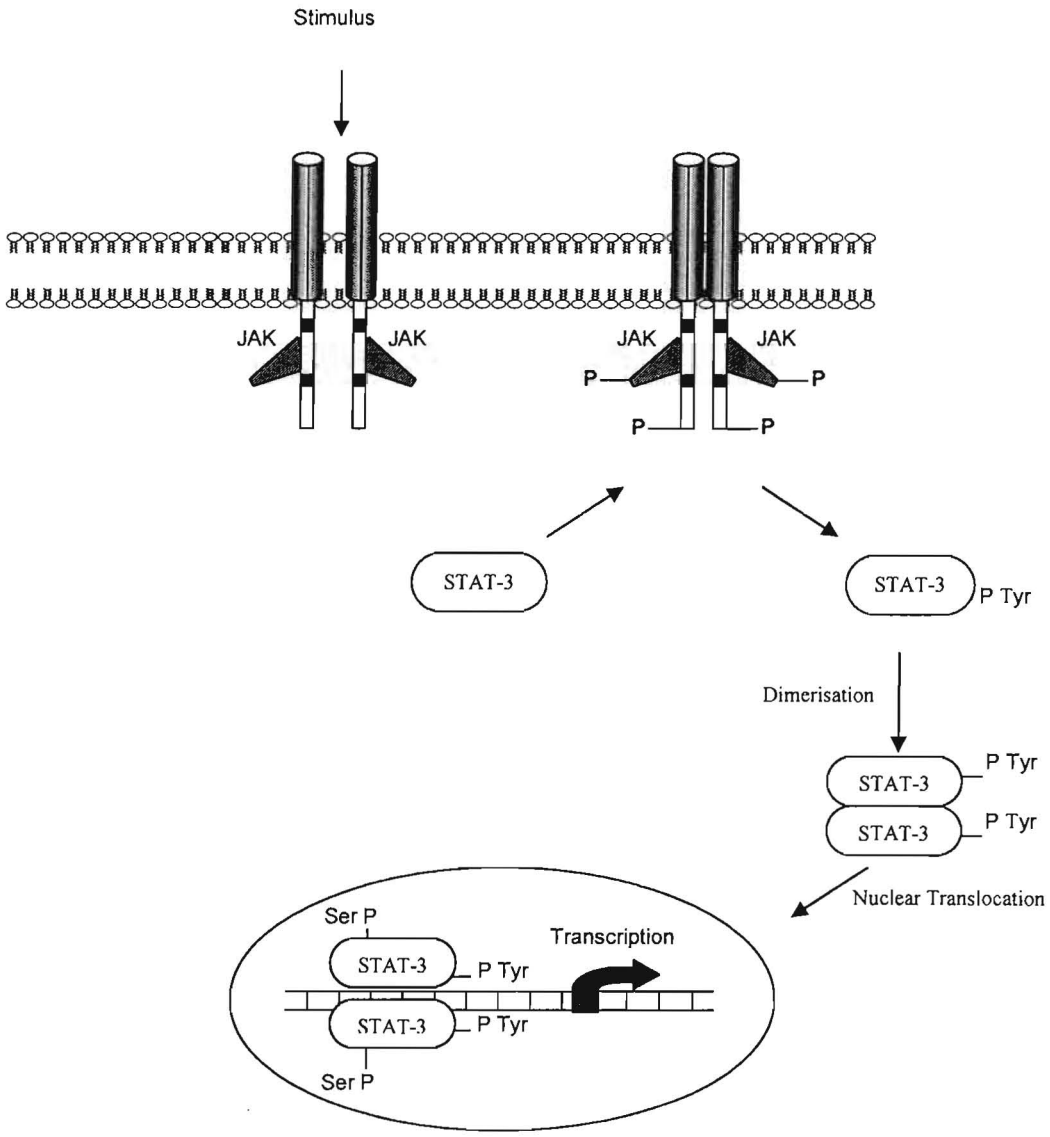

Figure 17: The JAK/STAT-3 signaling pathway. JAK-2 is activated in response to a stimulus. Activated JAK-2 recruits STAT-3 from the cytoplasm and phosphorylates STAT-3 on a tyrosine residue. This allows STAT-3 to form homodimers and translocate to the nucleus where it up regulates transcription. STAT-3 is further phosphorylated on a serine residue to improve DNA interaction. Ser- Serine, Tyr- Tyrosine. All other Abbreviations as described in text.

Activated STAT proteins are deactivated *via* several mechanisms. This can occur by dephosphorylation at the tyrosine residue or by degradation of STAT proteins

via the ubiquitine-proteosome pathway. In addition, two families of regulators control the JAK-STAT pathway intracellularly:

1. **Suppressor of cytokine signalling (SOCS)** - members of this family of proteins possess SH2 domains homologous to that of specific STAT peptides. These allow the SOCS to bind to the intracellular domain of activated receptors preventing STAT binding and subsequent activation (see review (Valentino *et al.* 2006)).
2. **Protein inhibitors of activated STAT (PIAS)** - these are a family of activated STAT-3 suppressor proteins. They influence regulation directly by binding to activated STAT molecules preventing dimerisation and inhibiting deoxyribonucleic acid (DNA) binding (see review (Valentino *et al.* 2006)).

3.4 STAT DNA binding

As transcription initiators, STAT proteins bind specifically to γ-interferon activated sequences (GAS) on target genes (see review Darnell (Darnell 1997)). These are TTC N_3/N_4 GAA binding motifs. In the nucleus, STAT proteins are further phosphorylated at a serine residue improving DNA binding and the rate of transcription (Figure 17). In addition, STAT proteins can interact with other transcription factors or other STAT isoforms to form higher oligomers and improve transcriptional control (see Review Darnell, (Darnell 1997)).

3.5 STAT-3

Of the two families (i.e. the JAK and the STAT families of proteins) forming the JAK/STAT signalling pathway, signal transduction *via* JAK2/STAT-3 has been observed to elicit many responses. It can be activated by numerous growth factors and cytokines. The physiological functions involving STAT-3 have been primarily studied through the generation of tissue specific STAT-3 KO mice, as whole body reduction of this peptide is embryo lethal. STAT-3 has been knocked out in:

1. **T-cells** – STAT-3 are involved in IL-6 mediated T-cell proliferation (Takeda *et al.* 1998).
2. **Skin cells**- STAT-3 are involved in skin remodelling including the hair formation cycle and wound healing (Sano *et al.* 1999).
3. **The central nervous system** – STAT-3 play an essential role in energy homeostasis *via* leptin signalling (Gao *et al.* 2004).
4. **Pancreatic β-cells** – these are the insulin producing cells of the pancreas. Here, STAT-3 play an important role in body weight control and glucose homeostasis (Gorogawa *et al.* 2004).
5. **The liver** – STAT-3 regulate the expression of gluconeogenic genes and is important for the maintenance of glucose homeostasis (Inoue *et al.* 2004).
6. **Cardiomyocytes** – STAT-3 play a central role with respect to resistance to cardiac inflammation, acute injury and the pathogenesis of age-related heart failure (Jacoby *et al.* 2003).

a) STAT-3 in the heart

STAT-3 is constitutively produced in myocytes as well as in cardiac fibroblasts and epithelial cells. It was first observed, in 1995 (Lowe *et al.* 1995), to be activated following exposure to leukaemia inhibitory factor (LIF). LIF treatment causes cardiac hypertrophy (Kodama *et al.* 1997), which is prevented *via* STAT-3 inhibition with AG490 (a tyrphostin that prevents JAK2 mediated activation of STAT-3). This role in cardiac hypertrophy was confirmed by overexpression of STAT-3 in cardiomyocytes (Kunisada *et al.* 2000). Here, genetic overexpression of STAT-3 in mice induced cardiac hypertrophy and the formation of new vessels contributing to cardiac adaptation under conditions of stress (Kunisada *et al.* 2000).

In the human failing heart, the phosphorylation levels of STAT-3 is reduced, suggesting a link between heart failure and the decrease in STAT-3 phosphorylation (Hilfiker-Kleiner *et al.* 2005). Also, STAT-3 KO mice have been observed to develop heart failure at a much earlier stage in their life cycle when compared to their wild type counterparts (Jacoby *et al.* 2003).

b) STAT-3 in ischaemia/reperfusion

STAT-3 is activated as part of a defence mechanism in response to ischaemia, hypoxia and ischaemia/reperfusion (Negoro *et al.* 2001; Oshima *et al.* 2005). In an *in vivo* model of sustained ischaemia, STAT-3 was observed to be phosphorylated following permanent coronary artery occlusion. (Negoro *et al.* 2001). Here, tyrosine phosphorylation of STAT-3 was sustained for up to 24 hours and inhibition of STAT-3 activation with AG490 increased the number of

apoptotic cells. This suggested a protective role for STAT-3 (Omura *et al.* 2001). In support of these data, Omura *et al* also observed an increase in STAT-3 phosphorylation following coronary artery ligation (Omura *et al.* 2001). In addition, STAT-3 activation exerts anti-apoptotic effects in cultured neonatal rat cardiomyocytes subjected to anoxia, metabolic inhibition and acidosis (Stephanou *et al.* 2000). Moreover, incremental expression of STAT-3 reduces ischaemia/reperfusion induced apoptosis in isolated myocytes (Stephanou *et al.* 2000).

In genetically modified mice, cardiac overexpression of STAT-3 protected isolated myocytes from induced hypoxia/reoxygenation and ischaemia/reperfusion injury (Kunisada *et al.* 2000). Here, the harmful burst of ROS, which occurs at the onset of reperfusion, was attenuated resulting in a decrease in the infarct size. This protection occurred in part due to the amplified production of the free radical scavengers metallothionein 1 (MT1) and metallothionein 2 (MT2) (Oshima *et al.* 2005).

c) STAT-3 in cardiac preconditioning

STAT-3 has been implicated in cardioprotection *via* the induction of anti-apoptotic signals by up-regulating the expression of the anti-apoptotic proteins b-cell leukaemia-2 (Bcl-2) (Hattori *et al.* 2001), Bcl-xL and free radical scavengers (Negoro *et al.* 2001). In addition, STAT-3 activation is linked with the phenomenon of cardiac preconditioning. Using an isolated working heart model of ischaemia/reperfusion injury, Hattori *et al*(Hattori *et al.* 2001) demonstrated that both JAK2 and STAT-3 are phosphorylated and activated during an

ischaemic preconditioning stimulus. This was correlated with improved functional recovery following index ischaemia, a decrease in the infarct size and the number of apoptotic myocytes as well as an increase in anti-apoptotic Bcl-2 and a decrease in pro-apoptotic Bax (a member of the Bcl-2 family of proteins which binds to Bcl-2 suppressing the anti-apoptotic activity of Bcl-2). In this model, the observed protective effect of the ischaemic preconditioning stimulus was reversed *via* the inhibition of JAK2/STAT-3 phosphorylation with AG490 (Hattori *et al.* 2001). Supporting this role in preconditioning, the pharmacological preconditioning mimetics LIF (Lowe *et al.* 1995; Funamoto *et al.* 2000) and cardiotrophin-1 (Funamoto *et al.* 2000; Brar *et al.* 2001) both enhanced the survival of the myocardium *via* STAT-3 phosphorylation (Negoro *et al.* 2001). This is followed by the upregulation of the free radical scavenger, MnSOD and the protective heat shock protein, HSP70 (Nelson *et al.* 1995; Negoro *et al.* 2001). Taken together these results present a strong case for the JAK2/STAT-3 signal transduction pathway in cardioprotection.

B. Objectives and Hypothesis

Ischaemic Preconditioning is a phenomenon whereby transient episodes of non-lethal ischaemia augments innate cardiac resistance towards a subsequent sustained ischaemia (Murry *et al.* 1986). TNFα is a multifaceted cytokine which is generally considered as detrimental to the heart. Recently, we have demonstrated that this cytokine is able to mimic the protective effects of cardiac preconditioning (Lecour *et al.* 2002). The intracellular signalling mechanisms involved in both ischaemic and TNFα-induced cardioprotection are unclear but they may share some similar signalling pathways. Delineating the intracellular mechanism of cardioprotection is needed to further the understanding of the mechanism by which the heart can be protected from ischaemia/reperfusion damage so that this knowledge can be translated to the clinical setting.

Recently the JAK2/STAT-3 signalling pathway has been suggested as a pivotal role in the protection afforded by ischaemic preconditioning (Hattori *et al.* 2001). In addition, a previous study indicates that TNFα can induce the phosphorylation of STAT-3 via TNFR1 in 3T3-L1 adipocytes (Guo *et al.* 1998).

Therefore, in this thesis, we hypothesized that:

Both ischaemic and TNFα-induced preconditioning require the activation of JAK2/STAT-3 pathway to confer cardioprotection.

To demonstrate our hypothesis, the following objectives will be pursue:

1. **To generate a cardiac specific STAT-3 KO mouse:** Using Cre-Lox P technology, we aim to generate our own cardiomyocyte specific STAT-3 KO mouse as part of this study (Section C of this thesis).

2. **To demonstrate that cardiomyocyte specific STAT-3 KO mice can not be preconditioned with an ischaemic or a TNFα stimulus** (Section D of this thesis).

3. **To demonstrate that both ischaemic and TNFα induced preconditioning require the activation of STAT-3 during the preconditioning stimulus to confer cardioprotection** (Section E of this thesis) .

4. **To demonstrate that both ischaemic and TNFα induced preconditioning require the activation of STAT-3 at the time of reperfusion to confer cardioprotection** (Section F of this thesis).

Is STAT-3 activation required in the protection afforded by ischaemic and TNFα-induced preconditioning?

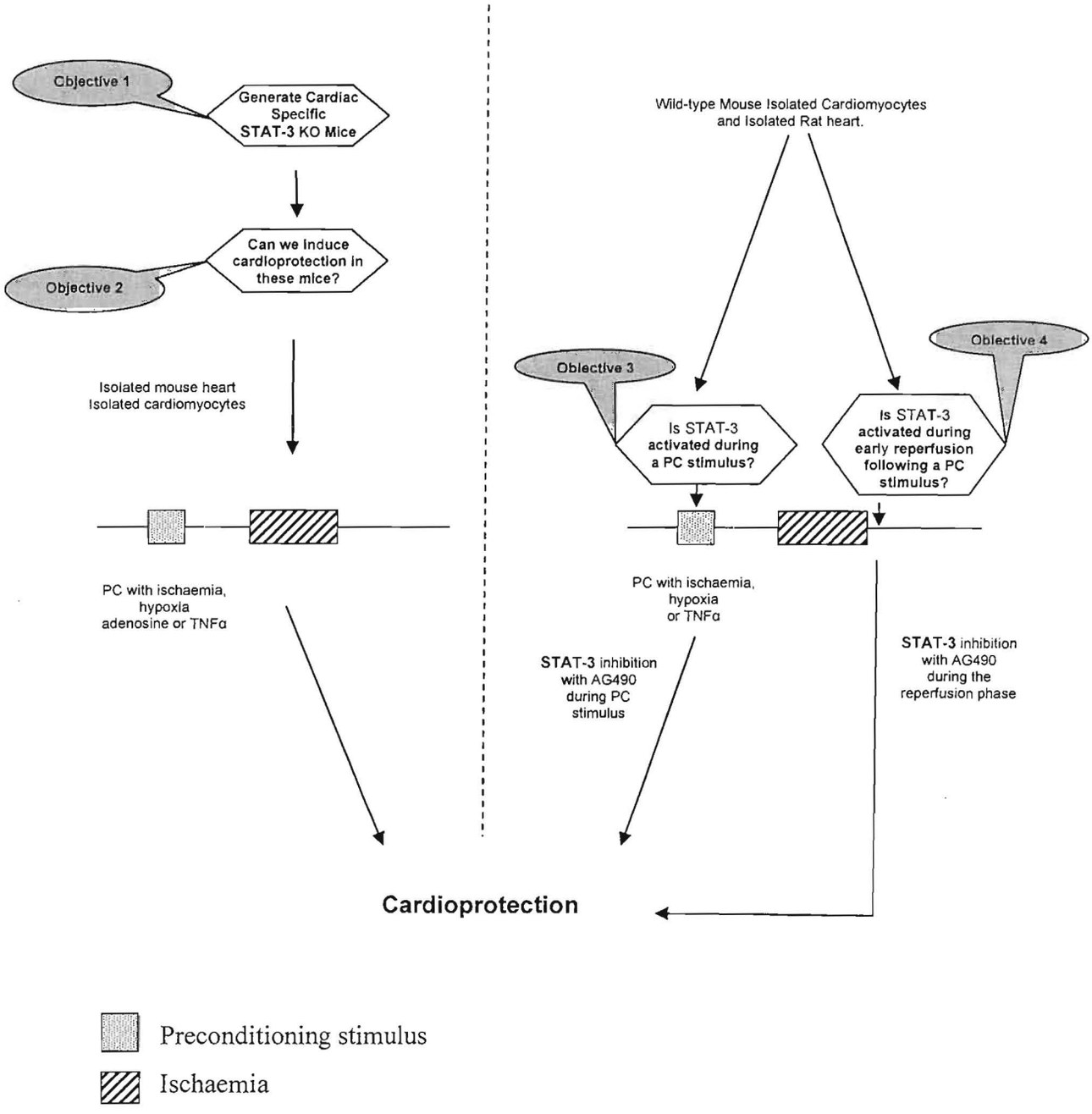

Figure 18: Hypothesis and Objectives. STAT-3 is known to be activated in cardiac preconditioning. We determine whether STAT-3 is activated in TNFα-induced cardioprotection and during early reperfusion following either an ischaemic or TNFα preconditioning stimulus. In addition we created cardiac specific STAT-3 knockout mice to confirm the protective role of STAT-3 in cardiac preconditioning. PC – preconditioning.

C. Generation of the cardiac specific STAT-3 knockout mouse

1. Introduction

Initial attempts at generating a traditional (whole body) STAT-3 knockout (KO) mouse were found to be unsuccessful. Here, genetic ablation of STAT-3 resulted in rapid degeneration of STAT-3 knockout (KO) embryos between 6.5 and 7.5 days of gestation (Takeda *et al.* 1997). This is indicative of the necessity for early STAT-3 expression during embryonic development. Subsequently, further investigations exploring the role of STAT-3 in cardiac preconditioning required the generation of a conditional, organ specific, STAT-3 KO mouse. This can be achieved with the Cre-Lox P system of conditional genetic ablation.

1.1 Cre-Lox P technology

The Cre-Lox P system of conditional gene targeting requires two separate transgenic mouse lines, a homozygous STAT-3 floxed mouse ($STAT\text{-}3^{F/F}$; F-Floxed) and an organ specific heterozygous Cre recombinase-expressing mouse ($Cre^{T/N}$; T – Transgene and N – Normal). These two strains of mice are then crossed to produce the organ specific STAT-3 KO animal ($Cre^{T/N}$ $STAT\text{-}3^{F/F}$) (Chien 2001).

1.2 STAT-3 floxed mouse

Floxed mice are defined as animals that have a specific deoxyribonucleic acid (DNA) sequence, the Lox P (Locus of X-ing over P) site, integrated into the germ line DNA. Two Lox P sites are integrated into the DNA of the protein to be disrupted, flanking a gene segment to be deleted. Lox P sites consists of 2 thirteen base pair repeats separated by an 8 base pair asymmetric spacer region (Figure 19). When the Lox P sites are arranged in an opposite orientation to each other,

they serve to invert the DNA between them (Figure 20A). When arranged in a direct orientation they act as targets for excision of the DNA between them (Figure 20B) (Wagner 2000).

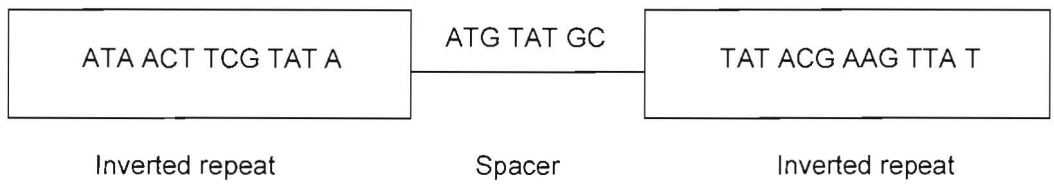

Figure 19: Lox P site. The DNA sequence of a Lox P site consists of 13 base pair repeats which are inverted and separated by a single 8 base pair asymmetric spacer region. DNA- deoxyribonucleic Acid.

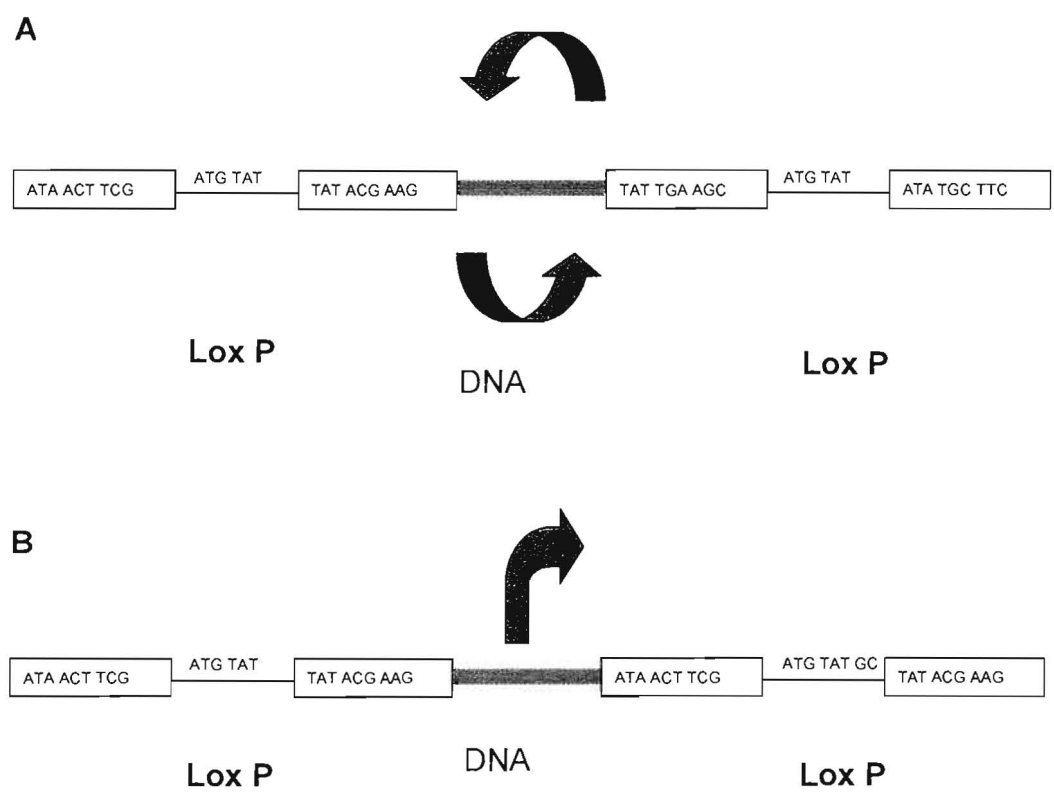

Figure 20: Functional orientation of Lox P sites. The orientation in which Lox P sites are integrated into the genome determines whether the DNA is inverted (A) or excised (B). DNA- deoxyribonucleic acid, Lox P- locus of x-ing over P site.

1.3 Cardiac specific Cre Recombinase expressing mouse

DNA excision between a pair of Lox P sites is carried out by the 38kD bacteriophage P1 Cre recombinase protein (Chien 2001). This enzyme directs the removal of the DNA between the Lox P sites leaving one Lox P site behind (Figure 21C). By placing the enzyme under the control of a tissue specific promoter, tissue specific expression of Cre recombinase occurs and hence tissue specific depletion of the target floxed protein. The transcriptional activity of the promoter used also determines the extent to which Cre recombinase is expressed and subsequently the attenuation of the target protein in the STAT-3 KO animal. A high constitutively active promoter would result in prominent expression of Cre recombinase and consequently a high rate of excision at Lox P site. Furthermore, as Cre recombinase cannot cross the cellular membrane, the effects of this protein are limited to the cell in which it is produced.

Heterozygous mice which had Lox P sites integrated into the STAT-3 germ line ($Cre^{N/N}$ $STAT\text{-}3^{F/N}$) in a direct orientation (Figure 21B) were obtained from Professor S Akira of the University Osaka in Japan (Takeda *et al.* 1997). Here, Lox P sites were introduced into introns 21 and 22 (non-coding regions of DNA) which flank the tyrosine phosphorylation site of exon (coding region of DNA) 21 (Figure 21B). As the Lox P sites are situated within the introns, they have no effect on the expression of STAT-3 in these floxed mice. Removal of exon 21 (Figure 21B) by Cre recombinase will result in the transcription and translation of a truncated non-functional STAT-3 protein (Figure 21C).

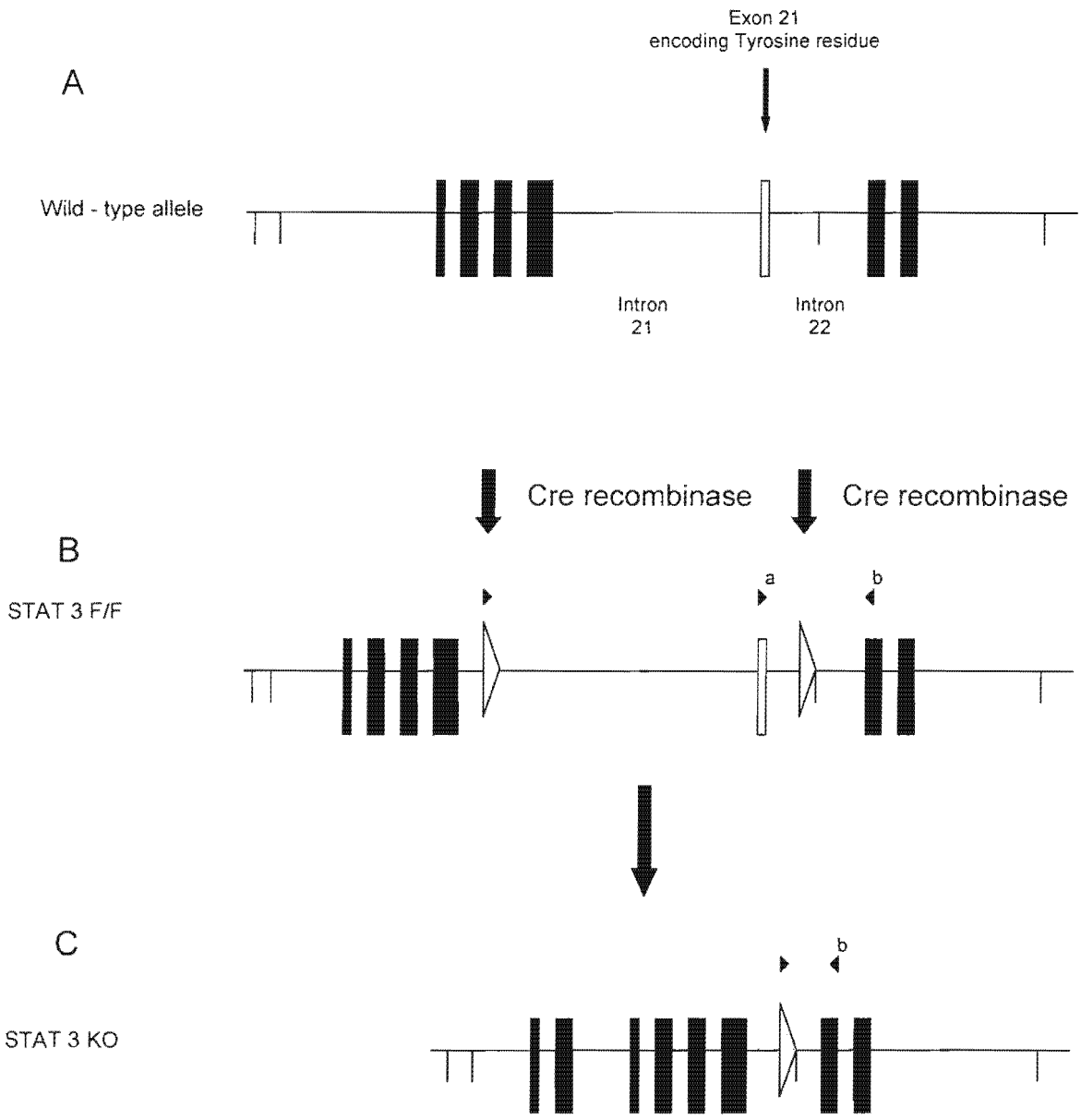

Figure 21: Targeted deletion of STAT-3. Gene organisation in STAT 3 $^{F/F}$ mice before and after Cre mediated disruption. Exon 21 (represented as ▯ in the figure) of STAT 3 was flanked by identical lox P sites (represented as ⟩ in the figure) sites (B). Each site is a target for Cre recombinase which is used to disrupt the STAT 3 gene, effectively knocking STAT 3 out. "a" and "b" are the primers used to determine whether the mouse is harboring a floxed STAT 3 gene. F- floxed, KO- knouckout.

The cardiac specific Cre recombinase expressing mouse used to generate our STAT-3 KO animal was a generous gift from Professor Kenneth R Chien of the University of California, San Diego School of Medicine. Here, the enzyme (Cre recombinase) was placed under the control of the Myosin Light Chain 2 Ventricle (MLC-2V) promoter ultimately substituting for one of the MLC-2V alleles (allele – one of 2 forms of the same gene). This promoter is only active in the ventricles of adult mice and is highly expressed ensuring an elevated expression of Cre recombinase (Chen *et al.* 1998).

2. Methods

2.1 Breeding strategy for the establishment of a cardiac specific STAT-3 KO mouse colony

The breeding strategy used to generate a cardiac specific STAT-3 KO with littermate controls is illustrated in figure 22. The original pairs of STAT-3 floxed mice had no Cre insert and were heterozygous for the STAT-3 floxed allele ($Cre^{N/N}$ $STAT\text{-}3^{F/N}$). These mice were crossed and the genotypes generated are illustrated in the punnett square (Lubey 1999) (punnet squares are simple way to determine the resulting genetic variance as a result of cross-breeding) of figure 23A and the subsequent genetic distribution of genotypes in figure 23B. Of this range of genotypes, the $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ mice were selected for establishing a stable breeding colony.

Subsequently, $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ mice were crossed with the transgenic Cre recombinase expressing strain ($Cre^{T/N}$ $STAT\text{-}3^{N/N}$) to generate $Cre^{T/N}$ $STAT\text{-}3^{F/N}$ mice (Figure 22A). Cross breeding these mice gave us 2 distinct genotypes, $Cre^{T/N}$ $STAT\text{-}3^{F/N}$ and $Cre^{N/N}$ $STAT\text{-}3^{F/N}$ mice (Figure 24A) in a 50/50 ratio (Figure 24B). Of these mice, $Cre^{T/N}STAT\text{-}3^{F/N}$ mice were selected to be crossed $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ mice (Figure 22B and Figure 25) to spawn $Cre^{T/N}$ $STAT\text{-}3^{F/F}$ mice, the genotype of cardiac specific STAT-3 KO mice. This breeding approach also gave rise to 4 distinct genetic backgrounds (Figure 25A) with the STAT-3 KO mice making up a quarter (Figure 25B) of the offspring.

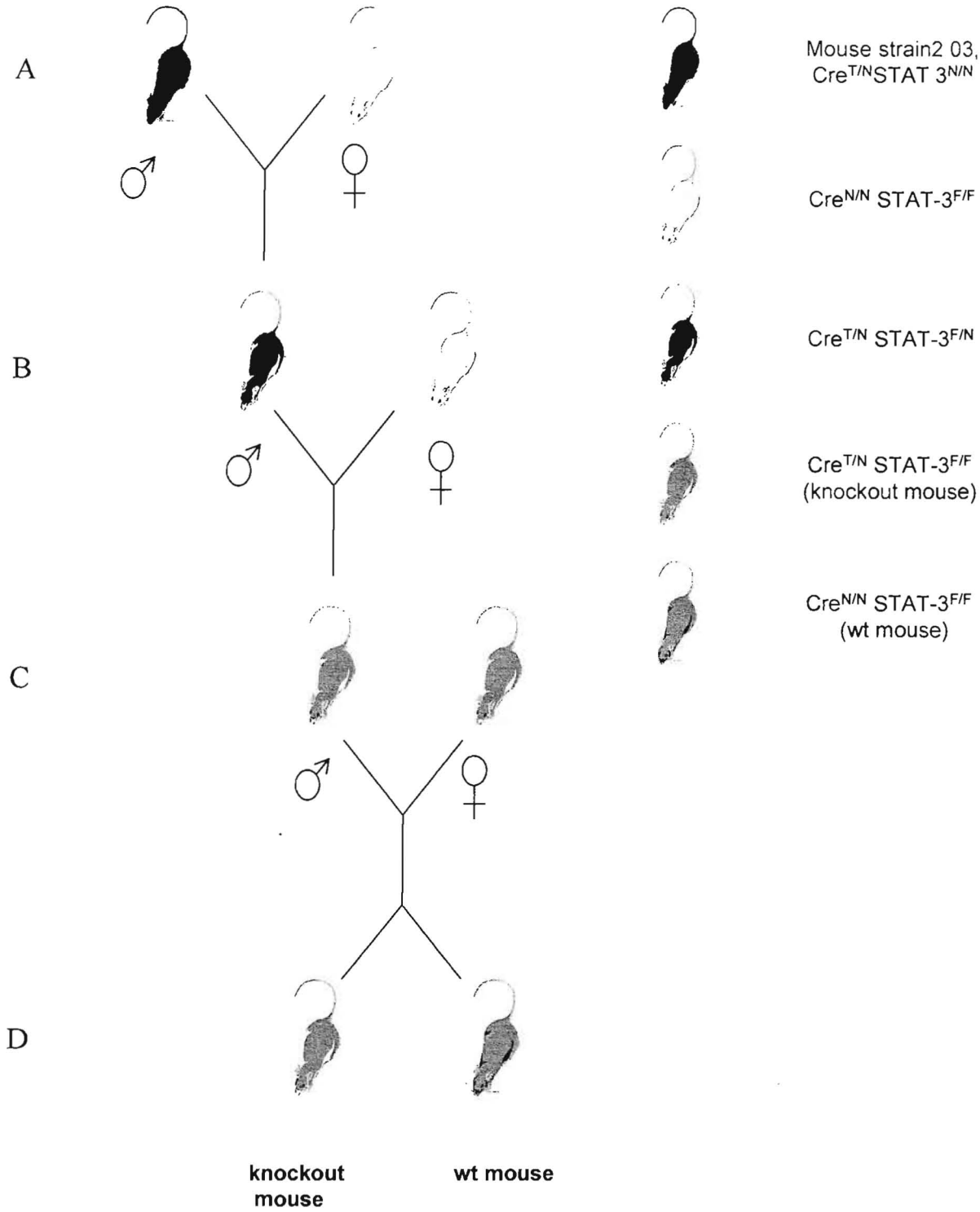

Figure 22: Breeding strategy used for the generation of cardiac specific STAT-3 knockout mice and wildtype (wt) littermate controls (D) .N - normal, T - Transgenic Cre insert, F-Floxed STAT-3.

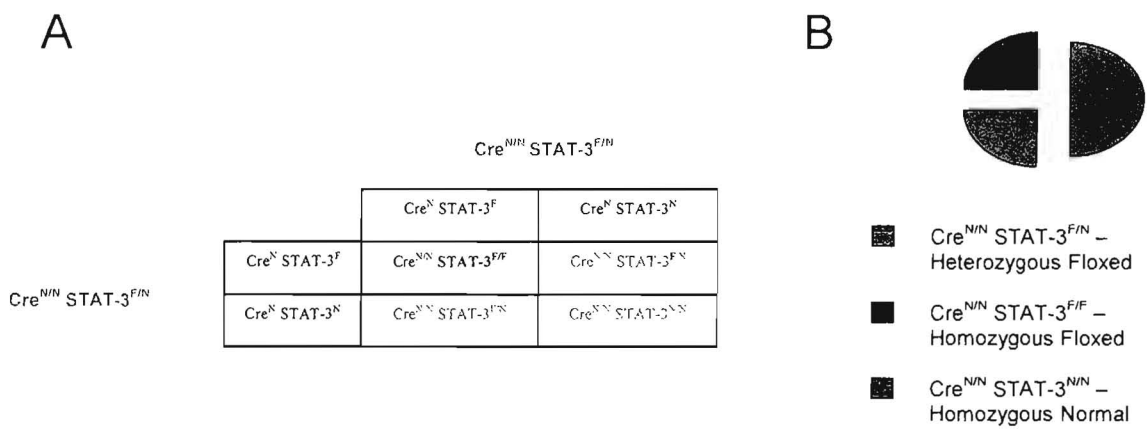

Figure 23: **Mouse Breeding: $Cre^{N/N}$ $STAT\text{-}3^{F/N}$ with $Cre^{N/N}$ $STAT\text{-}3^{F/N}$.** The punnett square (A) is used to illustrate the genetic distribution of the floxed allele in offspring of $Cre^{N/N}$ $STAT\text{-}3^{F/N}$ parents. Crossing heterozygous floxed mice ($Cre^{N/N}$ $STAT\text{-}3^{F/N}$) resulted in 3 distinctive genotypes. Homozygous floxed mice ($Cre^{N/N}$ $STAT\text{-}3^{F/F}$), heterozygous floxed mice ($Cre^{N/N}$ $STAT\text{-}3^{F/N}$) and homozygous normal ($Cre^{N/N}$ $STAT\text{-}3^{N/N}$) mice. The resulting Mendelian ratios of the offspring is shown in B. Abbreviations are as defined in text.

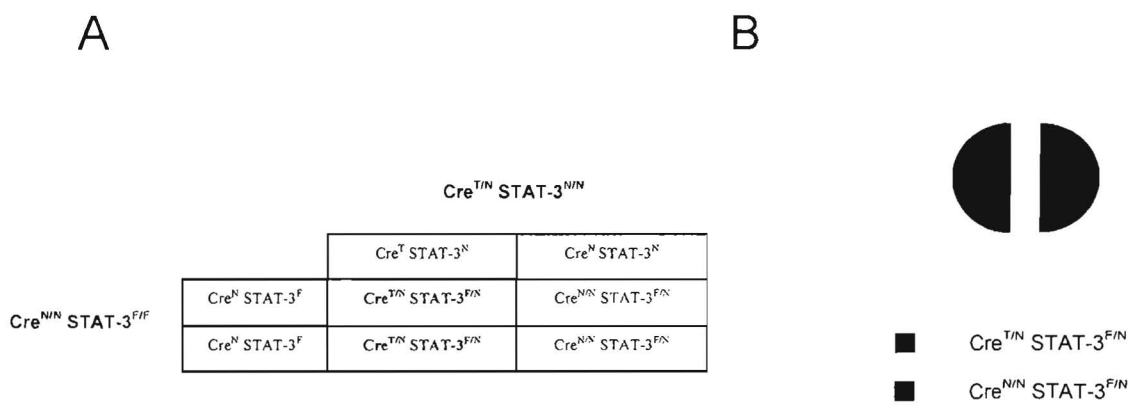

Figure 24: **Mouse Breeding: $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ with $Cre^{T/N}$ $STAT\text{-}3^{N/N}$.** Cross breeding $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ and $Cre^{T/N}$ $STAT\text{-}3^{N/N}$ gave 2 variant mouse strains (A), $Cre^{T/N}$ $STAT\text{-}3^{F/N}$ as well as $Cre^{N/N}$ $STAT\text{-}3^{F/N}$ mice in equal ratios (B). Abbreviations are as in text.

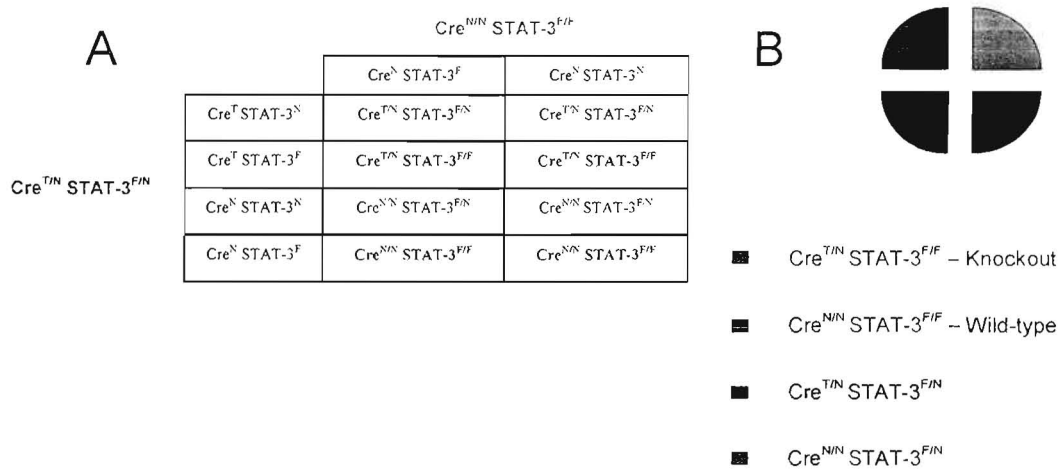

Figure 25: **Mouse Breeding: Cre$^{T/N}$ STAT-3$^{F/N}$ with Cre$^{N/N}$ STAT-3$^{F/F}$.** Cross breeding Cre$^{T/N}$ STAT- $^{F/N}$ and Cre$^{N/N}$ STAT-3$^{F/F}$ mice (A) gave rise to 4 (B) distinct genotypes of which 2, Cre$^{T/N}$ STAT-3$^{F/N}$ and Cre$^{N/N}$ STAT-3$^{F/N}$ were discarded as they did not possess the correct genetic configuration for both wild-type or STAT-3 KO mice. Abbreviations are as defined in text.

Cre$^{T/N}$ STAT-3$^{F/F}$ mice were selected and used to set up and maintain a stable breeding population to generate STAT-3 KO as well as littermate control mice (Figure 26A). The Mendelian STAT-3 KO to wild-type ratio of this breeding strategy produces 25% wild-type (Cre$^{N/N}$ STAT-3$^{F/F}$) and 50% STAT-3 KO (Cre$^{T/N}$ STAT-3$^{F/F}$) mice (Figure 26B). In addition, 25% of the embryos are homozygous for the Cre insert, i.e., Cre$^{T/T}$ STAT-3$^{F/F}$ which is embryo lethal, as both alleles of the MLC-2V gene are replaced, effectively knocking this critical gene out (Chen *et al.* 1998).

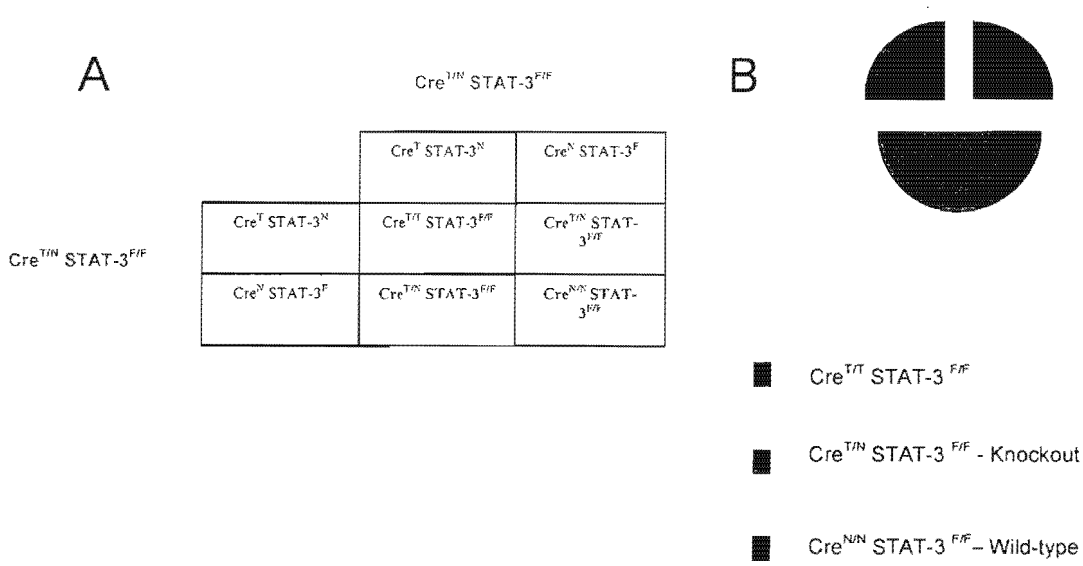

Figure 26: **Mouse Breeding: Cre$^{T/N}$ STAT-3$^{F/F}$ with Cre$^{T/N}$ STAT-3$^{F/F}$.** The breeding strategy used to maintain a stable mouse colony is illustrated in (A). The offspring ratio is shown in (B). Abbreviations are as in text.

2.2 Genotyping

a) Murine Genomic DNA extraction

The genomic DNA required to genotype mice was extracted from the respective tails (Disch *et al.* 1996). Approximately 1cm of a candidate mouse tail or the ventricle was removed from the mouse being tested and digested in 400µl digestion buffer (1M Tris pH 8.0; 5M NaCl; 0.5M EDTA pH 8.0; autoclaved then 10% SDS added) with Proteinase K (1mg/ml) and added fresh to the digestion buffer upon usage. Tails were then digested overnight, at 55°C. The following day, 400µl of Phenol: Chloroform: Alcohol (25:24:1) (Sigma) mixture was added to each digested tail, which was then gently mixed and cellular debris removed via centrifugation, 13 000 revolutions per minute (rpm) at room temperature for 10 minutes. This resulted in 2 separate aqueous layers, the lower layer containing

cellular debris and the upper layer containing the required DNA. To separate the 2 layers, without contaminating the samples, the bottom layer was removed and discarded using barrier tips (Quality Scientific Plastics) and 200µl of chloroform (Sigma) added to the remaining upper phase and the tube inverted. This was then spun in a centrifuge at 13 000rpm for 5 minutes. Once again 2 separate phases occurs and the bottom phase is removed and discarded while the upper phase is kept. Samples were then spun at 13 000rpm for 10 minutes to force the remaining debris into a pellet at the bottom of the tube. The fluid phase was then transferred to a new sterile 1.5ml eppendorff and 50µl of 7.5M ammonium acetate (Merck) and 1ml 100% ethanol (KIMIX) added and the tube inverted to precipitate the DNA. This white stringy DNA precipitate was removed, using sterile yellow tips, to a new 1.5ml eppendorff tube containing 70% ethanol to hydrate the DNA. The tube with the DNA precipitate is now spun at 13 000rpm for 10 minutes, so that the DNA may form a pellet and the bottom, and the alcohol decanted carefully so as not to dislodge the DNA pellet. Tubes are then left to air dry for 1 hour and sterile water added to dissolve the DNA.

b) Polymerase Chain Reaction (PCR) analysis of genetically altered animals

i) Detection of floxed STAT-3 mutated allele

Three primers were used to determine whether or not the animals harboured the floxed STAT-3 allele:

"a" : 5'- CCT GAA GAC CAA GTT CAT CTG TGT GAC –3' ;

is specific for exon 21 of the STAT-3 gene (Figure 21b).

"b" : 5'- CAC ACA AGC CAT CAA ACT CTG GTC TCC -3' ;

is specific for exon 22 of the STAT-3 gene (figure 21b).

PCR protocol used with primers "a" and "b" was performed in 25µl volumes containing magnesium free buffer (Promega), 4mM magnesium (Promega), 100µM Deoxy Nucleotide Tiphosphates (dNTP's) (Sigma), 0.2µM pimer mixture, 0.625U Taq polymerase (Promega) and 1.0µl DNA. The reaction mixtures were incubated at 94°C for 7 minutes then run for 35 cycles at 94°C for 30 seconds, 67°C for 1 minute and 74°C for 1 minute finishing with a 10 minute incubation at 74°C (Figure 27A) in the GeneAmp PCR system 2700 from Applied Biosystems.

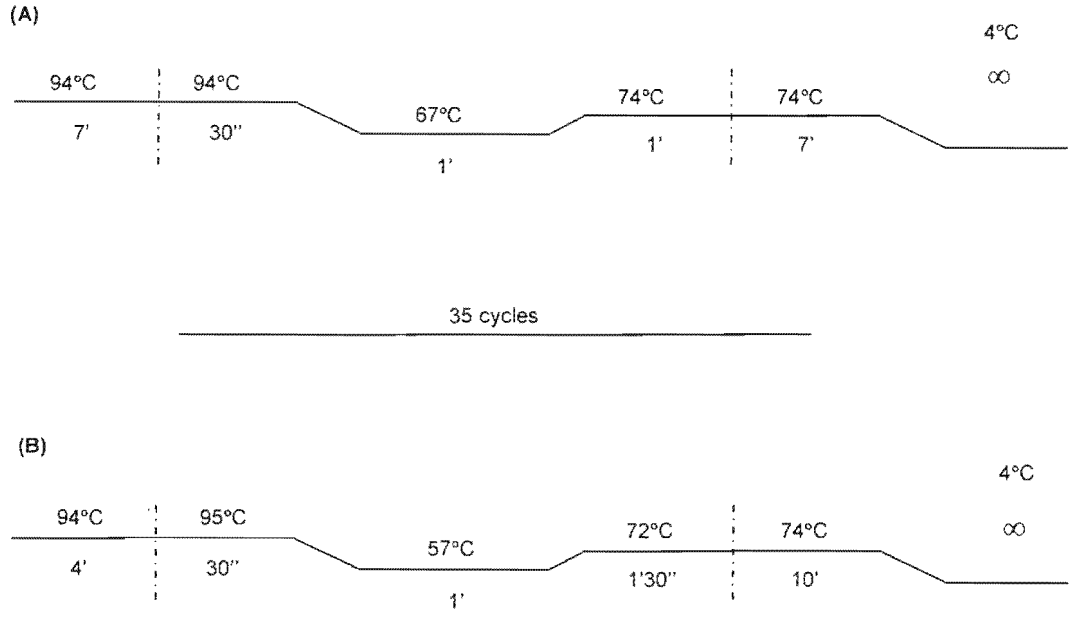

Figure 27: PCR protocols. Temperature cycling protocols used for genotyping. Distinct protocols were needed for individual sets of primers. (A) Detection of the STAT-3 floxed allele from DNA isolated from mouse tails using primers a+b. (B) Detection of the Cre transgene with primers specific for the Cre transgene and the wild-type MLC-2V gene. PCR- polymerase chain reaction, MLC-2V- myocyte light chain-2 ventricle, '- minute, " - second.

ii) Detection of Cre transgene animals

Animals which were heterozygous for the Cre recombinase gene were genotyped for Cre recombinase and the MLC-2V gene. MLC-2V served as a positive control for the PCR reaction. The following sense and anti-sense primers were used to determine the genotype of each animal:

Cre sense: 5'- GTT CGC AAG AAC CTG ATG GAC A -3'

Cre anti-sense: 5'- CTA GAG CCT GTT TTG CAC GTT C -3'

MLC-2V sense: 5'- GGC AAC CCT CAG ACA CCA T -3'

MLC-2V anti-sense: 5'- TGT GGA GGC TCT GGA TCA GGA C -3'

The reaction was performed in 50µl volumes containing Mg free buffer, 4mM Mg, 100µM dNTP's, 0.2µM of all primers, 1.25 U *Taq* polymerase and 1.5µl DNA. Reaction mixtures were incubated at 94°C for 4 minutes then run for 35 cycles at 95°C for 30 seconds, 57°C for 1 minute and 72°C for 1 minute and 30 seconds finishing with a 10 minute incubation at 72°C (Figure 27B).

12µl of each PCR mixture was mixed with 2µl gel loading buffer (0.25% Bromophenol Blue–Xylene Cyanole Dye Solution, Sigma) and electrophoresed on a 2% agarose (Whitehead Scientific) gel. Gels were stained with ethidium bromide (Sigma) (0.1µg/ml) and photographed.

2.3 Western blot analysis

To confirm the organ specific genetic depletion of STAT-3, the heart and liver was removed from wild-type and STAT-3 KO mice, at age 14 weeks, put to sleep with carbon dioxide. The tissue was washed to remove blood and freeze clamped using Wallenberger tongs precooled in liquid nitrogen. This instantly froze the tissue sample to –196°C preventing protein degradation. This tissue was then stored at -80°C until protein extraction was performed. Briefly, cytosolic proteins were extracted from isolated myocytes or tissue in lysis buffer containing 10% Nonidet P-40, 4M NaCl, 1M Hepes (pH 7.9), 500 mM EDTA and 400µl of an EDTA-free protease inhibitor cocktail (Roche Systems, USA). Tissue samples were homogenised while isolated myocytes were sonicated and then centrifuged at 3 000 rpm for thirty seconds and the supernatant transferred to a new tube. This was then spun at 5 000 rpm for 5 minutes to remove cellular debris. The resultant supernatant represented the total cytosolic proteins. Proteins concentrations were determined in duplicate by the Lowry method (Lowry R 1951). For all samples 100 µg of the total protein was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to PVDF membranes (Amersham Pharmacia Biotech) by electrophoretic transfer. Non-specific binding sites were blocked by incubation in Tris-Buffered Saline (TBS) containing 0.1% Tween 20 (Sigma) and 5% (wt/volume) fat free milk powder (Elite), for 2 hours at room temperature. Immunoblotting analyses were performed with the appropriate primary (Cell Signalling) and secondary anti-bodies (Santa Cruz, CA, USA) and visualised with chemiluminescent reagents (Amersham). Relative peptide levels were measured using densitometric analysis with UVI band (UVI Tech, Cambridge UK) software on a personnel computer.

2.4 Basal Characterisation of Wild-type and STAT-3 KO mice

Male mice (aged 14-36 weeks) were anaesthetised (sodium pentabarbitone, 60 mg/kg intraperitoneal and heparinized (25 IU intraperitoneal)). Once an adequate level of anaesthesia had been achieved the chest was opened and the heart removed to compare the wet weights of wild-type and STAT-3 KO to detect any phenotypical anomalies. In addition, basal metabolic characteristics of the mice were monitored with respect to weight, food intake and glucose levels (Accu-check). Measurements were made fortnightly and blood for glucose measurement was taken from the tip of the respective mouse's tail.

To explore the effect of the genetic depletion of STAT-3 on the morphology of the heart, we performed some histology analyses in the wild-type and STAT-3 KO mice. Briefly, 14 weeks male mice were anaesthetised (as described above), the hearts were rapidly excised, rinsed in cold saline solution, immersed in Jung's Tissue Medium (Jung, Germany) and frozen at -80°C. Frozen hearts were sectioned into 1μm thick cross sections and processed for Haemotoxylin and Eosin staining by the laboratory of Histology at the University of Cape Town.

3. Results

3.1 Generation of a myocardial specific STAT-3 KO mouse

To study the functional role of STAT-3 in cardioprotection, we utilised the Cre-Lox P technique of targeted gene excision. This strategy suited our needs, as we were able to obtain the mice necessary to create a unique strain of myocardial specific STAT-3 KO mice. Also, this technology has been shown to be effective in the generation of a wide variety of tissue specific STAT-3 KO mice (Jacoby *et al.* 2003; Gorogawa *et al.* 2004). The generation of a stable breeding colony of STAT-3 KO mice (as well as littermate controls) required numerous intermediate mouse strains, a process which took over 18 months.

3.2 Homozygous floxed STAT-3 mice - STAT-$3^{F/F}$

PCR analysis, of the floxed STAT-3 allele of DNA isolated from the original breeding mice received, showed that the mice were heterozygous for the floxed STAT-3 gene ($Cre^{N/N}$ STAT-$3^{F/N}$) (Figure 28A).

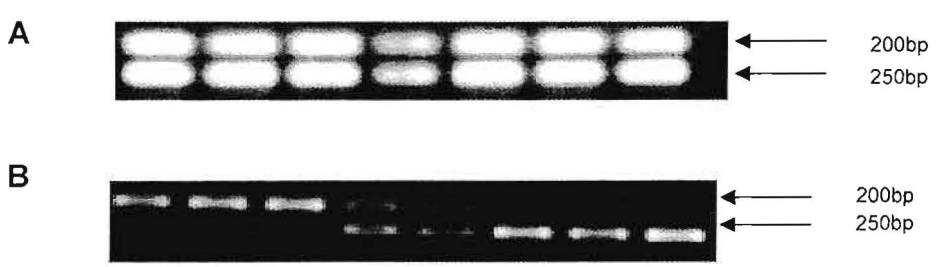

Figure 28: **PCR: Floxed STAT-3 allele genotyping.** PCR was performed on DNA isolated from mice using the a+b primers for the detection of STAT-3 floxed alleles. The 250 base pair (bp) band corresponds to the floxed STAT-3 allele and the 200 bp band corresponds to the non-floxed STAT-3 allele. Dual bands are representative of STAT-$3^{F/N}$ (A) and a single 250 or 200 bp band signifying either STAT-$3^{F/F}$ or STAT-$3^{F/N}$ respectively (B). Abbreviations are as

As our strategy required homozygous STAT-3 floxed mice, therefore these mice were crossbred and the offspring genotyped. The resulting litters produced homozygous as well as heterozygous STAT-3 floxed mice, $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ and $Cre^{N/N}$ $STAT\text{-}3^{F/N}$ (Figure 23A). Based on the PCR results (Figure 28B) $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ mice were selected to set up a homozygous floxed STAT-3 breeding colony and the $Cre^{N/N}$ $STAT\text{-}3^{N/N}$ and $Cre^{N/N}$ $STAT\text{-}3^{F/N}$ mice were discarded.

3.3 Cre producing but still heterozygous for the floxed STAT-3 allele - $Cre^{T/N}$ $STAT\text{-}3^{F/N}$

The $Cre^{T/N}$ mice obtained are lacking the STAT-3 floxed allele; therefore it has to be bred into the germ line. These $Cre^{T/N}$ $STAT\text{-}3^{N/N}$ mice were crossed with $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ mice (Figure 22A). The resulting brood was genotyped for the Cre recombinase transgene and found to be either $Cre^{N/N}$ or $Cre^{T/N}$ (Figure 29). Offspring were also genotyped for the presence of floxed STAT-3 genes. All mice were found to be $STAT\text{-}3^{F/N}$ in this regard. Of the genetic variations encountered, $Cre^{T/N}$ $STAT\text{-}3^{F/N}$ mice were selected to be crossed with $STAT\text{-}3^{F/F}$ mice (Figure 22B) to award us with the first myocyte specific STAT-3 KO mice.

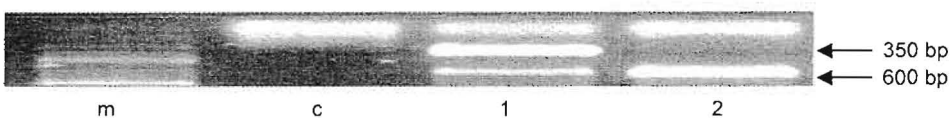

Figure 29: PCR Results: Cre genotyping. PCR analysis performed on the offspring of the $Cre^{T/N}$ $STAT\text{-}3^{N/N}$ and $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ crossing to detect the Cre recombinase insert. The 600 base pair (bp) band is the control PCR product of the wild-type MLC-2V gene and the 350 bp band is indicative of the Cre transgene (Lane 1). m – marker, C – water control.

3.4 Myocyte specific STAT-3 KO mice and there wild-type litter mate controls

As predicted in figure 25A, crossing the $Cre^{T/N}STAT\text{-}3^{F/N}$ and $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ mice produced the first of our STAT-3 KO and wild-type littermate control mice. These offspring displayed numerous genetic variations of which the $Cre^{T/N}$ $STAT\text{-}3^{F/F}$ mice were our first STAT-3 KO mice and the $Cre^{N/N}$ $STAT\text{-}3^{F/F}$ the first of our wild-type littermates. It would not have been practical to generate the required mice in this manner, as the other genetic variations encountered were not required for this study. As shown in the punnett square (Figure 25), the most practical strategy to generate superior STAT-3 KO mouse numbers would be to cross $Cre^{T/N}$ $STAT\text{-}3^{F/F}$ with each other (Figure 22C).

By crossing the STAT-3 KO ($Cre^{T/N}$ $STAT\text{-}3^{F/F}$)animals with each other we are able to generate both STAT-3 KOs as well as wild-type littermate controls for our study (Figure 22D). This breeding strategy also gives rise to the embryo lethal $Cre^{T/T}$ $STAT\text{-}3^{F/F}$ genotype (Figure 25A) (Chen *et al.* 1998). Due to this 66% of mice born are STAT-3 KOs and 33% born are wild- type animals.

3.5 Genetic depletion of STAT-3

Cre mediated recombination leads to removal of exon 21, which contains the functionally relevant tyrosine residue of STAT-3. This results in ablation of STAT-3 in ventricular myocytes. The depletion of STAT-3 was confirmed biochemically with western blot analysis for the presence of STAT-3 in wild-type and STAT-3 KO mice. Ventricular tissue was removed from wild-type and STAT-3 KO mice and probed for STAT-3 (Figure 30A). STAT-3 levels are seen to be extensively depleted in STAT-3 KO mice. Using the liver we alsoIconfirm

the organ specificity of our STAT-3 KO. As shown in figure 30B, STAT-3 is not detected in STAT-3 KO mouse ventricular tissue but still detected in the liver. These results serve to confirm the status of our mouse as being a cardiac specific STAT-3 KO mouse.

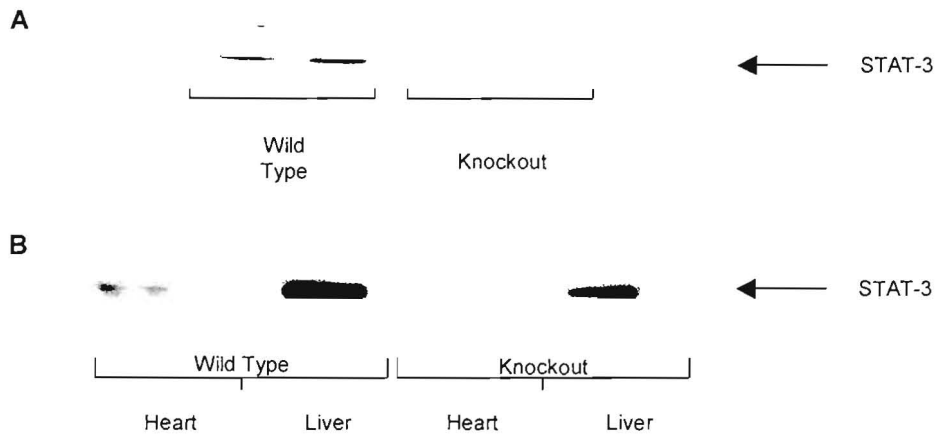

Figure 30: Western Blot Analysis: Wild-type vs STAT-3 KO. Representative western blots of protein isolated from wild-type and knockout mice. Proteins were extracted from cardiac and liver tissue from wild-type and STAT-3 KO mice and the STAT-3 levels compared.

3.6 Basal characterization of Wild-type and myocyte specific STAT-3 KO mice

Wild-type and STAT-3 KO mice were found to be viable, fertile and visually indistinguishable from the wild-type mice (Figure 31). Animals were monitored to determine if genetic depletion of STAT-3 had any effect on basal rudimentary metabolism. As seen in figure 32A the comparative body weights of wt and ko mice were found to have no significant difference over the course of 52 weeks. With respect to food intake and glucose levels, statistically significant differences were observed at weeks 22 and 30 respectively (Figure 32B and C).

Figure 31: Photograph of wild-type and STAT-3 KO mice. No phenotypical distinguishing features can be observed between wild-type and STAT-3 KO mice. Abbreviations are as in text.

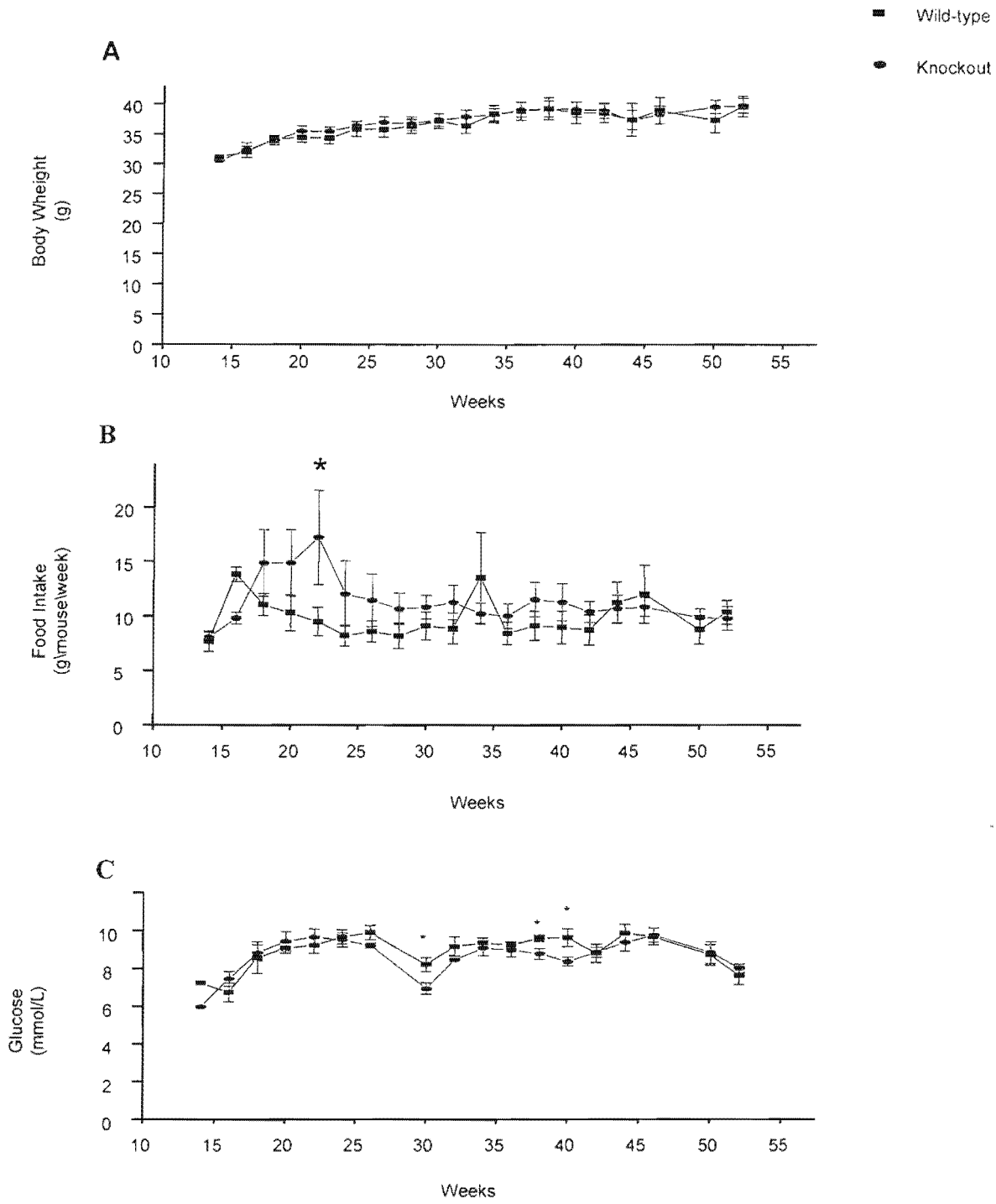

Figure 32: Comparative body weights, food intake and glucose levels of wild-type and STAT-3 KO mice. Basal characterisation of wild-type and knockout mice. Body weights (A) food intake (B) as well the blood glucose levels (C) of wild-type and STAT-3 knockout mice was measured every 2 weeks over a 52 week period ($p < 0.05$ vs matched ko mouse; $n \geq 6$.

In addition, the wet weights of wild-type and STAT-3 KO mice hearts were compared. No significant differences were observed between these weights (Figure 33).

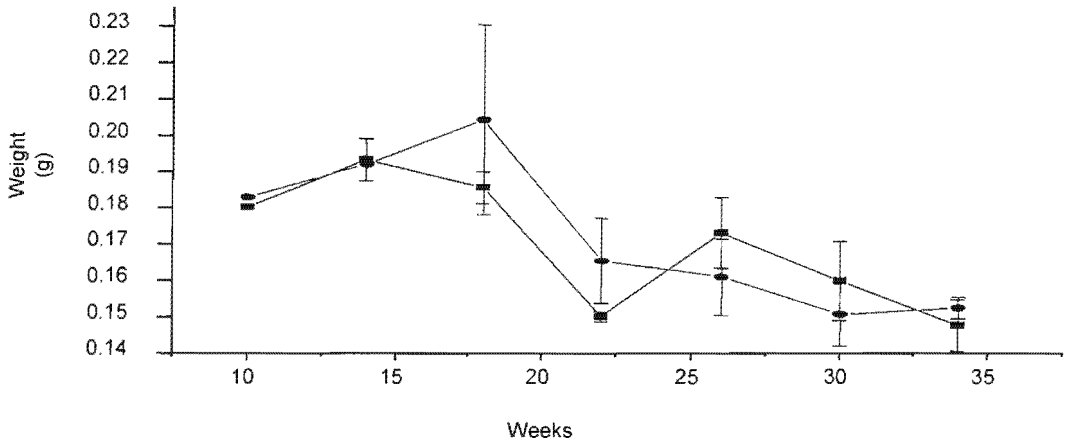

Figure 33: Comparative heart weights of wild-type and STAT-3 KO mice. Hearts were excised from wild-type and STAT-3 KO mice, rinsed and blotted dry before being weighed. KO- knockout.

Finally, histology analysis performed in 14 weeks old heart mice did not show any changes in the cellular structure of the genetically depleted STAT-3 mice compared to their littermate controls. (Figure 34).

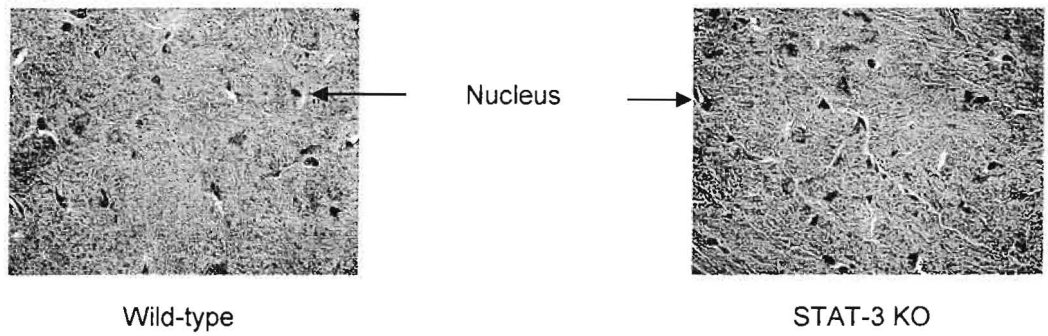

Figure 34: Cross sections of hearts from wild-type and STAT-3 KO mice, magnified (100x). No apparent difference was noticeable in the morphology between the wild-type and STAT-3 KO mice hearts.

4. Discussion

In the present section, we have described the successful generation and breeding of cardiomyocyte specific STAT-3 KO mice using the Cre-Lox P recombinant technology. The organ specific and proteomic depletion of STAT-3 in these animals was confirmed in hearts and livers by western blot analysis. The removal of functional STAT-3 in the heart did not alter the body and heart weights, the morphology of the heart, the food intake and the blood glucose levels compared to their litter mate controls.

Molecular techniques now allow the design of precise genetic modifications in the mouse (Sauer 1998). Cre-Lox P technology has become an important tool in the precise manipulation of the genome, and its adaptation has led to the development of increasingly accurate mouse models for the understanding of the gene function. Although much of current work exploits the alacrity and precision with which Cre catalyses excisive DNA recombination, Cre also is adept at the insertion of heterologous DNA in the genome (see review (Sauer 1998)).

STAT-3 is known as a critical transcription factor implicated in the embryonic development of mice (Takeda *et al.* 1997). The generation of mice deficient in STAT-3 (whole body) by gene targeting revealed that embryos degenerated between embryonic days 6.5 and 7.5 probably due to the result of a defect in functions of visceral endoderm, such as nutritional insufficiency (Takeda *et al.* 1997). For this reason, we created a cardiac specific STAT-3 KO mouse to evaluate the role of STAT-3 in cardioprotection.

The organ specific nature of Cre recombinase expression was achieved by having the transcription of Cre recombinase under the control of the MLC-2V promoter which is a highly conserved, cardiac exclusive and constitutively active promoter region (Chen *et al.* 1998b). Interestingly, MLC-2V has a unique function in the maintenance of cardiac contractility and ventricular chamber morphogenesis during mammalian cardiogenesis and total cardiac depletion of MLC-2V gene is embryo lethal (Chen *et al.* 1998b). The Cre recombinase expressing mouse that we have used in our model has been characterised by its creator (Professor Ken Chien) (Chen *et al.* 1998b) and has already been successfully used to deplete the retinoic acid receptor gene in ventricular cardiomyocytes (Chen *et al.* 1998a). In all the specific KO models, the targeted protein was found to b significantly depleted in the heart.

The floxed mouse that we have used in our model (gift from Professor Akira) has floxed sites inserted into the introns flanking the exon coding for the tyrosine activation residue of STAT-3 (Takeda *et al.* 1998). These flox sites serve as DNA recognition sites for cleavage by Cre recombinase and removal of the intervening DNA segment. Therefore, by crossing these floxed mice with other organ specific Cre recombinase expressing mice, numerous organ specific STAT-3 KO mice can be created. The floxed mice used in this study have been successfully used for the organ specific genetic depletion of STAT-3 in pancreatic B-cells (Gorogawa *et al.* 2004) and in T-cells (Takeda *et al.* 1998).

To explore the role of STAT-3 in the heart, alternative methods can be performed such as the silent interfering ribonucleic acid technique (siRNA).This technique

inhibits and translation of target proteins effectively in cell culture. By allowing constitutive degradation of the target protein to continue and inhibiting the translation of the target protein, the levels of the target protein is depleted (Jones *et al.* 1999). This technique is well suited for the silencing of genes in established cell lines (Zhang *et al.* 2005; Frias *et al.* 2006). STAT-3 has been successfully "silenced" in T-cell lymphomas (Zhang *et al.* 2005) as well as neonatal rat ventricular cardiomyocytes (Frias *et al.* 2006). The recent commercial availability of STAT-3 siRNA kits (from Upstate UK) will further facilitate the depletion of STAT-3 in cell lines.

Using Cre-LoxP technology, two other research teams have recently used cardiac specific STAT-3 KO mice to explore the role of STAT-3 in the heart. In 2003, Jacoby et al generated a cardiac specific STAT-3 KO mouse with floxed sites inserted into the introns flanking exons 18, 19 and 20 (Jacoby *et al.* 2003). In addition, the Cre recombinase was placed under the control of the α-myosin heavy chain promoter, which also resulted in tissue specific expression of Cre recombinase. As the exons they excised contained the essential src-2 homology domain (SH-2 domain) of STAT-3 (this domain is required for the recruitment of STAT-3 to the receptor for phoshorylation and activation by JAK2), a non-functional STAT-3 protein is produced. Young STAT-3 KO mice created in this manner also did not display any signs of cardiac damage in line with our model but these STAT-3 KO mice developed higher sensitivity to inflammation, heart dysfunction and cardiac fibrosis with advancing age (after the age of 7 months) (Jacoby *et al.* 2003). In 2004, Hilifiker-Kleiner et al, also created cardiac STAT-3 KO mice using Cre-LoxP technology (Hilfiker-Kleiner *et al.* 2004) . Similar to

the STAT-3 KO mouse produced by Jacoby *et al* (Jacoby *et al.* 2003), these mice were considered normal when compared to their wild-type counterparts until 9 months of age. After this age, STAT-3 KO mice were found to display signs of heart failure. As we did not examine our mice at advanced ages, we can only speculate that our STAT-3 KO mouse would also develop spontaneous heart failure as they get older. No cardiac alteration was observed at 14 weeks old, age that we have chosen to perform our further experiments.

The depletion of STAT-3 in the heart was confirmed via western blot analysis of proteins from isolated cardiomyocytes and liver tissue from wild-type and STAT-3 KO mice aged 14 weeks (Figure 12). Cre recombinase is only expressed in the adult mouse heart and initially pups born still express a functional STAT-3. However, with the progression of time, the levels of STAT-3 produced decline significantly with maximum depletion of cardiac STAT-3 observed at 3 months (Hilfiker-Kleiner *et al.* 2004).

Previously, it has been reported that genetic ablation of STAT-3 in the brain resulted in obese mice (Gao *et al.* 2004) due to a loss of leptin signalling. Therefore, we compared the food intake and body weights of cardiac specific STAT-3 KO mice and their littermate wild-type controls but no significant difference was observed between the two groups. Similarly, genetic depletion of STAT-3 in the β-cells of the pancreas has been linked to impaired glucose secretion (Gorogawa *et al.* 2004). We analysed blood glucose levels in cardiac specific STAT-3 KO and wild-type mice but no major difference between wild-type and STAT-3 KO mice could be detected. In addition, the wet weights of

wild-type and STAT-3 KO mouse hearts were measured to determine the effects of genetic depletion of STAT-3 on the size of the heart. Again, no significant differences occurred between wild-type and STAT-3 KO mouse hearts. Excessive fibrosis in STAT-3 KO hearts has been linked with an increase in the heart weights of STAT-3 KO but only with advanced age (Jacoby *et al.* 2003). The basal characterisation of our STAT-3 KO model, as well as the literature, indicated that the best time to use our STAT-3 KO mice would be around the age of 14 weeks. To finally confirm this, histological analysis was performed on cardiac tissue slices from wild-type and STAT-3 KO mice. No differences were observed to occur under high magnification of the cross sections.

In conclusion, using the Cre-LoxP technology, we have successfully created a cardiac specific STAT-3 KO mouse. This mouse will now allow us to explore the critical role of STAT-3 in cardiac preconditioning. For further studies, we will use 14 weeks old mice as no alteration has been noticed at this age by us and other groups (Jacoby *et al.* 2003; Hilfiker-Kleiner *et al.* 2004). However, further characterization of our STAT-3 KO mouse model (such as ultrastructural analysis by electron microscopy) would be required to confirm the impact of this technology on the heart.

D. STAT-3 Knockout mice failed to be protected from ischaemia/reperfusion injury

(Part of the work in this section has been published in ***Cardiovasc Res, 2004. 63(4): p. 611-6.****)*

1. Introduction

Cardiac preconditioning is a phenomenon whereby the myocardium is trained to resist sustained ischaemia and reperfusion injury. It can be induced with physical stimuli such as ischaemia or pharmacologically with agents such as adenosine (Auchampach *et al.* 1993; Smith *et al.* 2002) or TNFα (Lecour *et al.* 2002) given prior to the sustained ischaemia. The damage caused during reperfusion can also be prevented by staged reperfusion with exogenous administration of pharmacological agents. The administration of insulin, at the onset of reperfusion, has been successfully demonstrated to prevent the damage caused during reperfusion (Jonassen *et al.* 2000; Jonassen *et al.* 2001).

Ischaemic preconditioning and staged reperfusion, by exogenous administration of insulin, is known to protect via some common mechanisms. Akt plays a pivotal role in ischaemic and pharmacological preconditioning (Jonassen *et al.* 2000; Jonassen *et al.* 2001; Krieg *et al.* 2002; Mocanu *et al.* 2002; Hausenloy *et al.* 2004; Uchiyama *et al.* 2004) and it is activated when insulin is given at the onset of reperfusion (Jonassen *et al.* 2000; Jonassen *et al.* 2001). However, the exact protective signalling cascade still remains unclear for both protective mechanisms. Recent evidence supports a role for the JAK2/STAT-3 signal transduction pathway in protecting the myocardium from damage (Hattori *et al.* 2001; Negoro *et al.* 2001).

Using the cardiac specific STAT-3 deficient mice that we are breeding in our laboratory (discussed in section C), we aim to confer cardioprotection in these mice by preconditioning or by insulin treatment at the time of reperfusion using two different models:

- **The isolated Langendorff perfusion model**

The isolated heart perfusion model of ischaemia/reperfusion injury has been shown to be effective in studying cardiac preconditioning. This model is both reliable and highly reproducible for the dissection of signalling pathways. In addition, this is an established model in our laboratory.

- **The isolated adult cardiomyocytes model**

Freshly isolated and cultured adult cardiomyocytes have been successfully used to demonstrate and investigate the powerful effects of ischaemic preconditioning. In cell culture, ischaemia can be simulated *via* hypoxia and the use of substrate deprivation buffers (media lacking glucose and a reduction of the buffer pH to 6.2). In the STAT-3 knockout (KO) mice model that we have generated, STAT-3 is depleted in the cardiomyocytes only, making the use of this model appropriate as only cardiomyocytes are used.

Of note, in this thesis, the ischaemic insult in isolated cells will be termed "simulated ischaemia" and the ischaemic preconditioning will be named "hypoxic preconditioning".

2. Methods

2.1 Cardiomyocyte STAT-3 deficient mice

STAT-3 deficient mice (STAT-3 KO) were generated as described in the previous chapter. These KO mice were used in conjunction with wild-type littermate control animals in all experiments. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Health 1996), and all procedures were approved by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town (UCT). Animals were bred and stored at the animal unit of UCT until the day of the experiment.

2.2 Isolated, perfused mouse hearts

Male mice (aged 14-16 weeks) were anaesthetised (sodium pentabarbitone, 60 mg/kg intraperitoneal injection) and heparinized (25 IU intraperitoneal injection). Once an adequate level of anaesthesia had been achieved, the chest was opened and the heart of the mouse was rapidly removed, placed in ice cold (4°C) Krebs-Henseleit buffer and the aorta was cannulated. Hearts were then perfused with a modified Krebs-Henseleit buffer (Sodium Chloride (NaCl) 118.0mM; Sodium Hydrogen Carbonate ($NaHCO_3$) 24.0mM; Potassium Chloride (KCl) 4.0mM; Sodium Dihydrogen Phosphate (NaH_2PO_4) 1.0mM; Calcium Chloride ($CaCl_2$) 2.5mM; Magnesium Chloride ($MgCl_2$) 1.2mM; di-sodium Ethylenediaminetetra-acetic Acid (EDTA) 0.5mM; glucose 10mM; gassed with 95% Oxygen (O_2)/ 5% Carbon Dioxide (CO_2) at 37 °C) in a retrograde fashion with a constant pressure of 110cm water (H_2O). In this modified Krebs-Henseleit buffer EDTA was added in order to lower the final calcium concentration to the physiological range of

1.5mM. Temperature was measured by the placement of a fine thermocouple wire in the left ventricle (Physitemp, NJ, USA) and monitored on a Digitron 2600T (Torquay, UK). Hearts were fastened, *via* a rigid lightweight lexan coupling rod, to a force displacement transducer (Grass FT03C, Mass. USA) by means of a 4-0 silk (on a 20mm curved atraumatic needle) placed through the apex of the heart. Diastolic tension was adjusted to 2g and hearts were paced at 600 beats per minute. Pacing was induced in the hearts as the spontaneous heart rate is significantly lower than the physiological rate. The developed tension was recorded on a chart recorder (Lectromed Multitrace-2, Letchworth, UK) and the coronary flow rate was measured by timed collection.

2.3 Experimental protocol: Isolated mouse heart

For the normoxic control, isolated mouse hearts were perfused for 100 minutes under normoxic conditions. Other isolated mouse hearts were subjected to 35 minutes of global ischaemia at 37 °C and 45 minutes of reperfusion (Figure 36). Hearts were preconditioned either with a low dose of TNFα (0.5ng/ml), adenosine (100mM), administered for 7 minutes followed by a 10 minute washout period, or with 4 cycles of 5 minutes of global ischaemia/reperfusion prior to index ischaemia (Figure 36). At the end of each experimental protocol, the infarct size was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Briefly, 2ml of a 1% solution of TTC was perfused through the heart followed by immersing the heart in TTC solution at 37°C for 1 minute with gentle shaking. Hearts were frozen at -20 °C and sectioned into 1.5 mm slices. These slices were laid out and compressed between thin glass plates 0.5mm apart. The sections were scanned,

enlarged, and infarct size assessed using computerised planimetry (Planimetry+, Boreal Software, Norway) by a researcher blinded to the groups.

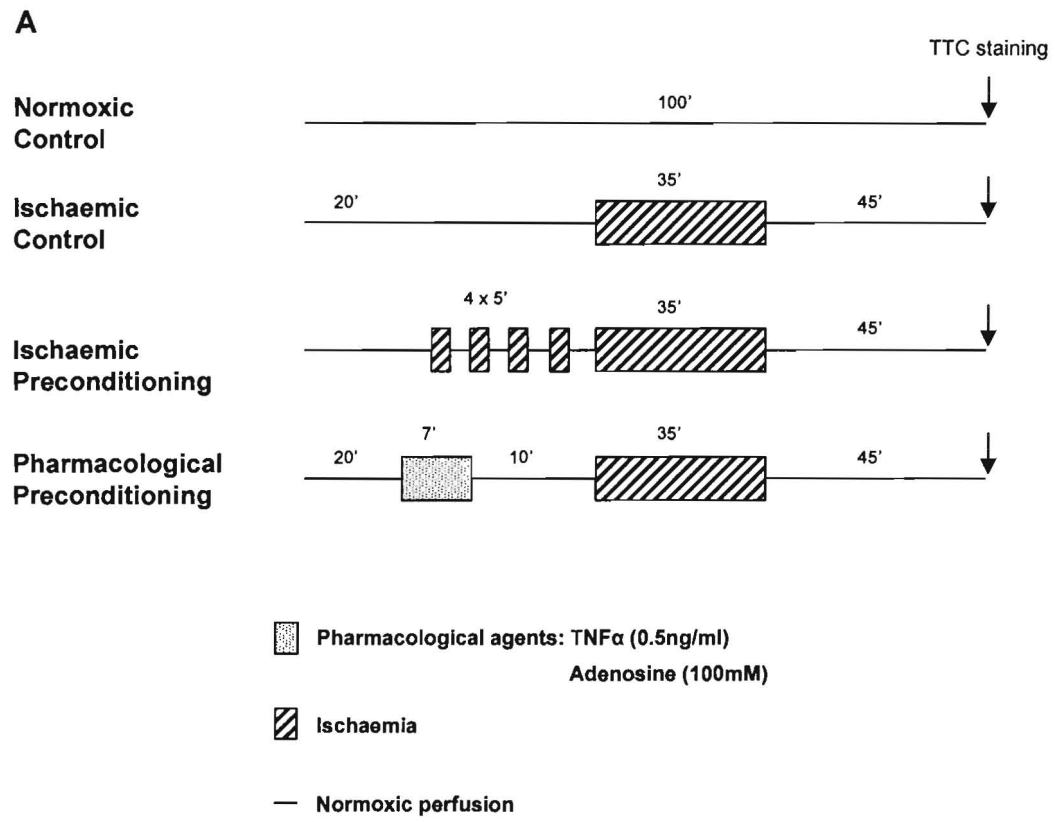

Figure 36: **Langendorff perfusion of isolated mouse hearts.** Hearts were subjected to an index ischaemia of 35 minutes followed by 45 minutes of reperfusion (A). Ischaemic preconditioning was conferred by 4 cycles of 5 minutes of ischaemia and reperfusion prior to the index ischaemic insult. The pharmacological preconditioning agents were administered for 7 minutes followed by a 10 minute washout prior to the index ischemia. Infarct size was measured at the end of each protocol by TTC staining. (B) Langendorf mouse perfusion set up. TTC- 2,3,5-triphenyltetrazolium chloride.

2.4 Cardiomyocyte isolation

Cardiomyocytes were isolated using the modified method of Zhou *et al* (Zhou *et al.* 2000). In brief, male wild-type and STAT-3 KO mice, aged between 14-16 weeks were anesthetized as described above. Hearts were removed and perfused at 37°C through an aortic cannula with a calcium-free HEPES-buffered solution (Buffer 1, pH 7.4, composition: 116.0mM NaCl, 17.4mM HEPES, 0.9mM NaH_2PO_4, 5.6mM glucose, 5.4mM KCl, 0.4mM Magnesium Sulphate ($MgSO_4$). Cannulated hearts were perfused with Buffer 1 containing 0.025mM EGTA and 0.1% BSA for 10 minutes to remove all blood (Figure 37).

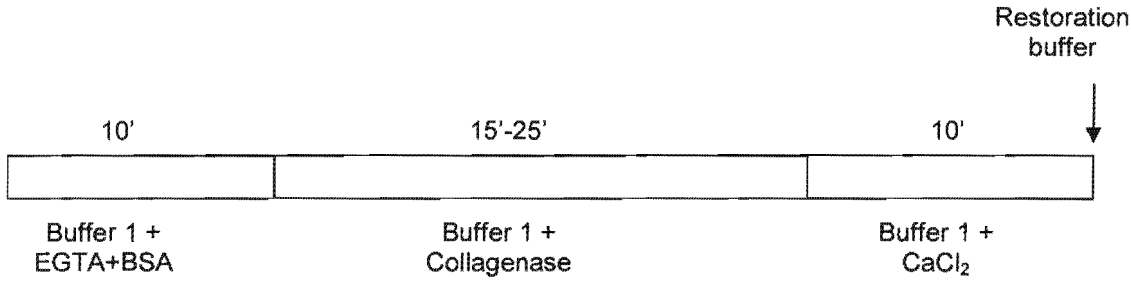

Figure 37: **Cardiomyocyte extraction protocol.** Isolated and cannulated hearts were perfused in a step wise manner. First, they were perfused with Buffer 1+ EGTA+BSA to remove the blood. Then collagenase was used to digest the heart tissue. Finally the collagenase was washed out in Buffer 1 + $CaCl_2$ before hearts were transferred to a restoration buffer. $CaCl_2$- calcium chloride, EGTA- ethidium glycine tetra-acetic acid.

Hearts were then perfused with Buffer 1 containing collagenase type II (0.14mg.ml^{-1}) (Worthington Biochemicals) and $CaCl_2$ (25μM), in a recirculating manner for 15 – 25 minutes to digest the hearts. The collagenase was washed out from well digested hearts with Buffer 1 with 25μM $CaCl_2$ for 10 minutes, before being removed from the cannula and minced finely with scissors in 25ml restoration buffer (Buffer 1 with 10.0mM Na pyruvate, 10.0mM EGTA, 1.6mM

carnitine, 5.0mM taurine, 5.0mM creatine and 0.1mM $CaCl_2$ and 1% BSA, pH 7.4) (Figure 37). This minced tissue suspension was shaken vigorously at 37°C for 15 minutes, the resulting suspension was filtered through a sterile nylon mesh (40μm mesh size, Spectrum Laboratories Inc.) and the cells pelleted 1500rpm for 10 minutes, Sanyo Harrier 18/80). The supernatant was decanted and the cells resuspended in 25ml restoration buffer. 100mM calcium was added in an incremental manner to achieve a final concentration of 1.25mM over a period of 12 minutes. Cells were spun down at 1500rpm for 10 minutes and the resulting pellet of cardiomyocytes was resuspended in modified, insulin free, KSLMS (Kawamoto, Sato, Le, McClure, and Sato) (Kawamoto *et al.* 1986) media. Isolated cardiomyocytes were quantified and a threshold of 60% viable rod shaped myocytes was considered to be a good quality preparation of isolated cardiomyocytes. Isolated myocytes were cultured in standard tissue culture flasks for 16-24hrs at 37°C in a humidified 5% CO_2 atmosphere prior to experimentation.

2.5 Experimental protocol: Isolated cardiomyocytes

Isolated cardiomyocytes were exposed to simulated ischemia (SI) in a modified Esumi Buffer (Esumi *et al.* 1991) containing : 118.0mM NaCl, 3.6mM KCl, 0.5mM $MgCl_2$, 0.9mM $CaCl_2$, 4.0mM HEPES and 20.0mM 2-deoxy-D-glucose (2-DG) at pH 6.2 for 26 hours, at 37°C, in a humidified environment containing 1% O_2, 5% CO_2 and the balance N_2. This was followed by 2 hours of reoxygenation in insulin free KLSMS media (Figure 38) under normoxic conditions. Normoxic myocytes were maintained in insulin free KSLMS media,

pH 7.4, in a humidified environment containing 20% O_2, 5% CO_2 and balance N_2 for 28 hours (Figure 38). Myocytes were preconditioned by exposing the cells to 1% O_2 in Esumi buffer without 2-DG at pH 6.4 for 30 minutes. This was followed by 60 minutes reoxygenation under normoxic conditions in KSLMS, 26 hours of index simulated ischaemia, in Esumi buffer without 2-DG and 2 hours reoxygenation in normoxic media (Figure 38). Cardiomyocytes were pharmacologically preconditioned by adding 0.5ng/ml TNFα or 100mM adenosine to KSLMS media for 30 minutes under normoxic conditions followed by a 60 minutes washout period prior to 26 hours of simulated ischaemia and 2 hours of reoxygenation (Figure 38).

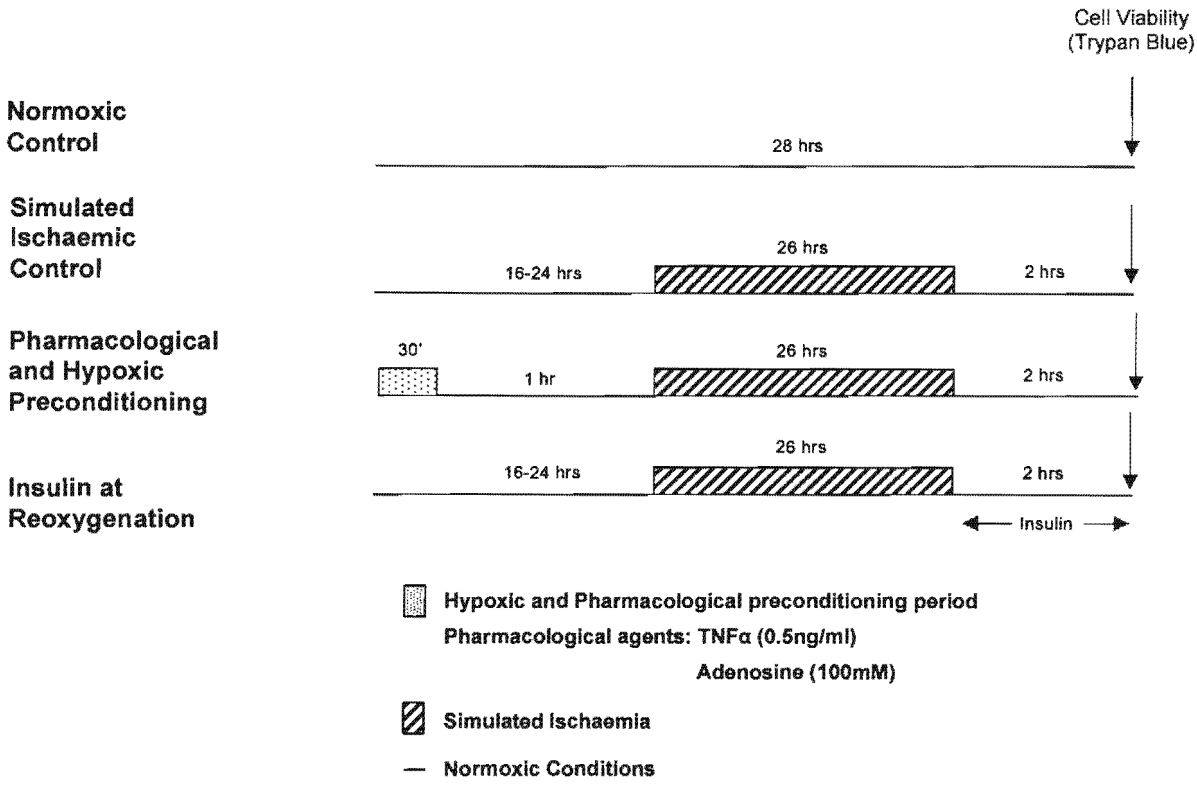

Figure 38: Experimental protocol in the isolated cardiomyocytes. The index simulated ischaemia lasted for 26 hours and the final reoxygenation time was 2 hours. Cells were preconditioned for 30 minutes with hypoxia, TNFα or adenosine followed by one hour washout period prior to the index ischaemia. Myocyte cell viability was measured at the end of each protocol via trypan blue exclusion.

2.6 Measurement of cells viability

Cardiomyocyte viability was measured at the end of the 2 hour reoxygenation period using trypan blue exclusion as well as cell morphology (Zhou *et al.* 2000; Marais *et al.* 2001) by a researcher blinded to the groups. Results are expressed as percentage (%) of viable myocytes.

2.7 Pharmacological Agents

Collagenase, CLS 2, was purchased from Worthington Biochemicals (New Jersey, USA). Insulin free KSLMS medium was obtained from Highveld Biologicals (Johannesburg, South Africa). Recombinant murine TNFα was obtained from Peprotech Inc (Rocky Hills, NY). Actrapid HM (ge) insulin (Novo Nordisk) was purchased at the local pharmacy. All other reagents including adenosine were obtained from Sigma Biochemicals (St. Louis, Mo, USA).

2.8 Statistical Analysis

Results are expressed as mean values ± standard error of the mean (S.E.M.) and were analyzed by one-way ANOVA with Dunn's post-test, using GraphPad InStat version 3.01 (Graphpad software, San Diego, California, USA). Differences were considered significant at values of $p<0.05$.

3. Results

3.1 The Role of STAT-3 in Ischaemic Preconditioning

To evaluate the physiological significance of STAT-3 in ischaemic preconditioning we used the isolated perfused heart model of ischaemia/reperfusion injury as well as isolated cardiomyocytes.

a) Isolated mouse heart

Following an ischaemia/reperfusion insult, the infarct size (expressed as a percentage of the whole heart) was similar between wild-type and STAT-3 KO mice (wild-type; 35.8±1.8% and STAT-3 KO; 38.4±2.6%; n≥6, ns) (Figure 39). Ischaemic preconditioning reduced the infarct size in wild-type mice to 14.4±2.2% ($p<0.05$ vs ischaemic control; n≥6) but not in the STAT-3 KO mice (43.9±6.7%; ns vs ischaemic control; n≥6) (Figure 39).

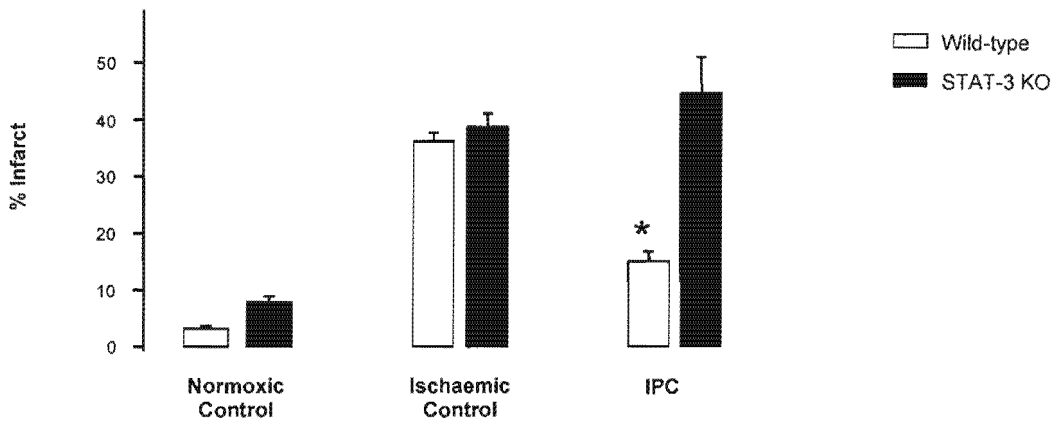

Figure 39: Effect of ischaemic preconditioning (IPC) in isolated wild-type and STAT-3 KO mouse heart. Hearts were subjected to 30 minutes of global index ischemia and 45 minutes of reperfusion (Ischaemic control). Hearts were preconditioned with four cycles of 5 minutes of ischemia and 5 minutes of reperfusion (IPC) prior to the index ischemia. Wild-type and STAT-3 KO hearts were found to have similar infarct sizes in the ischaemic control group. Wild-type hearts preconditioned prior to the index ischemia had significantly smaller infarcts than those of control hearts. STAT-3 KO mice could not be protected by IPC. *$p<0.05$ vs ischaemic control. n≥6. IPC- ischaemic preconditioning.

b) Isolated Cardiomyocytes

Similar results were obtained using isolated cardiomyocytes (Figure 40). Exposure of isolated cardiomyocytes to index simulated ischaemia resulted in a similar decrease of myocyte viability in wild-type and STAT-3 KO myocytes (wild-type and STAT-3 KO from 100% myocyte viability to 56.3±2.1% and 58.6±3.5% respectively; n≥6). Preconditioning the cardiomyocytes with hypoxia improved viability in wild-type mice (88.5±3.6%; *p<0.001 vs ischaemic control; n≥6) but had no protective effect on the myocytes of STAT-3 KO animals (60.6±3.3% vs ischaemic control; ns; n≥6).

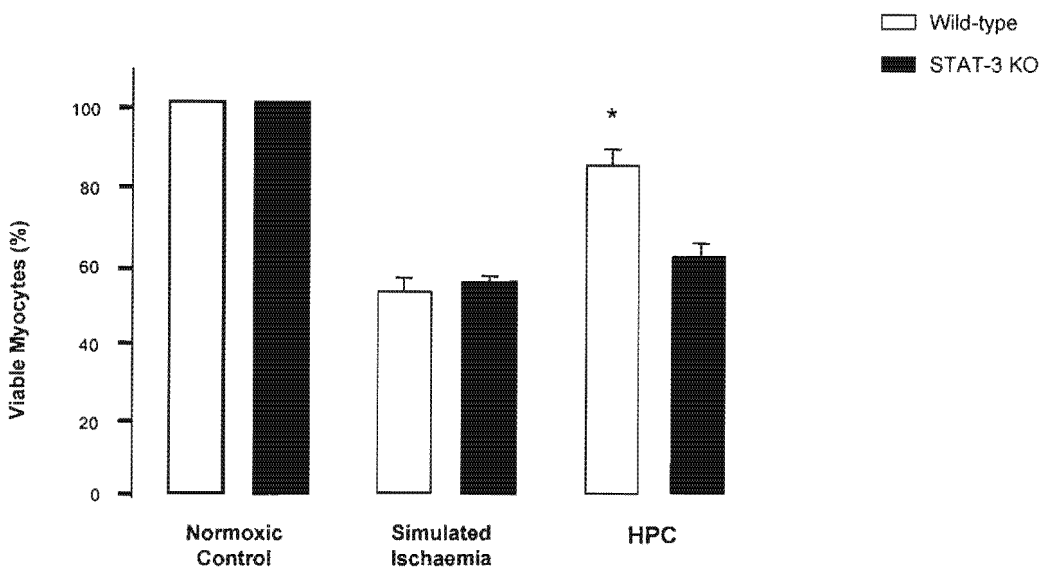

Figure 40: Effect of hypoxic preconditioning (HPC) in isolated wild-type and STAT-3 KO mouse cardiomyocytes. Myocytes were isolated from intact wild-type and STAT-3 KO hearts and were subjected to either 26 hours of simulated ischaemia and 2 hours of reoxygenation (Ischaemic Control). Cells were preconditioned with one cycle of 30' Hypoxia and 1 hour reoxygenation prior to the simulated ischaemia (HPC) protocol. Simulated ischaemia decreased myocyte cell viability in both wild-type and STAT-3 KO myocytes. HPC improved cell viability of wild-type myocytes but not of myocytes from STAT-3 KO animals. *p < 0.01 vs simulated ischaemic. n≥6. HPC- hypoxia-induced preconditioning.

3.2 The Role of STAT-3 in Pharmacological Preconditioning

To investigate the role of STAT-3 in pharmacological preconditioning, we used adenosine and TNFα as preconditioning mimetics.

a) Isolated mouse heart

Adenosine and TNFα significantly reduced the infarct size compared to ischaemic controls (17.2±3.1 and 24.6±2.5% respectively; $p<0.01$ vs ischaemic control; $n\geq5$) (Figure 41). In contrast, these preconditioning mimetics failed to precondition the STAT-3 KO hearts against sustained ischaemia (31.0±2.9% and 41.6±7.1% respectively; ns; $n\geq5$) (Figure 41).

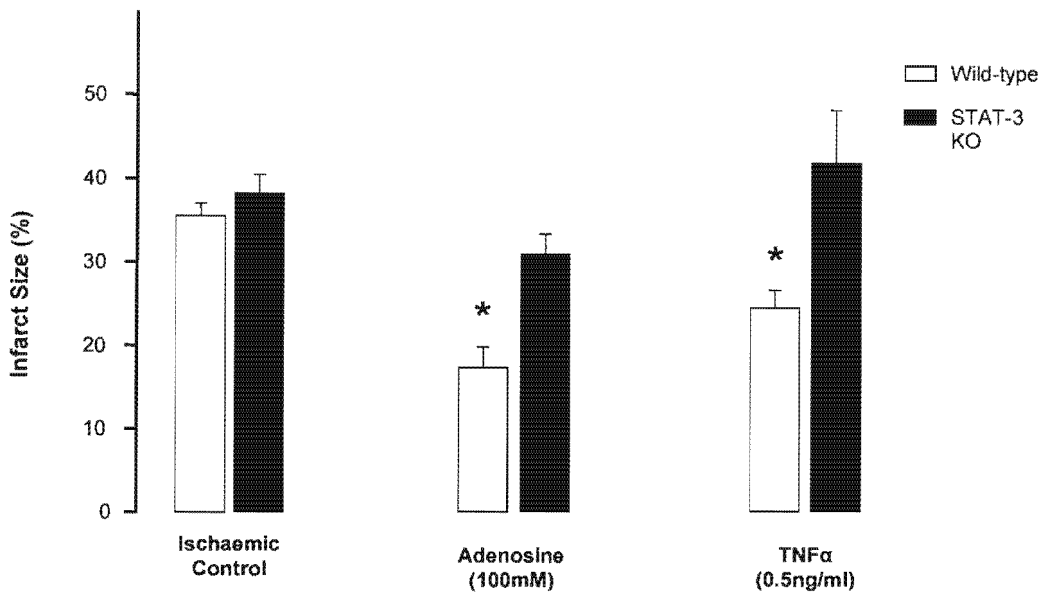

Figure 41: Effect of pharmacological preconditioning, with adenosine or TNFα, in isolated wild-type and STAT-3 KO mouse heart. Wild-type mice were preconditioned against ischaemia/reperfusion injury with either 100mM adenosine or 0.5ng/ml TNFα. Both agents decreased the infarct size when compared to the ischaemic control in wild-type mice. In contrast, neither adenosine nor TNFα had a protective influence on the STAT-3 KO hearts. *$p<0.01$ vs ischaemic control. $n\geq5$.

b) Isolated Cardiomyocytes

Similar results were obtained in the isolated cardiomyocytes model. Here, adenosine and TNFα improved cardiomyocytes viability in wild-type hearts (83.0±2.6% and 83.0±3.8% respectively; $p<0.01$ vs ischaemic control; $n\geq6$) (Figure 42). However, these agents failed to protect the STAT-3 KO cardiomyocytes (54.6±1.3% and 52.1±4.0% respectively; ns; $n\geq6$) (Figure 42).

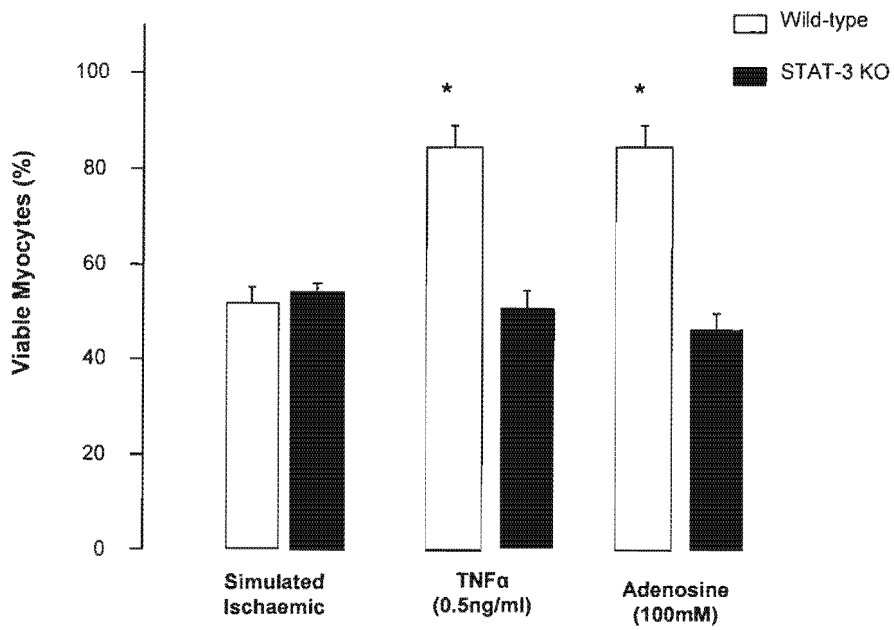

Figure 42: Effect of pharmacological preconditioning, with adenosine or TNFα, in isolated wild-type and STAT-3 KO mouse cardiomyocytes. Myocytes were PC with a 30' of exposure of either adenosine (100mM) or TNFα (0.5ng/ml). Both PC mimetics improved cell viability in wild-type myocytes but failed to protect STAT-3 KO myocytes. * $p < 0.01$ vs simulated ischaemia. $n\geq6$.

3.3 The role of STAT-3 in reperfusion injury

Reperfusion injury can be reduced with the administration of insulin at the onset of reperfusion. Here, 0.3mU, 3mU and 30mU of insulin improved cell viability in wild-type myocytes (85.2±3.4%, 81.2±6.2% and 96.2±5.7%; $p<0.001$ vs ischaemic control; $n>6$) (Figure 43). However, this improvement in cardiomyocytes viability was not observed in cells isolated from STAT-3 knockout hearts (51.8±5.6%, 52.8± 5.3% and 50.2± 4.7%; ns vs ischaemic control) (Figure 43).

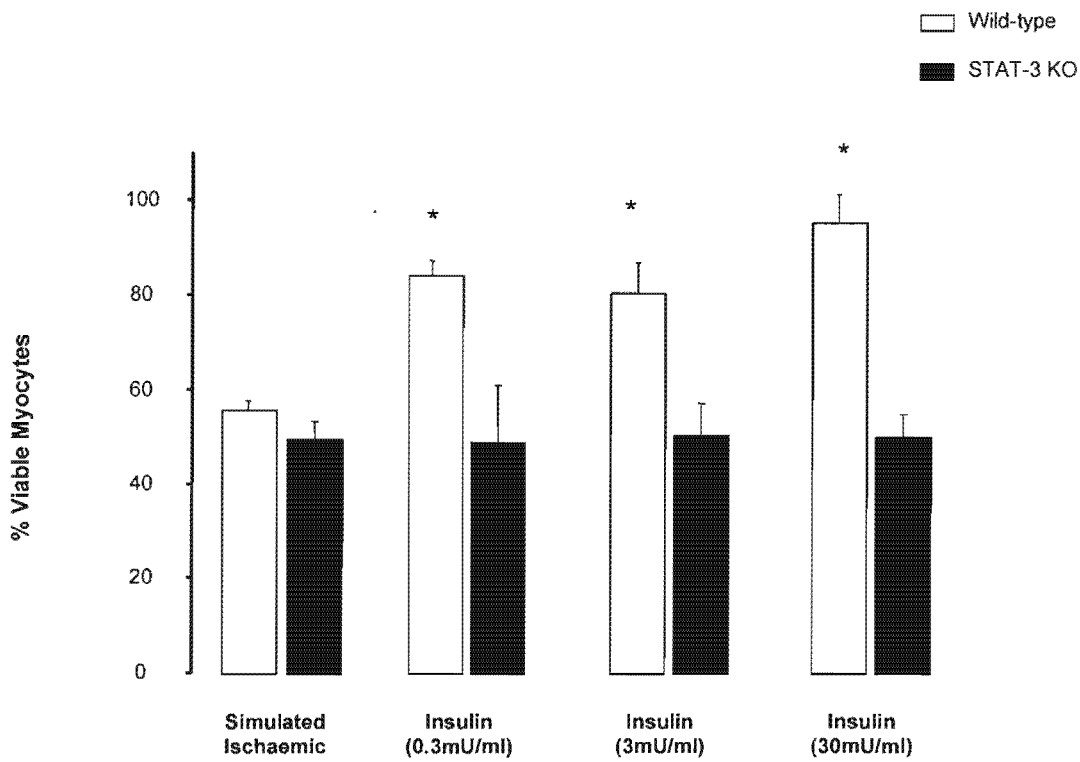

Figure 43: Effect of with insulin given at the time of reperfusion in isolated wild-type and STAT-3 KO mouse cardiomyocytes. Insulin improved the myocyte survival rate in wild-type mice, but had no protective effect on myocytes from STAT-3 KO animals. *$p<0.01$ vs ischaemic control. $n\geq6$.

4. Discussion

The major finding of this study is that genetic depletion of STAT-3 in cardiomyocytes abolishes the capacity to activate classical ischaemic preconditioning. In addition, pharmacological preconditioning with adenosine or the innate cytokine TNFα, was unable to activate this cardioprotective mechanism in the isolated perfused mouse heart and isolated cardiomyocytes. Furthermore, the deleterious effects of reperfusion induced injury was not attenuated by staged reperfusion with insulin in isolated STAT-3 KO cardiomyocytes. Taken together, these data strongly suggest that STAT-3 signalling is required for cardioprotection.

The activation of STAT-3, in response to cellular stress, has been reported as an important event in pro-survival signalling (Hattori *et al.* 2001). STAT-3 is activated by the receptor bound tyrosine kinase JAK2 following an ischaemic or oxidative stress (Negoro *et al.* 2001). This JAK2/STAT-3 pathway is an important part of the signalling cascade from the membrane to the nucleus in which tyrosine phosphorylation of STAT-3 leads to dimeristaion of monomeric STAT-3 (Mascareno *et al.* 2001), translocation to the nucleus and the binding to promoter regions of DNA and regulation of gene expression (Ihle 1996).

The inability to activate the cardioprotective program by ischaemic and pharmacological preconditioning as well as staged reperfusion with insulin in these STAT-3 deficient cardiomyocytes, raises several interesting possibilities. The first is that STAT-3 may be acting as a signalling molecule and activating

other signalling pathways or interacting with other signalling organelles besides the nucleus. The interaction of STAT-3 with other signal transduction pathways is incompletely explored, although there is an interaction of STAT and MAPK signalling (Ihle 1996) and STAT-3 and Akt signalling (Gross *et al.* 2006). Recently, Gross *et al* have suggested that PI3K mediated Akt activation, in opioid-induced preconditioning, is mediated by JAK2/STAT-3 (Gross *et al.* 2006). The obligatory role of Akt in both ischaemic and pharmacological preconditioning as well as staged reperfusion is well documented (see review by Hausenloy (Hausenloy *et al.* 2004)). The second possible function is that STAT-3 may be acting as a distal final common mediator of preconditioning-mediated cell survival signalling. However, this possibility is unlikely as pharmacological inhibition of downstream cardioprotective signalling mediator GSK3β still induces cardioprotection (Gross *et al.* 2006). The third possibility is that cardiomyocytes lacking functional STAT-3 are deficient in one or more STAT-3 regulated proteins which may play obligatory role in cardioprotection. In brief, STAT-3 has been shown to be involved in the regulation of carbohydrate metabolism (Inoue *et al.* 2004) and the lack of functional STAT-3 may lead to perturbations in metabolism within these cells that prevents the metabolic adaptations associated with preconditioning (Opie *et al.* 2002). Moreover, STAT-3 activates proteins involved in regulating apoptosis including Bcl-2 and Bax (Hattori *et al.* 2001). Here, lack of STAT-3 may alter the balance of anti-apoptotic versus pro-apoptotic signalling within cardiomyocytes. Finally, STAT-3 signalling modulates the regulation of the antioxidant defence system *via* the upregulation of manganese superoxide dismutase (Negoro *et al.* 2001) and the attenuation of anti-oxidant

defences, abolishes the preconditioning program (Das *et al.* 2003). Furthermore, cardioprotection observed in STAT-3 over-expressing mice has been attributed to up regulation of the anti-oxidants metallothionien 1 and 2 (Oshima *et al.* 2005).

As STAT-3 is considered to act in a large part, *via* transcriptional regulatory control, it is intriguing that the genetic depletion of this regulatory protein can nullify the classical preconditioning program that is thought to be predominantly orchestrated *via* post-translational events. It is interesting to note that multiple other organs can be preconditioned including liver (Hardy *et al.* 1996), kidney (Islam *et al.* 1997) and brain (Gidday *et al.* 1994). Other cell types that are present in the intact heart including epithelial cells (Minners *et al.* 2001), endothelial cells (Andjelkovic *et al.* 2003), vascular smooth muscle cells (Nayeem *et al.* 2002) and fibroblasts (Han *et al.* 2001) also have the capacity to be preconditioned. Hence, as cardiomyocytes represent only about 30% of the cell number and approximately 75% of the volume (Erikson *et al.* 1996), and as preconditioning triggers applied to remote organs can also precondition the heart (Verdouw *et al.* 1996), we determined if the extra cardiomyocyte components of the heart could rescue the preconditioning phenotype in the intact isolated perfused mouse heart. The inability to protect the myocardium in the presence of all the cell types found in the heart suggests that STAT-3 in the cardiomyocytes is required to induce this protective phenotype on the heart.

To conclude, using functional genomic depletion studies, we have shown that STAT-3 is crucial for classical preconditioning and the attenuation of reperfusion

injury in cardiomyocytes. Furthermore, the loss of STAT-3 in cardiomyocytes cannot be compensated for by the non-cardiomyocyte cellular components of the intact heart.

The challenge of using data solely from KO animals is that genetic depletion of STAT-3 may modify cardiac development, or function of the heart, which are not related to cardiac preconditioning. Therefore, to reinforce our data and explore the role of STAT-3 as an intracellular signalling protein we used pharmacological agents to inhibit STAT-3 activation and confirm that the loss of cardioprotection observed is specifically due to the loss of STAT-3 signalling. In addition, whether STAT-3 exerts a protective effect during the trigger phase and not the mediator phase is yet unknown and needs to be addressed.

E. STAT-3 as a trigger of cardiac preconditioning

1. Introduction

In the previous section, we have shown that cardiac specific STAT-3 KO mice failed to be protected from ischaemia/reperfusion injury, hence demonstrating the importance of STAT-3 activation in both ischaemic and pharmacological preconditioning. However, the challenge of using data from KO animals is that genetic depletion of STAT-3 may modify cardiac development or functions which are not related to triggering cardiac preconditioning. Furthermore, this data does not identify at which time point (during the preconditioning stimulus or at the time of reperfusion) STAT-3 activation is required for cardioprotection to occur. Therefore, in this chapter:

Using a specific pharmacological inhibitor of the JAK/STAT-3 pathway, we hypothesized that activation of STAT-3 during the preconditioning stimulus (ischaemia, hypoxia or TNFα) is required for preconditioning to confer cardioprotection. In addition, we aim to delineate a possible cross-talk between the JAK2/STAT-3 pathway and the already described PI3K/Akt pathway in ischaemic preconditioning (Figure 44).

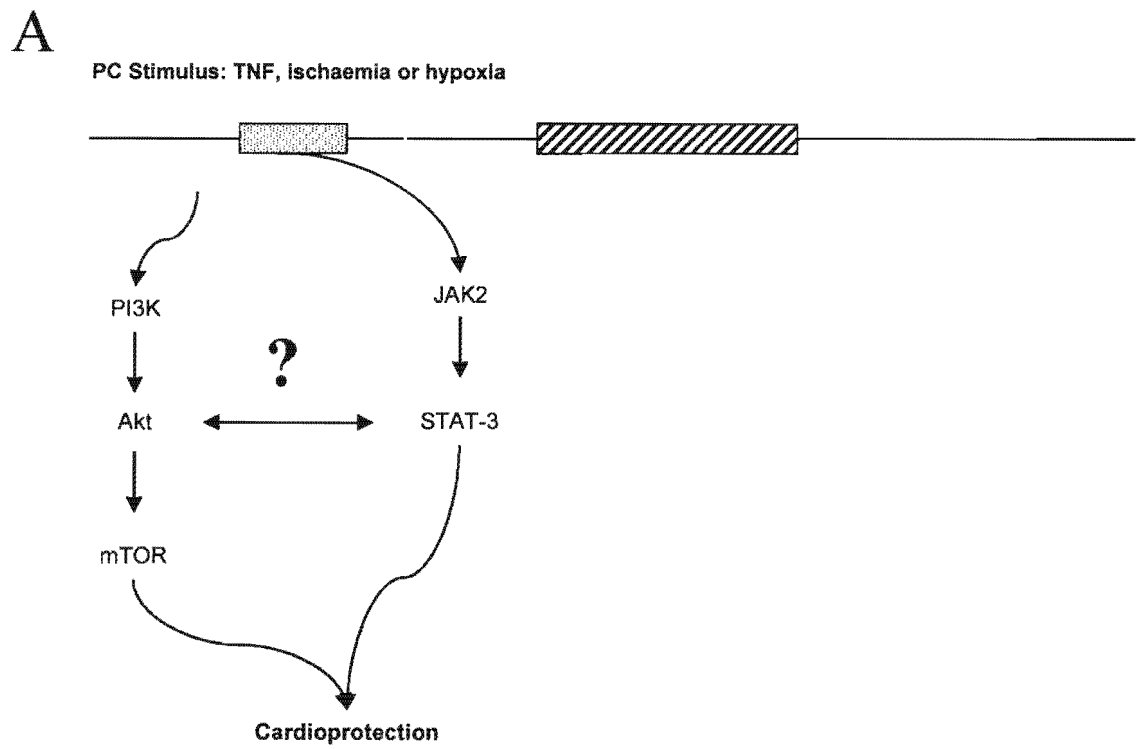

Figure 44: Hypothesis: The role of STAT-3 and Akt as preconditioning stimuli in ischaemia- and TNF-induced preconditioning. JAK2- janus kinase 2, mTOR- mammalian target of rapamycin, PI3K- phosphotidylinositol-3-kinase, STAT-3- signal transducer and activator of transcription-3.

Inhibition of the JAK2/STAT-3 pathway is achieved with the tyrphostin AG490 (Figure 45). Previous findings have shown the pharmacological inhibition with AG490 abolishes the acute protection seen in ischaemic preconditioning as well as significantly depresses STAT-3 phosphorylation levels in the isolated rat heart when given prior to an ischaemic preconditioning stimulus (Hattori *et al.* 2001). AG490 binds to JAK2 hence, preventing the binding and subsequent activation of STAT-3 (Figure 45). Inhibition of the PI3K/Akt pathway is achieved with either wortmannin or rapamycin (Figure 45) (Jonassen *et al.* 2001). The actions of both inhibitors are well documented in their abilities to inhibit this signalling pathway. Wortmannin prevents the phosphorylation of PI3K mediated phosphorylation and consequently the activation of Akt (Figure 46). Rapamycin inhibits downstream of

the PI3K/Akt signaling pathway by inhibiting mammalian target of rapamycin (mTOR) a central transcriptional regulator of the PI3K/Akt pathway (Figure 46).

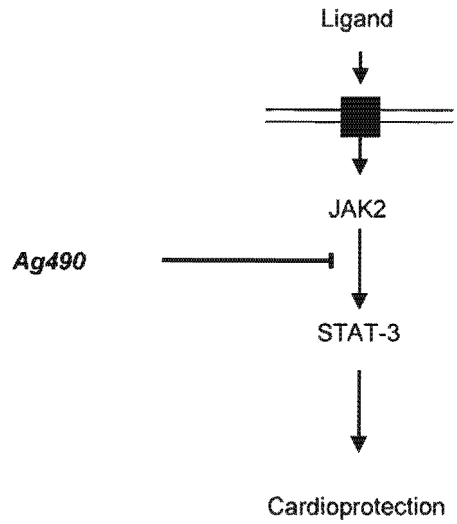

Figure 45: AG 490 mediated inhibition of the JAK2/STAT-3 signaling pathway. Signal transduction through the JAK2/STAT3 pathway can be inhibited with AG490 which prevents JAK2 mediated activation of STAT-3. JAK2- janus kinase 2, STAT-3- signal transducer and activator of transcription-3.

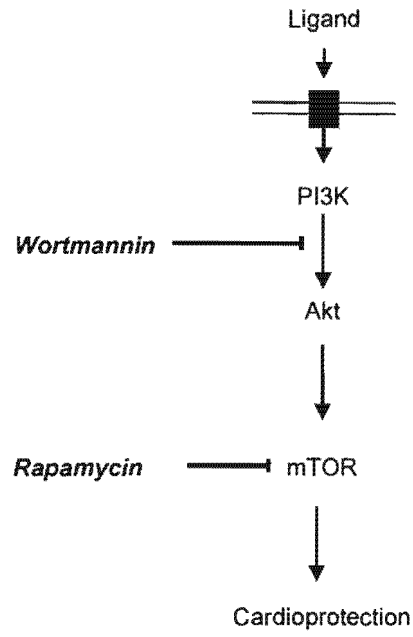

Figure 46: Wortmannin and rapamycin mediated inhibition of the PI3K/Akt signaling pathway. Signal transduction through the PI3K/Akt pathway can be inhibited with wortmannin which prevents PI3K mediated activation of Akt or with rapamycin which blocks mTOR, a downstream mediator of Akt signaling, mTOR. mTOR- mammalian target of rapamycin, PI3K- phosphatidylinositol-3-kinase.

2. Methods

2.1 Isolated mouse cardiomyocytes

The protocol used to isolate cardiomyocytes is described in section D 2.4. Isolated myocytes were exposed to simulated ischaemia/reperfusion injury and the preconditioning protocols described in section D 2.5 (Figure 38). Pharmacological inhibition of the PI3K/Akt pathway was achieved with, wortmannin (100mM) or rapamycin (0.5nM) and STAT-3 activation was inhibited with AG490 (100mM). All these inhibitors were given during the preconditioning stimulus with a washout period of one hour prior to the simulated ischaemia (Figure 47). The concentrations of wortmannin and rapamycin used have been successfully demonstrated to inhibit the PI3K/Akt signaling pathway in both cells and the isolated rat heart (Jonassen *et al.* 2001). The concentration used for STAT-3 corresponds to the IC_{50} of the drug, which has been demonstrated to inhibit the phosphorylation levels of STAT-3 in cell culture models (Dowlati *et al.* 2004). Cardiomyocyte viability was measured at the end of the 2 hour reoxygenation period using trypan blue exclusion as well as cell morphology (Zhou *et al.* 2000; Marais *et al.* 2001) by a researcher blinded to the groups. Results are expressed as percentage (%) of viable myocytes.

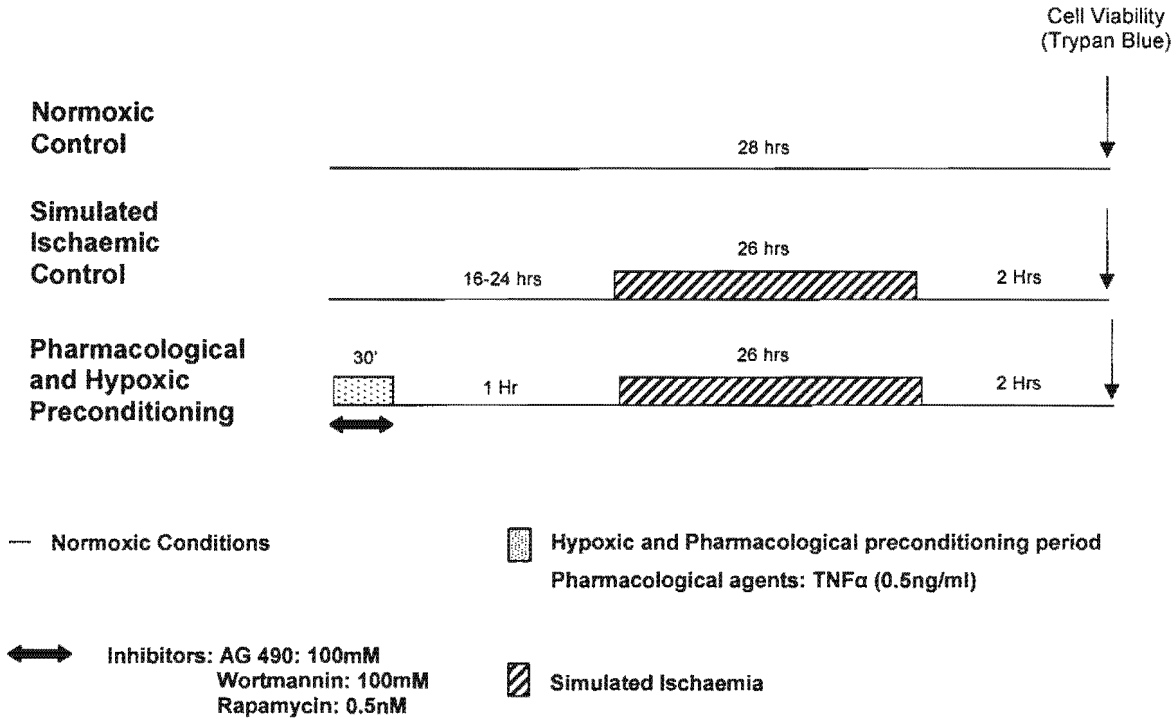

Figure 47: **Tissue culture protocols of isolated cardiomyocytes.** Cells were exposed to a 26 hours simulated ischaemia followed 2 hours of reoxygenation. Cells were preconditioned with 30 minutes hypoxia or TNFα (0.5ng/ml) followed by a 1 hour washout period prior to the simulated ischaemia. Myocyte cell viability was measured at the end of each protocol via trypan blue exclusion. The inhibitors AG490, rapamycin or wortmannin were added to the buffer during preconditioning stimulus. IPC- Ischaemic Preconditioning.

2.2 Isolated perfused rat hearts

Male Long-Evans rats (250 to 300g) were anesthetised (sodium pentobarbitone 60mg/kg; intraperitoneal) and heparinised (200IU intraperitoneal). Hearts were excised rapidly and perfused retrogradely by the Langendorff technique at a constant pressure (100cm H_2O) with oxygenated Krebs Henseleit buffer (118.0mM NaCl, 25.0mM $NaHCO_3$, 11.0mM Glucose, 4.7mM KCl, 1.2mM $MgSO_4.7H_2O$, 1.2mM KH_2PO_4 and 1.4mM $CaCl_2.2H_2O$). A balloon was inserted

through the left atrium into the left ventricle and the left ventricular end diastolic pressure was adjusted between 4 and 8mmHg. All hearts were subjected to 30 minutes of regional ischaemia by occlusion of the left coronary artery followed by 120 minutes of reperfusion (Figure 48). Hearts were preconditioned either with a low dose of TNFα (0.5ng/ml) that was given for seven minutes followed by a 10 minute washout period before standard ischaemia or with 2 cycles of 5 minutes of global ischaemic/reperfusion prior to standard ischaemia (Figure 48). AG490 (100mM), wortmannin (100mM) and rapamycin (0.5nM) were given concurrently with the preconditioning triggers ischaemia and TNFα (Figure 48). For infarct size measurements, the coronary artery was re-occluded at the end of the reperfusion period, and a solution of 2.5% Evans blue was perfused to delineate the area at risk. Hearts were then frozen overnight and sliced into 5 equal sections the following day. The infarcted area was stained with a 1% TTC solution at 37°C for 5-7 minutes. The sections were then scanned, enlarged, and infarct size was assessed using computerised planimetry (Planimetry+, Boreal Software, Norway) by a researcher blinded to the groups.

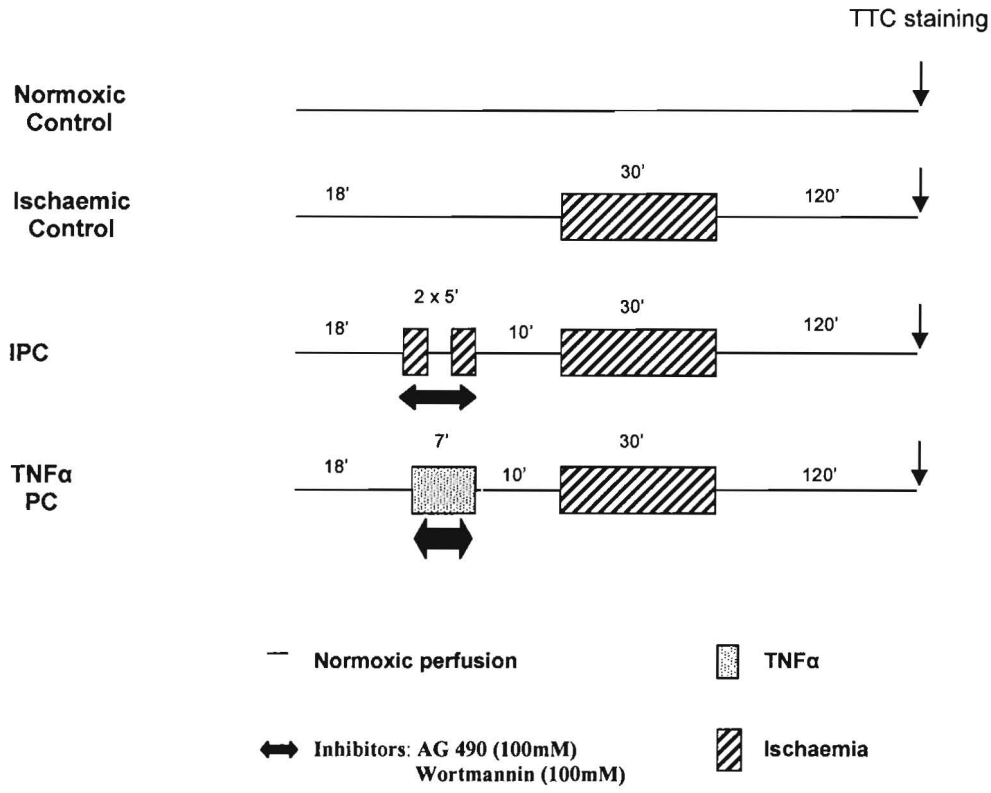

Figure 48: **Rat heart Langendorff perfusion protocols.** Rats were subjected, to an index ischaemia of 30 minutes and the final reperfusion time was 120 minutes. Ischaemic preconditioning was conferred by 2 cycles 5 minutes of ischaemia and reperfusion prior to the index ischaemic insult. TNFα was administered for 7 minutes followed by a 10 minute washout period prior to the index ischemia. The inhibitors AG490, rapamycin and wortmannin were infused during the IPC or TNFα administration period. Infarct size was measured at the end of each protocol via TTC staining. IPC- Ischaemic Preconditioning, PC- Preconditioning.

2.3 Western blot analysis

Control isolated myocytes were cultured in KSLMS media for 16-24 hours, spun down at 1500 rpm for 10 minutes and resuspended in ice-cold PBS before being pelleted, by spinning the cells down at 1500rpm for 10 minutes, and stored at –80°C for extraction. Myocytes were subjected to either the hypoxic

preconditioning or TNFα-induced preconditioning protocols and they were collected after 15 minutes or 30 minutes of treatment in the same conditions than the control group. Cytosolic proteins were extracted as described previously section C 2.3.

2.4 Pharmacological agents and anti-bodies

Recombinant murine TNFα was obtained from Peprotech Inc (Rocky Hills, NY). Anti-bodies for phospho-Akt, phospho-STAT-3 and total Akt were obtained from Cell Signalling and anti-bodies for total STAT-3, β-Actin and all secondary anti-bodies were obtained from Santa Cruz (Santa Cruz Biotech, CA, USA). All other reagents were obtained from Sigma Biochemicals (St. Louis, Mo, USA).

2.5 Statistical analysis

Results are expressed as mean values ± standard error of the mean (S.E.M.) and were analyzed by one-way ANOVA with Dunn's post-test, using GraphPad InStat version 3.01 (Graphpad software, San Diego, California, USA). Differences were considered significant at values of $p<0.05$.

3. Results

3.1 Inhibition of STAT-3 activation during preconditioning abrogates the protection due to ischaemic- and TNFα-induced cardioprotection

To investigate the role of STAT-3 during ischaemia- (isolated rat heart), hypoxia- (isolated mouse cardiomyocytes) and TNFα-induced (both models) preconditioning we administered AG490, the STAT-3 inhibitor, during the hypoxic or TNFα preconditioning phase. As previously reported in section C, myocytes cell viability following a simulated ischaemia/reperfusion insult was 55.9±1.9% (Figure 49). Both hypoxia- and TNFα-induced preconditioning improved myocytes viability to 88.5±3.6% and 83.0±3.6% respectively (***$p<0.001$ vs simulated ischaemia for both preconditioning stimuli; $n\geq6$) (Figure 49). Administration of AG490 inhibited the cardioprotective effects of both hypoxic and TNFα-induced preconditioning in isolated cardiomyocytes (myocyte viability in hypoxia-induced preconditioning+AG490: 62.9±2.8% and TNFα+AG490: 47.6±4.4%, ns vs control, $n\geq6$) (Figure 48).

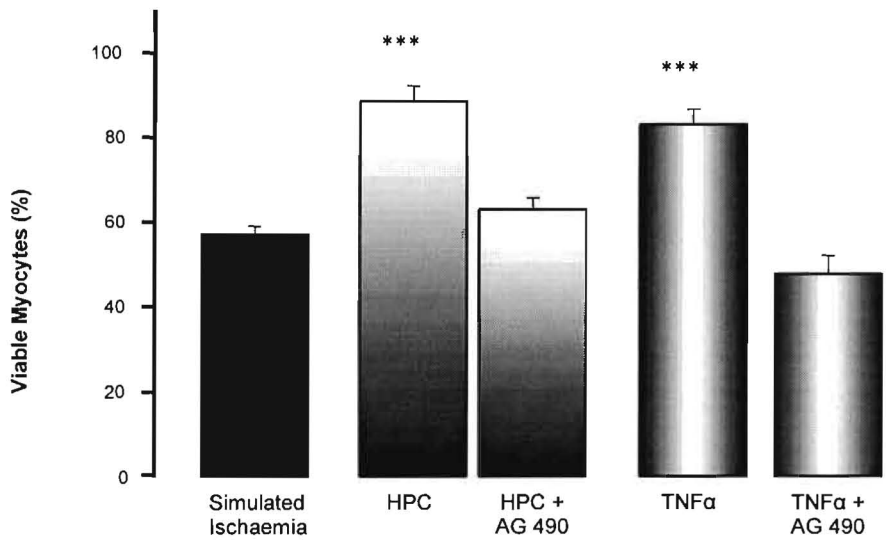

Figure 49: The role of STAT-3 in hypoxia- and TNFα-induced preconditioning using an isolated mouse myocytes model. Inhibition of STAT-3 with AG490 given during the hypoxia or TNFα preconditioning period. abrogates the protection due to both preconditioning stimuli. ***$p<0.001$ vs control; $n \geq 6$.HPC- Hypoxia-induced preconditioning.

Similar results were obtained in the isolated rat heart. Both ischaemic preconditioning and TNFα administration reduced the infarct size compared to the control group and this protective effects were lost in the presence of AG 490 (Infarct sizes for Control: 32.7±3.0%, ischaemic preconditioning: 6.5±2.2%, ischaemic preconditioning+AG490: 29.6±5.0%, TNFα: 9.4±2.9% and TNFα+AG490: 41.64±4.6%, n≥6) (Figure 50). Of note, AG490 on its own did not affect the infarct size

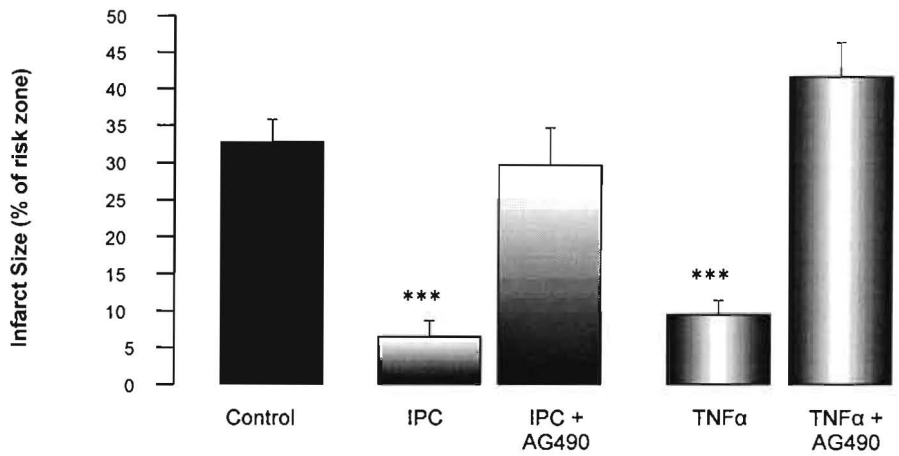

Figure 50: The role of STAT-3 in ischaemia- and TNFα-induced preconditioning in the isolated rat heart model. Inhibition of STAT-3 with AG490 given during the trigger phase abrogates the protection due to Ischaemic and TNFα-induced PC. ***p< 0.001 vs Control. n≥6 per group. IPC- Ischaemic Preconditioning.

Western blot analysis of STAT-3 phosphorylation showed an increase in STAT-3 activation during TNFα administration (Figure 51A: expressed in Arbitrary Units (AU) from 174.3±35.6 for the control to 514.3±10.7 after 30 minutes of TNFα exposure; ***p<0.001 vs control; n≥6) as well as during a hypoxic preconditioning stimulus (Figure 52A: in AU from 102.1±16.7 for the control to 180.5±5.1). Administration of AG490 during both preconditioning stimuli abolished the activation of STAT-3 (Figure 51B and 52B respectively).

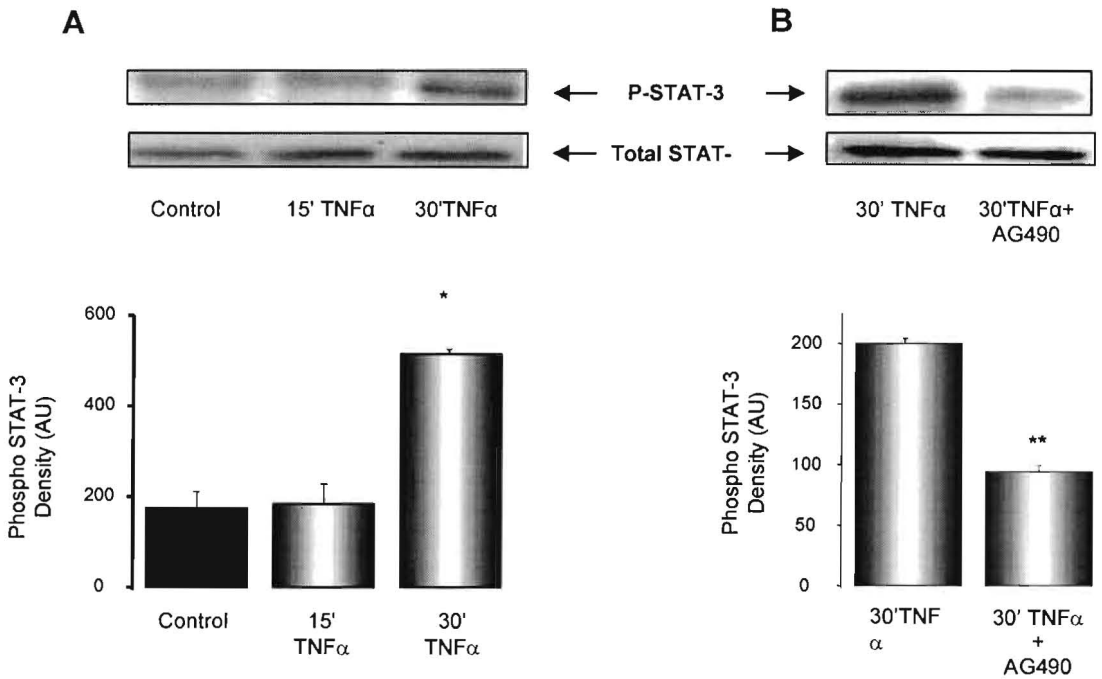

Figure 51: STAT-3 activation in cardiomyocytes exposed to TNFα. Densities expressed in Arbitrary Units (AU) showed that STAT-3 phosphorylation was increased during TNFα (0.5ng/ml) exposure (A). This increase was lost in the presence of AG 490 (B). *p<0.05 vs control, **p<0.01 vs 30' TNFα. n≥4.

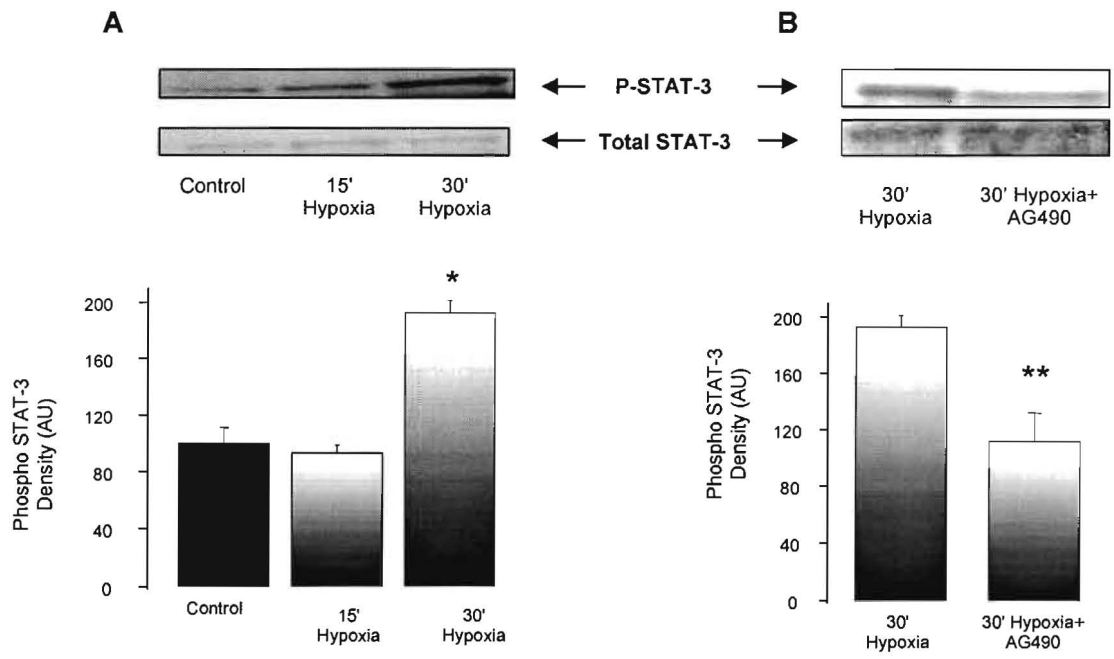

Figure 52: STAT-3 activation during a hypoxia. Densities expressed in AU show that STAT-3 phosphorylation was increased during a hypoxic preconditioning stimulus (A). This increase was lost in the presence of AG 490 (B). A-*p<0.05 vs control, B- **p<0.05 vs 30' hypoxia, n≥4 per group.

3.2 Inhibition of Akt activation during the preconditioning phase abrogates the protection due to ischaemic- but not TNFα-induced cardioprotection

To study the role of the PI3K/Akt pathway in ischaemic and TNFα-induced preconditioning, we determined myocytes viability following inhibition of the PI3K/Akt signaling pathway during the preconditioning stimulus (wortmannin was used to inhibit PI3K and rapamycin was used to inhibit mTOR kinase). Both wortmannin and rapamycin, given during the hypoxic preconditioning stimulus, abolished the protective effect afforded by preconditioning (myocytes viability 55.2±4.0% and 53.2±4.1% respectively, ns vs simulated ischaemia; n>6) (Figure 53). In contrast, these inhibitors failed to reverse the protection afforded by TNFα (85.0±5.9% and 93.4±2.3% respectively, ***p<0.001vs simulated ischaemia, n≥6) (Figure 53).

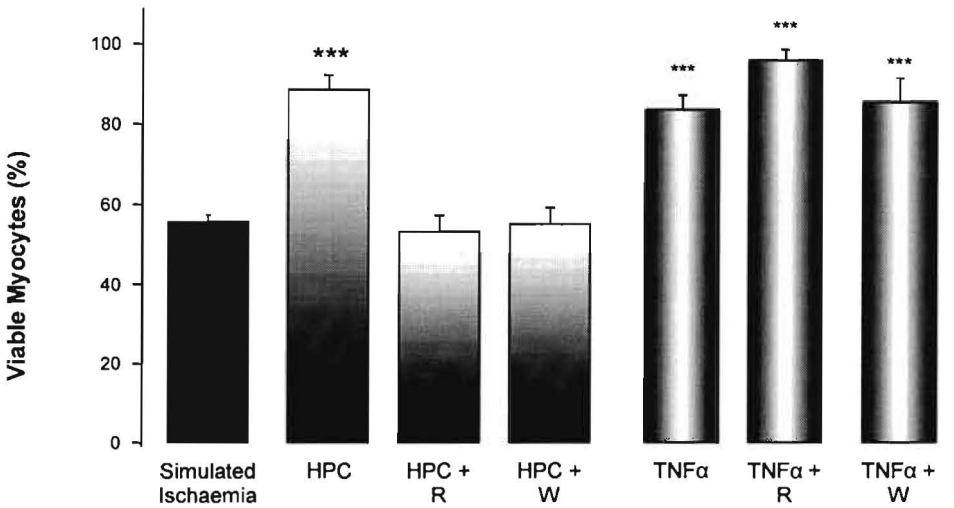

Figure 53: The role of Akt in hypoxia- and TNFα-induced preconditioning using the isolated mouse myocytes model. Inhibiting the activation of PI3K/Akt pathway with mammalian Target of Rapamycin (mTOR) with rapamycin (R) or wortmannin (W) given during the trigger phase prevented the protection due to HPC in isolated cardiac myocytes. In contrast, TNFα-induced protection was not abrogated in the presence of these inhibitors. ***p<0.001 vs control. n≥6. HPC- hypoxia-induced preconditioning, R- rapamycin, W- wortmannin.

Similar results were observed in the isolated rat heart with ischaemic and TNFα preconditioning. Here, wortmannin inhibited the protective effect of hypoxic preconditioning but not of TNFα (Control-32.7±3.0%; Ischaemic Preconditioning-6.5±2.2%; Ischaemic Preconditioning+W-16.9±3.5%; TNFα-9.4±2.9; TNF+W-8.8±2.9%; n≥6) (Figure 54).

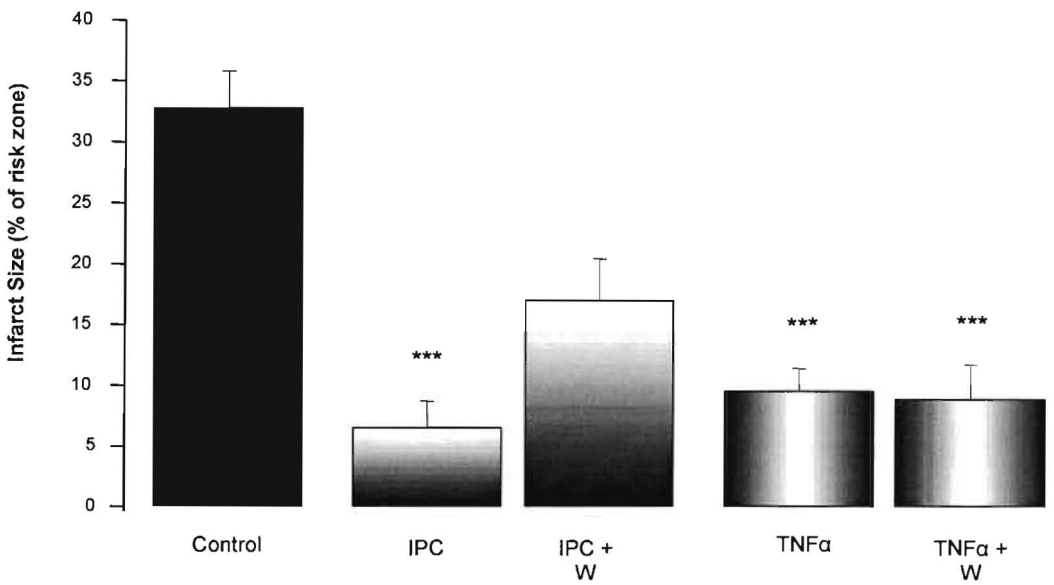

Figure 54: The role of Akt in ischaemia- and TNFα-induced preconditioning using the isolated rat heart model. Inhibition of Akt activation during the trigger phase abrogates the protection due to ischaemic but not TNFα-mediated cardioprotection. ***p< 0.001vs control. n≥6. IPC-ischaemic preconditioning, W- wortmannin.

3.3 Hypoxia but not TNFα triggers Akt activation

To confirm the role of Akt during the trigger phase, we examined the phosphorylation of Akt during the hypoxic and TNFα preconditioning stimulus in isolated mouse cardiomyocytes. An increase in Akt phosphorylation was observed

following 15 minutes and 30 minutes of hypoxia (in AU from 100 in the control to 158.4±3.1 and 192.2±7.3 following 15 minutes and 30 minutes of hypoxia respectively; $p<0.001$ vs control; $n\geq6$) (Figure 55A). In contrast, TNFα administration did not affect the phosphorylation of Akt at both time points (Figure 55B).

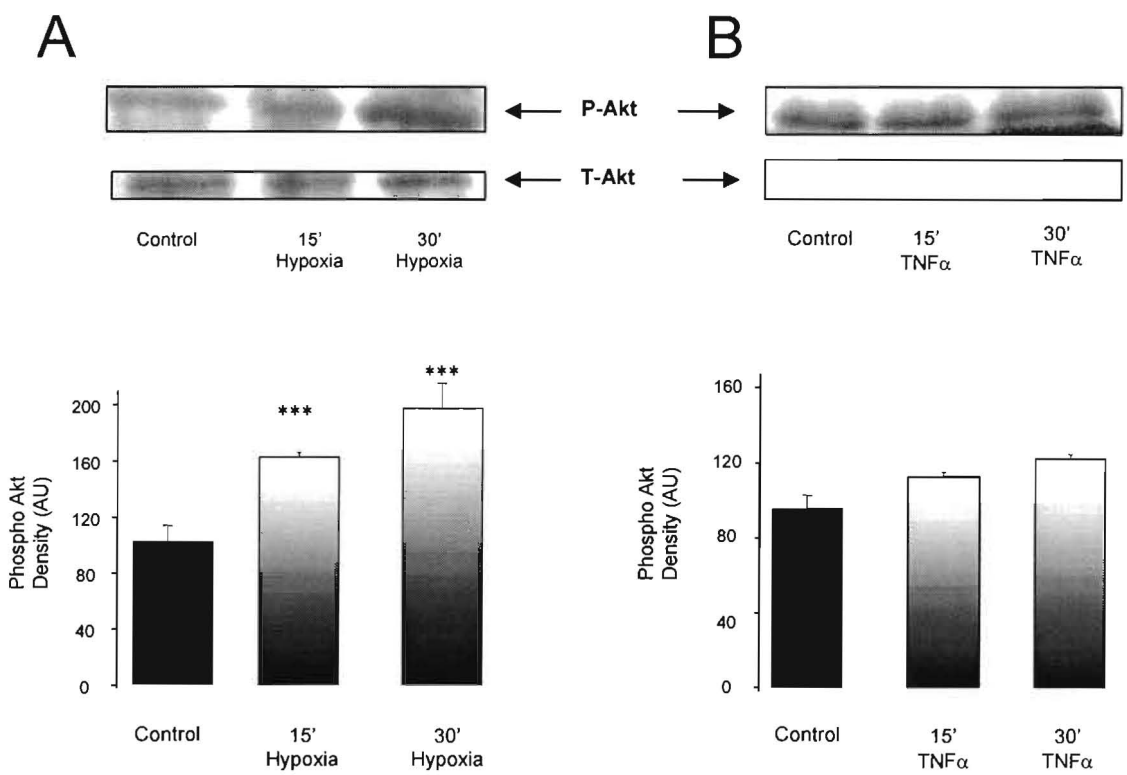

Figure 55: Akt activation during a hypoxic or a TNFα preconditioning stimulus. Densities expressed in Arbitrary Units (AU) showed that a HPC stimulus (A) and not a TNFα (0.5ng/ml) stimulus (B) increased Akt phosphorylation Akt. ***$p<0.001$ vs control. $n\geq4$ per group. Abbreviations are as defined in text.

3.4 Cross-talk between Akt and STAT-3

To determine whether a cross-talk between Akt and STAT-3 activation may exist, we determined STAT-3 phosphorylation in the presence of the PI3K/Akt inhibitor (wortmannin) and the phosphorylation of Akt in the presence on the JAK2/STAT-3 inhibitor (AG490). In addition, we explored Akt phosphorylation in cardiac

specific STAT-3 KO mice following a hypoxic preconditioning stimulus. Western blot analysis of STAT-3 phosphorylation showed that wortmannin decreased STAT-3 activation during a hypoxic preconditioning stimulus (265±45 AU for 30' of hypoxia and 120±28 AU for 30' of hypoxia + wortmannin, $p<0.05$ vs 30'hypoxia; $n\geq6$) (Figure 56). Addition of AG490 during the hypoxic preconditioning stimulus prevented Akt phosphorylation. (156±19.8 AU following a 30 minute hypoxic stimulus to 64.1±12.3 AU, $p<0.01$ vs 30' hypoxia) (Figure 57). Similarly, genetic depletion of cardiac STAT-3 prevented Akt phosphorylation following 30 minutes of a hypoxic preconditioning stimulus (set to 100 AU in the control to 101±5.6 AU following 30' hypoxia. ns, $n\geqslant4$) (Figure 58).

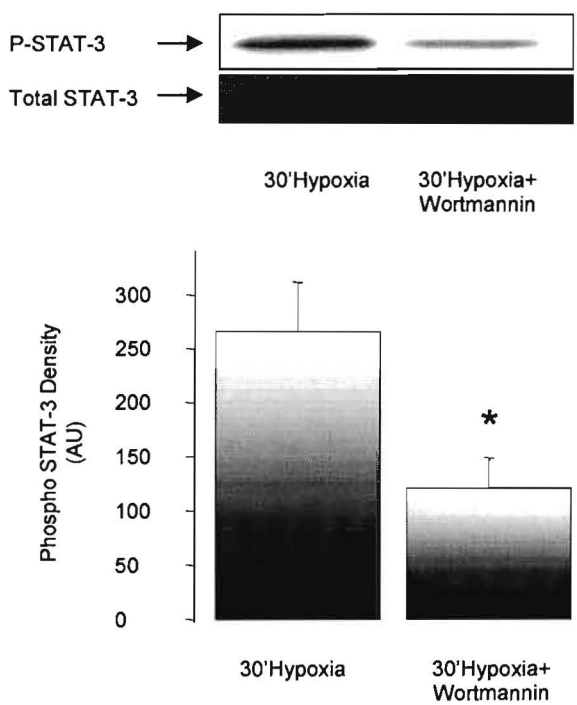

Figure 56: **STAT-3 inhibition with wortmannin during hypoxia.** Densities expressed in AU show that wortmannin prevents an increase in STAT-3 phosphorylation *$p<0.05$ vs 30'hypoxia. $n\geq4$ per group.

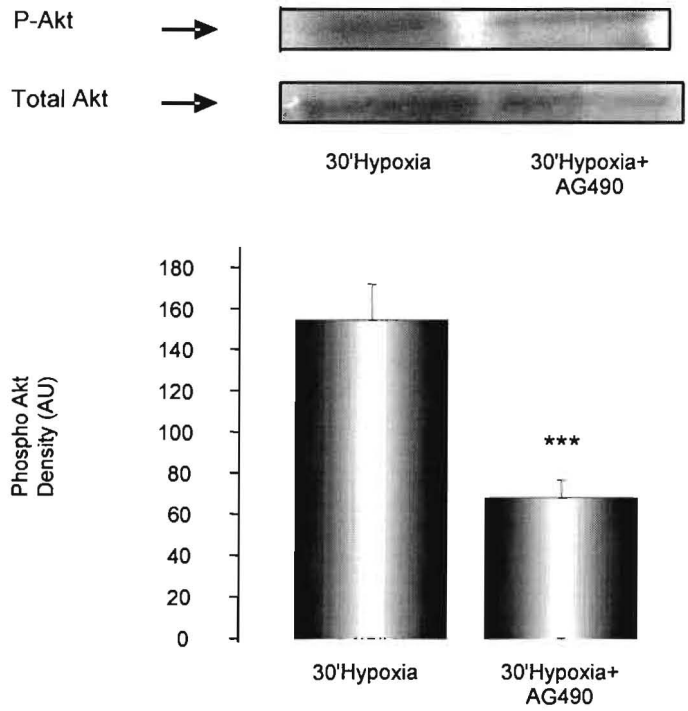

Figure 57: Effect of AG490 on Akt phospholation during hypoxia. Densities expressed in arbitrary units (AU) showed that AG490 prevents an increase in Akt phosphorylation following an hypoxic insult. ***p<0.001 vs 30' hypoxia. n≥4 per group.

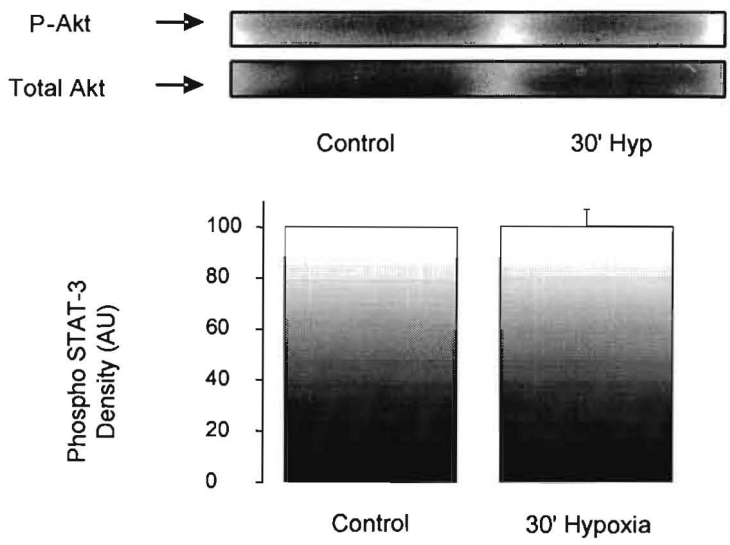

Figure 58: Akt phosphorylation in STAT-3 KO mice during hypoxia. Densities expressed in arbitrary units (AU) showed that genetic ablation of STAT-3 prevented hypoxia-induced Akt phosphorylation. n≥4 per group.

4. Discussion

Using different models and animal species, the novel data observed in this study show for the first time that pharmacological preconditioning with the cytokine TNFα requires STAT-3 phosphorylation during a preconditioning stimulus. The protection observed occurs independently of the Akt activation suggesting parallel protective signaling cascades. Furthermore, we confirm previous findings that describe a protective role for both Akt and STAT-3 as triggers of ischaemic preconditioning. In addition, we demonstrate the possibility for a cross-talk between Akt and STAT-3 activation in ischaemic preconditioning.

During ischemia (Schulz *et al.* 2004), in heart failure (Aker *et al.* 2003) or following coronary microembolization (Thielmann *et al.* 2002), myocardial and/or serum TNFα is increased and causally involved in contractile dysfunction. In contrast, *in vitro* experiments conducted in rats or mice hearts have shown that exogenous TNFα could also confer cardioprotection against an ischemia/reperfusion insult in a time- and dose- dependent manner (Lecour *et al.* 2002; Smith *et al.* 2002). However, these *in vitro* models represent limitations in that they do not take into account the intracoronary activation of leukocytes which may result in an opposite effect of TNFα in the setting of myocardial ischemia. We have previously confirmed that TNFα could mimic ischemic preconditioning *in vivo* to a similar extent as in the isolated rat heart model (Lecour *et al.* 2005b). Interestingly, the protective dose of TNFα (0.1μg/kg) used *in vivo* is within the range 1) of the dose used to mimic ischemic preconditioning in the isolated rat heart model (0.5ng/ml) and 2) of the amount of TNFα endogenously released in the isolated heart following an ischemia-reperfusion insult (Gurevitch *et al.* 1996).

The concept of multiple pathways in preconditioning has already been suggested in relation to the trigger phase (Yellon *et al.* 2003). Using an isolated rat heart model with global ischemia/reperfusion, we found that sphingolipids are involved with TNFα-induced preconditioning but not necessarily with ischemic preconditioning (Lecour *et al.* 2002). In contrast, activation of tyrosine kinase is required in ischemic preconditioning (Vahlhaus *et al.* 1998; Fryer *et al.* 1999) but not necessarily in pharmacological preconditioning (Ebel *et al.* 2004). We investigated the role of the JAK2/STAT-3 signaling pathway as the possible signaling pathway which results in TNFα-induced preconditioning. The role of STAT-3 has previously been reported during the trigger phase in ischaemic preconditioning (Bolli *et al.* 2001; Hattori *et al.* 2001) but not in TNFα preconditioning. Using two models of cardiac ischaemia/reperfusion, we were able to abolish the cardioprotective effect associated with ischaemic or TNFα-induced preconditioning when the STAT-3 inhibitor AG490 was given during the preconditioning stimulus. Furthermore, the protection observed with both preconditioning stimuli was associated with phosphorylation of STAT-3 during and after the preconditioning stimuli. TNFα transmits a signal via two distinct receptors, TNFR1 and TNFR2 which both lack intrinsic kinase activity in their intracellular domains (Guo *et al.* 1998). Due to this, they signal via interactions with signaling kinases, such as JAK2, which is known to be constitutively associated with the intracellular domain of TNFα receptors. STAT-3 is activated by direct phosphorylation of a tyrosine residue by JAK2 (Darnell *et al.* 1994).

The signaling cascade associated with the activation of STAT-3 following a preconditioning stimulus still remains to be elucidated. We have previously reported that TNFα-induced cardioprotection is dependent of ROS (Lecour *et al.* 2005a) and recent work by Sauer *et al* (Sauer *et al.* 2004) demonstrated that treatment of cardiomyocytes with cardiotrophin-1 (also a cardioprotective cytokine) stimulated STAT-3 phosphorylation in a ROS dependent manner. Further experiments will be required to explore whether a cross-talk may exist between ROS and STAT-3.

During the preconditioning stimulus, it is now well established that activation of Akt is require to confer cardioprotection (Mocanu *et al.* 2002). In addition, many pharmacological agents such as adenosine (Auchampach *et al.* 1993), opioids (Cohen *et al.* 2001; Gross *et al.* 2006), bradykinin (Cohen *et al.* 2001) all protect via the activation of Akt during the trigger phase. Our data show for the first time that a pharmacological preconditioning compound can protect independently of the activation of Akt during the trigger phase. This data supports the concept of multiple signaling pathways in cardiac preconditioning.

As both STAT-3 and Akt activation are required in ischaemic preconditioning, we aimed to explore the possible cross-talk between these two intermediates of the protective signaling cascade. First, we showed that inhibition of Akt with wortmannin also inhibited STAT-3 phosphorylation thus suggesting that STAT-3 activation may occur downstream of Akt activation. In contrast, inhibition of STAT-3 with AG490 also inhibited Akt phosphorylation thus suggesting that Akt activation may occur downstream of STAT-3 activation. In addition,

phosphorylation of Akt did not occur in cardiac specific STAT-3 KO mice exposed to an ischaemic preconditioning stimulus. Taken all together, these results clearly suggest the presence of a cross-talk between Akt and STAT-3 in ischaemic preconditioning. In 1997, Pfeffer at al. demonstrated that the p85 regulatory subunit of PI3K can promote the serine phosphorylation of STAT-3 which is critical for the formation of stable STAT-3 homodimers (Pfeffer *et al.* 1997). In pulmonary artery endothelial cells, STAT-3 activation with carbon monoxide exposure is dependent on PI3K/Akt pathway (Zhang *et al.* 2005). In 2001, it was demonstrated that JAK2 can phosphorylate Akt after binding to the component p85 of PI3K (Nguyen *et al.* 2001). More recently, Gross et al, used an *in vivo* rat model of ischaemia-reperfusion and a cell culture model (H9C2 cardiomyoblasts) to show that opioids induced cardioprotection via the activation of JAK/STAT-3 and PI3K/Akt pathways and that STAT-3 phosphorylation was dependent on PI3K activation (Gross *et al.* 2006). They suggest that the observed protection is due to JAK2 regulated, PI3K dependent phosphorylation of STAT-3, Akt and GSK3β, with GSK3β playing a central role in cardioprotection. Further experiments into the role of GSK3β in TNFα-induced preconditioning will be required to determine whether GSK3β plays a role in TNFα-induced preconditioning. This is in concordance with our data where STAT-3 knockout mice, in contrast to their littermate control failed to increase Akt phosphorylation following an ischaemic preconditioning insult. Taken together these results provide an answer to the puzzling role of STAT-3, which is a transcription factor, in classical preconditioning which predominantly, occurs via post-translational control of proteins.

In summary, TNFα-induced preconditioning requires the activation of STAT-3 during the preconditioning stimulus but occurs independently of PI3K/Akt signaling. In contrast, ischaemia-induced preconditioning occurs via the dual activation of both the PI3K/Akt and JAK2/STAT-3 signaling pathways with the recruitment of STAT-3 to the receptor by JAK2 forming part of a signaling complex required for PI3K mediated activation of Akt.

F. The Role of STAT-3 and Akt as mediators of cardiac preconditioning

(Part of the work presented in this section has been published Circulation. 2005 Dec 20; 112(25):3911-8.)

NB: Part of the langendorff perfusions and western blot analysis presented in this section has been performed with Dr Sandrine Lecour

1. Introduction

Current interest surrounding cardiac preconditioning is centered on the events that occur at the onset of reperfusion. As yet, no investigation has taken place to explore the role of the JAK2/STAT-3 signalling pathway during early reperfusion following both ischaemia and pharmacologically induced preconditioning. In the previous section, we demonstrated that STAT-3 activation is required during the ischaemic and TNFα preconditioning stimuli for cardioprotection to occur. In addition, we show that this protection occurs independently to the activation of the PI3K/Akt pathway during the trigger phase in TNFα-induced preconditioning. Akt is found to be phosphorylated and activated during early reperfusion following both ischaemic (Hausenloy *et al.* 2002) and pharmacological (Jonassen *et al.* 2001; Krieg *et al.* 2002) preconditioning. In addition, its inhibition during the early phase attenuates the protection afforded by ischaemic preconditioning (See review (Hausenloy *et al.* 2004c)).

Therefore, in this section we aim to investigate

The role of STAT-3 and Akt during early reperfusion in ischaemia, hypoxia and TNFα-induced cardiac preconditioning (Figure 59).

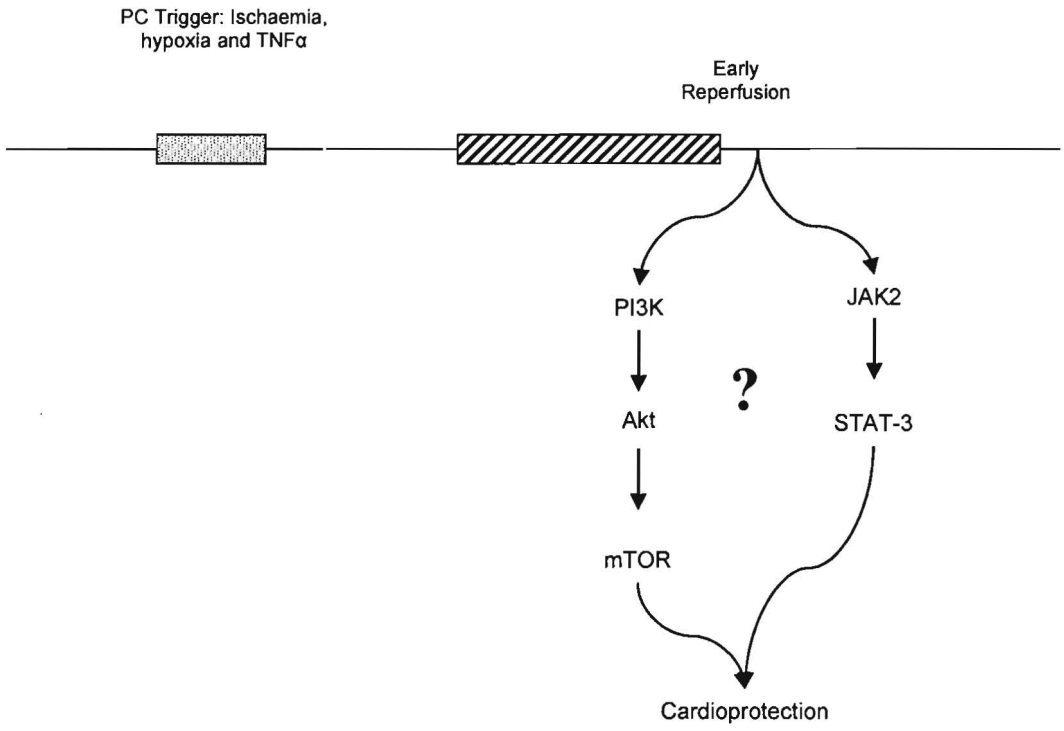

Figure 59: Hypothesis: The role of STAT-3 and Akt during early reperfusion in ischaemia- and TNF-induced PC. JAK2- janus kinase 2, mTOR- mammalian target of rapamycin, PI3K- phosphotidylinositol-3-kinase, STAT-3- signal transducer and activator of transcription-3.

2. Methods

2.1 Isolated mouse cardiomyocytes

The protocol used to isolate cardiomyocytes is described in section C 2.4. Isolated myocytes were exposed to either simulated ischaemia, preconditioning with hypoxia or TNFα as described in section C 2.5. Pharmacological inhibition of the PI3K/Akt pathway was achieved with wortmannin (100nmol/L) or rapamycin (0.5nmol/L) (Figure 60) added at the onset of reoxygenation in both preconditioning groups (Figure 60). Inhibitors were used at the previously specified concentrations (Section E 2.1). Similarly, STAT-3 activation was inhibited with AG490 (100nmol/L) administered at the onset of reoxygenation in both hypoxic preconditioning and TNFα-induced preconditioning (Figure 60). Cardiomyocyte viability was measured as previously described in section D 2.6.

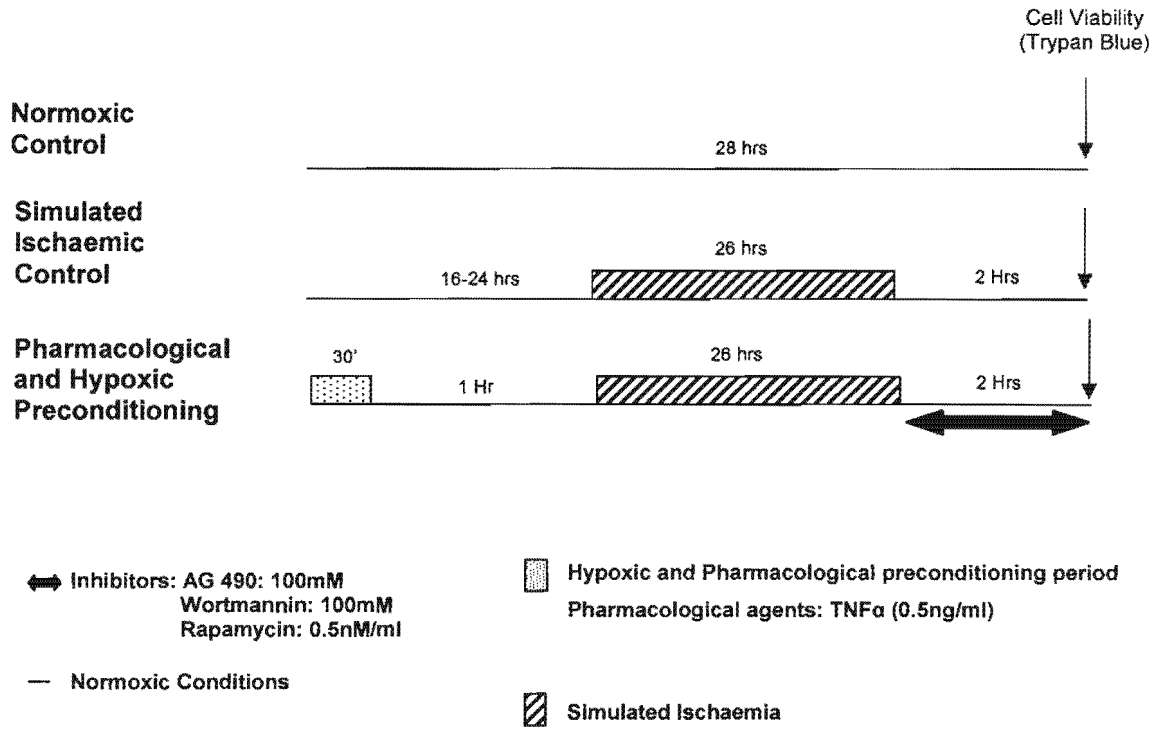

Figure 60: Tissue culture protocols for isolated cardiac myocytes. In all experiments, cells were subjected to an index simulated ischaemia for 26 hours and 2 hours of reoxygenation. Cells were preconditioned with either 30 minutes of hypoxia or low dose (0.5ng/ml) TNFα followed by a 1 hour washout period prior to the index ischaemia. Myocyte cell viability was measured at the end of each protocol via trypan blue exclusion. The inhibitors AG490, rapamycin or wortmannin were added to the buffer at the time of reoxygenation.

2.2 Isolated perfused rat hearts

Hearts from adult, male Long-Evans rats (250 to 300g) were excised rapidly and perfused retrogradely by the Langendorff technique as described in section E 2.2. All hearts were subjected to 30 minutes of regional ischaemia by occlusion of the left coronary artery and 120 minutes of reperfusion. Hearts were preconditioned with either ischaemic preconditioning or low dose TNFα (0.5ng/ml) as described previously (see Section E 2.2) and the inhibitors, AG490 (100nmol/L), wortmannin (100nmol/L) or rapamycin (0.5nmol/L) were given for the first 15 minutes of reperfusion only (Figure 61). Infarct size was determined as described in Section E 2.2.

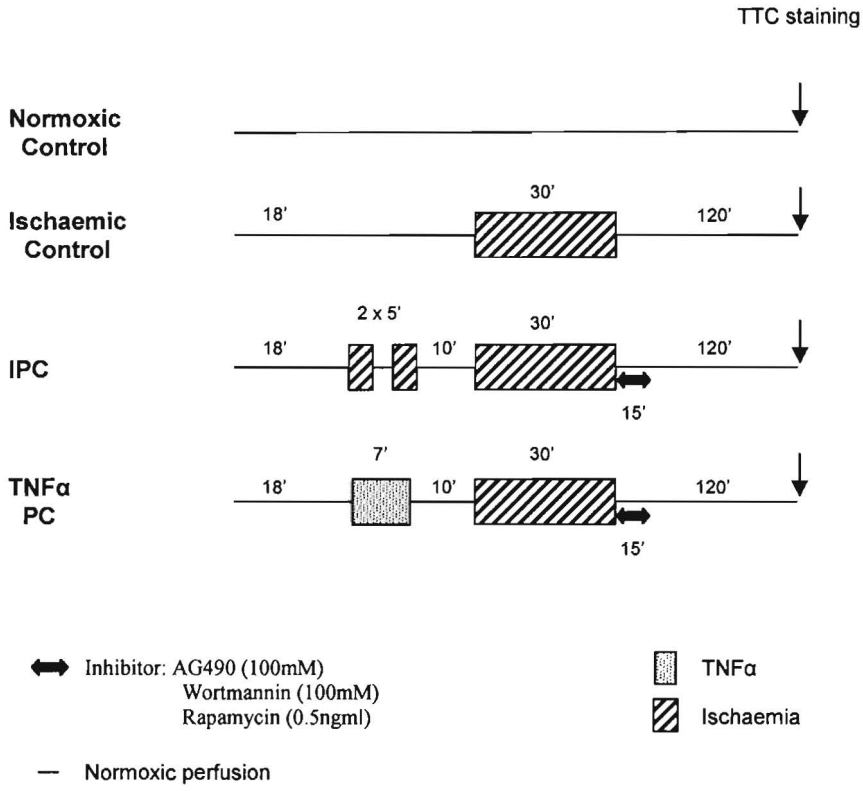

Figure 61: **Rat heart Langendorff perfusion protocols.** In all experiments, the index ischaemia lasted for 30 minutes and the final reperfusion time was 120 minutes. Ischaemic preconditioning was conferred by 2 times 5 minutes of ischaemia and reperfusion prior to the index ischaemic insult. TNFα was administered for 7 minutes followed by a 10-minute washout prior to the index ischemia. The inhibitors AG490, rapamycin and wortmannin were infused for the first 15 minutes of reperfusion. Infarct size was measured at the end of each protocol via TTC staining.

2.3 Western blot analysis

Control or preconditioned rat hearts were subjected to 30 minutes of regional ischaemia and 5 minutes of reperfusion. At the end of reperfusion, the ventricular tissue at risk was excised and freeze clamped in liquid nitrogen before being stored at –80°C. Cytosolic proteins were extracted as described previously section E 2.3. Phosphorylated states of Akt (phospho-Ak; serine 473) and STAT-3 (phospho-STAT-3 tyrosine 705) as well as the total levels of Akt, STAT-3 and β–

actin were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to PVDF membranes by electrophoretic transfer. Equal loading was verified by Ponceau staining or β-actin, and phosphorylated levels of proteins were normalised to their total protein levels in the same samples and under the same conditions but on a separate membrane. Relative peptide levels were measured using densitometric analysis with UVI band (UVI Tech, Cambridge UK) software on a personal computer.

2.4 Pharmacological agents and anti-bodies

Recombinant murine TNFα was obtained from Peprotech Inc (Rocky Hills, NY). Anti-bodies for phospho-Akt, phospho-STAT-3 and total Akt were obtained from Cell Signalling and anti-bodies for total STAT-3, β-Actin and all secondary anti-bodies were obtained from Santa Cruz (Santa Cruz Biotech, CA, USA). All other reagents were obtained from Sigma Biochemicals (St. Louis, Mo, USA).

2.5 Statistical analysis

Results are expressed as mean values ± standard error of the mean (S.E.M.) and were analyzed by one-way ANOVA with Dunn's post-test, using GraphPad InStat version 3.01 (Graphpad software, San Diego, California, USA). Differences were considered significant at values of $p<0.05$.

3. Results

3.1 Inhibition of STAT-3 activation during early reperfusion abrogates the protection due to ischaemic and TNFα-induced preconditioning

To investigate the role of STAT-3 at the time of reperfusion in hypoxic (isolated mouse cardiomyocytes), ischaemic (isolated rat heart) and TNFα-induced preconditioning (both models) we administered AG490 at the time of reperfusion. As reported previously in section C, myocytes cell viability was 55.9±1.9% following index ischaemia (Figure 62). Both hypoxia- and TNFα-induced preconditioning improved myocytes viability to 88.5±3.6% and 83.0±3.6% respectively ($p<0.001$ vs simulated ischaemia for both preconditioning stimuli; $n>6$) (Figure 61). AG490 administration inhibited the cardiac protective effects of both hypoxic and TNFα-induced preconditioning in isolated cardiomyocytes (myocyte viability 49.1±7.5% and 62.7±8.5% respectively; $p<0.001$ vs simulated ischaemia, $n\geq5$) (Figure 62).

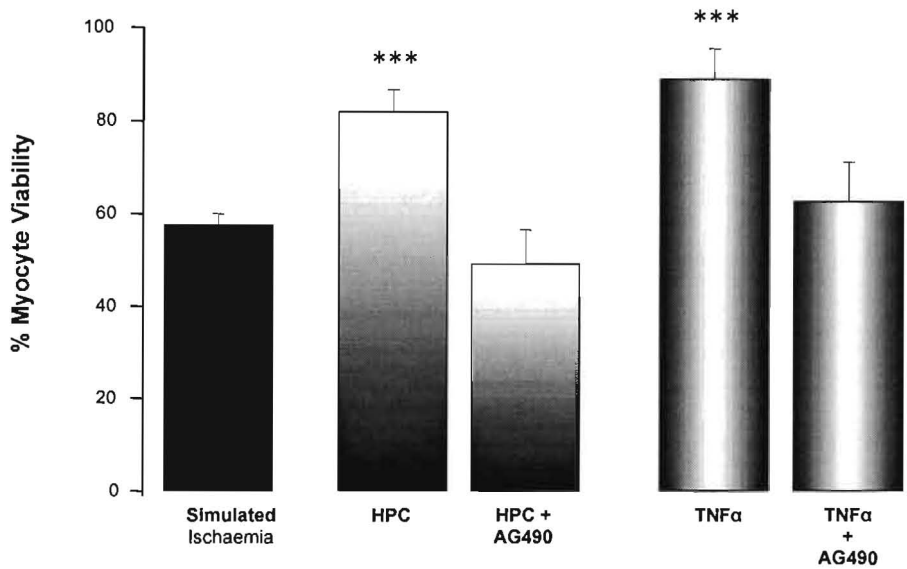

Figure 62: The Role of STAT-3 during early reperfusion in hypoxia- and TNFα-induced preconditioning in isolated mouse cardiomyocytes. STAT-3 activation was inhibited with AG490 added during the 2 hours of reperfusion in both the hypoxia or TNFα preconditioned groups. This abrogated the protection due to both hypoxia- and TNFα-induced cardioprotection (***$p<0.001$ vs control, $n\geq5$). HPC-hypoxia-induced preconditioning.

Similar results were obtained in the isolated rat heart (Figure 63).

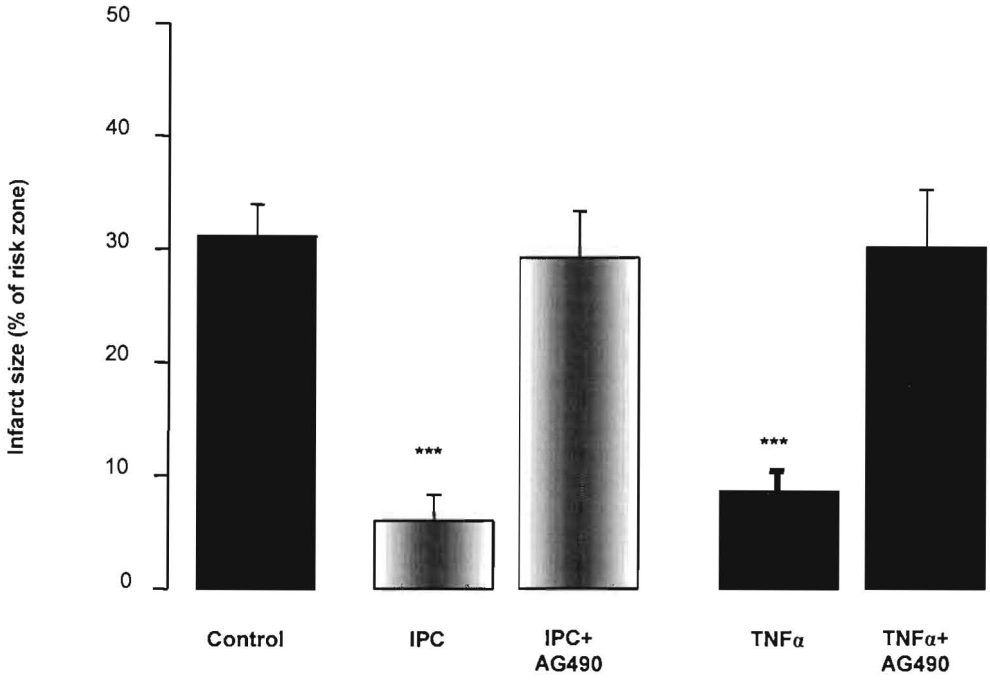

Figure 63: **The role of STAT-3 during early reperfusion ischaemic and TNFα-induced preconditioning in the isolated rat heart.** Inhibition of STAT-3 with AG490 during early reperfusion abrogates the protection due to ischaemic and TNFα-induced preconditioning (***p< 0.001 vs control, n≥6). IPC- ischaemic preconditioning.

Western blot analysis of STAT-3 phosphorylation performed in tissue collected after 5 minutes of reperfusion in the isolated rat heart showed an increase of STAT-3 phosphorylation in both the ischaemic and TNFα preconditioned groups (Figure 64A). However, this increase was reversed following the addition of AG490 (Figure 64B).

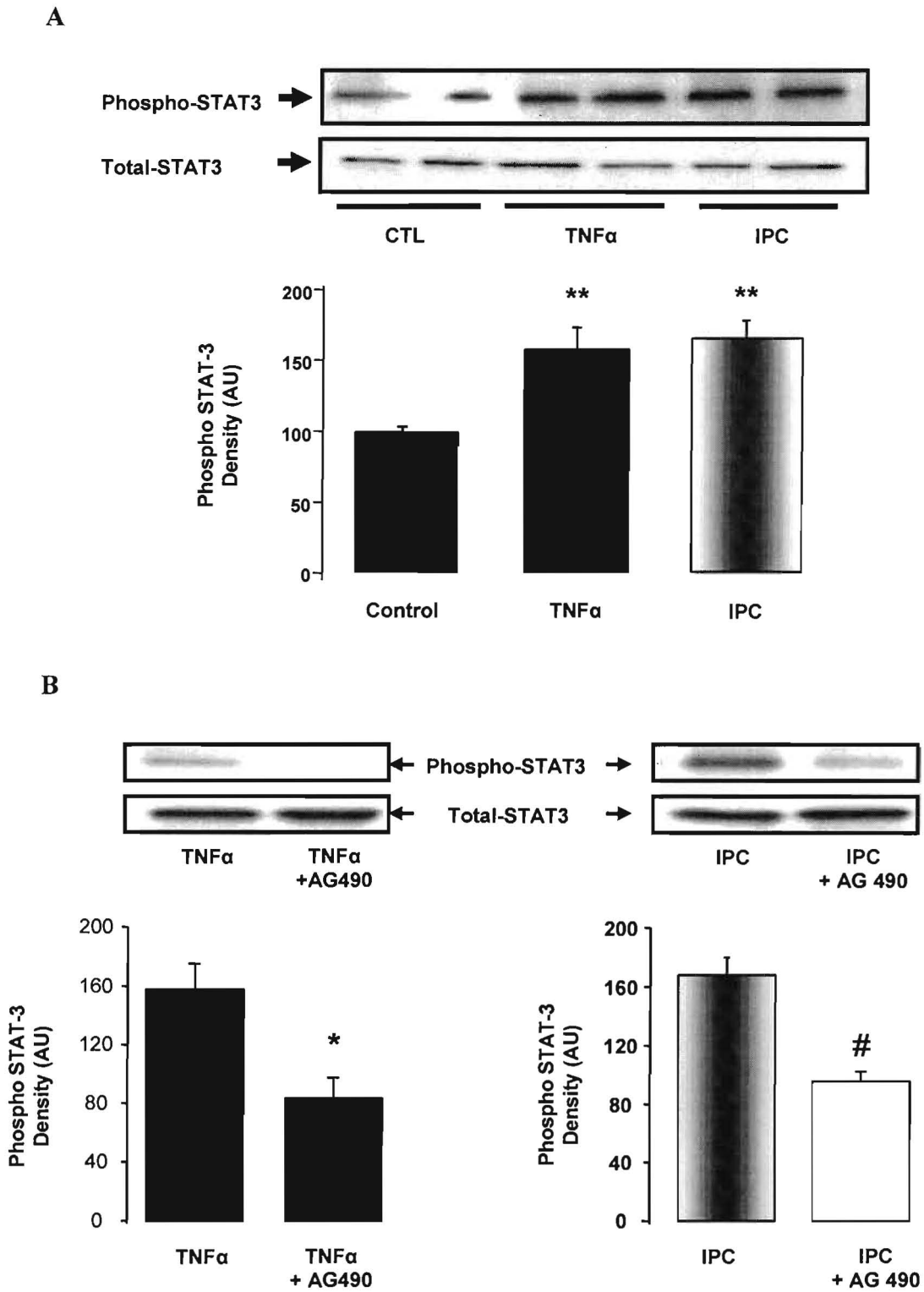

Figure 64: STAT-3 phosphorylation during early reperfusion. Densities expressed in Arbitrary Units (AU) show that STAT-3 is activated during early reperfusion following an ischaemic or TNFα preconditioning stimulus (A). This activation is inhibited with AG490 (B) (A: **p<0.01 vs control, B: *p<0.05 vs TNFα and #p<0.05 vs IPC, n=4 in all groups). IPC- ischaemic preconditioning.

3.2 Inhibition of Akt activation at reperfusion abrogates the protection due to ischaemic preconditioning but not TNFα-induced cardioprotection

To study the role of the PI3K/Akt pathway, we determined myocytes viability following inhibition of the PI3K/Akt signaling pathway (wortmannin was used to inhibit PI3K and rapamycin was used to inhibit mTOR kinase) at reoxygenation in isolated myocytes following hypoxia- or TNFα-induced preconditioning. When wortmannin or rapamycin was given at the onset of reoxygenation in hypoxic preconditioned cardiomyocytes, the cardioprotective effect was lost (myocytes viability 45.3±9.8% and 42.1±5.1% respectively; ***$p<0.001$ vs hypoxia-induced preconditioning; $n\geq5$) (Figure 65). In contrast, wortmannin and rapamycin failed to reverse the protection afforded by TNFα (96.3.0±6.6% and 78.4±5.7% respectively, # ns vs TNFα, $n\geq5$) (Figure 65).

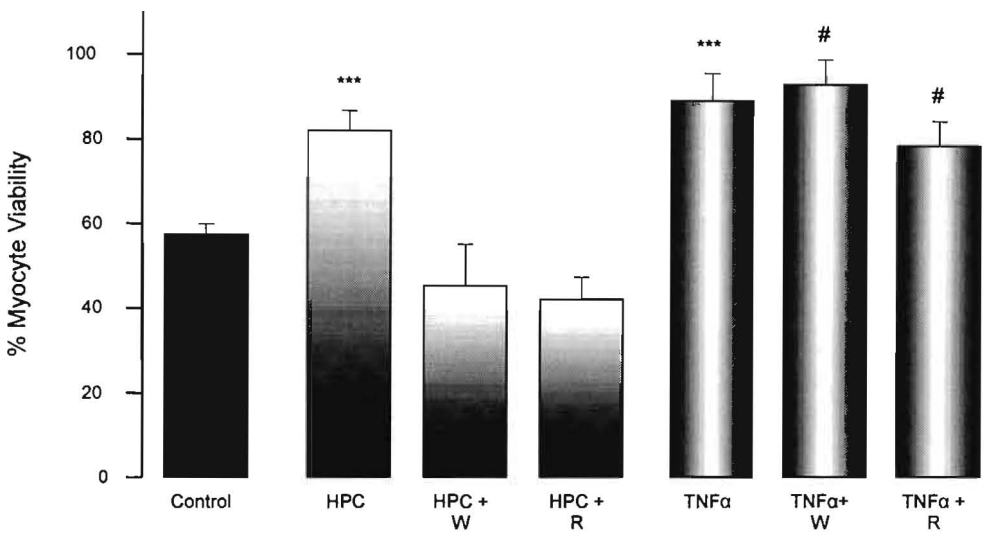

Figure 65: The Role of the PI3K/Akt pathway during early reperfusion in Hypoxia- and TNFα-induced preconditioning in isolated mouse cardiomyocytes. Inhibiting the activation of Akt with wortmannin (W) or mammalian Target of Rapamycin (mTOR) with rapamycin (R) during early reperfusion prevented the protection due to hypoxic preconditioning in isolated cardiomyocytes. In contrast, TNFα-induced protection was not abrogated (***$p<0.001$ vs control, $n\geq5$, # ns vs TNFα, $n\geq5$). HPC- hypoxia-induced preconditioning, R- rapamycin, W- wortmannin.

Similar results were observed in the isolated rat heart with ischaemic preconditioning and TNFα inducing cardioprotection (***p<0.001 vs control, n≥6) (Figure 66) and wortmannin and rapamycin abrogating the protective effect of ischaemic but not TNFα-induced preconditioning (Figure 66).

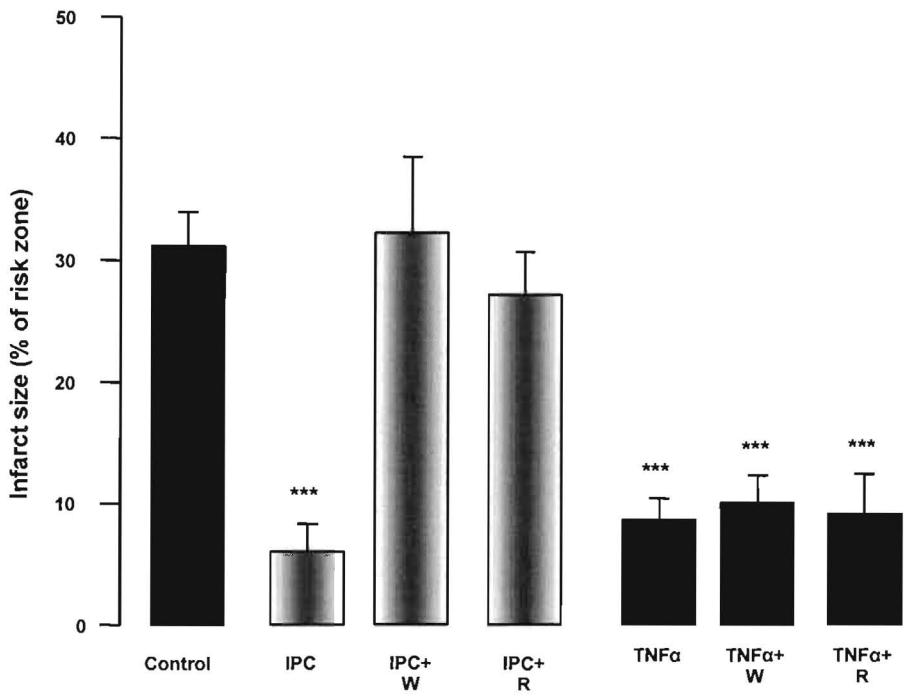

Figure 66: The role of the PI3K/Akt pathway during early reperfusion ischaemic and TNFα-induced preconditioning in the isolated rat heart. Inhibition of Akt during early reperfusion abrogates the protection due to ischaemic but not TNFα-induced preconditioning (***p< 0.001, n≥6). IPC-ischaemic preconditioning, R- rapamycin, W-wortmannin..

3.3 Hypoxia but not TNFα triggers Akt activation

We examined if Akt was phosphorylated during early reperfusion in ischaemic and TNFα-induced preconditioning. Ischaemic preconditioning increased Akt phosphorylation (from 100.0±3.0AU in the ischaemic control to 134.0±5.8AU in ischaemic preconditioning, **p<0.01 vs control; n=6) following 5 minutes of reperfusion in the rat heart. In contrast, TNFα did not affect the phosphorylation of Akt (Figure 67).

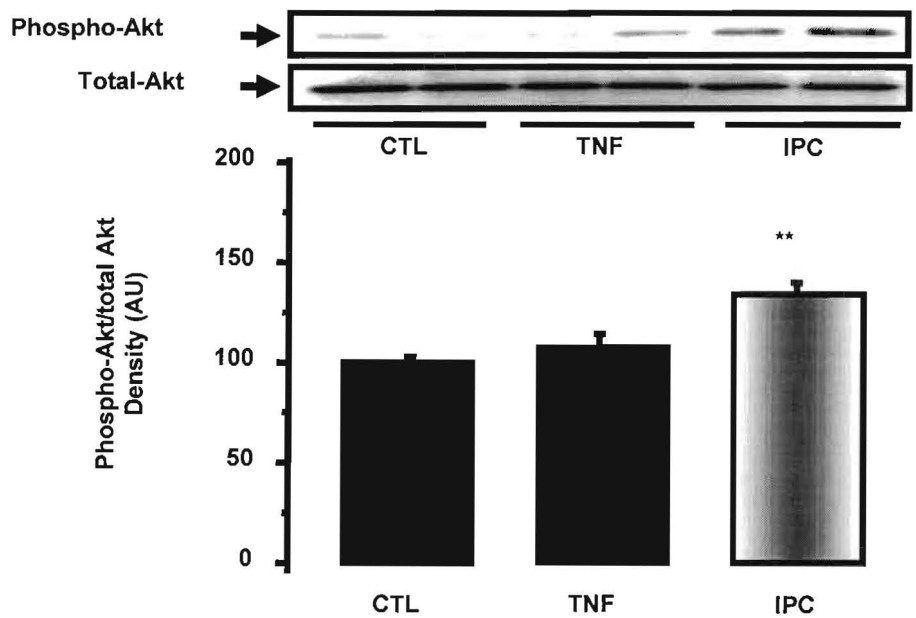

Figure 67: **Akt activation during early reperfusion.** Densities expressed in Arbitrary Units (AU) show that Akt is phosphorylated during early reperfusion following an ischaemic or TNFα preconditioning stimulus (**p< 0.01 vs control, n= 6 per group). IPC-ischaemic preconditioning.

4. Discussion

Using different models and animal species, our novel data show, apparently for the first time, that cardioprotection at reperfusion by TNFα is independent of activation of the pro-survival kinase Akt (part of the RISK pathway) (Hausenloy *et al.* 2004c). More importantly, we have shown the existence of an alternative protective pathway that involves activation of the transcription factor STAT-3 at the time of reperfusion in response to both TNFα and ischaemic preconditioning. The main data leading to these conclusions are as follows:

1. TNFα-induced preconditioning did not phosphorylate the kinase Akt at the time of reperfusion.
2. Specific inhibitors of Akt given during the reperfusion phase did not inhibit protection by TNFα.
3. TNFα and classic ischaemic preconditioning phosphorylated STAT-3 during early reperfusion.
4. Addition of the STAT-3 inhibitor AG490 abolished the protection afforded by both TNFα and ischaemic preconditioning as preconditioning stimuli.

As already mentioned in section E, the concept of multiple pathways in preconditioning has already been suggested in relation to the trigger phase (see review by Yellon *et al* (Yellon *et al.* 2003)). During the early phase of reperfusion, it is now well established that ischaemic preconditioning activates the RISK pathway (Tsang *et al.* 2004; Hausenloy *et al.* 2005). In addition, many pharmacological agents, such as adenosine agonists, bradykinin, atorvastatin, urocortin, insulin and cardiotrphin-1, all protect the heart by activating the RISK

pathway when given at the beginning of reperfusion (Hausenloy *et al.* 2004c). However, now for the first time, to our knowledge, we have found that a pharmacological preconditioning agent (TNFα) could confer cardioprotection independently of activation of the RISK pathway during early reperfusion. By contrast, we found that an alternative and RISK-independent pathway is activated by both classic ischaemic preconditioning and TNFα preconditioning. This novel pathway requires phosphorylation of STAT-3 at the time of reperfusion.

In section E, we have demonstrated the role of STAT-3 during the trigger phase in both TNFα preconditioning (Smith *et al.* 2004) and ischaemic preconditioning but not during the early reperfusion phase. Because we have already demonstrated that TNFα acts as a trigger in classic ischaemic preconditioning (Smith *et al.* 2002), we now postulate that ischaemic preconditioning activates STAT-3 *via* TNFα. However, further experiments will be required to determine whether STAT-3 may be activated in a TNFα-independent manner in ischaemic preconditioning. Our data with different pharmacological inhibitors suggest that Akt and STAT-3 are all essential for cardioprotection at reperfusion and that activation of these 2 components is required to reach a postulated threshold of protection in ischaemic preconditioning. Inhibiting one of these components means that the threshold for protection cannot be reached. As an example, Hausenloy *et al* (Hausenloy *et al.* 2004b) have shown that inhibiting Akt at the time of reperfusion with wortmannin blocks ischaemic preconditioning, even if the activity of ERK is not inhibited. These experiments demonstrated that, although ERK is not a component of a linear pathway nor is it downstream of Akt, the protection can be lost if Akt is blocked. Similarly, with TNFα, activation of

Akt is not essential to reach the threshold for protection by TNFα. Of note, other experiments that we have conducted in our Institute have also demonstrated that, unlike ischaemic preconditioning, TNFα-induced cardioprotection is independent of the activation of ERK at the time of early reperfusion (Lecour *et al.* 2005). Another possibility would be that STAT-3 is a downstream target of ERK and/or Akt. TNFα could directly activate STAT-3, whereas ischaemic preconditioning would activate STAT-3 *via* ERK and/or Akt. Further investigations will be required to determine the exact link or possible cross-talk between these different pathways. However, based on the results that we have obtained in section E and based on the work that has been described by Gross et al (Gross *et al.* 2006) during the trigger phase, we can postulate that STAT-3 activation may mediate Akt activation, during the early reperfusion phase following an ischaemic preconditioning protocol.

Although BAD, BAX, p70S6K and eNOS appear to be the downstream components responsible for mediating the protection associated with the activation of the RISK pathway at the time of reperfusion, (Hausenloy *et al.* 2004a) the cell survival components activated by the RISK-independent pathway still remain to be determined. Work that we have performed in our laboratory delineates the inhibition of the apoptotic promoting factor BAD as one of the components responsible for mediating the protection at reperfusion that is associated with the activation of STAT-3 (Lecour *et al.* 2005). Many of the anti-apoptotic pathways activated by prosurvival kinase cascades converge to the mitochondria. Juhaszova *et al* (Juhaszova *et al.* 2004) have recently shown that preconditioning prevents mitochondrial pore opening by activating ERK and Akt.

We have previously reported that pharmacological preconditioning with TNFα involves the mitochondrial potassium ATP-dependent channel (Lecour *et al.* 2002). It would be interesting to investigate whether STAT-3 activation, like the RISK pathway, confers protection by preventing the mitochondrial permeability transition pore opening, now postulated to be the end point of ischaemic preconditioning (Hausenloy *et al.* 2002; Hausenloy *et al.* 2004a).

In summary, the present study demonstrates that pharmacological preconditioning induced by TNFα does not require activation of the RISK pathway at early reperfusion. Moreover, we provide evidence for an alternative protective pathway that involves the activation of STAT-3 during early reperfusion. Delineating the exact pro-survival components involved in this RISK-independent pathway, as well as a possible link between these two pathways, is of current interest, as it may have potential therapeutic significance in the mitigation of reperfusion-induced damage.

G. Conclusion

Summary

Ischaemic Preconditioning was first described in 1986 as a procedure giving powerful protection against myocardial infarction (Murry *et al.* 1986). Despite extensive research, the cellular pathways and mechanisms involved in this protective phenomenon have not been fully clarified (see review (Yellon *et al.* 2003)), nor is it clear whether they converge mechanistically (Heusch 2004). TNFα is a pleiotropic cytokine that, in a dose- and time-dependent manner mimics ischaemic preconditioning (Lecour *et al.* 2002). STAT-3 is a transcription factor mediating intracellular signalling that is initiated at the cytokine cell surface receptor and can activate prosurvival pathways.

To further understand the signalling mechanisms of cardiac preconditioning, we aimed to explore the role of JAK2/STAT-3 signalling pathway in both ischaemic and TNFα-induced preconditioning.

The main and novel findings presented in this thesis are as follows:

1. Cre-LoxP technology can be used to successfully create cardiomyocyte specific STAT-3 knockout (KO) mice.

2. Genetic depletion of cardiac specific STAT-3 disrupts the intracellular signalling pathways of ischaemic and pharmacological preconditioning and abrogates its cardioprotective effect. Furthermore, cardioprotection by staged reperfusion is also lost in the STAT-3 KO mice.

3. STAT-3 phosphorylation is required during both the trigger and mediator phases in ischaemic and TNFα-induced preconditioning.

4. In contrast to ischaemic preconditioning, TNFα-induced preconditioning does not require Akt phosphorylation during the trigger or mediator phase to confer cardioprotection.

5. During ischaemic preconditioning, activation of Akt is dependent of the JAK/STAT-3 pathway.

In summary, we have shown the existence of an alternative protective pathway that involves activation of the transcription factor STAT-3 in response to both ischaemic and TNFα preconditioning. In addition, we have shown for the first time that cardioprotection with preconditioning can occur independently of the activation of the prosurvival kinase Akt. We also suggest that JAK2/STAT-3 is a key regulator of Akt in ischaemic preconditioning.

Study Limitations

A substantial part of this work was done on STAT-3 KO mice. The challenge of using data solely from KO animals is that genetic depletion of STAT-3 may modify cardiac development or function of the heart. Therefore, further characterisation of this STAT-3 KO mouse model is required. A detailed metabolic analysis of the STAT-3 KO hearts, in both young and older mice, is required to determine if the loss of cardioprotection may result from metabolic deficiencies. Hence, STAT-3 has been linked to carbohydrate metabolism (Inoue *et al.* 2004) and we can speculate that the regulation of carbohydrate metabolism in STAT-3 KO mouse hearts may be impaired. Furthermore, a detailed histology analysis of wild-type and STAT-3 KO mice hearts will be required to determine the presence of any structural irregularities due to STAT-3 depletion. An

increased susceptibility to heart disease has been reported in separate cardiac specific STAT-3 KO mice with advanced age (Jacoby *et al.* 2003).

To supplement data from STAT-3 KO mice, we used the pharmacological agent AG490 to inhibit STAT-3 phosphorylation. This inhibitor binds to JAK2, thereby preventing STAT-3 phosphorylation. Similarly, wortmannin inhibits PI3K mediated Akt phosphorylation. These two inhibitors do not target directly the proteins of interest. Direct inhibitors of these proteins are needed to confirm our results and better delineate the cross-talk between Akt and STAT-3. However, these are not commercially available yet. An alternative approach is the use of the silent interference ribonucleic acids (siRNA) technique (Frias *et al.* 2006) or over-expression of mutant dominant negative forms of STAT-3 within cardiomyocytes (Zhang *et al.* 2005). Based on our findings and the literature, we can speculate that direct, pharmacological, activation of STAT-3 will lead to cardioprotection. This can be achieved with the use of STAT-3 over-expressing mice (Negoro *et al.* 2001).

The models that we have used in this study are both *ex vivo* models of ischaemia/reperfusion injury. These models represent limitations in that they do not take into account the intracoronary activation of leukocytes, which may interfere in the pathways. It is important to confirm these results in an *in vivo* mouse model of cardiac ischaemia/reperfusion injury and determine whether genetic ablation and pharmacological inhibition of STAT-3 abrogates ischaemic and TNFα cardiac preconditioning.

Future Directions

STAT-3 is required for the transcriptional control of many proteins. To identify these proteins a proteomic study of basal and preconditioned STAT-3 KO mice is required. Target proteins for analysis include metallothionein-1 and 2 (Oshima *et al.* 2005), manganese superoxide dismutase (Negoro *et al.* 2001) and the Bcl-2 family of proteins (Hattori *et al.* 2001). These proteins have been linked with the protection induced by STAT-3 activation. In addition, the mitochondrial permeability transition pore (MPTP) is currently thought to be the end effector of cardiac preconditioning (see review by Hausenloy *et al* (Hausenloy *et al.* 2002)). A study of the effect of STAT-3 depletion on mitochondrial bioenergetics and the MPTP may provide a common end effector of both the JAK2/STAT-3 and PI3K/Akt signalling pathways.

The data described in this thesis demonstrates STAT-3 as an important signalling protein in cardiac preconditioning. However, in the clinical setting of acute myocardial infarction, the unpredictability of as ischaemic event prevents the successful application of preconditioning. Recently, Vinten-Johansen *et al* ((Zhao *et al.* 2003) have described a new cardioprotective phenomenon known as postconditioning. Postconditioning is a form of 'staged reperfusion' whereby the ischaemia/reperfusion cycles used for conditioning the heart are applied immediately after the index ischaemia. Similar to ischaemic preconditioning, ischaemic postconditioning limits the resultant infarct size (Zhao *et al.* 2003). Since it was first described in the canine heart model of ischaemia/reperfusion, ischaemic postconditioning has also been successfully applied as a mean of cardioprotection in rats (Zhu *et al.* 2006), mice (Heusch *et al.* 2006) and pigs

(Iliodromitis *et al.* 2006) models. More recently, ischaemic postconditioning has been applied clinically (Staat *et al.* 2005; Loukogeorgakis *et al.* 2006). Compared to preconditioning, ischaemic postconditioning provides a more attractive clinical potential and has therefore become a new focus of research and attention. Thus far, studies into the signalling mechanism of ischaemic postconditioning have revealed similarities between ischaemic preconditioning and postconditioning. For example, Yellon *et al* have identified the RISK (Reperfusion Injury Salvage Kinase pathway comprising of Akt and ERK 1/2 activation) as a point of convergence for the cardioprotective phenomena of ischaemic preconditioning and ischaemic postconditioning (Hausenloy *et al.* 2005). Therefore, our future experiments will aim to explore the role of STAT-3 in postconditioning as it may have an important application for the development of future drugs to protect against myocardial infarction.

H- Publications arising from this work

Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. RM Smith, **N Suleman**, L Lacerda, LH Opie, S Akira, KR Chien and MN Sack. Cardiovascular Research 2004 Sept 63:4: 611-6.

Pharmacological preconditioning with TNFα activates STAT-3 at reperfusion without involving the prosurvival kinases (Akt and Erk). S Lecour, **N Suleman,** GA Deuchar, S Somers, L Lacerda, B Huisamen and L H Opie. Circulation. 2005 Dec 20;112(25):3911-8.

Conference Outputs

Obligatory role for Signal Transducer and Activator of Transcription-3 in ischemic preconditioning. **N Suleman**, LH Opie, MN Sack and RM Smith. The 31st Annual Congress of the Physiological Society of Southern Africa 2003.

Obligatory role for Signal Transducer and Activator of Transcription-3 in ischemic preconditioning. **N Suleman**, LH Opie, MN Sack and RM Smith. South African Heart Association Meeting 2003.

Genetic ablation of cardiomyocyte STAT-3 abolishes classical pre-conditioning. **N Suleman**, LH Opie, MN Sack and RM Smith. International Society for Heart Research Satellite Meeting. Cellular Injury in Ischemia 2004.

TNFα protects cardiomyocytes via STAT-3 activation independently of PI3K/Akt. **N Suleman**, LH Opie, RM Smith and S Lecour . International Society for Heart Research European Section, Norway 2005. JMCC, 2005:38,997.

Genetic depletion of cardiomyocyte STAT-3 abolishes insulin induced cytoprotection. **N Suleman**, BN Fuglesteg, L Lacerda, C Tiron, R Smith, S Lecour, MN Sack, OD Mjøs and AK Jonassen. International Society for Heart Research European Section, Norway 2005. JMCC, 2005:38,997. (oral presentation)

Role of STAT-3 and PI3K/Akt as triggers in TNFα- or hypoxia-mediated preconditioning. **N Suleman**, LH Opie, RM Smith and S Lecour. American Heart Association 2005. Circulation, 2005, 112(17):II-228. (oral presentation)

TNFα protects cardiomyocytes via STAT-3 activation independently of PI3K/Akt. **N Suleman**, LH Opie, RM Smith and S Lecour. The 33rd Annual Congress of the Physiological Society of Southern Africa 2005

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