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Cardioprotective role of Signal Transducer Activator of Transcription 3 (STAT-3) against ischaemia reperfusion injuries

"At the heart of research"

University of Cape Town

Jonathan Chan King

KNGJON005

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Supervisor: Associate Professor Sandrine Lecour PharmD, PhD *

Co-supervisor: Professor Lionel H. Opie MD, DPhil, DSc, DM, FRCP, FCP*

* Hatter Cardiovascular Research Institute, Department of Medicine

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Please note that this thesis was served in concurrent registration with an MBChB. Hence at the start of the thesis much was unknown regarding the following work presented in this thesis. However, over the years, other researchers have made significantly advancements in the knowledge of ischaemia-reperfusion.

Plagiarism declaration

I, *Jonathan Chan King*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate 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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Date: 15 March 2011

Student Name: Jonathan Chan King

Student Number: KNGJON005

Signature: ....

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List of abbreviations

ADMIRE	AndreView Myocardial Imaging for Risk Evaluation
AMISTAD	Acute Myocardial Infarction STudy ADenosine
ANP	atrial natriuretic peptide
ANOVA	analysis of variance
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
Bcl-Xl	B-cell lymphoma-extra large
BH	Bcl-2 homology domain
BIM	Bcl-2-interacting mediator of cell death
BSA	Bovine serum albumin
cGMP	cyclic guanosine monophosphate
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DHS	dl-threo-dihydrosphingosine
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ERK	extracellular signal-regulated kinases
EDTA	ethylenediaminetetraacetic
FCS	fetal calf serum
FoxO	Forkhead box O
GAS	gamma activated sequence
GSK-3B	glycogen synthase kinase-3β
HDL	high density lipoprotein
IONA	Impact of Nicorandil in Angina
JAK	Janus kinase
JCAD	Japanese Coronary Artery Disease
J-WIND	Japan-Working groups of acute myocardial Infarction for the reduction of Necrotic Damage
JNK	Jun N-terminal kinase

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KO	knock out
LPP	lipid phosphate phosphatase
LVDP	left ventricular developed pressure
MAPK	mitogen activated protein kinase
MEK 1/2	mitogen activated protein kinase 1/2
MnSOD	manganese superoxide dismutase
MPG	N-2mercaptopropionylglycine
MPTP	mitochondrial permeability transition pore
mKATP	mitochondrial potassium ATP
NaOH	sodium hydroxide
NO	nitric oxide
NF-κB	nuclear factor kappa B
PBS	phosphate buffered saline
POST-AMI	POSTconditioning during Coronary Angioplasty in Acute Myocardial Infarction
PKC	protein kinase C
p38MAPK	p38 mitogen activated protein kinase
PMSF	phenylmethylsulfonyl fluoride
P-STAT-3	phosphorylated STAT-3
RPP	rate pressure product
SAFE	survivor activating factor enhancement
SDS	sodium dodecyl sulfate
Skp2	S-phase kinase-associated protein-2
Sgk	serum and glucocorticoid inducible protein kinase
Sirt1	silent information regulator 1
STAT	signal transducer and activator of transcription
TNFα	tumour necrosis factor alpha
TNFR1	TNFα receptor 1
TNFR2	TNFα receptor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRAF2	TNF receptor-associated factor 2

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TTC	triphenyltetrazolium chloride
T-STAT-3	total STAT-3
RISK	reperfusion injury salvage kinase
2-DG	2-Deoxy-d-Glucose

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Abstract

Introduction: Sphingosine 1 phosphate (S1P) is a major constituent of high density lipoprotein (HDL) cholesterol. Both S1P preconditioning and ischaemic postconditioning reduce myocardial damage following an ischaemia-reperfusion insult but the mechanisms involved remain unclear. Janus kinase/Signal transducer and activator of transcription 3 (JAK/STAT-3) form part of a recently discovered powerful prosurvival path termed as the Survivor Activating Factor Enhancement (SAFE) pathway. The SAFE pathway plays a critical role in ischaemic preconditioning to promote cell survival but whether activation of STAT-3 is required for S1P preconditioning and ischaemic postconditioning induced cardioprotection is unknown.

Hypothesis: Activation of the STAT-3 is required for S1P preconditioning and ischaemic postconditioning.

Methods: Mouse fibroblast L-cells were cultured and subjected to 8.5 hours of simulated ischaemia. Cells were preconditioned for 30 minutes with S1P with/without AG490, a STAT-3 pathway inhibitor. Isolated hearts from male Wistar rats were subjected to ischaemia-reperfusion, and preconditioned with a 7 minute episode of S1P with/without AG490. Hearts undergoing ischaemic postconditioning were subjected to 30 minutes of ischaemia followed by 6 cycles of intermittent 10 second cycles of ischaemia reperfusion at the onset of reperfusion with/without AG490. Cell survival, haemodynamic parameters, functional parameters, infarct size and western blots analysis were assessed as end points.

Results: S1P preconditioning improved cell survival in fibroblasts subjected to simulated ischaemia (38.1±1.5% vs. 30.0±0.0%, $p<0.05$ vs. control), an effect abolished in the presence of AG490 (29.9±2.7%, ns vs. control). S1P preconditioning also decreased infarct size (4.6±1.4% vs. 25.8±3.1%., $p<0.05$ vs. control), but this effect was abolished with AG490 (29.5±4.0%. ns vs. control). Similarly, ischaemic postconditioning decreased infarct size (11±1% vs. 32±5%., $p<0.05$ vs. control) but this infarct sparing effect was abolished with AG490 (26±4%, ns vs. control). Finally,

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ischaemia postconditioning resulted in the concomitant decrease in the cytosolic fraction and increase in the nuclear fraction of tyrosine phosphorylated STAT-3 (0.5±0.1A.U. vs. 1.6±0.3A.U., $p<0.05$ vs. control and 2.5±0.2A.U. vs. 1.3±0.4A.U., $p<0.05$ vs. control, respectively).

Conclusion: Our data suggest that both S1P preconditioning and ischaemic postconditioning can protect the heart similar to ischaemic preconditioning. This protection requires the activation of STAT-3, therefore suggesting that activation of the SAFE pathway is critical to conditioning of the heart. Our findings may lead to the development of novel therapies to protect the heart against ischaemic heart disease.

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Chapter One: Introduction

Cardiovascular disease is projected to be the leading cause of global mortality in 2020 according to the World Health Organisation (Murray and Lopez, 1997). South Africa is currently in a transitional state, burdened by the growing infectious diseases such as the HIV pandemic and emerging chronic diseases. Cardiovascular disease is the leading cause of chronic diseases and is currently the second most important cause of death in South Africa (Mayosi et al., 2009, Bradshaw et al., 2003) (Figure 1).

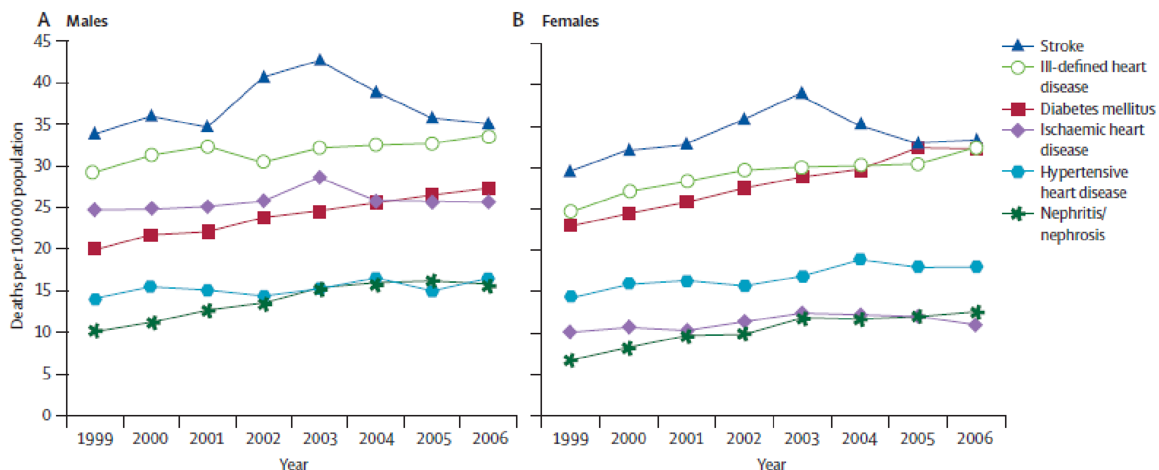

Figure 1: Mortality rate of the South African population due to cardiovascular disease

Males (A) and females (B) aged 15-64 years old die from different causes of cardiovascular disease represented deaths per 100 000 population (Mayosi et al., 2009).

The most prominent cardiovascular disease is coronary heart disease (Yusuf et al., 2004). It refers to the accumulation of an atherosclerotic plaque in coronary arteries, resulting in either partial or total vessel obstruction. The obstruction results in a reduction of blood flow, nutrients and oxygen to the cardiac tissue distal to the obstruction. Should the obstruction continue, the cardiac tissue will progressively become ischaemic and place the ischaemic tissue at risk of cell death. Should no intervention take place to relieve the obstruction, the ischaemic tissue will undergo

irreversible cell death and hence would be referred to as having undergone infarction (Figure 2).

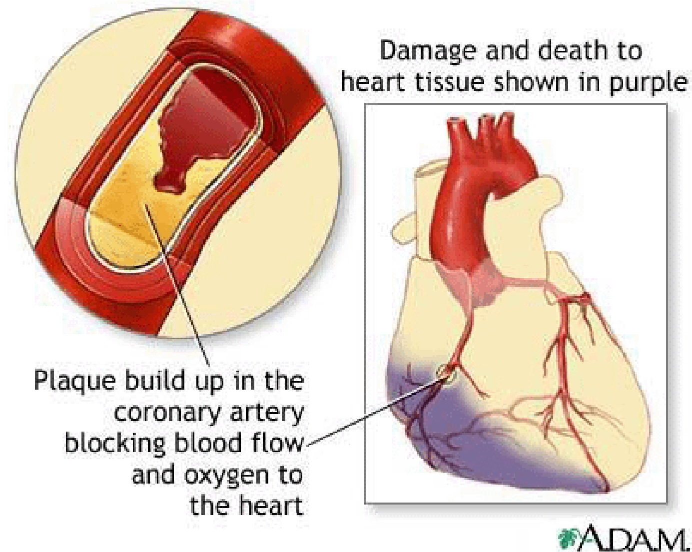

Figure 2: Graphic representation of an acute myocardial infarction

Patients with coronary artery disease can develop a blocked coronary artery, typically the result of atherosclerotic build up within the coronary with or without the addition of a thrombus (from www.adam.about.com/encyclopedia/acute.mi.html)

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The extent and severity of a myocardial infarct has long been known to prognosticate a patients outcome (Braunwald, 1974) and extensive research was focused on how to decrease the amount or size of myocardial infarction as a result of ischaemia.

Early research indicated that the best intervention to salvage ischaemic cardiac tissue was to restore blood flow or reperfuse the ischaemic tissue (Maroko et al., 1972). Clinically, reperfusion can be achieved with the use of thrombolytic drugs or by coronary balloon angioplasty (Vinten-Johansen et al., 2007). However, whilst reperfusion is overall beneficial, it does paradoxically lead to additional tissue damage, an effect known as ‘reperfusion injury’ (Murry et al., 1986). Reperfusion injury can account for up to 50% of the final infarcted tissue as a result of endothelial damage, arrhythmias and stunned myocardium (Yellon and Hausenloy, 2007).

1. Concept of conditioning

1.1 Preconditioning versus postconditioning

In 1986, Murray et al. discovered an intrinsic cardioprotective mechanism against reperfusion injury and called the phenomenon 'ischaemic preconditioning'. It involved four cycles of five minutes of ischaemia with intermittent reperfusion prior to the main ischaemic insult. The result was a decrease in infarct size by 75% (Murry et al., 1986).

Brief repetitive episodes of ischaemia decreased the rate of adenosine triphosphate (ATP) depletion during subsequent ischaemic episodes (Reimer et al., 1986) and intermittently washed away many harmful metabolites such as lactate, hydrogen ions and ammonia (Murry et al., 1986). As a result, more cardiac tissue was salvaged which was reflected as a decrease in infarct size.

Seventeen years later, Vinten-Johansen and colleagues (2003) discovered that similar cycles of ischaemia and intermittent reperfusion occurring after the main ischaemic insult, resulted in a similar decrease in infarct size as seen in ischaemic preconditioning. This new phenomenon was called ischaemic postconditioning (Zhao et al., 2003) (Figure 3).

The protective effect of ischaemic postconditioning was hypothesized to be the result of delayed realkalinisation of the acidic ischaemic tissue, which in turn, delays the accumulation of intracellular calcium ions. Hence, ischaemic contractures due to intracellular calcium ion overload are avoided (Piper et al., 2003).

Ischaemic preconditioning allows the heart to initiate not only protective mechanisms prior to ischaemia but also after ischaemia and hence during the reperfusion phase (Hausenloy et al., 2005). In contrast, postconditioning can only activate protective mechanisms at the onset of reperfusion (Crisostomo et al., 2006). Despite these differences, many signalling pathways have been found to be shared (Crisostomo et al., 2006, Vinten-Johansen et al., 2007, Hausenloy and Yellon, 2007).

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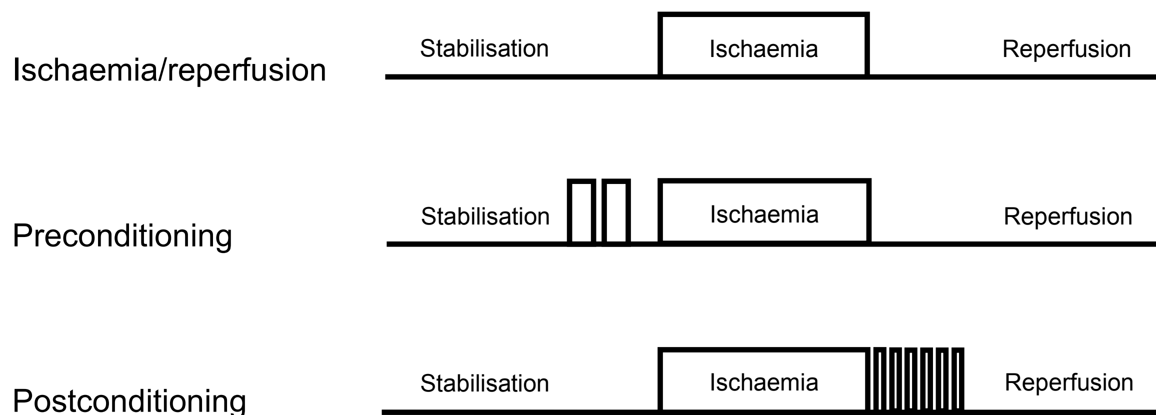

Figure 3: Schematic representation of preconditioning and postconditioning

Standard ischaemia reperfusion protocol involved 30 minutes of stabilisation, followed by 30 minutes of index ischaemia and reperfusion for 2 hours. Preconditioning protocol involved 2 cycles of 5 minutes of ischaemia and reperfusion followed by 10 minutes of reperfusion. Postconditioning involved 6 cycles of ischaemia and reperfusion for 10 seconds each.

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1.2 Classical versus delayed conditioning

Classical preconditioning or the first window of protection has been discussed and refers to the initial phase of protection from ischaemia. It is robust and lasts between one and two hours after the main ischaemic event. Delayed or the second window of protection refers to the period in which protection from ischaemia can occur 24 hours after the main ischaemic event, lasting between 48-72 hours (Bolli et al., 2007) (Figure 4).

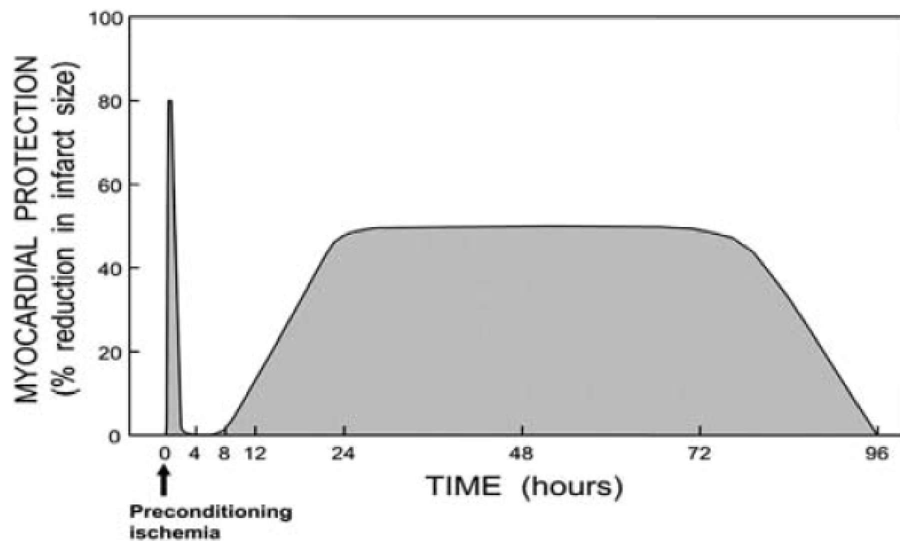

Figure 4: Schematic diagram showing two phases of protection from ischaemia

The classical preconditioning occurring early lasting 2 to 4 hours. The delayed phase occurring only 24 to 48 hours later and lasting 3 to 4 days (Marber et al., 1993).

There are important distinctions between classical and delayed preconditioning mechanisms. The former is the result of immediate change of pre-existing proteins whilst the latter involves newly synthesized proteins. As a result, the former only limits the progression of the final infarct size while the latter will also preserve the functional capacity of the myocardium (Bolli et al., 2007).

Similar to classical preconditioning, classical postconditioning requires intermittent episodes of ischaemia to be applied ideally immediately after the onset of reperfusion or postponed until up to one minute after reperfusion. Thereafter, protection from postconditioning was lost (Kin et al., 2004).

Delayed postconditioning has been sparsely explored in the heart with a few studies in the brain. Delayed postconditioning has been found to be effective in the brain following an episode of a stroke three to six hours after the main ischaemic event (Ren et al., 2008, Berdeaux, 2007, Ren et al., 2009).

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1.3 Pharmacological conditioning

Certain pharmacological compounds can substitute ischaemia as triggers for preconditioning like protection (Bolli, 2001). Such pharmacological compounds allow easier clinical application via medical rather than surgical methods (Granfeldt et al., 2009). Many of these pharmacological compounds mimic the innate production of compounds that otherwise would have been produced as a result of ischaemic preconditioning.

Compounds that can imitate preconditioning include adenosine (Liu et al., 1991), bradykinin (Goto et al., 1995), opioid agonists (Schultz et al., 1995), ethanol (Krenz et al., 2001), atrial naturetic peptide (ANP) (Okawa et al., 2003), cyclosporine (Yang et al., 2003), nicorandial (Mizumura et al., 1997), statins (Jones and Lefer, 2001) and tumour necrosis factor alpha (TNFα) (Lecour et al., 2002).

Since many signalling pathways are shared between preconditioning and postconditioning, not surprisingly many of these compounds can initiate postconditioning. These include adenosine (Yang et al., 2005) , bradykinin (Penna et al., 2007), opioid agonists (Zatta et al., 2008), ANP (Takagi et al., 2000), cyclosporine (Argaud et al., 2005a) and TNFα (Lacerda et al., 2009). Statins however have not been found to not be effective in postconditioning (Kocsis et al., 2008).

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1.4 Remote conditioning

1.4.1 Remote preconditioning

In 1993, Pryzyklenk demonstrated that ischaemic preconditioning in one region of the heart conferred protection to another region of the heart. Both regions were distinct in their different blood supply and the modified form of preconditioning was called remote preconditioning (Przyklenk et al., 1993).

Subsequently, it was shown that preconditioning other organs conferred protection to heart. These other organs include skeletal muscle (Addison et al., 2003), intestine (Gho et al., 1996) and kidney (Pell et al., 1998).

Whilst the mechanism of action of remote preconditioning is almost identical to ischaemic preconditioning, there are three hypotheses regarding the communication of remote ischaemia to protect other organs. The first hypothesis is the neuronal hypothesis where local release of adenosine and bradykinin can stimulate local nerve endings to signal other organs via neuronal signals. The second hypothesis is the humoral hypothesis where local release of adenosine and bradykinin can be released into the blood stream for systemic signalling. The third hypothesis is the 'inflammatory suppression hypothesis' in which a systemic anti-inflammatory and anti-apoptotic phenotype is induced resulting in protection. It is likely that all three hypotheses play a role, however the mechanisms involved are still unclear (Hausenloy and Yellon, 2008).

1.4.2 Remote postconditioning

Application of ischaemia in remote postconditioning is similar to remote preconditioning but it can occur during two time points. The first is referred to as 'perconditioning' whereby ischaemic postconditioning is applied to the remote organ during myocardial ischaemia (Kerendi et al., 2005). The second refers to the traditional sense of ischaemic postconditioning where intermittent ischaemia is applied immediately after the onset of reperfusion locally (Andreka et al., 2007).

The lower limbs (Andreka et al., 2007) and the kidney (Kerendi et al., 2005) have been demonstrated to confer protection to the heart in ischaemic postconditioning. Similar to Pryzyklenk experiments, remote postconditioning of one region of the heart conferred protection to another region of the heart undergoing the main ischaemic event (Gritsopoulos et al., 2009).

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1.4.3 Other forms of preconditioning

Rapid right ventricular pacing (Koning et al., 1996), transient hyperthermia (Yamashita et al., 2000) and acute myocardial stretch (Ovize et al., 1994) due to acute volume overload have also been shown to induce preconditioning.

Gene therapy has been studied to impart long term beneficial effects of preconditioning. Specific genes relating to the pharmacological compounds that are already known to be protective are used to up regulate certain beneficial enzymes including cyclo-oxygenase-2 (Bolli et al., 2007), endothelial nitric oxide synthase and inducible nitric oxide synthase (Bolli, 2001).

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1.5. Clinical application of conditioning

1.5.1 Clinical application of ischaemic preconditioning and postconditioning

Many clinical studies have been undertaken to exploit the clinical benefit of modifying reperfusion injury to decrease infarct size. Most of these early studies used ischaemia as a preconditioning or postconditioning stimulus (Von Klemperer, 2010).

Clinical applications of ischaemic preconditioning included intermittent reperfusion prior to coronary artery bypass grafting (Yellon et al., 1993) and ischaemic preconditioning as an adjunct to cardioplegic arrest (Illes and Swoyer, 1998). These studies showed a 75% improvement of ATP preservation in cardiac biopsy and a 32% improvement of functional outcomes at 12 hours post bypass respectively. In particular, the latter did not show any improvement in morbidity or mortality.

Clinical application of ischaemic postconditioning included intermittent reperfusion with coronary balloon angioplasty within six hours after the onset of a myocardial infarction (Staat et al., 2005). In a similar study, long term follow up was conducted (Thibault et al., 2008). These studies showed a 36% reduction in infarct size as

measured as the area under the creatine kinase curve and a 7% increase in left ventricular ejection fraction at 1 year follow up respectively. A retrospective analysis of such trials showed that either three (Darling et al., 2007), or four or more balloon inflations were beneficial when applied at reperfusion (Wang et al., 2009a). The most recent trial employing ischaemic postconditioning is the upcoming POSTconditioning during Coronary Angioplasty in Acute Myocardial Infarction (POST-AMI) trial, the results of which are pending since the authors are still collecting data (Tarantini et al., 2010).

Due to the success of these clinical trials, researchers have collaborated to form guidelines and position papers on the future of conditioning in heart disease. The aim is to hopefully enhance the success of other novel bench to bedside translational strategies for cardiac protection (Hausenloy et al., 2010a, Ovize et al., 2010).

1.5.2 Clinical evidence of delayed conditioning

Similar to classical preconditioning, many clinical applications of delayed preconditioning are restricted to events involving reperfusion injury which can be reliably predicted. Pre-infarct angina defined as angina experienced within 24-48 hours of an acute myocardial infarct has been used as a surrogate for ischaemic preconditioning. Thus, patients who experienced pre-infarct angina prior to coronary balloon angioplasty had a significantly better regional wall motion as compared to their control (Noda et al., 1999). Similar patients who underwent coronary artery bypass grafting had reduced creating kinase release (Vahlhaus et al., 2005). Unfortunately, no clinical application of delayed postconditioning has been explored.

1.5.3 Clinical application of pharmacological conditioning

The most notable pharmacological preconditioning compound first tested was adenosine which was perfused prior to thrombolytic therapy. Adenosine was studied in the Acute Myocardial Infarction STudy ADenosine (AMISTAD) trial (Mahaffey et al., 1999) and only after subgroup analysis did results show that adenosine was

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protective in large infarcts involving the anterior descending coronary artery (Ross et al., 2005). Hence AMISTAD II was conducted (Ross et al., 2005) but again, only after subgroup analysis did results show that adenosine was protective if infused within three hours of onset of a myocardial infarction (Kloner et al., 2006). The AndreView Myocardial Imaging for Risk Evaluation (ADMIRE) trial tested the synthetic adenosine agonist AMP579 which did not reduce infarct size (Kopecky et al., 2003).

Another preconditioning mimetic nicorandil was tested in the Impact of Nicorandil in Angina (IONA) study (2002) and most recently the Japanese Coronary Artery Disease (JCAD) study (Horinaka et al., 2010). Both studies showed that nicorandil, an activator of mitochondrial potassium ATP channels and nitric oxide synthesis, significantly decreased major coronary events. However, the relative contribution between its two effects has yet to be determined and how does the former effect compare to traditional nitric oxide donors and modification of disease, rather than just symptomatic treatment (Kojda, 2002).

The peptide hormone, ANP has been used as a postconditioning agent in the Japan-Working groups of acute myocardial Infarction for the reduction of Necrotic Damage (J-WIND) trial. Interestingly, the results of trial showed that nicorandil did not have any beneficial effect but ANP did (Kitakaze et al., 2007). It has been proposed by the same authors that nicorandil should be administered chronically and ANP should be perfused acutely for cardioprotection (Asakura and Kitakaze, 2010).

Cyclosporine has been evaluated as a postconditioning agent in the setting of standard coronary balloon angioplasty which has resulted in a decrease in infarct size as measured by area under the curve of creatine kinase (Piot et al., 2008). Cyclosporine is a known inhibitor of the mitochondrial permeability transition pore (Argaud et al., 2005a, Griffiths and Halestrap, 1993) and the pore itself has been postulated as the site of convergence for all conditioning stimuli (Hausenloy et al., 2002).

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1.5.4 Clinical application of remote preconditioning

The clinical advantage of remote preconditioning is the removal of placing additional ischaemia at the site that is already ischaemic. In 2000, Günaydin explored the effect of inflation/deflation of blood pressure cuffs prior to coronary artery surgery in patients. His results showed that only lactate dehydrogenase levels were significantly increased after the removal of the aortic cross clamp as compared to the control which implied that preconditioning protected the heart via anaerobic glycolysis (Gunaydin et al., 2000). Similar methods of inducing remote preconditioning have been used in paediatric patients (Cheung et al., 2006), adults undergoing coronary artery bypass grafts (Hausenloy et al., 2007), coronary stenting (Hoole et al., 2009) or abdominal aneurysm repairs (Ali et al., 2007).

1.5.5 Clinical application of remote postconditioning

The effect of remote postconditioning has been evaluated in healthy patients and in patients with atherosclerotic disease. Only endothelial function was assessed showing benefit of preserving flow mediated vasodilation after ischaemia (Loukogeorgakis et al., 2006), an effect blocked by the potassium ATP channel blocker glibenclamide (Loukogeorgakis et al., 2007). The protective role of remote postconditioning during cardio pulmonary resuscitation is still under debate (Mouton et al., 2010).

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1.6 Signalling pathways in conditioning

Since there are a variety of mechanisms involved in conditioning, they have conveniently been divided into cell surface receptor activation, signal transduction pathways and end effectors.

1.6.1 Cell surface receptors

Common to preconditioning and postconditioning, are the activation of cell surface receptors. These receptors are commonly G-protein coupled receptors which activate intracellular signal transduction pathways (Hausenloy and Yellon, 2007) (Hausenloy, 2009).

Adenosine was one of the first agents to be discovered as a preconditioning mimetic (Liu et al., 1991) and later as a postconditioning mimetic (Kin et al., 2005). Of the four subtypes, adenosine receptors A1 and A3 are involved in preconditioning and A2B in postconditioning (Jacobson, 2009). The outcome of adenosine's many beneficial effects include coronary artery dilation and negative inotropy (Meldrum et al., 1996).

Bradykinin has been described in preconditioning (Wall et al., 1994) and postconditioning (Penna et al., 2007). Bradykinin acts via its B2 receptor and signals through many mediators such as cyclooxygenase, prostacyclin (Penna et al., 2008), cyclic guanine monophosphate and reactive oxygen species (Penna et al., 2007). Similar findings have now been extended to human atrial trabeculae studies (Lemoine et al., 2010). Bradykinin has been used as a preconditioning mimetic in patients undergoing coronary artery bypass grafting resulting in a decreased infarct size as measured by the area under the curve of creatine kinase release (Wang et al., 2009b)

Statins or hydroxymethylglutaryl-coenzyme A reductase inhibitors are protective in preconditioning (Thuc et al., 2010) but not in postconditioning (Kocsis et al., 2008). Whilst statins do not bind directly onto receptors, they induce intracellular changes

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that result in the increased expression of low density lipoprotein cell surface receptors. In preconditioning, statins mediates its effects via protein kinase C dependent nitric oxide production (Bao et al., 2007), dampening the reactive oxygen species burst, hence preventing mitochondrial potassium ATP (mKATP) channel opening (Thuc et al., 2010). Their beneficial effects include lowering cholesterol levels, releasing nitric oxide (Laufs et al., 1998) and decreasing neutrophil infiltration (Lefer et al., 2001). In postconditioning, it is hypothesised that the protocol for inducing postconditioning is too brief for statins to have a measurable benefit (Kocsis et al., 2008, Iliodromitis et al., 2010).

Ethanol is a known preconditioning (Krenz et al., 2001) and postconditioning mimetic (Li et al., 2010). Ethanol cardioprotects via protein kinase C activation but is independent of free radicals and mKATP channels (Krenz et al., 2002). However if ethanol is also perfused during the index ischaemia, the cardioprotective effect of ethanol is lost (Churchill et al., 2008, Krenz et al., 2002). Such findings are in agreement with human studies were patients treated with ethanol during percutaneous coronary intervention had adverse effects (Niccoli et al., 2008).

TNFα can precondition the heart albeit at low doses (Eddy et al., 1992). TNFα is also a necessary requirement for ischaemia preconditioning (Smith et al., 2002) and most recently it was confirmed to be involved in ischaemic postconditioning (Lacerda et al., 2009). This protective effect of TNFα is thought to be mediated via the activation of its cell surface receptors type 2 (Lacerda et al., 2009).

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1.6.2 Signal transduction pathways

Several signal transduction pathways have been elucidated for preconditioning but only few have been involved in preconditioning and postconditioning. These include the survivor activating factor enhancement (SAFE) pathway (see section 2), the reperfusion injury salvage kinase (RISK) pathway, reactive oxygen species, protein kinase C and the nitric oxide-cGMP-PKG signalling cascade (Figure 5).

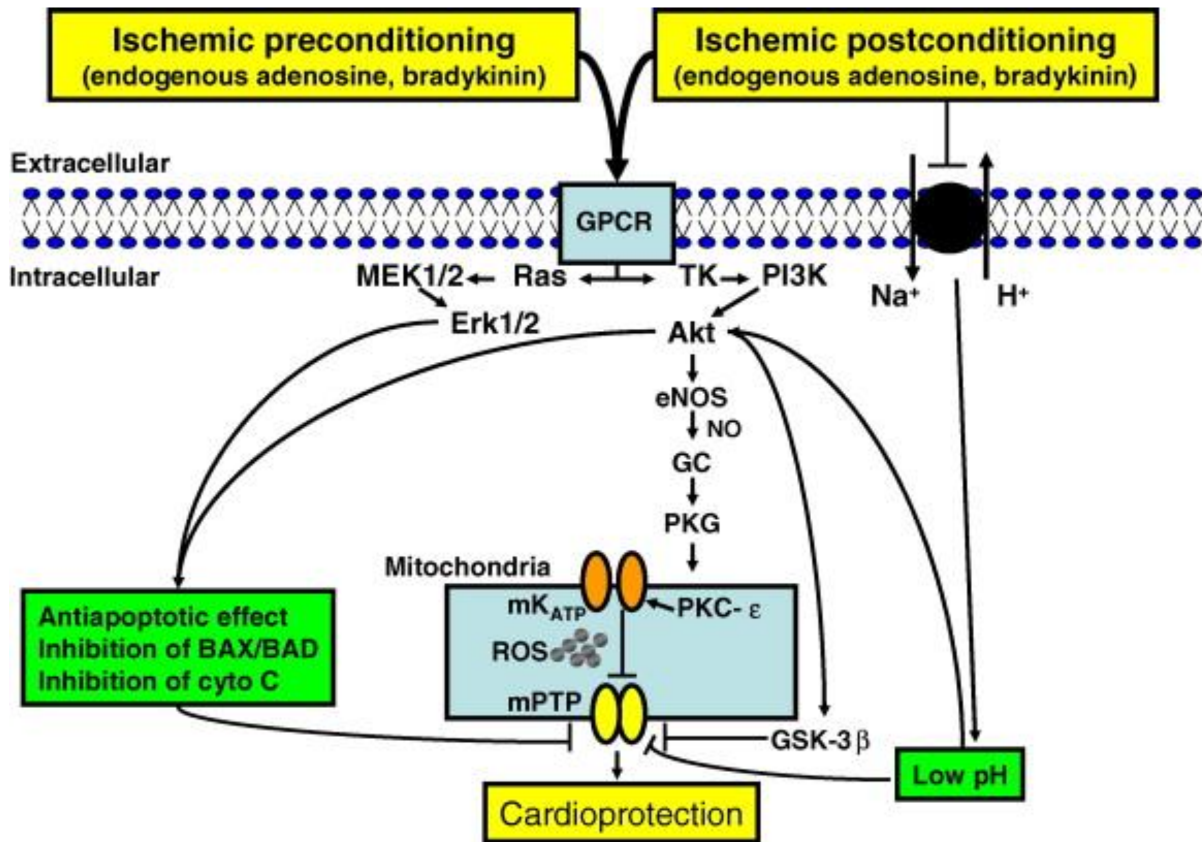

Figure 5: Schematic diagram representing the signalling pathways of ischaemic preconditioning and postconditioning

The signalling pathways are proposed to merge at the mitochondrial permeability transition pore as the common end effector. Abbreviations are provided in the text (Hausenloy and Yellon, 2007).

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1.6.2.1 The reperfusion injury salvage kinase (RISK) pathway

Activation of the RISK pathway occurs after ischaemic preconditioning (Tong et al., 2000) and postconditioning (Tsang et al., 2004). Comprised of phosphatidylinositol-3-kinase, protein kinase B (PI3K-Akt), and extracellular signal-regulated kinases (ERK 1/2), these prosurvival kinases form part of the reperfusion injury salvage kinase (RISK) pathway (Hausenloy and Yellon, 2004). Later, other mediators were included as downstream mediators, such as protein kinase C (PKC), protein kinase G (PKG), p38 mitogen activated protein kinase (p38MAPK), Jun N-terminal kinase (MAPK), and glycogen synthase kinase-3β (GSK-3B) (Hausenloy et al., 2010b).

During preconditioning, the RISK pathway was found to have a bi-phasic response. The first phase occurs immediately after the preconditioning stimulus and prior to the index ischemia. The second phase occurs immediately after the index ischaemia during the onset of reperfusion (Hausenloy et al., 2005). Both phases were required to mediate protection with preconditioning (Hausenloy et al., 2005).

During postconditioning, activation of the RISK pathway is analogous to the second phase, as previously described in preconditioning. PI3K-Akt is implicated in ischaemic postconditioning as selective inhibition of PI3K by either wortmannin (an irreversible inhibitor of PI3K) or LY 294002 (a reversible less potent inhibitor of PI3k as compared to wortmannin) abrogated its protective effects (Tsang et al., 2004). Similarly, addition of PD-98059, an indirect ERK 1/2 antagonist via the direct antagonism of upstream mitogen-activated protein kinase 1/2 (MEK1/2), abrogated the protective effects of ischaemic postconditioning (Darling et al., 2005).

1.6.2.2. Reactive oxygen species

Reactive oxygen species (ROS) have concentration dependent effects. At low concentration, ROS are protective in preconditioning and postconditioning (Penna et al., 2009). At high concentration, ROS are deleterious resulting in membrane lipid and DNA oxidation (Zweier et al., 1987). Low ROS concentrations act as signalling molecules to activate survival kinases (Samavati et al., 2002), an effect also seen in human studies (Loukogeorgakis et al., 2010). Blockade of low ROS concentration with oxygen radical scavengers (such as superoxide dismutase) (Tanaka et al., 1994) or N-2mercaptopropionylglycine (MPG) (Baines et al., 1997) abolish the protective role of preconditioning.

1.6.2.3 Protein Kinase C

Protein kinase C (PKC) has been demonstrated to have a protective role in ischaemia reperfusion (Yamashita et al., 1994), delayed preconditioning (Baxter et

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al., 1995), ischaemic preconditioning (Hausenloy et al., 2005) and postconditioning (Penna et al., 2006b). Following adenosine A2B receptor stimulation, PKC is activated (Philipp et al., 2006) resulting in PKC localising to the mitochondria to inhibit mitochondrial permeability transition pore (MPTP) opening (Jaburek et al., 2006).

Alternatively, PKC can activate the PI3K-Akt pathway downstream and affect the ratio of antiapoptotic and proapoptotic proteins to promote cell survival (Hausenloy and Yellon, 2004). It remains unknown whether the change in apoptotic protein is as a result of direct interaction with PKC or via the PI3K-Akt pathway (Basu and Sivaprasad, 2007).

1.6.2.4 The nitric oxide-cGMP-PKG-signalling cascade

This pathway has been implicated in preconditioning (Lochner et al., 1998) and postconditioning (Penna et al., 2006a). Nitric oxide (NO) can activate guanylate cylase to result in the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). In turn, low concentrations of cGMP inhibit phosphodiesterase while high concentrations of cGMP activates PKG (Schulz et al., 2004). PKG mediates the protective effects of this pathway via regulation of calcium homeostasis (Piper et al., 2004), mediate MPTP opening (Costa et al., 2005) and prevent apoptosis via anti-apoptotic B-cell lymphoma 2 (Bcl-2) (Razavi et al., 2005).

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1.6.3 End effectors

Mitochondria provide energy for the cell but also play a critical role in determining the cells fate (Zamzami et al., 1996). In fact, the signalling pathways converge on the mitochondria to initiate either prosurvival or apoptotic pathways during ischaemia reperfusion injury (Baines, 2009). Research has primarily focused on the opening of mitochondrial potassium ATP (mKATP) channel and closure of the MPTP as prosurvival mechanisms during reperfusion (Mykytenko et al., 2008, Yang et al., 2004).

1.6.3.1 Mitochondrial potassium ATP channel

Despite disagreement on the existence of the putative mKATP channel (Halestrap et al., 2007), many researchers have continued to explore the protective effect of the mKATP channel (Costa and Garlid, 2009). Opening of the mKATP channel was found to be beneficial in ischaemia reperfusion (Garlid et al., 1997), ischaemic preconditioning (Garlid et al., 1996) and postconditioning (Krolikowski et al., 2005). The physiological role of the mKATP channel is proposed to help stabilise the mitochondria during increased inotropy demand by the heart. Hence, with increase demand for ATP, opening of the mKATP channel results in stabilisation of the inner mitochondrial membrane to prevent disruption of the voltage dependent anion channel and hence allow efficient energy transfer (Costa and Garlid, 2009).

During reperfusion, it is proposed that numerous signals converge to open the mKATP channel via either PKG or protein kinase C epsilon (PKCε) which then phosphorylate receptors 1 and 2 on the outer mitochondrial membrane respectively. These receptors then phosphorylate PKCε on the inner membrane to open the mKATP channel, resulting in potassium influx and ROS production via complex I to alkalinise the inner membrane (Costa and Garlid, 2009, Costa et al., 2006). In addition to mKATP opening, ROS signalling results in the inhibition of MPTP opening (Krolikowski et al., 2005, Costa and Garlid, 2009). Hence, many are now considering the mKATP to have a signalling role rather than being an end effector (Pain et al., 2000).

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1.6.3.2. Mitochondrial permeability transition pore (MPTP)

MPTP closure is important in ischaemic preconditioning (Hausenloy et al., 2002) and postconditioning (Argaud et al., 2005b). MPTP closure prevents dissipation of the proton electrochemical gradient necessary for aerobic energy generation and prevents influx of small proteins and water inside the mitochondria. Hence, mitochondrial swelling is prevented, with no rupture and release of pro-apoptotic proteins into the cytosol (Baines, 2009) and a concomitant reduction in the

availability of ATP (Halestrap et al., 2004). Therefore many signalling pathways converge on the MPTP including PKG, PKC, mKATP, Akt, Erk 1/2 and GSK-3β (Hausenloy et al., 2009).

2 The Survivor Activating Factor Enhancement (SAFE) pathway

The Survivor Activating Factor Enhancement (SAFE) pathway has recently been described (Lecour, 2009b) and is activated during ischaemic preconditioning (Lecour et al., 2005b). Evidence has demonstrated that the SAFE pathway is an alternative protective pathway as compared to the RISK pathway. However, cross-talk or additive effects between the two pathways have not been clarified (Lecour, 2009a, Lecour, 2009b).

The SAFE pathway is comprised of TNFα, its cell surface receptors and its signal transduction pathway, the Janus kinase/Signal transducer and activator of transcription 3 (JAK/STAT-3).

2.1 TNFα and the SAFE pathway

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TNFα and its similar isoform TNFβ, have many functions including their regulation of inflammation, cell survival, growth, differentiation and apoptosis (Meldrum, 1998). TNFα is not only produced by immune cells but also by nucleated cells, including myocardial cells (Mann, 2003).

2.1.1. TNFα receptors

TNFα has two cell surface receptors, namely TNFα receptor 1 (TNFR1) and 2 (TNFR2) otherwise known as CD120a/p55/60 or CD120b/p75/80 respectively. (Krown et al., 1995, Torre-Amione et al., 1995). In general, TNFR1 mediates proinflammatory and programmed cell death pathways while TNFR2 mediates tissue repair and angiogenesis (Kleinbongard et al., 2010). In fact, it was recently described that TNFα binds to TNFR2 to mediate its protective effect via the SAFE pathway (Lacerda et al., 2009).

Interestingly, a TNFα binding protein on the mitochondria has been demonstrated, suggesting a direct link of TNFα to the mitochondria, independent of cell surface receptor activation (Ledgerwood et al., 1998, Busquets et al., 2003, Lacerda et al., 2010).

2.1.2. TNFα and ischaemia reperfusion

The role of TNFα in ischaemia reperfusion is controversial in humans. Although it is elevated after an acute myocardial infarction, interventions aimed at reducing TNFα by anti-TNFα antibodies resulted in increasing mortality (Mann, 2002). Conversely, animal models have shown beneficial effects of TNFα blockade (Gurevitch et al., 1997, Belosjorow et al., 2003) or genetic lack thereof (Maekawa et al., 2002). Hence there is a disparity in clinical evidence versus basic research.

Such disparities can be explained by the differential effects of concentration, timing, duration of treatment, or recruitment of different receptors (Schulz and Heusch, 2009). High concentrations of TNFα can be deleterious as opposed to low concentrations (Deuchar et al., 2007, Sack, 2002, Lecour et al., 2005b, Lecour et al., 2002). With increasing duration of ischaemia, TNFα is released initially from resident mast cells (Gilles et al., 2003) and macrophages but later from the myocardium (Dorge et al., 2002).

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2.1.3 TNFα and conditioning

The role of TNFα in humans has been implicated in remote ischaemic preconditioning (Zhou et al., 2010) and ischaemic postconditioning (Lin et al., 2010). Remote ischaemic preconditioning was conducted in paediatric patients undergoing open heart surgery resulting in a significant post-operative decline of TNFα. Post-operative morbidity and mortality were not affected. However, it was postulated that sicker patients are likely to benefit from the reduced post-operative ischaemia reperfusion injury and systemic inflammatory response syndrome (Zhou et al., 2010). Ischaemic postconditioning after an acute myocardial infarction resulted in

decreased TNFα levels and it was proposed that TNFα concentration and wall motion score index were valuable prognostic markers (Lin et al., 2010).

TNFα in animal models have been demonstrated to acts as a preconditioning (Lecour et al., 2005b) and postconditioning (Lacerda et al., 2009) mimetic. Similarly, blockade of TNFα with antibodies (Yamashita et al., 2000) or use of TNFα knockout mice during preconditioning (Smith et al., 2002) can increase infarct size. Similarly, TNFα knockout mice cannot be postconditioned (Lacerda et al., 2009). Interestingly, exogenous TNFα can only protect the heart if there is a washout period prior to the main ischaemic event suggesting that TNFα induced protection is time dependent (Lecour, 2009a, Lecour et al., 2002). Furthermore low concentrations of TNFα can preconditioning the heart but higher concentrations of TNFα are deleterious, demonstrating that TNFα's cardioprotective effect is also concentration dependent (Lecour et al., 2002).

2.2 JAK/STAT-3 and the SAFE pathway

Activated during episodes of stress, the JAK/STAT-3 pathway transmits signals from the cell surface to the nucleus to modulate gene expression (Bolli et al., 2003). Various ligands can bind to their cell surface receptors including interleukin 6, growth factors and TNFα. Thereafter, the associated cytosolic JAK homodimerises and phosphorylates to transphosphorylate and activate STAT-3 (Meyer et al., 2003). Activated STAT-3 can then form homodimers or heterodimers with other members of the STAT family. These include STAT-1 to STAT-6, however only STAT-3 will be considered in this thesis because thus far only STAT-3 has been proposed to be involved in the SAFE pathway (Lecour, 2009a). However, it does not preclude the importance of other STAT isoforms such as STAT-1, the benefits of which are discussed elsewhere (Boengler et al., 2008b). Dimerised STAT-3 translocates to the nucleus to bind to interferon gamma activated sequence (GAS) motifs to promote end target genes (Levy and Darnell, 2002) (Figure 6). In addition, STAT-3 has the potential to regulate mitochondrial function and thus is proposed to regulate MPTP opening (Boengler et al., 2010).

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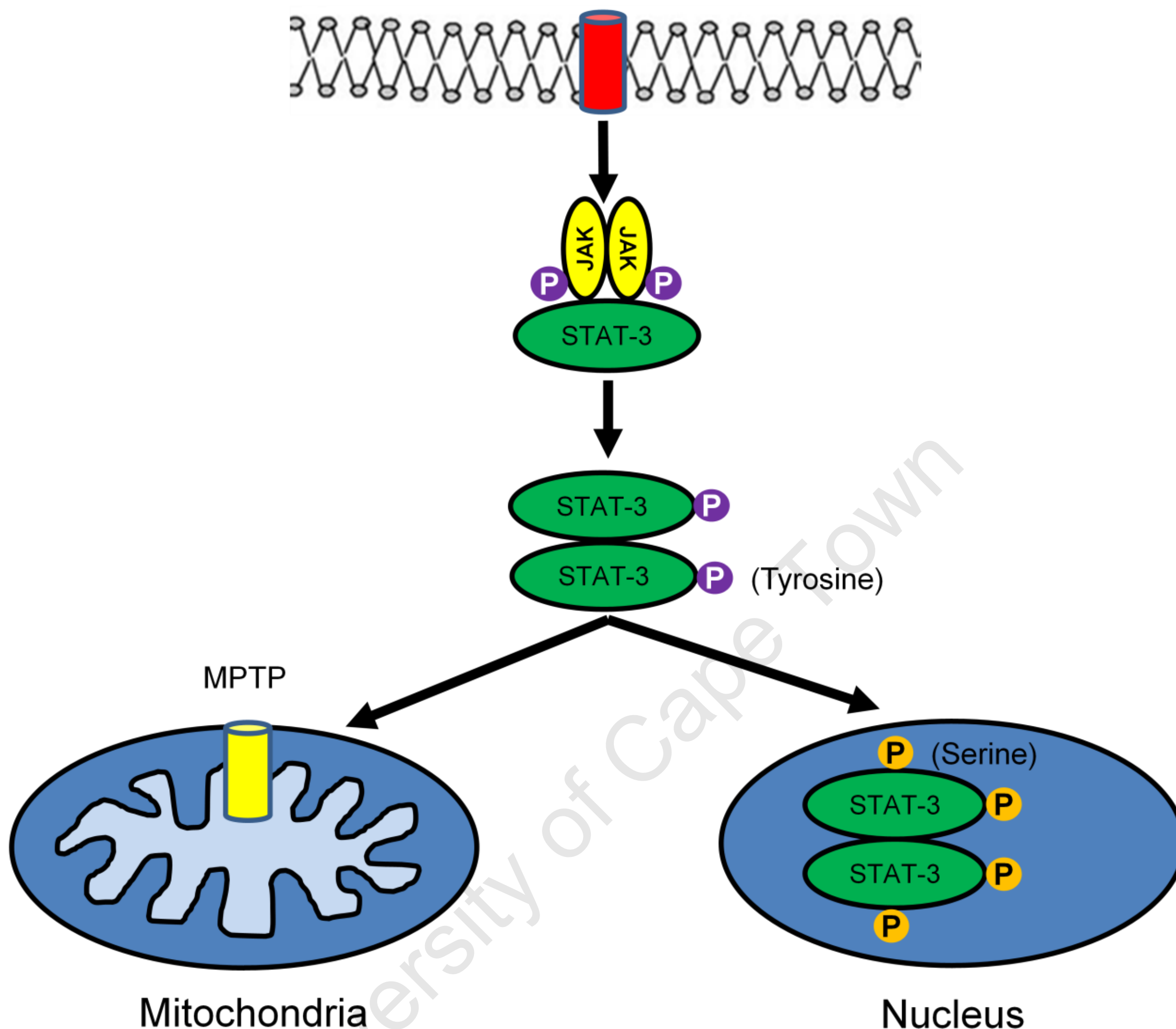

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Figure 6: Schematic diagram of JAK/STAT-3 pathway

Please refer to text for details. Modified from Lecour et al. (Lecour, 2009a).

Although gene expression intuitively may require hours to initiate protection, the JAK/STAT-3 pathway can initiate protection with minutes of ischaemic preconditioning (Lecour et al., 2005b) and postconditioning stimuli (Boengler et al., 2008a). Such immediate protection has been postulated to be the result of cross talk with other signalling cascades (Hausenloy and Yellon, 2007) or direct effects on end effectors such as the mitochondria (Wegrzyn et al., 2009, Lecour, 2009a, Lacerda et al., 2010).

Current controversies regarding the paradigm of JAK/STAT-3 signalling include the following (Sehgal, 2008); First, nonphosphorylated STAT-3 dimers exist in the cytosol. Second, nonphosphorylated STAT-3 shuttles constitutively between cytosol and nucleus. Third, nonphosphorylated STAT-3 can be transcriptionally active but activate a different set of genes. Fourth, cytosolic to nuclear transport can take place via endocytic trafficking. Fifth, not all STAT-3 mediate effects are via the nucleus but includes the cytoplasm, of which its association with mitochondria is one of many examples (Wegrzyn et al., 2009). The effects of such shifts in the current paradigm of JAK/STAT-3 signalling have yet to be determined.

2.2.1 JAK/STAT and ischaemia reperfusion

The JAK/STAT pathway is activated following ischaemia reperfusion and pharmacological blockade of JAK by AG490 results in inhibition of STAT-3 phosphorylation and increased apoptosis (Negoro et al., 2001, Negoro et al., 2000). Cardiac specific over expression of STAT-3 decreased infarct size (Oshima et al., 2005) and cardiac specific deletion of STAT-3 increased infarct size (Smith et al., 2004). Both studies emphasise the protective nature of STAT-3 against ischaemia reperfusion. In contrast, some studies have found that addition of AG490 protected the heart (Hwang et al., 2005, Mascareno et al., 2001).

2.2.2 JAK/STAT and conditioning

During ischaemic preconditioning both JAK/STAT and PI3K-Akt (RISK) pathways are required to confer protection (Suleman et al., 2008, Hausenloy et al., 2009). However, if TNFα is used as a preconditioning mimetic, only the JAK/STAT pathway is activated and not the RISK pathway because Erk1/2 inhibition by PD98059 (Lecour et al., 2005b) and PI3K-Akt inhibition by wortmannin (Suleman et al., 2008) could not abrogate TNFα preconditioning. Similar results were achieved with TNFα postconditioning (Lacerda et al., 2009).

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STAT-3 knock out (KO) mice cannot be preconditioned (Smith et al., 2004). Likewise, pharmacological blockade of the JAK/STAT pathway by AG490 in wild type mice prevents any protective effects of the SAFE pathway during preconditioning (Suleman et al., 2008). At the time I started my MSc degree, the role of STAT-3 via postconditioning was unknown.

Therefore, the use of TNFα to activate the JAK/STAT pathway resulting in protective effects against reperfusion injury, has been termed the SAFE pathway and is an independent signalling pathway to the already described RISK pathway (Lecour, 2009a) (Figure 7).

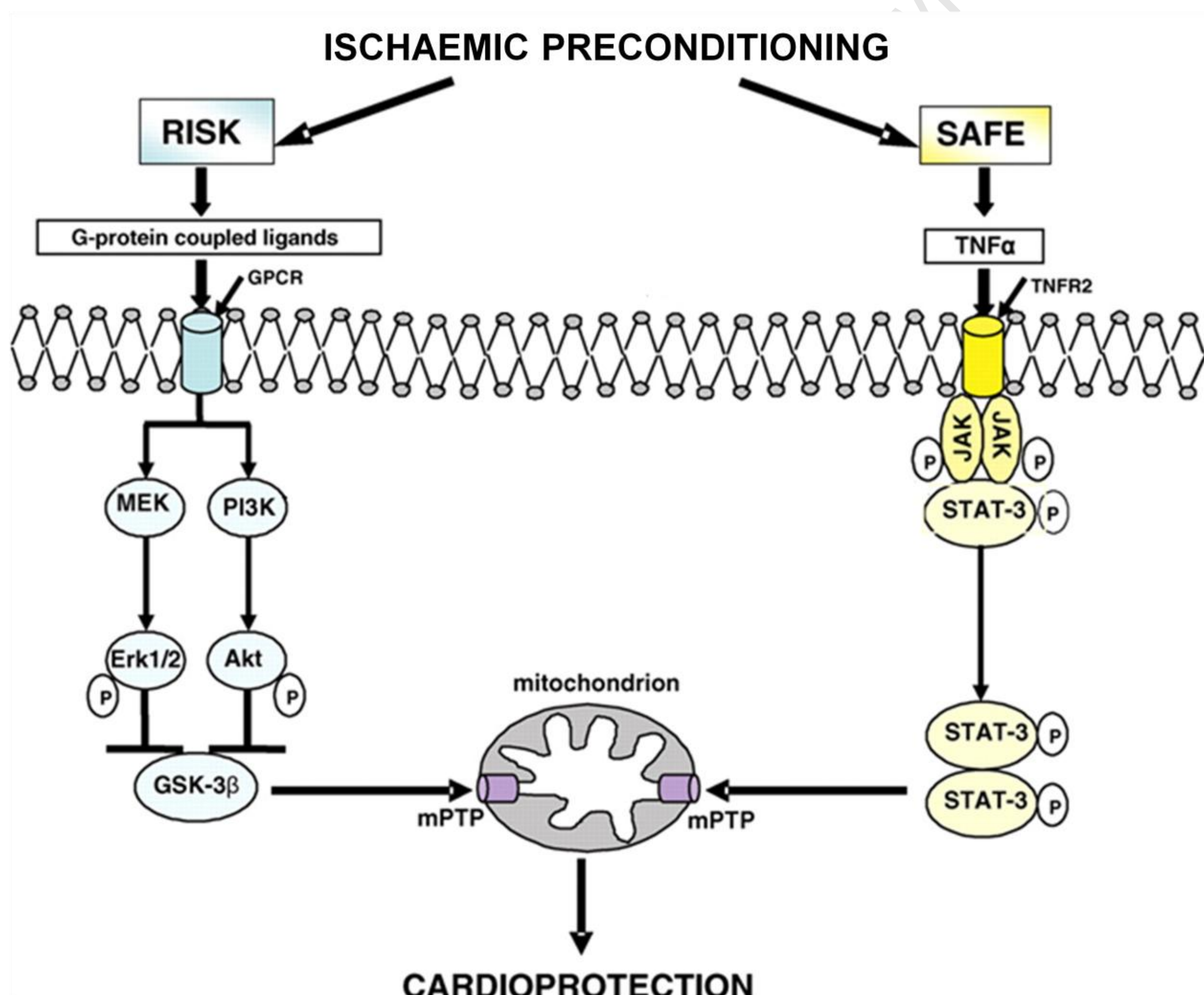

Figure 7: Schematic diagram of the SAFE and RISK pathway

See text for abbreviations (Lacerda et al., 2009).

2.3 Downstream effectors of the SAFE pathway

Known downstream targets include genes activated by STAT-3. These include inducible nitric oxide synthase and cyclooxygenase 2 although these genes were elucidate in a mouse model undergoing delayed preconditioning by interleukin 6 signalling (Dawn et al., 2004). Similarly, antiapoptotic Bcl-2 and B-cell lymphoma-extra large (Bcl-Xl) are activated by STAT-3 via interleukin 6 signalling in myeloma cells (Schwarze and Hawley, 1995) and pharmacological postconditioning with opioids (You et al., 2011).

Other known end effectors of the SAFE pathway include the mKATP channel (Lecour et al., 2002), NFκ-B (Somers, 2005) and inhibition of proapoptotic BAD (Lecour et al., 2005b). In contrast, neither Akt, Erk, p38MAPK nor GSK3β are involved in the SAFE pathway (Lacerda et al., 2009, Lecour et al., 2005b, Tanno et al., 2003).

Recent research has focused on the MPTP as the final downstream effector for the SAFE pathway. It was recently discovered that STAT-3 can regulate mitochondrial metabolic function (Gough et al., 2009, Boengler et al., 2010) and proposed to have a direct interaction with complex I/II on the inner mitochondrial membrane (Wegrzyn et al., 2009). However, other researchers disagree and state that STAT-3 mediates its effects via nuclear transcription, or at the very least, in close association with mitochondria but indirectly (Phillips et al., 2010). Interestingly STAT-3, specifically its serine phosphorylation motif, has been implicated as a regulator in mitochondrial respiration, independent of its nuclear transcription effects (Wegrzyn et al., 2009). Finally, the MPTP has recently been proposed to be common end protector between the RISK and SAFE pathways (Hausenloy et al., 2010b).

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3 The FOXO pathway and conditioning

Forkhead box O (FoxO) has many diverse functions including apoptosis and mediating the response to oxidative stress (Maiese et al., 2008). FoxO has many subtypes including FoxO1, FoxO3, FoxO4 and FoxO6. Each subtype has a predilection for certain tissues such as adipose, liver, muscle and brain, respectively. Regarding muscle, FoxO4 is more specific to skeletal muscle and FoxO3 is expressed more than FoxO1 in cardiac muscle (Furuyama et al., 2000).

3.1 Apoptotic function of FoxO

One of the most important FoxO mediated pathways is apoptosis. Depending on the stimulus and hence the relevant signalling pathway, FoxO can either have an antiapoptotic or proapoptotic role.

3.1.1 Antiapoptotic role of FoxO

FoxO antiapoptotic pathways can broadly be divided into Akt or non-Akt dependent pathways. In response to ischaemia, Akt can phosphorylate and hence inactivate either the cytoplasmic or nuclear pool of FoxO3a. As a result, cytoplasmic FoxO3a will be bound to the 14-3-3 protein (Biggs et al., 1999) and nuclear FoxO3a will be exported out of the nucleus by the 14-3-3- protein (Brunet et al., 2002). Hence, nuclear FoxO3a is unable to mediate its transcription of genes to induce apoptosis such as Bcl-2-interacting mediator of cell death (Bim) (Huang and Tindall, 2007, Dijkers et al., 2000)

Non-Akt pathways include serum and glucocorticoid inducible protein kinase (Sgk) and S-phase kinase-associated protein-2 (Skp2). Similar to Akt, Sgk can phosphorylate FoxO3a to bind it to the 14-3-3-protein and hence prevent apoptosis (Leong et al., 2003, Brunet et al., 2001). Within the nucleus, Skp2 can degrade FoxO1 via an ubiquitin-dependant pathway (Huang et al., 2005). An inverse

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relationship exists between Skp2 and FoxO3a levels, although the mechanism of their interaction has yet be to described (Lu et al., 2009).

3.1.2 Proapoptotic role of FoxO

Proapoptotic pathways allow dephosphorylated FoxO to accumulate in the nucleus and hence activate proapoptotic pathways. The pathways which enable apoptosis can be divided into the extrinsic and intrinsic pathways.

The extrinsic pathway can be modulated by dephosphorylated FoxO3a following the genetic expression of TNF-related apoptosis-inducing ligand (TRAIL) (Modur et al., 2002, Obexer et al., 2007) and Fas/CD95 (Brunet et al., 1999, Barthelemy et al., 2004). These cell surface receptors can then activate caspases to induce apoptosis.

The intrinsic pathway can be modulated by dephosporylated FoxO3a by up regulation of genes encoding for Bim and Noxa (equivalent to phorbol-12-myristate-13-acetate-induced protein). These two proteins from part of a classification system that recognises the number of conservatively shared Bcl-2 homology (BH) domains, Hence Bim and Noxa are similar to BAD, all of which are BH3 only proteins. BH3 proteins can bind BH1234 proteins hence allowing BH123 proteins to induce outer membrane permeabilisation with subsequent cytochrome C release and caspase activation (Obexer et al., 2007).

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3.2 FoxO response to oxidative stress

In response to oxidative stress by hydrogen peroxide for example, FoxO3a is deacetylated by silent information regulator 1 (Sirt1) and together form a complex (Brunet et al., 2004). The Sirt1/FoxO3a complex will allow FoxO3a to bind promoters of genes that increase manganese superoxide dismutase (MnSOD) (Kops et al., 2002) and catalase (Nemoto and Finkel, 2002). These enzymes will subsequently combat the increase in ROS caused by the oxidative stress. In addition, the

Sirt1/FoxO3a complex will prevent FoxO3a from increasing BIM expression and hence, prevent apoptosis during oxidative stress (Motta et al., 2004). Recently it was demonstrated that in addition to FoxO3a, FoxO1 also localised to the nucleus in response to oxidative stress, resulting in an increase in MnSOD and catalase amongst others (Sengupta et al., 2010).

The response to starvation or ischaemia-reperfusion in cardiomyocytes has resulted in dephosphorylation of FoxO1 and FoxO3 which up regulates genes necessary for autophagy (Sengupta et al., 2009).

3.3 FoxO and ischaemia reperfusion

Following ischaemia reperfusion, myocardial overexpression of H11 kinase (a eukaryotic homologue of the viral protein kinase expressed after herpes simplex viral infection) resulted in cardioprotection, in part mediated by FoxO1a (Depre et al., 2006). Similarly, in the brain subjected to ischaemia reperfusion, Akt phosphorylation prevented FoxO3a from inducing Fas and BIM expression (Shioda et al., 2007).

Phosphorylated cytoplasmic FoxO3 was reduced and hence active in ischaemia reperfusion, resulting in nuclear FoxO3 to induce autophagy in cardiomyocytes (Sengupta et al., 2009). FoxO3 is also deacetlyated by Sirt1 and Sirt1 is increased in ischaemia reperfusion. Although Sirt1 is located in the cytosol and nucleus at rest (Tanno et al., 2007), following ischaemia and reperfusion Sirt1 locates predominately to the nucleus (Brunet et al., 2004). FoxO3 then activates primarily GADD45 (a DNA repair target gene) and not BIM expression (Brunet et al., 2004).

Recently it was described that FoxO1 could translocate to the nucleus and increase expression of manganese superoxide dismutase in response to Sirt1 induced cardioprotection following ischaemia reperfusion (Hsu et al., 2010).

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3.4 FoxO and conditioning

Very little is known about the role of FoxO in preconditioning and none is known in postconditioning. Bromelin, a proteolytic enzyme extracted from pineapple, is a preconditioning mimetic which reduced apoptosis following myocardial ischaemia reperfusion via Akt mediated phosphorylation of FoxO3a (Juhasz et al., 2008). Another study utilised protein H11 kinase (H11k) cardiac specific over expressing mice to demonstrate the cardioprotective role of H11k. H11k binded to Akt and 5' AMP-activated protein kinase (AMPK) to inhibit FoxO1, GSK3β, BAD amongst others (Depre et al., 2006). Similar effects were achieved in rat brain where ischaemic preconditioning resulted in activation and phosphorylation of Akt and hence sustained phosphorylation of FoxO1, FoxO3 and FoxO4 to prevent apoptosis (Zhan et al., 2010). The role of FoxO as a potential downstream effector of the SAFE pathway has yet to be explored.

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4 Sphingosine 1 phosphate and conditioning

Sphingolipids form part of all eukaryotic cell membranes, mechanically stabilising the membrane and chemically forming part of the hydrophobic lipid bi-layer (Takabe et al., 2008). One of the sphingolipids, sphingosine, is converted by sphingosine kinase (SpK) to form sphingosine 1 phosphate (S1P) (Takabe et al., 2008). In addition to its membrane location, 60% of plasma S1P concentrations are found in high density lipoprotein (HDL), where S1P can mediate HDL's protective effects (Keul et al., 2007).

S1P has many functions including cytoskeletal migration, vasculogenesis, cell growth, lymphoid tracking, intracellular calcium mobilisation and chemokine attractant (Cyster, 2005, Karliner, 2009). In particular, S1P has a regulatory role in cell survival (Olivera and Spiegel, 1993) and apoptosis (Cuvillier et al., 1996). The pleiotropic nature of S1P can be attributed to its intracellular and extracellular effects. Intracellular S1P production via sphingosine kinase enables S1P to act as a second messenger to increase DNA synthesis and mobilise calcium from internal stores (Payne et al., 2002). Importantly, via extracellular secretion, S1P can bind to its cell surface receptors (Spiegel and Milstien, 2003).

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S1P has 5 receptors named in numerical order. S1P receptors 1 to 3 ($S1P_1$, $S1P_2$ $S1P_3$) are found in cardiomyocytes with $S1P_1$ the most dominant. $S1P_4$ and $S1P_5$ are limited to immune and nervous systems (Means and Brown, 2009). In contrast, cardiac fibroblasts express more $S1P_3$ than $S1P_1$ and $S1P_2$ receptors (Landeen et al., 2008) and since they are the most abundant cell type in the heart, the role of S1P via the activation of it receptors is non negligible (Kacimi et al., 2007). In addition, cardiac fibroblasts can functionally organise the cardiomyocytes, enabling the cardiomyocytes to respond to stimuli (Means and Brown, 2009).

Following extracellular secretion, S1P can bind to and activate G-protein coupled S1P receptors. Thereafter, activation of either ERK or PI3K-Akt will lead to cell proliferation or cell survival respectively (Radeff-Huang et al., 2004). S1P has also

been shown to activate Akt and inactivate GSK-3β during cardiomyocyte hypoxia (Zhang et al., 2007).

Intracellular effects of S1P are mainly confined to its production from sphingosine by sphingosine kinase and its breakdown to ceramide (Melendez, 2008, Taha et al., 2006) (Figure 8). S1P is known to be pro-growth and anti-apoptotic, but sphingosine and ceramide have mainly anti-proliferative and pro-apoptotic effects (Cuvillier et al., 1996). Therefore, the concept of a 'sphingolipid rheostat' was proposed, indicating the dynamic role between the three compounds which ultimately determines the cells fate (Spiegel and Milstien, 2002).

Importantly, the two isoforms of SpK, namely SpK1 and SpK2 strongly influence the sphingolipid rheostat. SpK1 converts sphingosine to S1P and decreases ceramide levels, thereby promoting cell survival. Conversely, SpK2 increases ceramide levels thereby promoting cell death (Maceyka et al., 2005).

Interestingly, TNFα can promote cell survival by activating SpK1 directly, independent of S1P cell surface receptors but rather via the TNF receptor-associated factor 2 (TRAF2) (Xia et al., 2002). In addition, TNFα, by binding to TNFR2 and then via TRAF2, can induce nuclear factor kappa B (NF-κB) to prevent apoptosis (Rothe et al., 1995, Wang et al., 1998).

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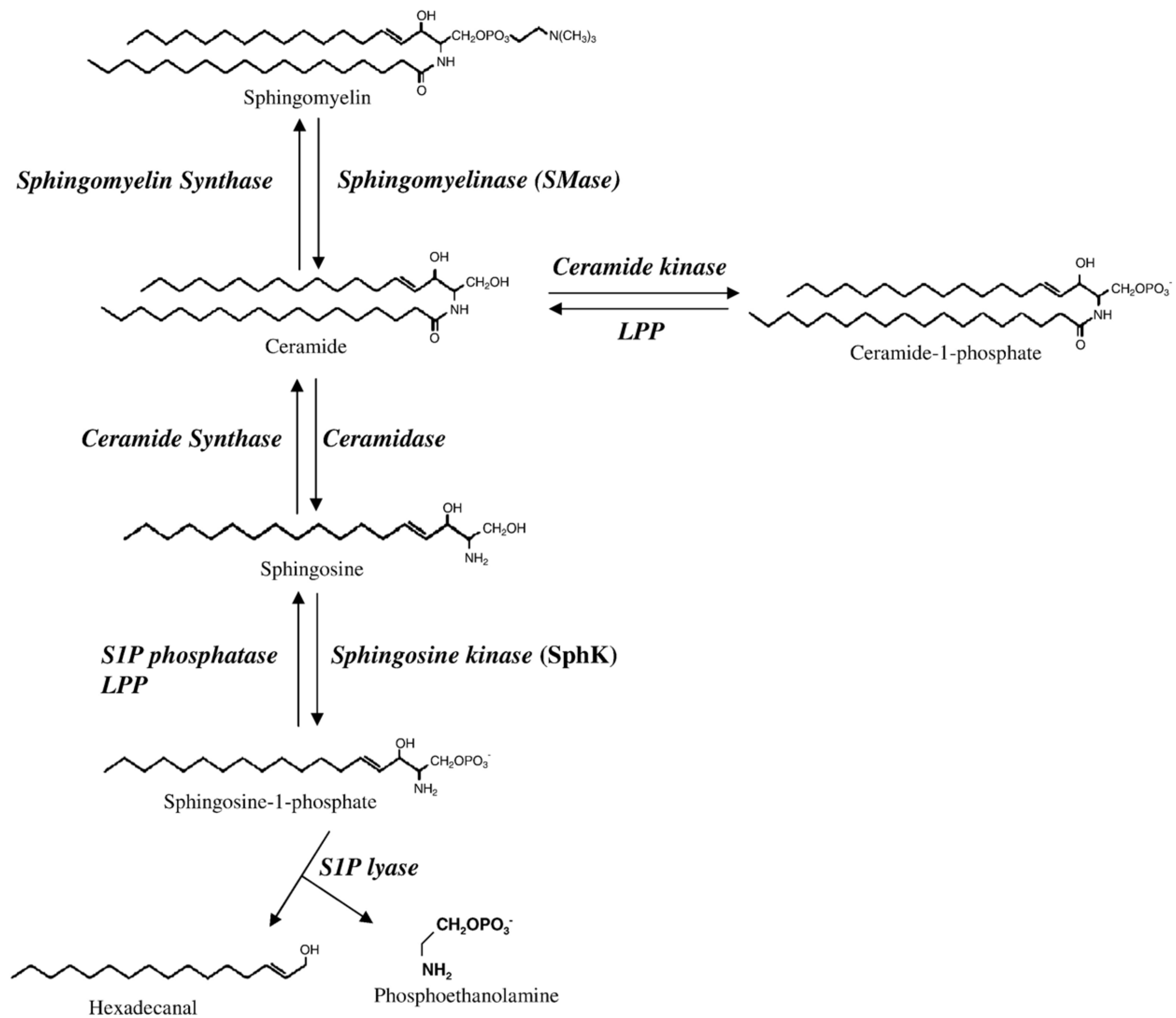

Figure 8: Sphingolipid metabolic pathway

Sphingomyelin is hydrolysed by sphingomyelinase to ceramide. Ceramide is metabolised by ceramidase to generate sphingosine, or ceramide can be phorphorylated by ceramide kinase to generate ceramide-1-phosphate. Alternatively, sphingomyelin synthase can convert ceramide back to sphingomyelin. Ceramide-1-phosphate can be dephosphorylated by lipid phosphate phosphatase (LPP) back to ceramide. Sphigosine-1-phosphate can be dephosphorylated to produce sphingosine (by S1P phosphatases or LPP) or it can be irreversibly cleaved by S1P lyase to hexadecanal and phosphoethanolamine (Melendez, 2008).

4.1 S1P and ischaemia/reperfusion

Following the protective effects of S1P in hypoxia (Karliner et al., 2001), its role in myocardial ischaemia/reperfusion was described (Jin et al., 2002, Lecour et al., 2002, Egom et al., 2010). Akt (Hofmann et al., 2009) and nitric oxide have thus far been implicated to mediate S1P's protective effects (Theilmeier et al., 2006). Interestingly, PKCε is also involved in S1P signalling but not via exogenous S1P administration. Rather, endogenous S1P production stimulates PKCε via addition of a ganglioside which in turn activates SpK1 (Jin et al., 2002).

4.2 S1P and conditioning

S1P has been shown to be both a preconditioning (Jin et al., 2002, Lecour et al., 2002, Vessey et al., 2009b) and a postconditioning mimetic (Hofmann et al., 2009, Vessey et al., 2009b, Somers, 2009). Furthermore, its interaction with one of its metabolites, sphingosine has been explored. Whilst S1P may mediate its effects through G protein coupled receptors, sphingosine mediates protection through protein kinase G or C. However, both S1P and sphingosine pathways unite through the activation of Akt in either preconditioning or postconditioning (Vessey et al., 2008b).

Similar research has focused on endogenous S1P production via SpK1 during ischaemia reperfusion. Knockout SpK1 mice failed to be protected by ischaemic preconditioning (Jin et al., 2007) and postconditioning (Jin et al., 2008, Hofmann et al., 2009).

Recently it was proposed that S1P and adenosine could independently protect the heart because VPC 23019, a $S1P_{1and3}$ antagonist and 8-SPT, a adenosine receptor antagonist, could abrogate their respective protective conditioning effect. However, if the ischaemic repetitive cycles in preconditioning and postconditioning were increased from two to three, and from four to five cycles respectively, VPC 23019 and 8-SPT could not individually inhibit the protective effect of ischaemic

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conditioning but together they could. Hence the authors suggest that in as much as one agent could be cardioprotective, if the strength of ischaemic conditioning were increased, it was no longer dependent on a single pathway (Vessey et al., 2009b).

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Chapter Two: Aim, Hypothesis and Objectives

Pharmacological conditioning and ischaemic conditioning are powerful tools to reduce damage following ischaemia reperfusion but the mechanisms still remains unclear. JAK/STAT, as part of the SAFE pathway has recently been delineated as a critical and important pathway in ischaemic preconditioning but whether activation of STAT-3 is required for the protection of pharmacological preconditioning with S1P and ischaemic postconditioning is unknown.

2.1 Aim

The aim of this thesis was to explore the role of STAT-3 in the cardioprotective effect of preconditioning and postconditioning.

2.2 Hypothesis

We hypothesised that S1P preconditioning and ischaemic postconditioning protect via activation of STAT-3.

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2.3 Objectives

To asses this hypothesis, the following objectives were pursued:

1. To investigate the role of STAT-3 in S1P preconditioning, we used a cell culture (fibroblast) model and an isolated rat heart model subjected to an ischaemic insult, in the presence of a pharmacological inhibitor of the STAT-3 pathway (AG490). Cell survival, infarct size and western blot analysis were used as an endpoint.

2. To investigate the role of STAT-3 in ischaemic postconditioning, we used an isolated rat heart model subjected to either a global or regional ischaemic insult in the presence of a pharmacological inhibitor of the STAT-3 pathway (AG490). Infarct size and western blot analysis were used as an endpoint.

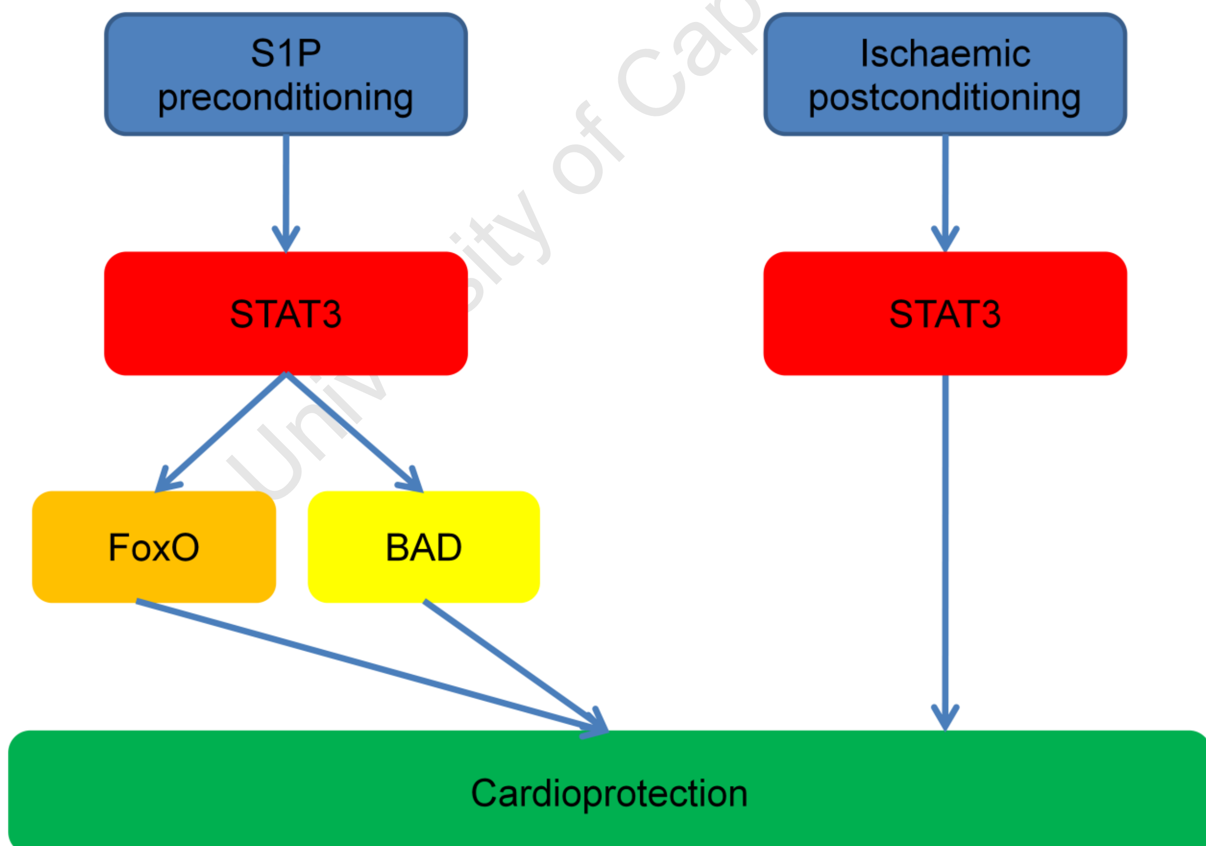

Figure 9: Schematic diagram of hypothesis

S1P preconditioning and ischaemic postconditioning activate STAT-3 as a result of ischaemia reperfusion to result in cardioprotection.

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Chapter Three: Material and methods

3.1 Isolated fibroblasts

Mouse L cell fibroblasts were initially grown from frozen vials stored in 1% dimethyl sulfoxide (DMSO) and seeded into flasks containing a medium of dimethyl sulfoxide (DMSO) (Highveld Biologicals, RSA) with 10% fetal calf serum (FCS) (Highveld Biologicals, South Africa) and 2 % L-glutamine (Sigma, Germany). The flasks were placed in a 5% CO_2 incubator maintained at 37°C and 95% humidity. After 6 hours, the medium was replaced with DMEM to remove the cytotoxic DMSO. Thereafter, the medium was replaced every 48 hours until 80-90% confluence (72 hours) was reached and then the cells were split.

In order to split the cells, the cells were trypsinised and hence incubated for 3 minutes with 0.25% Trypsin (w/v) and 0.2% ethylenediaminetetraacetic (EDTA) (w/v) (Sigma, Germany). After 3 minutes, the Tryspin/EDTA solution was neutralised with double the volume DMEM and 10% FCS. A 15µl sample solution was counted on the Neubauer hemocytometer and the remaining solution centrifuged for 5 minutes at 1000 rpm at 4°C, and the cell pellet resuspended in DMEM. The final concentration would be approximately 1 million cells per ml of solution.

Therefore, to maintain cell lines, 2 million cells or 2ml of cells in DMEM were seeded into 75cm^3 flasks. For experiments, 0.5 million cells or 0.5ml of cells in DMEM were seeded into 25cm^3 flasks and allowed to reach 80-90% confluence (48 hours).

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3.1.1. Simulated ischaemia

To replicate the metabolic changes associated with ischaemia, a modified Esumi or simulated ischaemia buffer was used (Esumi et al., 1991) and the cells were incubated in a hypoxic environment. The simulated ischaemia buffer consisted of 137 mM NaCl, 12 mM KCl, 0.5 mM MgCl, 0.9 mM $CaCl_2$, 20 mM Hepes, 20 mM 2-Deoxy-d-Glucose (2-DG) adjusted to a pH of 6.4. 2-DG is added because it irreversibly inhibits glycolysis and hence further mimics ischaemia. Furthermore, the cells were exposed to a hypoxic environment in a sealed incubation chamber set at 37°C and a gas controller (Gentronics, South Africa) was used to set oxygen levels at 1.5% ± 1.0% and carbon dioxide levels at 5.0% ± 2.5%.

3.1.2. Preconditioning protocol for isolated fibroblasts

A normoxic group consisted of cells not exposed to simulated ischaemia but exposed to the same amount of washes with phosphate buffered saline (PBS). The simulated ischaemia group was washed twice with PBS prior to incubation with simulated ischaemia buffer and the hypoxic environment for 8.5 hours. The S1P preconditioning group was incubated with S1P (10 nmol/L) in DMEM for 30 minutes, washed with PBS twice, incubated with drug free DMEM for 30 minutes before being washed again with PBS and subjected to simulated ischaemia. The S1P and AG490 preconditioning group was incubated with S1P (10 nmol/L) and AG490 (100 nmol/L) in DMEM for 30 minutes, washed with PBS twice, incubated with drug free DMEM for 30 minutes before being washed again with PBS and subjected to simulated ischaemia (Figure 10). At the end of the simulated ischaemia, cell viability was assessed.

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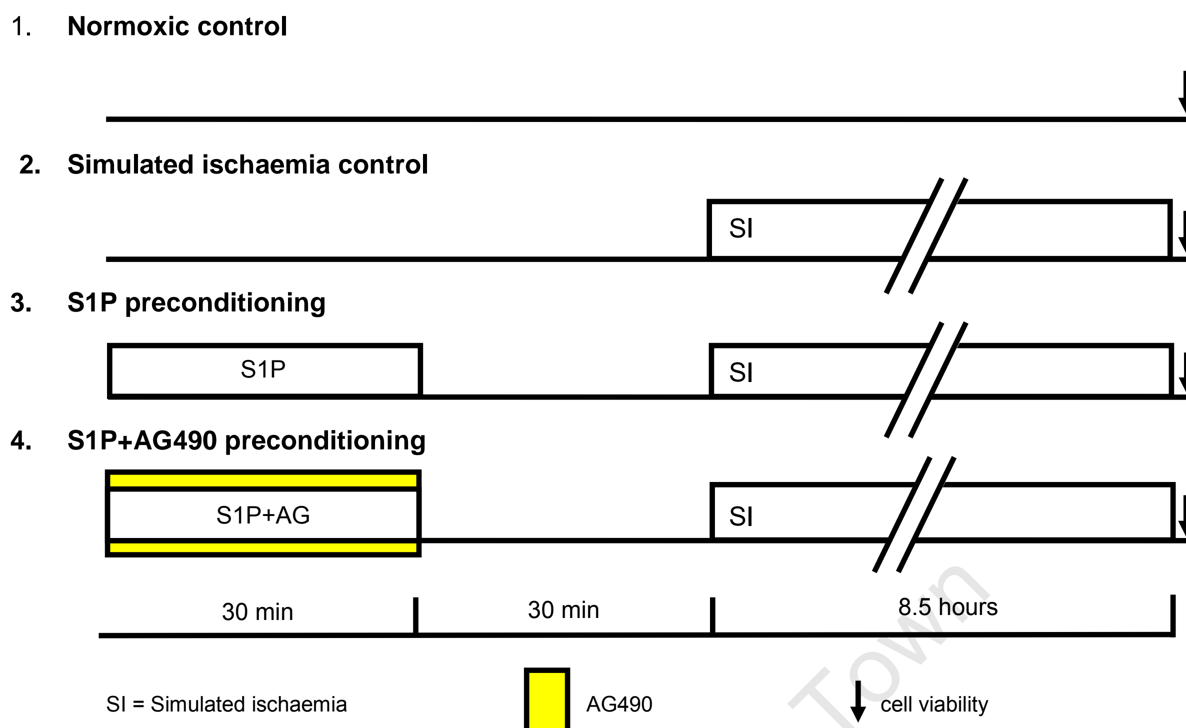

Figure 10: Schematic diagram of isolated fibroblasts undergoing preconditioning

See text for details.

3.1.3 Cell viability

The trypan blue exclusion technique for cell viability was used. The technique required that after the simulated ischaemia, cells were washed twice and incubated with 0.25% trypsin at 37°C for 3 minutes as previously described (Suleman et al., 2008). Trypsin was neutralised with double volume DMEM and centrifuged for 5 minutes at 1000rpm at 4°C. After discarding the supernatant and resuspending the pellet in 2ml of DMEM, 15µl of cell solution was stained with an equal volume of 0.4% Trypan blue (Sigma, Germany). The combination was analysed under a light microscope using a Neubauer hemocytometer.

Trypan blue will stain dead cells in blue due to their damaged membranes whilst alive cells would remain clear. Hence, the ratio of blue stained dead cells to unstained alive cells was calculated and expressed as a percentage.

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3.2 Animals

All experiments were conducted on Male Wistar (200-350g) rats as housed by the University of Cape Town Animal Unit. All protocols were carried out in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes for Health (NIH Publication No. 85 (23), revised 1996). The experimental protocol was approved by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town (06/031).

3.3 Isolated rat heart model

Rats were anaesthetised with sodium pentobarbital (50 mg/kg i.p.) and heparinised (500 IU) i.p. Hearts were rapidly excised, cannulated via the aorta and perfused retrogradely using the Langendorff isolated rat heart model. The system perfused the hearts at a constant pressure of 100cm H20 at 37°C with Krebs-Henseleit buffer equilibrated with oxygen/carbon dioxide ratio of 95:5%. The final composition of the buffer was as follows (in mM): 118.5 NaCl, 25 NaHCO3, 4.75 KCL, 1.18 MgSO4.7H2O, 1.2 KH2PO4 1.36 CaCl2.2H20 and 11.1 Glucose, pH of 7.4.

A water filled balloon was inserted into the left ventricle via the left atrium which was connected to a pressure transducer and data were recorded on a Powerlab Labchart (ADI Instruments, Australia). Temperature was measured with a fine thermocouple wire (Physitemp, NJ, USA), inserted into the left atrium and monitored with a Digitron 2600T temperature sensor (Torquay, UK). The left ventricular end diastolic pressure was adjusted between 4 and 12 mmHg. Various cardiac parameters were measured throughout the experiments, including heart rate, coronary flow rate and left ventricular developed pressure (LVDP). The latter is calculated as the difference between left ventricular end systolic pressure and left ventricular end diastolic pressure. Rate pressure product (RPP) was calculated as LVDP multiplied by heart rate and expressed as a percentage of baseline.

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3.3.1 Exclusion criteria

Rat hearts which did comply with the following criteria were excluded:

1) Left ventricular pressure greater than 80 mmHg.
2) Coronary flow rate at a minimum of 8 ml per minute and a maximum of 16ml per minute.
3) Heart rate at a minimum of 240 beats per minute and a maximum of 400 beats per minute.

A total of 22 rats were hence excluded from the study.

3.3.2 Isolated rat heart perfusion protocols

As previously described, isolated rat heart perfusions were divided into preconditioning and postconditioning stimuli. Furthermore, whilst preconditioning was conducted only under regional ischaemia, postconditioning was divided into global and regional ischaemia.

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3.3.2.1 Preconditioning protocol in isolated rat hearts

In the ischaemic control group, isolated rat hearts were subjected to 30 minutes of stabilisation, 30 minutes of regional ischaemia followed by 120 minutes of reperfusion. Regional ischaemia was induced by ligating the anterior descending coronary artery with a 3/0 silk suture snare.

In the ischaemic preconditioning group, 7 minutes of regional ischaemia followed by 10 minutes of reperfusion was performed prior to 30 minutes of index ischaemia. Similarly, S1P (10 nmol/L) was infused for 7 minutes followed by 10 minutes washout prior to the 30 minutes of index ischaemia in the S1P preconditioning group. Furthermore, in the S1P and AG490 preconditioning group, AG490 (100 nmol/L) was infused for 3 minutes before the concurrent 7 minutes infusion with S1P. Thereafter, only AG490 was infused for the next 5 minutes (hence a total of 15 min of AG490 was infused), followed by 5 minutes of washout. Finally the 30 min of index ischaemia was commenced followed by 120 minutes of reperfusion. Infarct size was assessed in all hearts at the end of the reperfusion period (Figure 11).

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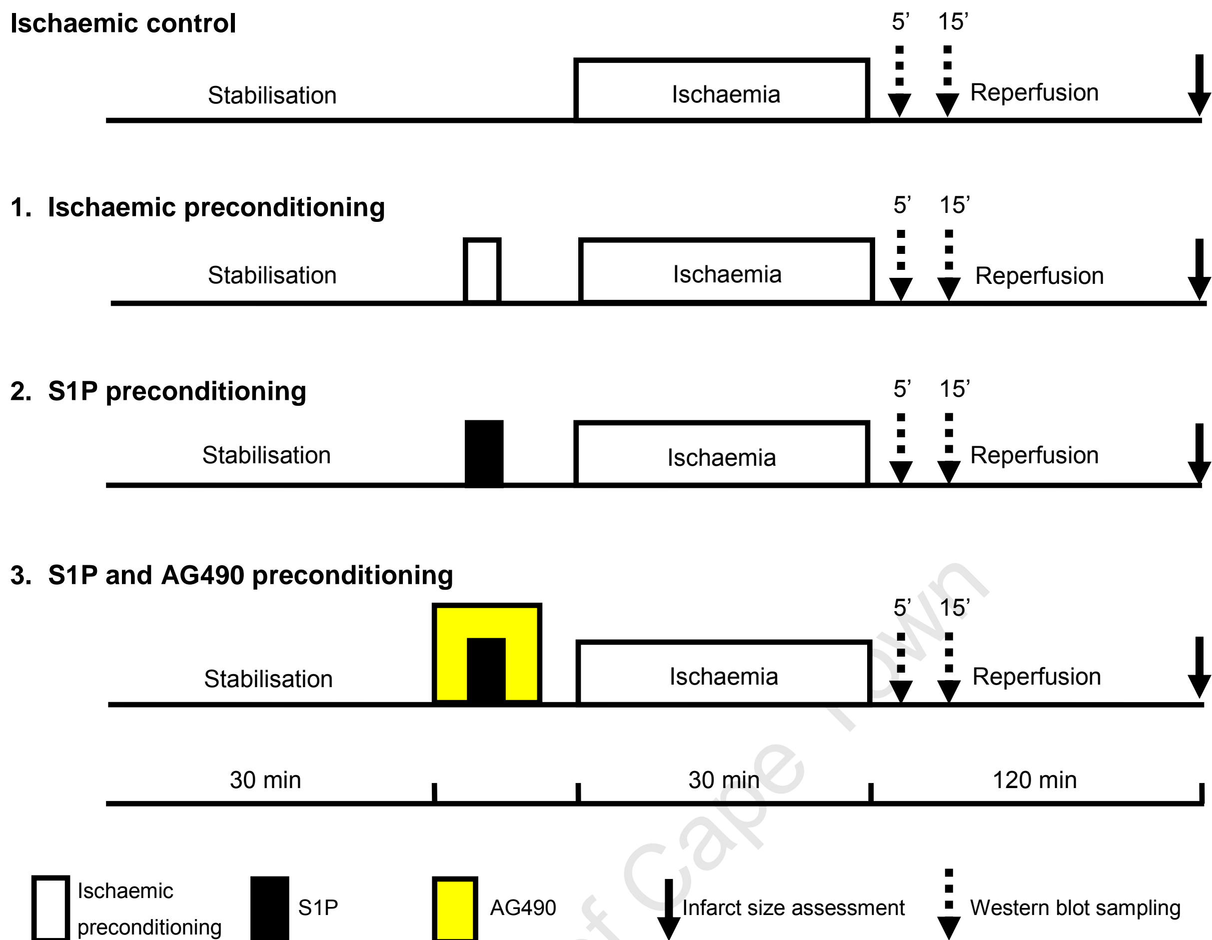

Figure 11: Schematic diagram of isolated rat hearts undergoing a preconditioning protocol

See text for details.

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3.3.2.2 Postconditioning protocol in isolated rat hearts

3.3.2.2.1 Regional ischaemia

The ischaemic control group in isolated rat hearts were subjected to 30 minutes of stabilisation, 30 minutes of regional ischaemia followed by 120 minutes of reperfusion. In the ischaemic postconditioning group, the postconditioning stimulus was performed immediately at the onset of reperfusion and consisted of 6 cycles of alternating 10 seconds reperfusion and 10 seconds of ischaemia. The ischaemic postconditioning and A490 group received AG490 (100 nmol/L) perfused for first 15 minutes of reperfusion together with the postconditioning stimulus. An AG490 control

group consisted of AG490 perfused for first 15 minutes of reperfusion only. At the end of each experiment, isolate rat hearts underwent infarct size assessment (Figure 12).

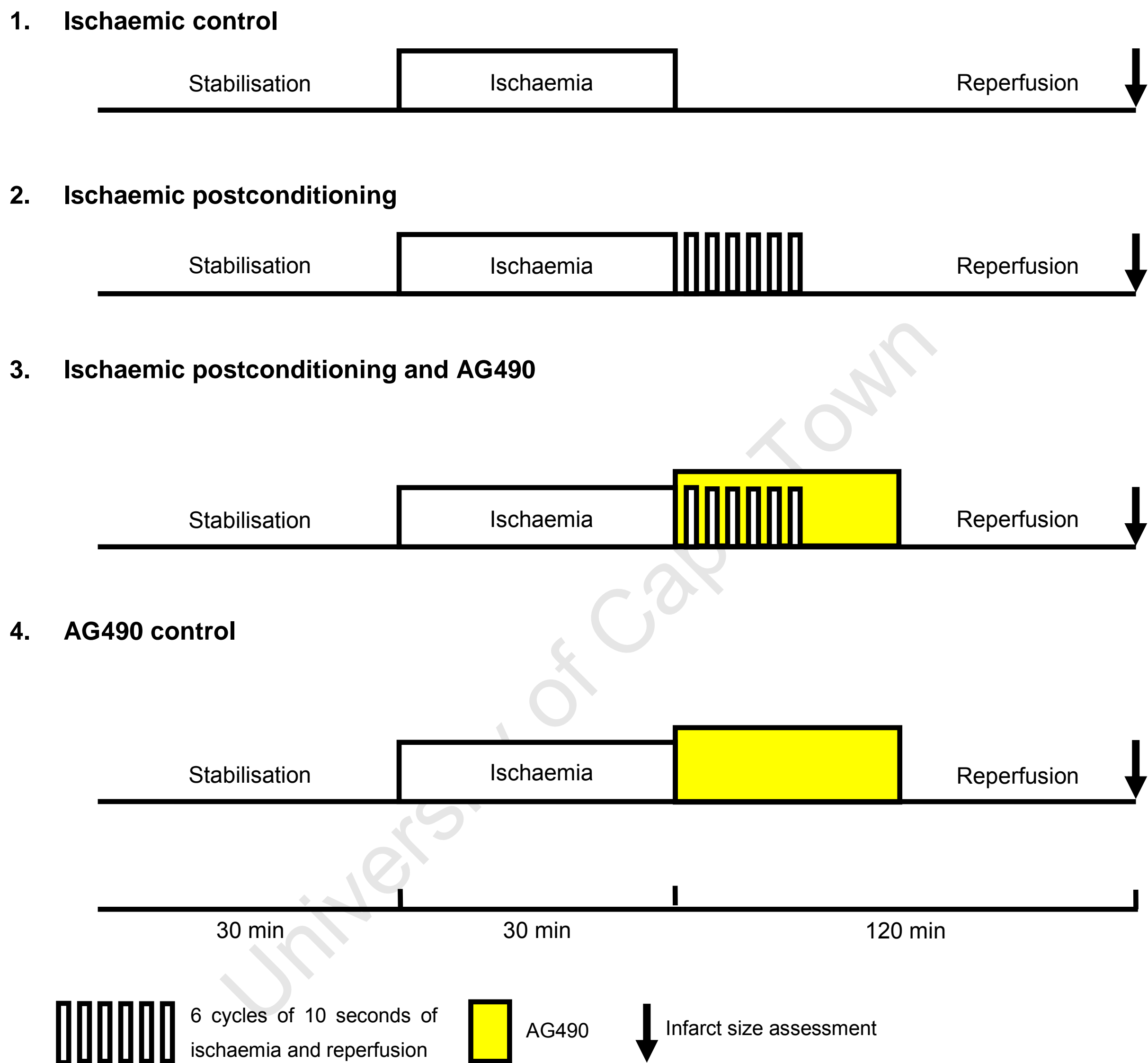

Figure 12: Schematic diagram of isolated rat hearts undergoing postconditioning and regional ischaemia

See text for details.

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3.3.2.2.2. Global ischaemia

In another set of experiments, the ischaemic control group in isolated rat hearts were subjected to 30 minutes of stabilisation, 30 minutes of global ischaemia followed by 30 minute of reperfusion. In the ischaemic postconditioning group, the postconditioning stimulus was performed immediately at the onset of reperfusion and consisted of 6 cycles of alternating 10 seconds reperfusion and 10 seconds of ischaemia. The ischaemic postconditioning and AG490 group received AG490 (100 nmol/L) perfused for first 15 minutes of reperfusion together with postconditioning stimulus. An AG490 control group consisted of AG490 perfused for 15 minutes at the onset of reperfusion. During the experiment, haemodynamic parameters such as heart rate, coronary flow rate and left ventricular developed pressures were recorded (Figure 13).

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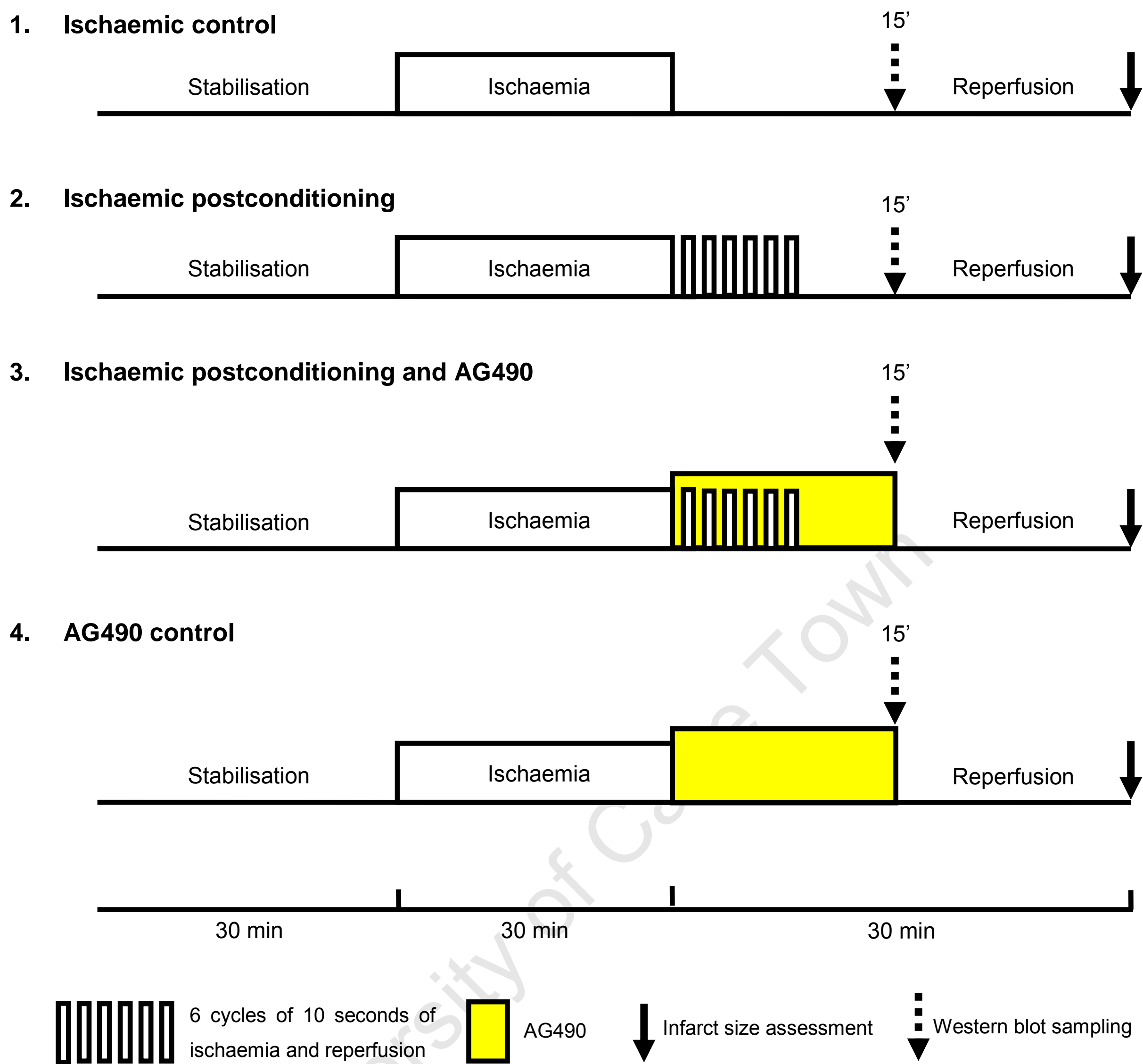

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Figure 13: Schematic diagram of isolated rat hearts undergoing postconditioning and global ischaemia

See text for details.

3.3.2.3. Infarct size assessment

Hearts which underwent regional ischaemia had the coronary artery re-occluded at the end of the protocol and 0.5ml of 2% Evans blue was perfused through the heart to delineate the area of the heart affected by the occlusion of the left descending coronary artery. All hearts were frozen for 24 hours, cut into 2mm thick slices, incubated in sodium phosphate buffer containing 1% w/v triphenyltetrazolium chloride (TTC) at 37°C, ph 7.4 for 15 min to visualize the area at risk of cardiac damage, with the remaining unstained area denoting the infarcted area in which the heart had irreversible died. Infarct and risk zone areas were determined with planimetry (Summa Sketch II; Summa Graphics) and infarct size was expressed as a percentage of the area at risk.

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3.4 Western blots

Western blotting was used to quantify changes, if any, in the level of protein expression as a result of preconditioning and postconditioning. Since two models were used, namely fibroblasts and the isolated rat heart model, two similar methods of protein extraction were used.

3.4.1 Protein extraction from fibroblasts

At the end of the preconditioning protocol and before the 8.5 hours of ischaemia, fibroblasts were immediately washed with 5ml cold phosphate buffered saline (PBS) and incubated in 5ml 0.25% trypsin for 5 minutes at 37°C. A further 9ml of cold PBS was added and placed in an eppendorf that was centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was discarded, the cell pellet was resuspended in 1ml cold PBS and transferred to another eppendorf.

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3.4.1.1 Cytosolic extract

Eppendorfs were centrifuged for 15 seconds at 8000rpm at 4°C, followed by removal of the supernatant and resuspension of the precipitate in 150µl of lysis buffer containing 10mM HEPES, 10mM KCL, 0.1mM EDTA and 0.1mM EGTA. The suspension was left on ice for 15 minutes before the addition of 30µl of 10% Igepal CA-630 (diluted in dH2O, Sigma) followed by brief vortexing and 30 seconds of centrifugation at 14000rpm at 4°C. The resulting supernatant contained the cytosolic protein extract and was appropriately aliquoted into eppendorfs. It was stored at -80°C until needed.

3.4.1.2 Nuclear extract

The pellet was resuspended in 50µl of nuclear lysis buffer containing 20mM HEPES, 400mM NaCl, 1mM EDTA, 1mM EGTA and 20% glycerol. The suspension was incubated in a shaker at 4°C for 15 minutes. After a brief vortex, it was returned to the shaker for a further 15 minutes. The tubes were then centrifuged at 14000rpm for 5 minutes at 4°C. The resulting supernatant contained the nuclear protein extract, and thus appropriately aliquoted into eppendorfs. It was stored at -80°C, until needed.

3.4.2 Protein extraction from rat hearts

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Rat hearts were harvested either immediately after the S1P preconditioning stimulus, or 5 or 15 minutes after reperfusion following the index ischaemia. Hearts were quickly removed from the experiemental rig, placed in ice cooled Krebs-Henseleit buffer whilst the atria were rapidly removed, separated into left and right ventricle and both ventricles freeze clamped in liquid nitrogen. The frozen rat ventricles were then stored at -80°C for later use.

Since the left ventricle is mainly affected during regional ischaemia, only the tissue from the rat left ventricle was used. All frozen hearts wrapped in aluminium foil were pulverized under liquid nitrogen with a hammer and approximately 200mg of powdered tissue was placed into 900µl of lysis buffer (20mM HEPES, 2.5mM MgCL2, 100µM EDTA, 20mM β- glycerophosphate, 0.5% Triton X-100, 500µM dithiothreitol (DTT), 100µM $NaVO_4$, 1mM phenylmethylsulfonyl fluoride (PMSF) and 75mM NaCl). The suspension of powered tissue in lysis buffer was ultrasonically homogenised by Polytron (Brinkmann Instruments, Canada) for 5 seconds.

3.4.2.1 Cytosolic extract

The homogenised samples were centrifuged at 10000g for 5 minutes at 4°C. The supernatant was decanted into another tube and represented the cytosolic extract (Williams et al., 2000).

3.4.2.2 Nuclear extract

The remaining pellet was resuspended in 500μl of lysis buffer (20mM HEPES, 2.5mM MgCL2, 100μM EDTA, 20mM β- glycerophosphate, 1% Triton X-100, 500μM DTT, 100μM $NaVO_4$, 1mM PMSF and 75mM NaCl). The suspension was centrifuged at 15000g for 30min 4°C with the supernatant decanted was into another tube and represented the nuclear extract (Williams et al., 2000).

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3.4.3 Protein quantification

The Lowry Assay was used to quantify the concentration of proteins (Lowry et al., 1951). The bovine serum albumin (BSA) standard curve ranged from a concentration of 5-200mg/l. Protein samples were added to 1 ml of distilled water in a 1:200 volume ratio. 1ml of Solution A containing a composite of three solutions in equal volumes; 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reagent, 10% sodium dodecyl sulfate (SDS) and 50 µM sodium hydroxide (NaOH) was added to each tube. CTC reagent contained 2% (w/v) sodium carbonate (Na_2CO_3) mixed in a ratio 100:1:1 (v:v:v) with 1% (w/v) copper sulphate ($CuSO_4.5H_2O$) and 1% (w/v) di-potassium tartrate hemihydrate ($C_4H_4K_2O_6.0.5H_2O$). After 10 minutes at room temperature, 0.02 N Folin phenol reagent was added to each tube. Following 30 minutes of incubation, the absorbance was measured with a spectrometer at 250nm.

3.4.4 SDS PAGE of extracted proteins

Lysates were diluted in Laemmli sample buffer, boiled for 5 min. 20µg of proteins were separated on 10% sodium dodecyl sulphate (SDS) PAGE using standard Bio-Rad Mini-PROTEAN II System for two hours at 0.2volts and transferred to nitrocellulose membrane (Amersham Bioscience Hybond P RPN 303F).

3.4.5 Immuno-blotting and detection

The membranes were stained with a Ponceau Red stain (Ponceau S solution Sigma, USA P7170) and the membrane was blocked with 5% of milk in tris-buffered saline (TBS-Tween) (0.1% Tween).

All blots were probed with phosphorylated states of STAT-3 (Tyr 705 and Ser 727), BAD (Ser 136) and FoxO (Ser 318/321) and their respective total levels of STAT-3, BAD and FoxO. After primary antibody incubation, blots were washed off with TBS -

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T (0.1% Tween) 3 times for 5 minutes and probed with their respective secondary antibody labelled with horseradish peroxidase.

All blots were developed and digitally captured in the Genegnome (SynGene, Cambridge, UK). All blots were normalised to either β-actin or ponceau, and the phosphorylated states of proteins were normalised by division with their respective total state. Relative densitometry was determined with GeneSnap and GeneTools software (SynGene, Cambridge, UK).

3.5. Chemicals and pharmacological agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemicals, Germany. Similarly, all antibodies were from Cell Signalling Technology, USA.

3.6 Statistical analysis

All data were expressed as the mean ± SEM. Statistical significance was determined by a student T-test when appropriate or a one way analysis of variance (ANOVA) with a Student Newman-Keul post hoc test (Graph Pad Instat). *$p < 0.05$ was considered significant.

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Chapter Four: Results

4.1 Cytoprotective effect of S1P preconditioning

4.1.1. Role of S1P preconditioning in fibroblasts

To explore the protective effect of S1P preconditioning, we initially conducted our studies in Mouse L cells. After 8.5 hours of simulated ischaemia, the survival rate was 30.0% (all data normalised to ischaemic control). This data was consistent with the literature (Lecour et al., 2006). S1P preconditioning significantly improved cell survival as compared to its respective ischaemic control at the end of the simulated ischaemia (38.1±1.5% vs. 30.0±0.0%, $p<0.05$ vs. control). Co-treatment of AG490 with S1P abolished the effect of S1P as compared to the control group at the end of simulated ischaemia (29.9±2.7%, ns vs. control). Surprisingly AG490 itself produced a protective effect as compared to the control group after simulated ischaemia (40.0±2.4%, $p<0.05$ vs. control, n=2) (Figure 14).

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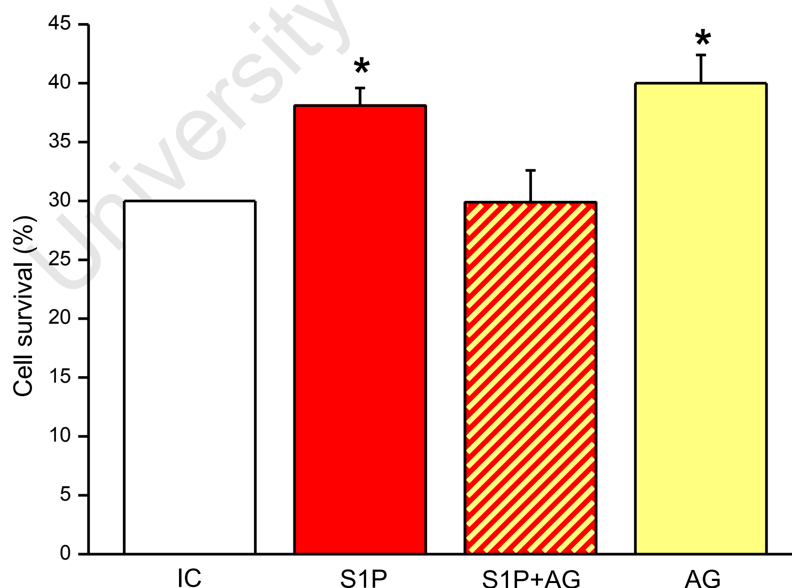

Figure 14: S1P increases cell survival in fibroblasts

IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490. * $p<0.05$ vs. IC. n≥5 per group except AG where n=2.

4.1.2. Role of S1P preconditioning on tyrosine phosphorylated STAT-3 in fibroblasts

Since S1P preconditioning significantly improved cell survival, we explored if the protective effect could be accounted by the activation and hence phosphorylation of tyrosine STAT-3.

In the cytosolic fraction, S1P preconditioning significantly decreased tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control at the end of simulated ischaemia (1.4±0.1A.U. vs. 0.6±0.1A.U., $p<0.05$ vs. control). Co-treatment of AG490 with S1P abolished the effect of S1P (1.1±0.2A.U., ns vs. control) and AG490 alone (1.5±0.2A.U., ns vs. control), had no effect on tyrosine phosphorylated STAT-3 as compared with the control group at the end of simulated ischaemia.

In the nuclear fraction, S1P preconditioning did not significantly affect tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control at the end of simulated ischaemia (1.1±0.2A.U. vs. 0.8±0.1A.U., ns vs. control). Co-treatment of AG490 with S1P (1.2±0.2A.U., ns vs. control) and AG490 alone (1.0±0.1A.U., ns vs. control), had no effect on tyrosine phosphorylated STAT-3 as compared with the control group at the end of simulated ischaemia (Figure 15).

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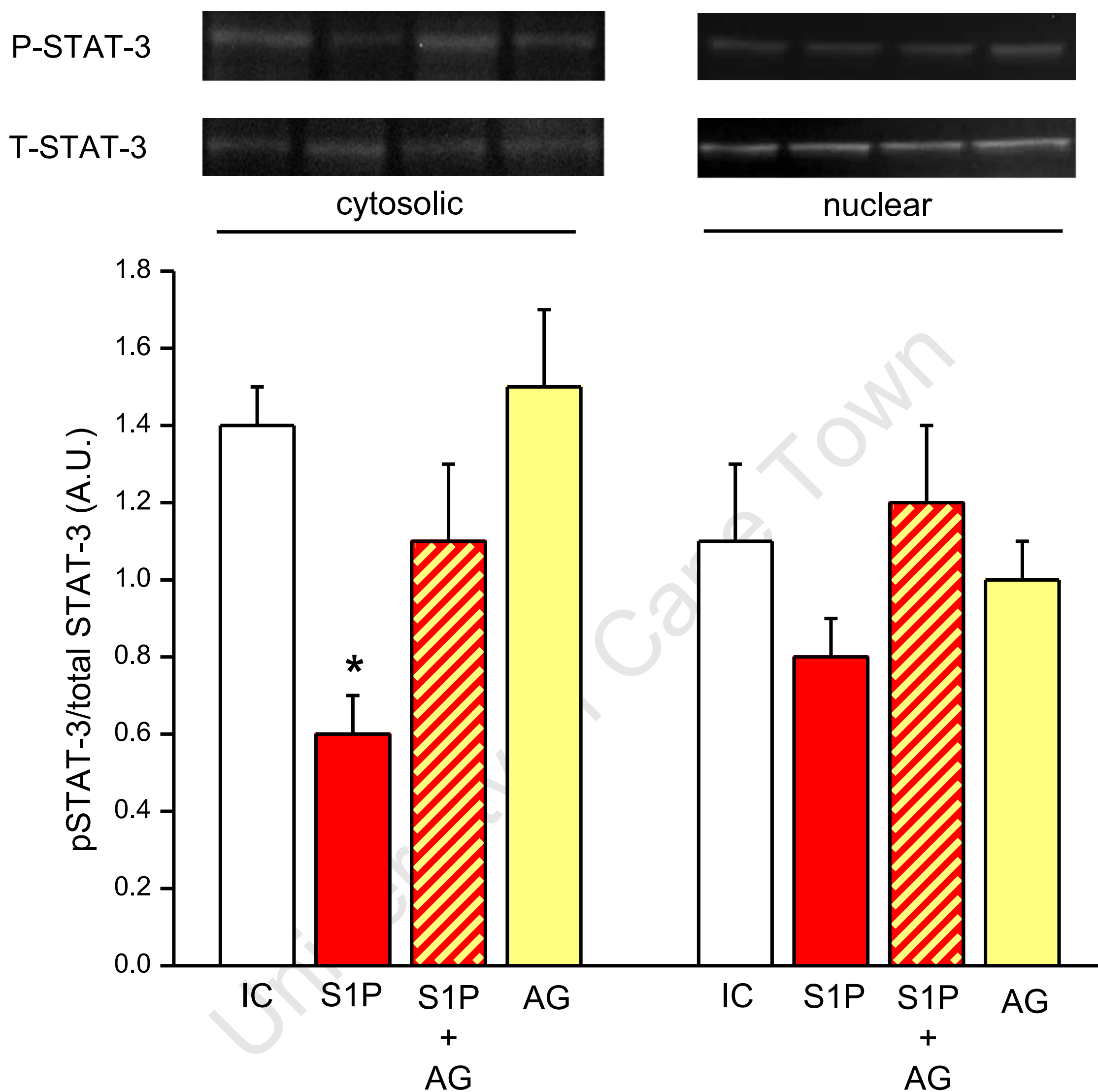

Figure 15: Tyrosine phosphorylated STAT-3 in the cytosol and nuclear fraction

Significant decrease in cytosolic tyrosine phosphorylated STAT-3 with S1P preconditioning compared to ischaemic control. Hearts were harvested 15 minutes after S1P pre-treatment and hence prior to the index ischaemia. IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. *$p<0.05$ vs. control. n=4 per group.

4.2 Cardioprotective effect of S1P preconditioning

4.2.1 Role of S1P preconditioning on haemodynamic parameters

4.2.1.1 Regional ischaemia

After 30 minutes of regional ischaemia and 120 minutes of reperfusion, the LVDP in the ischaemic control group was 46±8mmHg. This data was consistent with the literature (Lecour et al., 2005a). S1P preconditioning did not significantly change LVDP as compared to its respective ischaemic control at the end of reperfusion (45±7mmHg vs. 46±8mmHg, ns vs. control). Co-treatment of AG490 with S1P (67±3mmHg, ns vs. control) and AG490 alone (66±4mmHg, ns vs. control), had no effect on LVDP as compared with the control group at the end of reperfusion

S1P preconditioning did not significantly change heart rate as compared to its respective ischaemic control at the end of reperfusion (268±28 beats/minute vs. 293±11 beats/minute, ns vs. control). Co-treatment of AG490 with S1P (283±21 beats/minute, ns vs. control) and AG490 alone (257±24 beats/minute, ns vs. control), had no effect on heart rate as compared with the control group at the end of reperfusion.

SIP preconditioning did not significantly change coronary flow rate as compared to its respective ischaemic control at the end of reperfusion (5.9±0.8 ml/minute vs. 7.8±1.9 ml/minute, ns vs. control). Co-treatment of AG490 with S1P (5.8 ±0.7 ml/minute, ns vs. control) and AG490 alone (5.0±0.2 ml/minute, ns vs. control), had no effect on coronary flow rate as compared with the control group at the end of reperfusion (Table 1).

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Table 1: Haemodynamic parameters of isolated rat hearts exposed to regional ischaemia and S1P preconditioning

	Preischaemia	Reperfusion 5 minutes	Reperfusion 120 minutes
LVDP (mmHg)			
IC	86 ± 7	69 ± 8	46 ± 8
S1P	83 ± 5	71 ± 7	45 ± 7
S1P+AG	99 ± 3	81 ± 3	67 ± 3
AG	92 ± 5	75 ± 4	66 ± 4
Heart rate (beats/minute)			
IC	287 ± 18	270 ± 14	293 ± 11
S1P	280 ± 20	288 ± 42	268 ± 28
S1P+AG	273 ± 17	297 ± 18	283 ± 21
AG	293 ± 18	240 ± 15	257 ± 24
Flow (ml/minute)			
IC	10.8 ± 1.4	11.2 ± 1.7	7.8 ± 1.9
S1P	9.7 ± 0.9	9.8 ± 0.8	5.9 ± 0.8
S1P+AG	9.8 ± 1.0	8.4 ± 0.6	5.8 ± 0.7
AG	8.1 ± 0.3	8.2 ± 0.2	5.0 ± 0.2

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Parameters measured prior to ischaemia (preischaemia) and at 5 minutes or 120 minutes after reperfusion respectively. IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490. n=6 per group.

4.2.2 Role of S1P preconditioning on infarct size

After 30 minutes of regional ischaemia and 2 hours of reperfusion, the infarct size in the ischaemic control group was 25.8±3.1%. S1P preconditioning significantly decreased infarct size (4.6±1.4%, $p<0.05$ vs. control), an effect consistent with the literature (Vessey et al., 2008b, Vessey et al., 2009b, Lecour et al., 2002). Co-treatment of AG490 with S1P abolished the effect of S1P (29.5±4.0%. ns vs. control) and AG490 alone (25.7±5.9%, ns vs. control), had no effect on infarct size as compared with the control group at the end of reperfusion. All regional ischaemia data were normalised to the area at risk (data not shown).

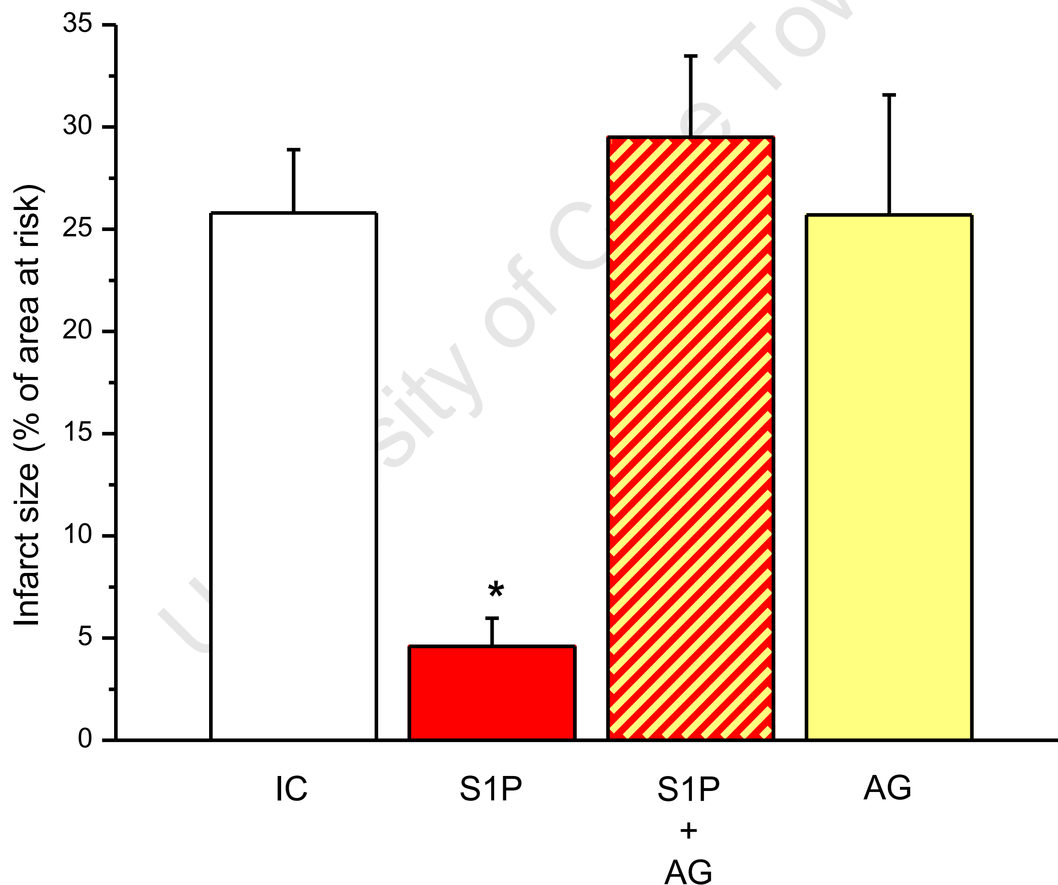

Figure 16: S1P preconditioning decreases infarct size and abolished with AG490 treatment

IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490. *p<0.05 vs. control. n=6 per group.

4.2.3 Role of S1P preconditioning on STAT-3/BAD/FoxO activation

Since S1P preconditioning decreased infarct size, we explored if the protective effect could be accounted by the activation of STAT-3 and its possible downstream effectors including BAD and FoxO. Western blot sampling initially occurred at 5 minutes during reperfusion in order to correctly identify the time period where one could detect changes in the proposed downstream effectors of S1P. Previous research from this lab has demonstrated changes in STAT-3 within 5 minutes of reperfusion (Lecour et al., 2005b). However in the setting of S1P preconditioning, other researchers have sampled protein levels after 40 minutes of reperfusion after global ischaemia (Vessey et al., 2009a).

Subsequent to the results of sampling at 5 minutes, we repeated the experiment at 15 minutes of reperfusion which has been used by many researchers as another optimal time period to detect changes in protein expression levels during reperfusion (Hausenloy et al., 2005, Fuglesteg et al., 2008).

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4.2.3.1.1 Role of S1P preconditioning on tyrosine phosphorylated STAT-3 at 5 minutes of reperfusion

After 30 minutes of regional ischaemia and at 5 minutes of reperfusion, S1P preconditioning did not significantly change tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control in the cytosolic fraction (1.3±0.3A.U. vs. 1.2±0.1A.U., ns vs. control). In the nuclear fraction, S1P preconditioning tended to decrease tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control at 5 minutes of reperfusion (1.0±0.1A.U. vs. 1.6±0.3A.U., $p=0.07$). However such a trend should be interpreted cautiously as no comparison was made with the AG490 co-treatment or AG490 alone (Figure 17).

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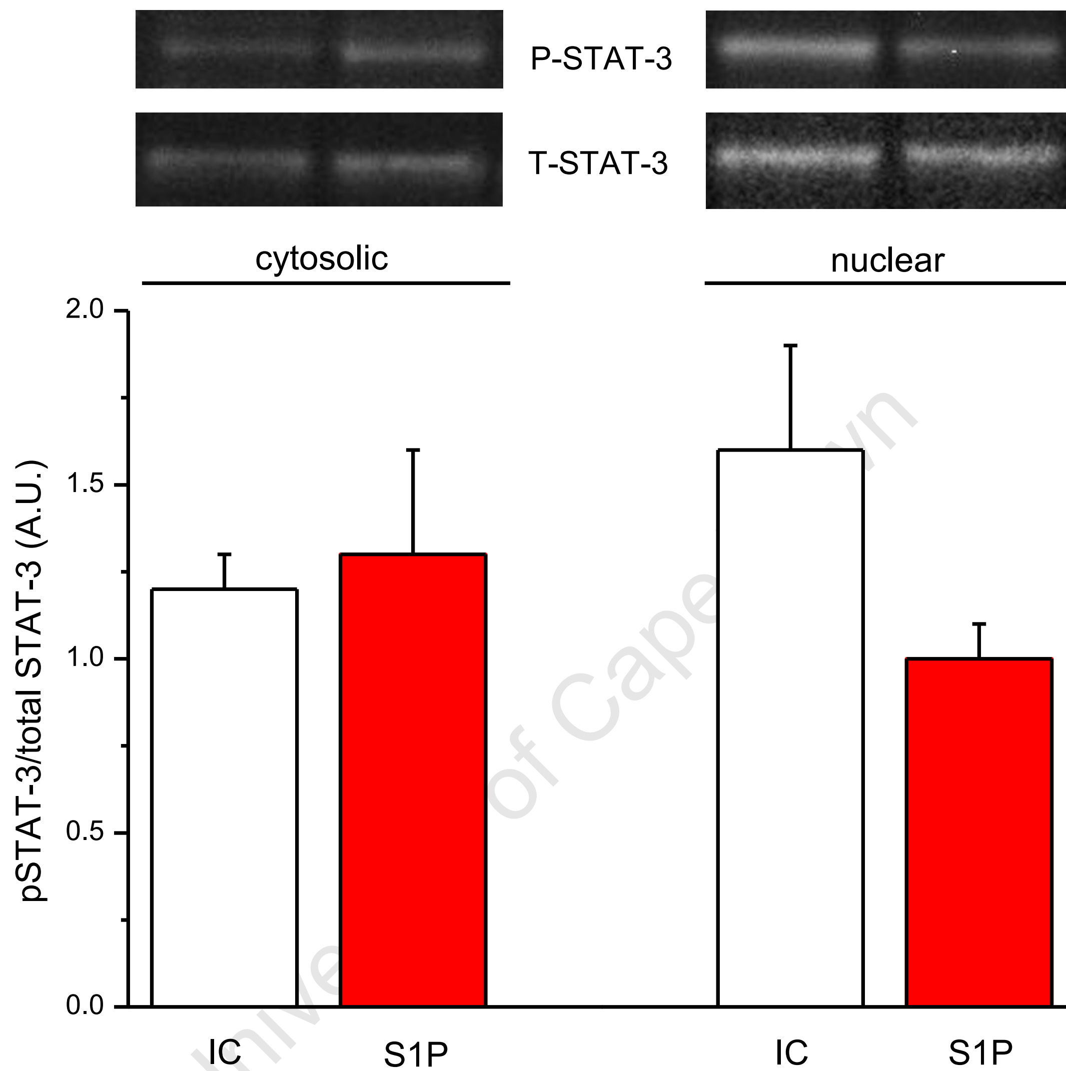

Figure 17: Tyrosine phosphorylated STAT-3 in cytosolic and nuclear fractions in isolated rat hearts undergoing regional ischaemia at 5 minutes of reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490. *p<0.05 vs. control. n=6 per group.

4.2.3.1.2. Role of S1P preconditioning on serine phosphorylated STAT-3 at 5 minutes of reperfusion

S1P preconditioning did not significantly change serine phosphorylated STAT-3 as compared to its respective ischaemic control in the cytosolic fraction (1.1±0.2A.U. vs. 1.2±0.3A.U., ns vs. control) or in the nuclear fraction at 5 minutes of reperfusion (1.5±0.4A.U. vs. 1.5±0.3A.U., ns vs. control) (Figure 18).

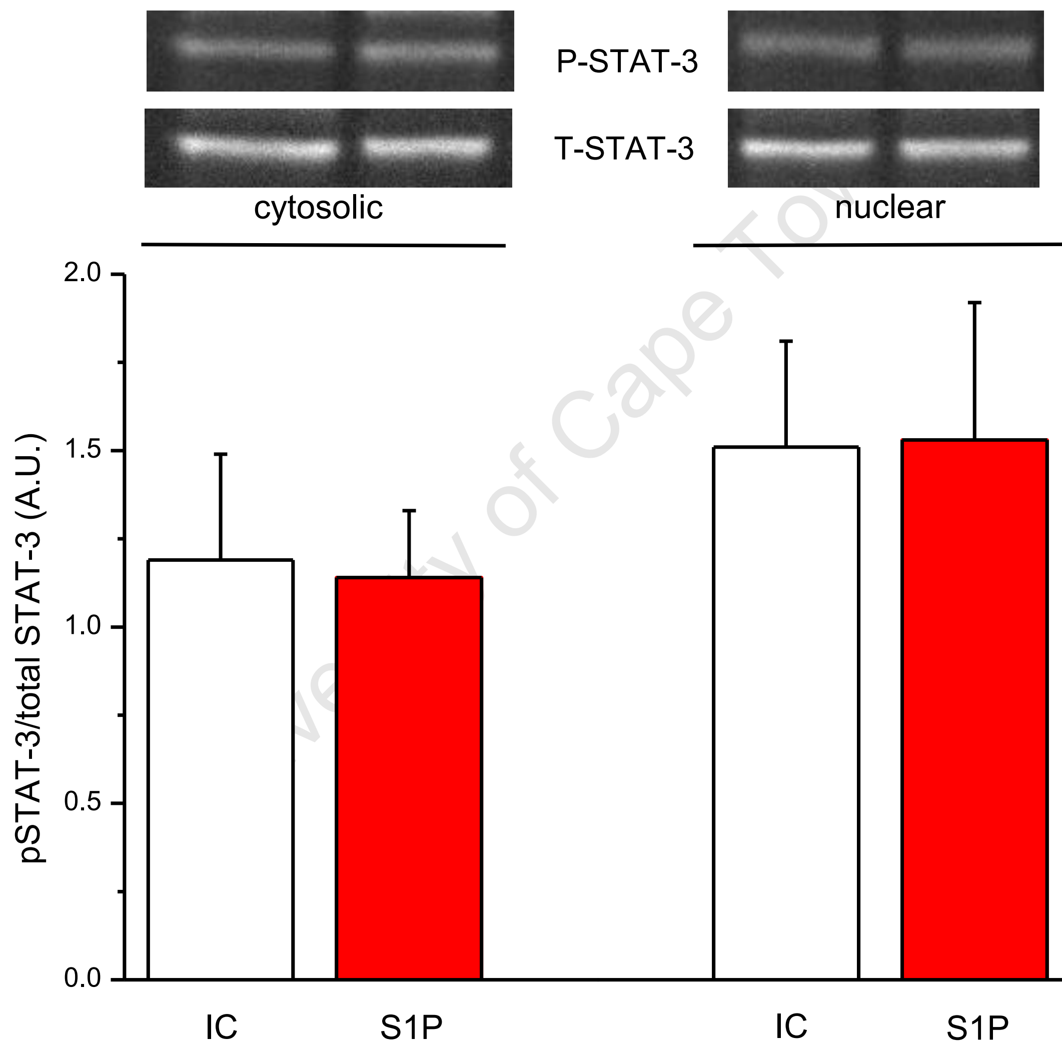

Figure 18: Serine phosphorylated STAT-3 in cytosolic and nuclear fractions of isolated rat hearts undergoing regional ischaemic at 5 minutes of reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. n=6 per group.

4.2.3.1.3 Role of S1P preconditioning on phosphorylated FoxO at 5 minutes of reperfusion

S1P preconditioning did not significantly change phosphorylated FoxO as compared to its respective ischaemic control in the cytosolic fraction (0.5±0.1A.U. vs. 0.8±0.2A.U., ns vs. control) or in the nuclear fraction at 5 minutes of reperfusion (1.3±0.3A.U. vs. 0.9±0.2A.U., ns vs. control) (Figure 19).

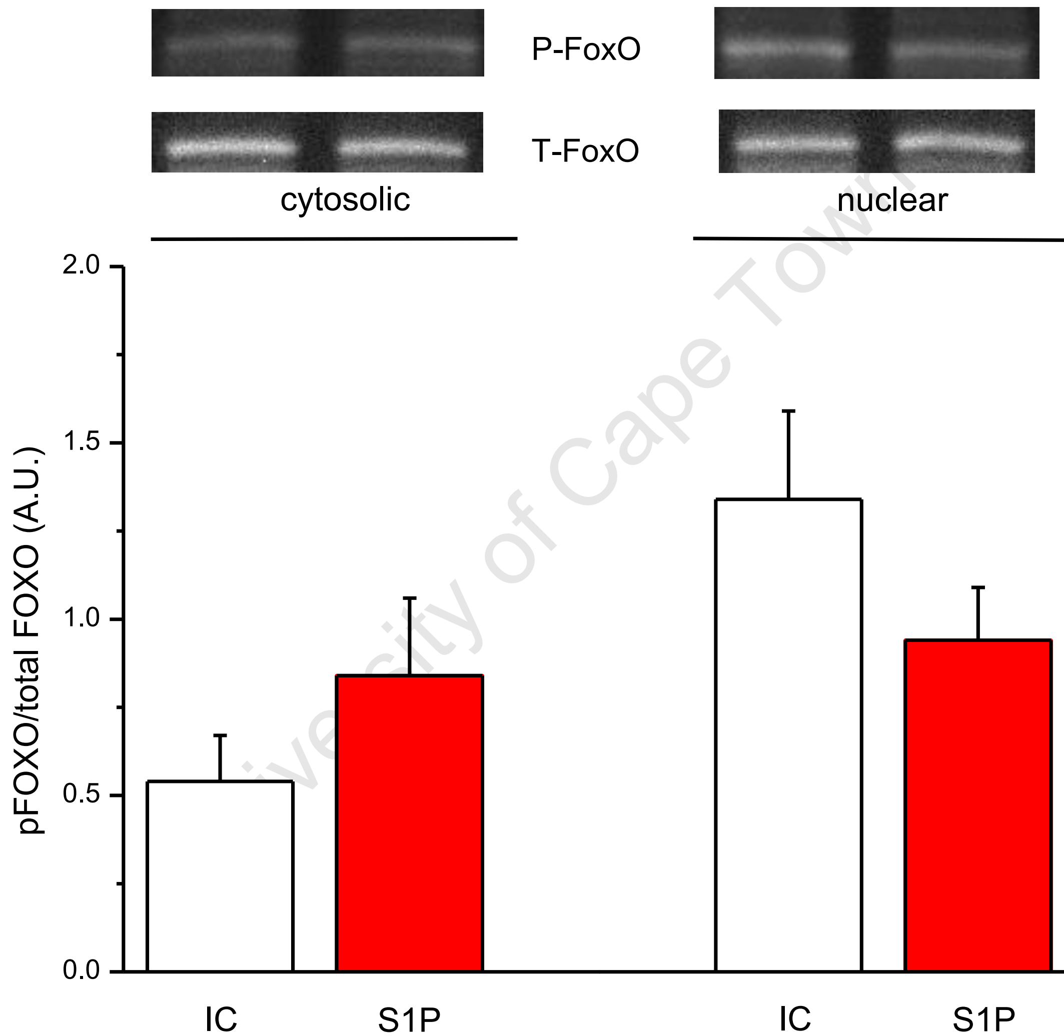

Figure 19: Phosphorylated FoxO in cytosolic and nuclear fractions in isolated rat hearts undergoing regional ischaemia at 5 minutes of reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. n=6 per group.

4.2.3.1.3 Role of S1P preconditioning on phosphorylated BAD at 5 minutes of reperfusion

In addition to FoxO, BAD was another possible downstream effector, whereby phosphorylation of proapoptotic BAD results in cardioprotection (Murphy, 2004). S1P preconditioning tended to decrease phosphorylated BAD as compared to its respective ischaemic control in the cytosolic fraction (1.6±0.4A.U vs. 0.7±0.3A.U., p=0.08). Again, such a trend should be interpreted cautiously as no comparison was made with the AG490 co-treatment or AG490 alone. In the nuclear fraction, S1P preconditioning did not significantly change phosphorylated BAD as compared to its respective ischaemic control at 5 minutes of reperfusion (1.4±0.4A.U. vs. 1.5±0.4A.U., ns vs. control) (Figure 20).

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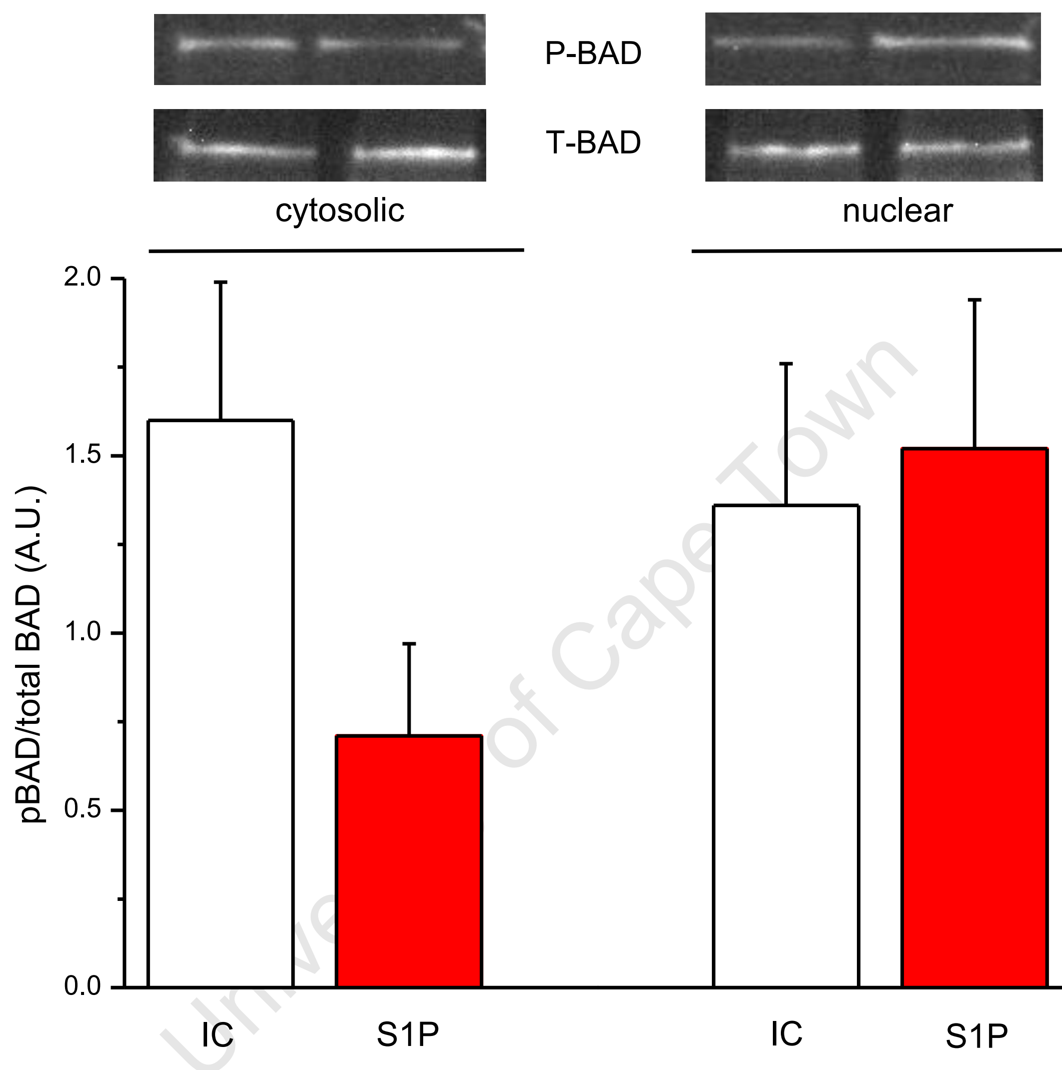

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Figure 20: Phosphorylated BAD in cytosolic and nuclear fractions in isolated rat hearts undergoing regional ischaemia at 5 minutes of reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. n=6 per group.

4.2.4.1.1 Role of S1P preconditioning on tyrosine phosphorylated STAT-3 at 15 minutes of reperfusion

Similar to the previous experiments conducted at 5 minutes of reperfusion, tyrosine phosphorylated STAT-3 levels were examined at 15 minutes of reperfusion. In the cytosolic fraction, S1P preconditioning did not significantly change tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control at 15 minutes of reperfusion (1.8±0.4A.U. vs. 1.6±1.0A.U., ns vs. control). Co-treatment of AG490 with S1P (1.6±1.0A.U., ns vs. control) and AG490 alone (1.4±0.7A.U., ns vs. control), had no effect on tyrosine phosphorylated STAT-3 as compared with the control group at 15 minutes of reperfusion.

In the nuclear fraction, S1P preconditioning did not significantly change tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control at 15 minutes of reperfusion (1.2±0.4A.U. vs. 0.5±0.1A.U., ns vs. control). Co-treatment of AG490 with S1P (0.7±0.3A.U., ns vs. control) and AG490 alone (1.1±0.4A.U., ns vs. control), had no effect on tyrosine phosphorylated STAT-3 as compared with the control group at 15 minutes of reperfusion (Figure 21).

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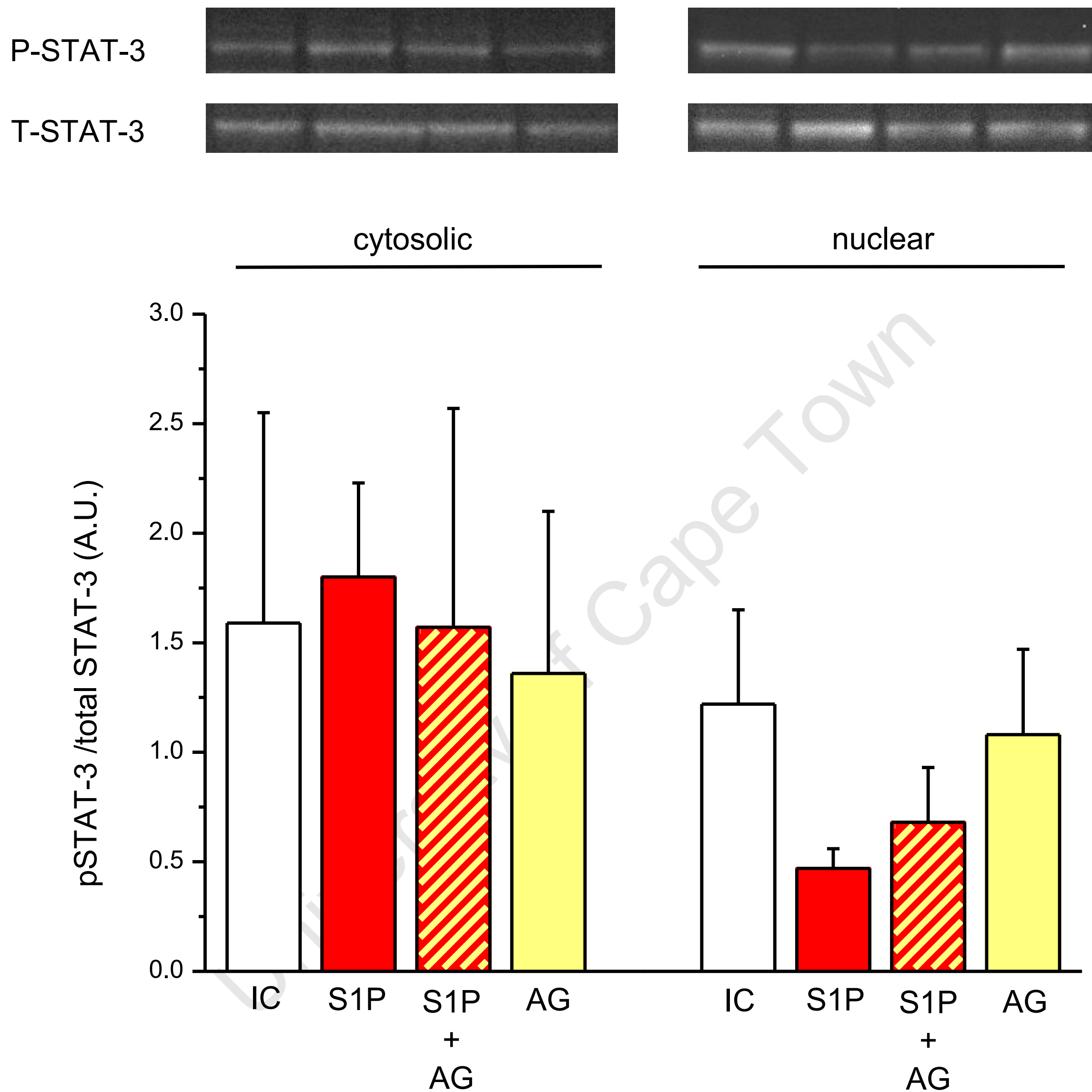

Figure 21: Tyrosine phosphorylated STAT-3 in cytosolic and nuclear fractions in isolated rat hearts undergoing regional ischaemia at 15 minutes of reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. n=4 per group.

4.2.4.1. 2 Role of S1P preconditioning on serine phosphorylated STAT-3 at 15 minutes of reperfusion

Similar to the previous experiments conducted at 5 minutes of reperfusion, serine phosphorylated STAT-3 levels were examined at 15 minutes of reperfusion. In the cytosolic fraction, S1P preconditioning did not significantly change phosphorylated serine STAT-3 as compared to its respective ischaemic control at 15 minutes of reperfusion (1.1±0.3A.U. vs. 1.4±0.4A.U., ns vs. control). Co-treatment of AG490 with S1P (0.6±0.3A.U., ns vs. control) or AG490 alone (0.9±0.4A.U., ns vs. control), had no effect of serine phosphorylated STAT-3 as compared with the control group at 15 minutes of reperfusion.

In the nuclear fraction, S1P preconditioning did not significantly change serine phosphorylated STAT-3 as compared to its respective ischaemic control at 15 minutes of reperfusion (0.7±0.3A.U. vs. 0.5±0.4A.U., ns vs. control). Co-treatment of AG490 with S1P (0.9±0.5A.U., ns vs. control) or AG490 alone (0.8±0.6A.U., ns vs. control), had no effect on serine phosphorylated STAT-3 as compared with the control group at 15 minutes of reperfusion (Figure 22).

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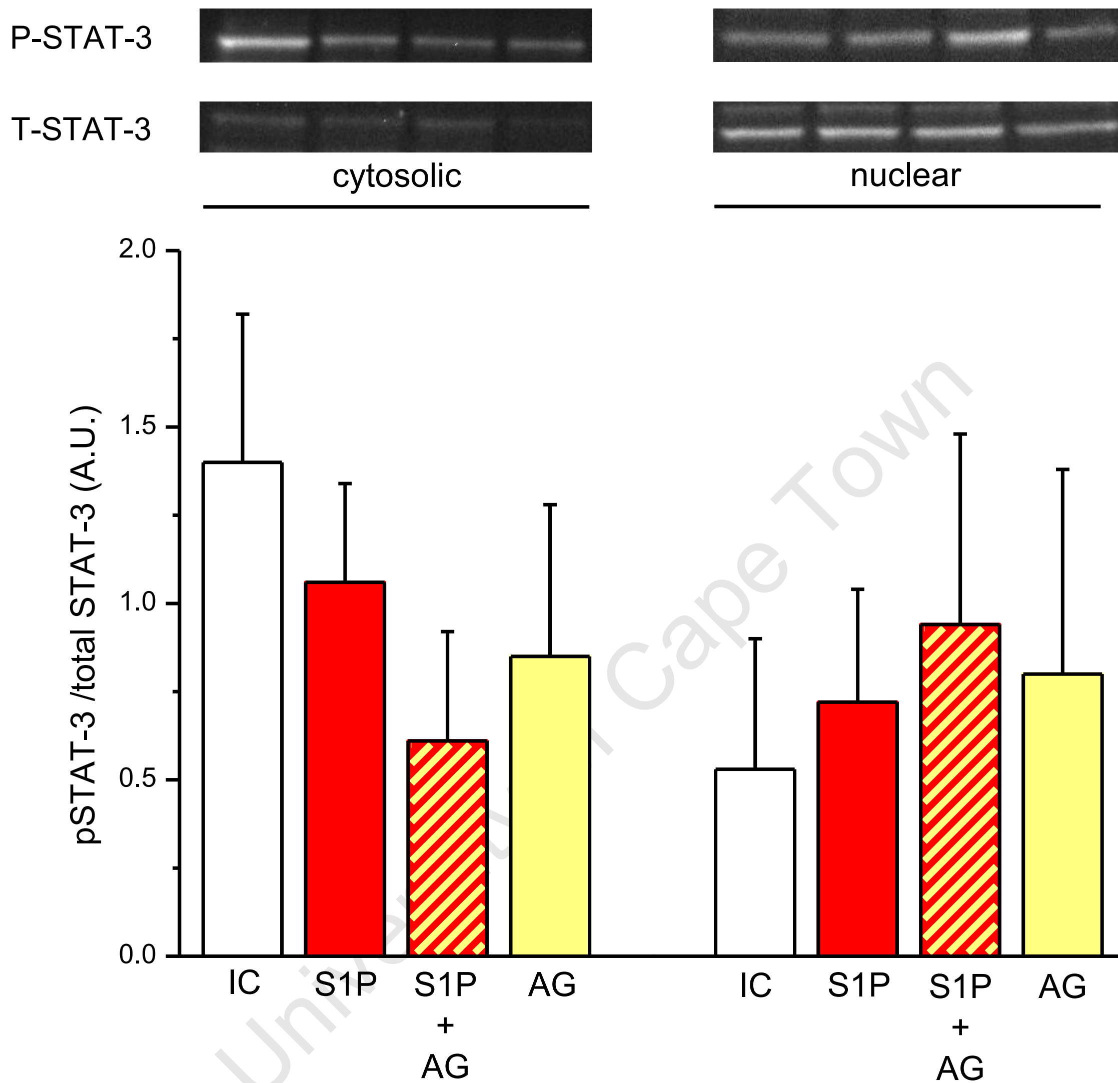

Figure 22: Serine phosphorylated STAT-3 in cytosolic and nuclear fractions in isolated rat hearts undergoing regional ischaemia at 15 minutes of reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. n=4 per group.

4.2.4.1. 3 Role of S1P preconditioning on phosphorylated FoxO at 15 minutes of reperfusion

Similar to the previous experiments conducted at 5 minutes of reperfusion, phosphorylated FoxO levels were examined at 15 minutes of reperfusion. In the cytosolic fraction, S1P preconditioning did not significantly change phosphorylated FoxO as compared to its respective ischaemic control at 15 minutes of reperfusion (1.4±0.9A.U. vs. 1.0±0.4A.U., ns vs. control). Co-treatment of AG490 with S1P (2.0±0.9A.U., ns vs. control) or AG490 alone (1.3±0.4A.U., ns vs. control), had no effect on phosphorylated FoxO as compared with the control group at 15 minutes of reperfusion.

In the nuclear fraction, S1P preconditioning did not significantly change phosphorylated FoxO as compared to its respective ischaemic control at 15 minutes of reperfusion (1.5±0.8A.U. vs. 1.5±0.5A.U., ns vs. control). Co-treatment of AG490 with S1P (1.2±0.5A.U., ns vs. control) or AG490 alone (1.5±0.4A.U., ns vs. control), had no effect on phosphorylated FoxO as compared with the control group at 15 minutes of reperfusion (Figure 23).

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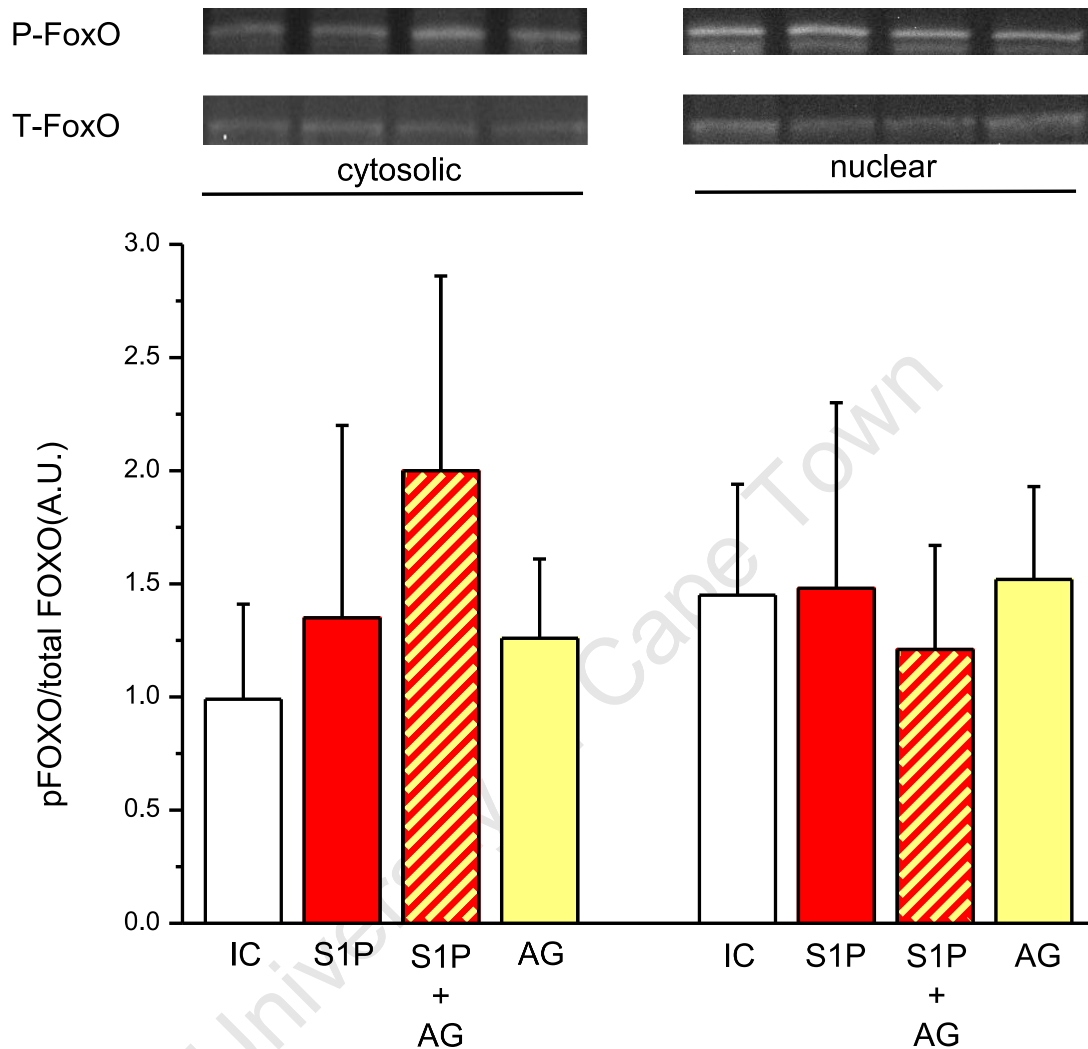

Figure 23: Phosphorylated FoxO in cytosolic and nuclear fractions in isolated rat hearts undergoing regional ischaemia at 15 minutes reperfusion

IC=ischaemic control, S1P=sphingosine 1 phosphate, AG=AG490, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. n=4 per group.

4.3 Ischaemic postconditioning protects the heart

4.3.1 Role of ischaemic postconditioning in hearts subjected to regional ischaemia

4.3.1.1 Haemodynamic parameters

After 30 minutes of regional ischaemia and 120 minutes of reperfusion, the LVDP in the ischaemic control group was 56±4mmHg. This data was consistent with the literature (van Vuuren et al., 2008, Lee et al., 2010). Ischaemic postconditioning did not significantly change LVDP as compared to its respective ischaemic control at the end of reperfusion (67±3mmHg vs. 56±4mmHg, ns vs. control). Co-treatment of AG490 with ischaemic postconditioning (49±5mmHg, ns vs. control) and AG490 alone (58±4mmHg, ns vs. control), had no effect on LVDP as compared with the control group at the end of reperfusion.

Ischaemic postconditioning did not significantly change heart rate as compared to its respective ischaemic control at the end of reperfusion (292±8 beat/minute vs. 283±13 beats/minute, ns vs. control). Co-treatment of AG490 with ischaemic postconditioning (253±12 beats/minute, ns vs. control) and AG490 alone (257±24 beats/minute, ns vs. control), had no effect on heart rate as compared with the control group at the end of reperfusion.

Ischaemic postconditioning did not significantly change coronary flow rate as compared to its respective ischaemic control at the end of reperfusion (8.0±0.6 ml/minute vs. 6.2±0.7 ml/minute, ns vs. control). Co-treatment of AG490 with ischaemic postconditioning (5.9±0.6 ml/minute, ns vs. control) and AG490 alone (5.0±0.2 ml/minute, ns vs. control), had no effect on coronary flow rate as compared with the control group at the end of reperfusion (Table 2).

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Table 2: Haemodynamic parameters of isolated rat hearts exposed to regional ischaemia and ischaemic postconditioning

	Preischaemia	Reperfusion 5 minutes	Reperfusion 120 minutes
LVDP (mmHg)			
IC	77 ± 2	70 ± 3	56 ± 4
Postcon	81 ± 4	75 ± 3	67 ± 3
Postcon+AG	90 ± 4	75 ± 8	49 ± 5
AG	94 ± 8	80 ± 7	58 ± 4
Heart rate (beats/minute)			
IC	314 ± 14	257 ± 29	283 ± 13
Postcon	307 ± 13	292 ± 10	292 ± 8
Postcon+AG	316 ± 12	253 ± 7	253 ± 12
AG	284 ± 15	267 ± 28	229 ± 21
Flow (ml/minute)			
IC	9.1 ± 0.4	8.1 ± 0.6	6.2 ± 0.7
Postcon	10.3 ± 0.8	9.8 ± 0.7	8.0 ± 0.6
Postcon+AG	10.7 ± 0.7	9.8 ± 0.6	5.9 ± 0.6
AG	10.7 ± 1.3	10.0 ± 0.8	6.1 ± 0.9

Parameters measured prior to ischaemia (preischaemic) and at 5 minutes or 120 minutes after reperfusion respectively. AG490 (100 nmol/L), LVDP=left ventricular developed pressure, IC=ischaemic control, postcon=postconditioning, AG=AG490. n=6 per group.

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4.3.1.2 Infarct size

After 30 minutes of regional ischaemia and 2 hours of reperfusion, the infarct size in the ischaemic control group was 32±5%. Ischaemic postconditioning with 6 cycles of alternating 10 seconds of reperfusion and 10 seconds of ischaemia significantly decreased infarct size (11±1%, $p<0.05$ vs. control), an effect consistent with the literature (You et al., 2011, Zhao et al., 2003). Co-treatment of AG490 with ischaemic postconditioning abolished the effect of ischaemic postconditioning (26±4%, ns vs. control) and AG490 alone (33±7%, ns vs. control), had no effect on infarct size as compared with the control group at the end of reperfusion (Figure 24). All regional ischaemia data were normalised to the area at risk (data not shown).

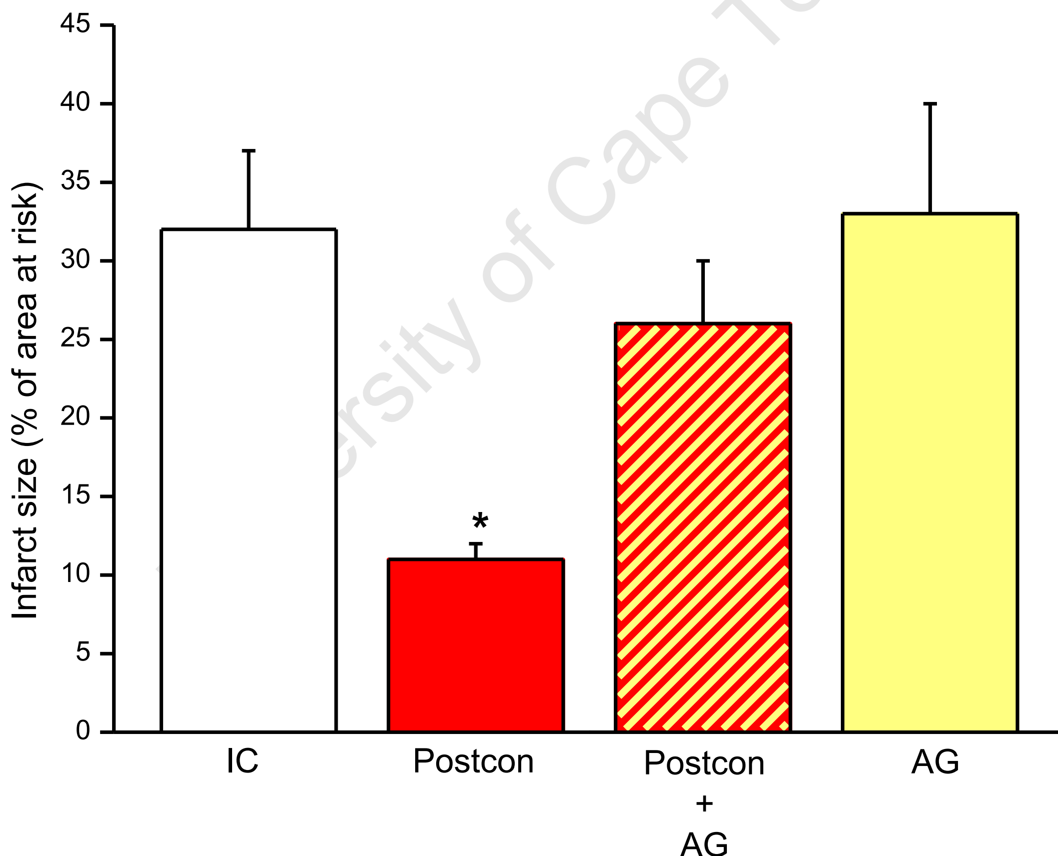

Figure 24: Ischaemic postconditioning decreases infarct size and abolished with AG490 treatment

IC=ischaemic control, AG=AG490. *p<0.05 vs. control. n=6 per group.

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4.3.2 Role of ischaemic postconditioning in hearts subjected to global ischaemia

4.3.2.1. Haemodynamic parameters

After 30 minutes of global ischaemia and 30 minutes of reperfusion, the LVDP was 25±3mmHg. Ischaemic postconditioning significantly increased LVDP (52±5mmHg vs. 25±3mmHg, $p<0.01$ vs. control), an effect consistent with the literature (Inamura et al., 2010, Jin et al., 2008). Co-treatment of AG490 with ischaemic postconditioning abolished the effect of ischaemic postconditioning (36±6mmHg, ns vs. control) and AG490 alone (21±4mmHg, ns vs. control), had no effect on LVDP as compared with the control group at the end of reperfusion.

Ischaemic postconditioning did not significantly change heart rate as compared to its respective ischaemic control at the end of reperfusion (248±15 beats/minute vs. 260±25 beats/minute, ns vs. control). Co-treatment of AG490 with ischaemic postconditioning (207±15 beats/minute, ns vs. control) and AG490 alone (273±35 beats/minute), had no effect on heart rate as compared with the control group at the end of reperfusion.

Ischaemic postconditioning did not significantly change coronary flow rate as compared to its respective ischaemic control at the end of reperfusion (8.3±2.0 ml/minute vs. 7.0±1.4ml/minute, ns vs. control). Co-treatment of AG490 with ischaemic postconditioning (9.8±1.1ml/minute, ns vs. control) and AG490 alone (8.0±0.6 ml/minute), had no effect on coronary flow rate as compared with the control group at the end of reperfusion (Table 3).

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Table 3: Hemodynamic parameters of isolated rat hearts exposed to global ischaemic postconditioning

	Pre-ischemic	Reperfusion 5 minutes	Reperfusion 30 minutes
LVDP (mmHg)			
IC	80 ± 1	22 ± 4	25 ± 3
Postcon	83 ± 3	40 ± 8 *	52 ± 5 *
Postcon+AG	83 ± 4	25 ± 2	36 ± 6
AG	87 ± 6	20 ± 3	21 ± 4
Heart rate (beats/minute)			
IC	313 ± 22	220 ± 38	260 ± 25
Postcon	304 ± 10	250 ± 44	248 ± 15
Postcon+AG	333 ± 8	200 ± 38	207 ± 16
AG	307 ± 13	180 ± 47	273 ± 35
Flow (ml/minute)			
IC	12.4 ± 1.6	9.0 ± 1.2	7.0 ± 1.4
Postcon	13.3 ± 1.3	10.0 ± 1.2	8.3 ± 2.0
Postcon+AG	12.0 ± 0.6	7.0 ± 1.0	9.8 ± 1.1
AG	12.7 ± 0.7	7.0 ± 1.0	8.0 ± 0.6

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Parameters measured prior to ischemia (pre-ischemic), 5 minutes and 30 minutes after reperfusion. AG490 (100 nmol/L). LVDP=left ventricular developed pressure, postcon=postconditioning, AG=AG490, * $p<0.01$ vs. IC, n≥4 per group.

4.3.2.2. Role of ischaemic postconditioning on rate pressure product (RPP)

After 30 minutes of global ischaemia, ischaemic postconditioning did significantly raise the rate pressure product (RPP) as compared to its respective ischaemic control at 5 minutes (48±1% vs. 19±3%, $p<0.05$ vs. control) and at 30 minutes of reperfusion (51±5% vs. 26±4%, $p<0.05$ vs. control). This data was consistent with the literature (Bopassa et al., 2006, Lee et al., 2010). Co-treatment of AG490 with ischaemic postconditioning abolished the effect of ischaemic postconditioning as compared to the control group at 5 minutes (16±4%, ns vs. control) and at 30 minutes of reperfusion (26±7%, ns vs. control). AG490 alone did not significantly change RPP as compared with control group at 5 minutes (13±5%, ns vs. control) and at 15 minutes of reperfusion (21±3%, ns vs. control) (Figure 25).

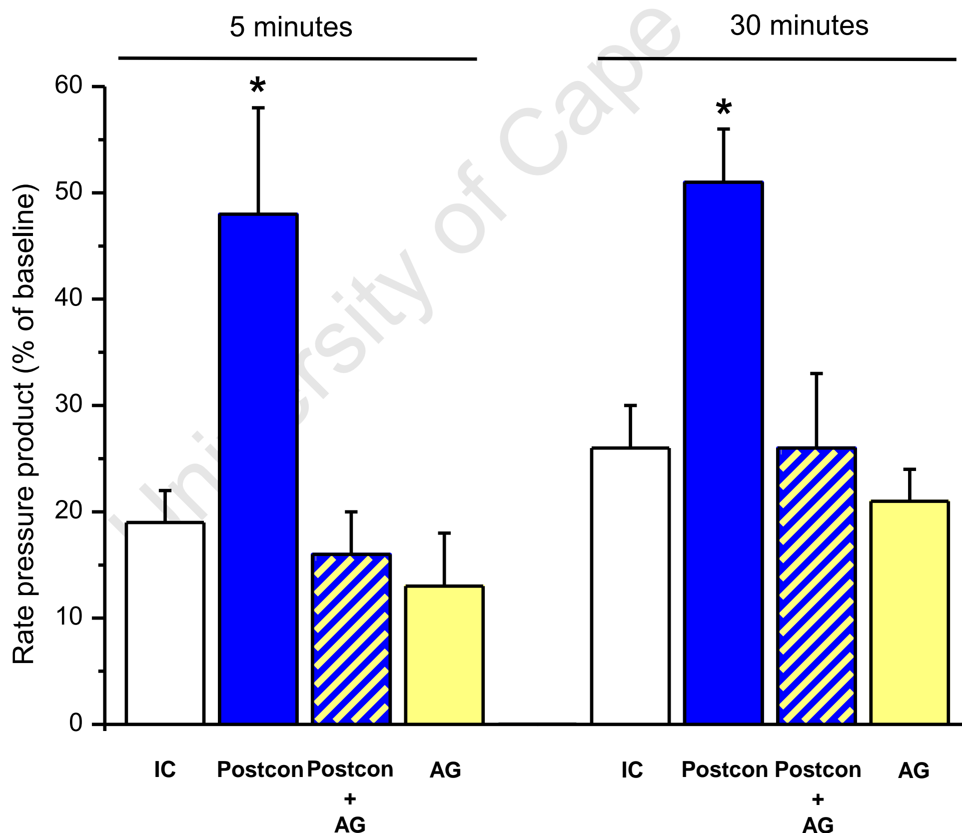

Figure 25: Postconditioning improves the rate pressure product at 5 and 30 minutes after reperfusion in isolated rat hearts after global ischaemia

IC=ischaemic control, postcon=postconditioning, AG=AG490. *$p<0.05$ vs. control. n≥4 per group.

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4.3.2.3 Role of ischaemic postconditioning on tyrosine phosphorylated STAT-3 at 15 minutes of reperfusion

After 30 minutes of global ischaemia and at 15 minutes of reperfusion, ischaemic postconditioning did significantly decrease tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control in the cytosolic fraction (0.5±0.1A.U. vs. 1.6±0.3A.U., $p<0.05$ vs. control). In the nuclear fraction, ischaemic postconditioning concurrently increase tyrosine phosphorylated STAT-3 as compared to its respective ischaemic control at 15 minutes of reperfusion (2.5±0.2A.U. vs. 1.3±0.4A.U., $p<0.05$ vs. control) (Figure 26).

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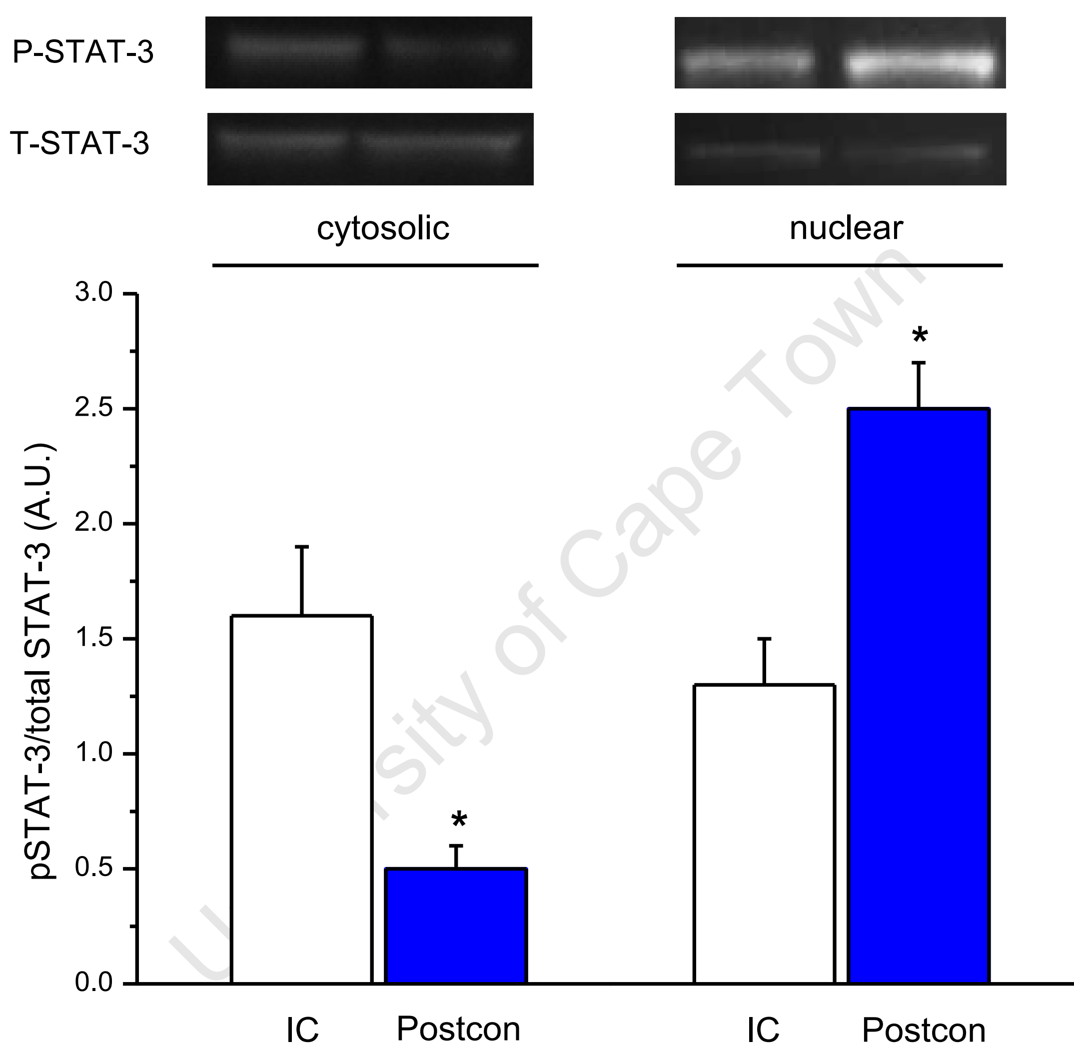

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Figure 26: Tyrosine phosphorylated STAT-3 in postconditioning isolate rat hearts including cytosolic and nuclear fractions

IC=ischaemic control, postcon=postconditioning, P-STAT-3=phosphorylated STAT-3, T-STAT-3=Total STAT-3. *p<0.05 vs. control. n=4 per group.

Chapter Five: Discussion

The results of this study have demonstrated that S1P preconditioning can mimic ischaemic preconditioning to result in cytoprotection in fibroblasts and cardioprotection in the isolated rat heart. Furthermore, the cell survival and infarct sparing effect of S1P preconditioning was abrogated by AG490, an inhibitor of the STAT-3 pathway therefore suggesting a role of STAT-3 in S1P preconditioning. Tyrosine phosphorylated STAT-3 was significantly decreased in the cell cytoplasm of fibroblasts as measured prior to the index ischaemia. In contrast, STAT-3 displayed no significant change after the index ischaemia in the isolated rat hearts. In addition, FoxO and BAD displayed no significant change after the index ischaemia suggesting that they are not involved as signalling mediators of S1P preconditioning. Ischaemic postconditioning could protect the isolated rat heart subjected to regional or global ischaemic reperfusion insults and western blot analysis suggest a role for STAT-3 in ischaemic postconditioning.

5.1 S1P confers cardioprotection in fibroblasts and isolated rat hearts

Using cell culture and isolated rat heart model, our data demonstrate that S1P confers cardioprotection against ischaemia reperfusion. Whilst our data is well validated in the isolated rat heart model (Vessey et al., 2009b, Tsukada et al., 2007), discrepancies arise in the use of the mouse L cell fibroblasts. Previous studies have successfully used the L cell line to validate preconditioning (Lecour et al., 2006) however it remains unknown how S1P may impact on mouse L cell fibroblasts and if these fibroblasts could be comparable to cardiac fibroblasts.

Other studies have noted discrepancies between cardiac fibroblasts and cardiomyocytes when studying the cellular effects of S1P. Cardiac fibroblasts express more $S1P_3$ receptors than $S1P_1$ and $S1P_2$ receptors. Cardiac fibroblasts also exhibit greater sphingosine kinase activity than cardiac myocytes. Hence S1P preconditioning will result in a greater increase in endogenous S1P production in

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fibroblasts as compared to cardiomyocytes respectively. (Landeen et al., 2008). As a result, there is a less robust response of exogenous S1P preconditioning in fibroblasts as compared to cardiomyocytes most likely because more S1P is already available intracellularly in fibroblasts.

Some researchers have used different dosages and vehicles for S1P. Such variation in drug concentration is due to the inherent physiological variation of S1P serum concentration which normally ranges from 0.2 to 1.1µM (Murata et al., 2000, Berdyshev et al., 2005), although these concentrations are most likely to vary during pathological conditions. The S1P dose used in our study was 10nM which is in agreement with some studies (Jin et al., 2002, Tsukada et al., 2007) but other studies used 0.4µM (400nM) (Vessey et al., 2009b). Our concentration was low in order to limit known haemodynamic effects such as coronary vasoconstriction via $S1P_2$ (Ohmori et al., 2003), bradycardia via $S1P_3$ (Sanna et al., 2004) and decreased blood pressure (Karliner, 2004). The vehicle for S1P delivery was DMSO in agreement with a previous study (Jin et al., 2002). Interestingly, a recent study used reconstituted HDL to demonstrate a cardioprotective effect of S1P against doxorubicin induced cardiotoxicity (Frias et al., 2009).

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5.1.1 Role of STAT-3 in S1P induced cytoprotection and cardioprotection

S1P is a known preconditioning mimetic using PKCε (Jin et al., 2002) or Akt (Vessey et al., 2008a) or ERK1/2 as signalling pathways. In fact, S1P itself has also been proposed to mediate the protective effects of TNFα preconditioning, via S1P's involvement in the sphingolipid signalling pathway (Lecour et al., 2002, Vessey et al., 2009b). However it was unknown whether the JAK/STAT-3 pathway can also mediate the effects of S1P preconditioning.

Our data has demonstrated that S1P mediates its cytoprotective and cardio-protective effects via the JAK/STAT-3 pathway. A link between S1P and JAK/STAT-3 has recently been demonstrated whereby S1P as part of HDL, protects against ischaemia reperfusion via STAT-3 activation (Frias et al., 2009). Furthermore, recent

work conducted in our lab showed that S1P can mimic ischaemic postconditioning via both STAT-3 and Akt (Somers, 2010).

While our data showed a decrease in cytosolic STAT-3 in fibroblasts exposed to S1P preconditioning, a reciprocal change in the nuclear fraction was not observed. STAT-3 translocation is important because nuclear translocation will result in the transcription of genes such as inducible nitric oxide synthase and cyclooxygenase 2 (Dawn et al., 2004) but cytosolic translocation, or more importantly mitochondrial translocation will result in the direct interaction of STAT-3 with mitochondria to prevent MPTP closure as currently postulated (Hausenloy et al., 2010b).

To our surprise, AG490 improved cell survival in fibroblasts as compared to the ischaemic control. Such findings are most likely attributed to the low 'n' value of 2 for the AG490 group as compared to the ischaemic control group of 5 or greater. In addition, whilst other studies have found that addition of AG490 protected the heart (Hwang et al., 2005, Mascareno et al., 2001), their respective concentrations were 50 μM and 5 μmol/L, which exceeds the 100nM dose used in this study and by others (Lecour et al., 2005b, Suleman et al., 2008, Wang et al., 2004). Hwang et al. and Mascareno et al. were also concerned with STAT-5 and STAT-6 activation, and not STAT-3 inhibition (Hwang et al., 2005, Mascareno et al., 2001). Lastly, since AG490 is an indirect pharmacological inhibitor of STAT-3, the non-specific effects of raising drug concentrations cannot be determined (see 'limitations').

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5.1.2 Possible end effectors of S1P preconditioning

Many researchers have proposed that the anti-apoptotic effect of preconditioning is critical in mediating reperfusion injury (Nakamura et al., 2000, Anversa et al., 1998). Hence our research has focused on whether S1P can also mediate these antiapoptotic effects.

5.1.2.1 BAD

Previous research has implicated the increase in antiapoptotic BCL-2 (Ding et al., 2010), the decrease in proapoptotic BAX (Nakamura et al., 2000) and BAD (Stein et al., 2007, Feng et al., 2005, Das et al., 2005). In fact, recent research has suggested that BAD is down regulated in response to TNFα preconditioning via the JAK/STAT-3 pathway (Lecour et al., 2005b).

Our results did not support a down regulation of proapoptotic BAD with S1P preconditioning. Such results are in agreement with another paper in which coronary effluent from preconditioned hearts induced a preconditioned state in donor hearts using the JAK/STAT-3 signalling cascade, but failed to detect a difference in BAD activation (Huffman et al., 2008). In contrast, other studies have noted the involvement of proapoptotic BAD but have either used different preconditioning stimuli such as bradykinin (Feng et al., 2005), resveratrol (Das et al., 2005) and late preconditioning (Stein et al., 2007), or have used non-cardiac models such as liver (Izuishi et al., 2006, Kulhanek-Heinze et al., 2004, Wang et al., 2008) or neurons (Miyawaki et al., 2008). In addition to these differences, activation of BAD was thought to have occurred via other signalling pathways including the Akt pathway (Das et al., 2005, Izuishi et al., 2006), extracellular signal-regulated kinase (ERK) pathway (Wang et al., 2008) and protein kinase A (Kulhanek-Heinze et al., 2004). However, our data needs to be interpreted with caution as only two time points were considered to measure the phosphorylation of BAD at the onset of reperfusion.

The importance of BAD translocation, in particular to the cytosol and specifically the mitochondria, is still postulated in cardiac preconditioning (Murphy, 2004). During normal physiological conditions, BAD is phosphorylated and hence bound to 14-3-3 therefore allowing Bcl-2 to inhibit apoptosis. Once unphosphorylated, apoptosis is allowed to proceed with BH123 protein inducing outer membrane permeabilisation of the mitochondria (Obexer et al., 2007, Letai et al., 2002).

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5.1.2.2 FOXO

Very few studies have examined the role of FoxO in ischaemic preconditioning. Of these four papers, most have recognised that Akt activation is central to phosphorylation and hence inactivation of the proapoptotic effects of FoxO.

Such evidence is provided in neuronal tissue, indicating that hypoxic preconditioning can activate Akt to phosphorylate FoxO1, FoxO3a and FoxO4. Interestingly, the phosphorylation of these FoxO's were decreased at 4 hours of reperfusion but increased at 14 and 48 hours of reperfusion (Zhan et al., 2010). Similarly, in PC12 cells (rat pheochromocytoma), ischaemic preconditioning induced phosphorylation of Akt and inactivation of GSK3β and FoxO4 (Hillion et al., 2006). Finally, ischaemic preconditioning in H11 kinase cardiac specific overexpressing mice resulted in an increase in Akt activity to inhibit and prevent the proapoptotic role of GSK3β, FoxO1a and BAD (Depre et al., 2006).

Our study did not support a change in phosphorylation status of FoxO at 5 and 15 minutes of reperfusion from S1P preconditioning. Again, the time point measuring FoxO may have been incorrect to measure the activation of FoxO by S1P preconditioning.

The importance of FoxO translocation, in particular to the nucleus, can be found from ischaemia reperfusion studies and not yet from ischaemic preconditioning studies. Whilst our results did not show FoxO translocation as a result of S1P preconditioning, ischaemia reperfusion studies have shown that FoxO can translocate to the nucleus. The result can be to an beneficial hence an increase in manganese superoxide dismutase as seen in Sirt1 mediated cardioprotection (Hsu et al., 2010) or activation of GADD45 (a DNA repair target gene) (Brunet et al., 2004).

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5.1.3. Clinical relevance of S1P preconditioning

Pharmacological preconditioning has been evaluated with adenosine (Ross et al., 2005), nicorandil (Horinaka et al., 2010), ANP (Kitakaze et al., 2007) and recently, cyclosporine (Piot et al., 2008). S1P presents a novel therapeutic target to activate STAT-3 as part of the SAFE pathway and results in cardioprotection.

5.2. Ischaemic postconditioning confers protection to hearts subjected to either regional or global ischaemia via STAT-3

Early data demonstrated the need for STAT-3 activation to result in Akt activation and subsequent RISK pathway activation (Goodman et al., 2008). Later, more evidence was gathered to support the preferential involvement of STAT-3 in ischaemic postconditioning (Lacerda et al., 2009). In fact, some have suggested that rather than the cytosolic or nuclear pool of STAT-3 accounting for the protective effects of postconditioning, the mitochondrial pool of STAT-3 was far more important (Lacerda et al., 2010, Boengler et al., 2010).

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Our data has confirmed the protective effect of STAT-3 activation in ischaemic postconditioning and indeed, in rat hearts undergoing global ischaemia. This is in agreement with previously published reports (You et al., 2011, Goodman et al., 2008).

The role of STAT-3 as part of the JAK/STAT-3 signalling pathway and its interaction with another significant pathway such as RISK is increasingly being explored. Since the seminal paper that proposed that the two pathways are distinct but interdependent on one another (Lecour et al., 2005b), another author has proposed that the JAK/STAT pathway may in fact regulate the RISK pathway (Goodman et al., 2008). In fact, very recent evidence is in support of this hypothesis, demonstrating that with STAT-3 activation, Akt and ERK are activated (Pedretti and Raddatz, 2011, Zhuo et al., 2011).

Despite the recent knowledge of elucidating how pathways interact with one another, further evidence is required to elucidate the role of STAT-3 on the mitochondria directly. Many researchers have found that the MPTP channel serves as the common end point of any pathway to determine cell survival. Maintaining closure of the MPTP channel prevents the release and activation of proapoptotic caspases, and activation of 14-3-3 which inactivates proapoptotic enzymes like FoxO and BAD (Halestrap et al., 2004, Hausenloy et al., 2009).

The use of regional and global ischaemia models were relevant because both models replicate either preconditioning or postconditioning in potentially clinically important situations. Those situations which mimic the regional ischaemia model include the use of coronary balloon angioplasty as a conditioning mimetic before coronary artery bypass surgery (preconditioning) or after a myocardial infarct (postconditioning). Furthermore, the situations which mimic the global ischaemia model include the use of aortic cross clamping and declamping as a conditioning mimetic before cardiac transplant (preconditioning) or, in combination with remote postconditioning, the use of a blood pressure cuff to inflate and deflate repetitively prior to cardiac surgery (remote preconditioning) (Hausenloy and Yellon, 2008, Granfeldt et al., 2009). The latter provides a non-invasive method of inducing a conditioning state globally which potentially can have local benefits to the heart as well as systemic benefits to other organs such as liver, spleen, gastrointestinal tract and kidney (Saxena et al., 2010, Hausenloy and Yellon, 2008).

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5.2.1 Clinical relevance of ischaemic postconditioning and STAT-3

Whilst many clinical studies have examined the protective effect of ischaemic postconditioning (Staat et al., 2005, Thibault et al., 2008), no studies have clinically evaluated the potential success of pharmacologically targeting the JAK/STAT-3 pathway with pharmacological postconditioning. This study hopes to add to existing scientific evidence to the growing knowledge of postconditioning and results in the development of novel therapeutic strategies of targeting the JAK/STAT-3 pathway to to protect the heart.

5.3 Limitations

5.3.1 Time point for western blot tissue collection

Many studies have sampled protein for western blot analysis prior to the index ischaemia in preconditioning studies. However, only one study has examined the state of protein phosphorylation levels after the index ischaemia (Hausenloy et al., 2005). Similar to the aforementioned study, we too examined the phosphorylation levels of STAT-3 after the index ischaemia despite using a preconditioning stimulus. Hence, we are only able to conclude that STAT-3 phosphorylation levels no longer have a dichotomy between cytosolic and nuclear fractions following preconditioning and reperfusion after the index ischaemia. However what remains unknown is the activity of mitochondrial STAT-3 at this time point. Furthermore, because our time point is different to the majority of other studies, comparison between our results and others must be cautioned.

5.3.1.1. Sample size

Throughout the experiment, numerous data demonstrated a trend in the difference amongst groups. Since statistical significance was not reached, it is compelling to increase the sample size for each group in order to achieve significance. Conversely, given the current small sample size, significant data should be interpreted cautiously but generally the p value was small enough to demonstrate that a significant difference was observed amongst groups despite the small sample size.

5.3.2. In vivo vs. in vitro studies

S1P has many pleiotrophic effects not restricted to cardiomyocytes or fibroblasts. These include S1P mediated effects on the endothelium, vascular smooth muscle, platelets and red blood cells (Karliner, 2009, Saba and Hla, 2004). Invariably, use of

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S1P in vivo rather than in vitro or ex vivo would enable us to account for these systemic effects which can impact on the overall outcome.

5.3.3. AG490 and use of knock out mouse model

Pharmacological blockade of the JAK/STAT-3 pathway by AG490 has not always lead to consistent results (Mascareno et al., 2001, Negoro et al., 2001). Such inconsistencies can be explained because AG490 is a JAK2 inhibitor and hence indirectly inhibits STAT-3. Recent creation of stattic in 2006 was a welcomed addition to the direct pharmacological blockade of STAT-3 (Schust et al., 2006). However, the use of cardiac specific STAT-3 genetic knock-out mice is far better in ensuring the blockade of STAT-3 (Smith et al., 2004).

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5.4 Future studies

To elucidate the role of STAT-3 in cardioprotection, it would be important to employ different models such as cardiac specific genetic knock-out mice for STAT-3 (Smith et al., 2004), with or without the use of direct pharmacological blockade with the recent development of stattic, a selective inhibitor of tyrosine phosphorylation of STAT-3 (Schust et al., 2006) or FLLL32, a derivative of curcumin which specifically reduces phosphorylation STAT-3 at its Tyrosine 705 site (Bill et al., 2010).

In addition, the use an in vivo rather than an in vitro model would be of benefit because it would include the systemic effects of STAT-3 activation on the endothelium (Hilfiker-Kleiner et al., 2005) and allow us to examine the long term effects of STAT3 on the myocardium (Haghikia et al., 2011).

With gaining interest in S1P and the sphingolipid pathway, additional pharmacological agonists and antagonists are being developed to target S1P directly or indirectly. Hence pharmacological agents such as FTY720 (a S1P receptor type 1 agonist) or VPC 23019 (a competitive antagonist of S1P receptors type 1 and 3) or

dl-threo-dihydrosphingosine (DHS), a competitive inhibitor of sphingosine kinase, could be used in the future (Takabe et al., 2008). It would be of interest to delineate which receptor is involved in S1P induced cardioprotection against ischaemia reperfusion.

There have been proposed challenges to the dogma of STAT-3 signalling (Sehgal, 2008) citing the existence of a mitochondrial pool of STAT-3 (Wegrzyn et al., 2009), which can directly influence mitochondrial function (Lacerda et al., 2010, Boengler et al., 2010). Such recent evidence merits further investigation to elucidate if STAT-3 modulates mitochondrial function in S1P induced cardioprotection.

5.5 Conclusion

Our data demonstrates that S1P preconditioning can protect the heart similar to ischaemic preconditioning via activation of STAT-3 as part of the SAFE pathway. The pathway is also involved in the cardioprotective effect of ischaemic postconditioning. Our data provides a unique therapeutic opportunity to target survival against ischaemic reperfusion injuries, especially since S1P forms part of high density lipoproteins. Addition of S1P to already existing synthetic HDL may be considered as a therapeutic option in the treatment of ischaemic heart disease.

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Publications and abstracts

1) Medical Research Council Presentation 2007: A critical role for phosphorylated STAT-3 in ischaemic postconditioning.

2) Medical Research Council Poster 2008: Delineation of mechanisms involved in sphingosine-1-phosphate mediated preconditioning.

3) International Society of Heart Research Poster (Athens) 2008: Delineation of mechanisms involved in sphingosine-1-phosphate mediated preconditioning.

4) University of Cape Town 2008. Delineation of mechanisms involved in sphingosine-1-phosphate mediated preconditioning.

5) AstraZeneca Medical Research Day 2008. Delineation of signalling pathways in ischaemic postconditioning.

6) South African Heart Journal Poster 2008: Myocardial preconditioning with Sphingosine-1-Phosphate, a major component of HDL, protects against ischaemia via STAT-3 activation.

7) Kelly R, King J, Lecour S. 2003. Sphingosine-1-phosphate induced cardioprotection is mediated by STAT-3. *Journal of Molecular and Cellular Cardiology*. (abstract), 44, 740.

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