Role of Tumour Necrosis Factor Alpha (TNFα) in

Ischaemic and Pharmacological Postconditioning

Lydia Lacerda

Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY

(Medicine)

Faculty of Health Sciences, University of Cape Town

August 2010

Supervisor: Professor S. Lecour, PharmD, PhD

(Hatter Cardiovascular Research Institute, Faculty of Health Science, University of Cape Town)

Co-supervisor: Professor L. H. Opie, PhD; DSc; DPhil

(Hatter Cardiovascular Research Institute, Faculty of Health Science, University of Cape Town)

Declaration

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

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature

_____

Date

_____

Acknowledgements

The guidance, motivation and support of my supervisor, Professor Sandrine Lecour has enabled me to complete this degree. Merci beaucoup, mille grazie, muito obrigada and a thousand thanks! The encouragement and opportunity to do this degree in the laboratory of the Hatter Cardiovascular Research Institute has been a privilege for which I am humbly grateful.

A thousand and one thanks to my co-supervisor, Professor Lionel Opie – his sound advice on all matters, scientific or not, kept my head above water and his sense of humour kept me going even when the going got tough! It has been a real honour to work under your auspices – many, many thanks.

My thanks also go to those colleagues who were always willing to listen, advise and encourage me in my quest. Without their support I would not have run so far!

A very special word of thanks goes to my husband, Jorge: he has supported me through thick and thin and when I wanted to give up, he gave me endless encouragement, a shoulder to cry on and the will to complete this thesis. I apologise for all the late suppers, the half-cooked suppers and the no suppers at all!!! I thank you from the bottom of my heart for all the support and love you have shown.

To my sons, Miguel and Philippe – although you have grown up and moved out, I know that I can always count on you to be there for me. I am forever grateful to have two such wonderful human beings as part of my family! Also to Candice and Nadia – thank you for your support in my quest.

My brother, Frank, and my sister, Theresa and their respective spouses, Janice and Derrick, deserve a special mention. You have always believed in me and because of that, I believe in myself. A greater gift you could not have given – I thank each and every one of you.

Thank you also to Sylvia Dennis – you do so much and are always willing to help and you do it with a smile – what would we do without you?!!!!

Many thanks also to Pat van der Walt and Victor Claasens - these are two individuals without which the lab would be a poorer place!

Lastly, to my three very special friends, Liz, Vicky and Jeanette – we have climbed many mountains together, one of which was this thesis! I am lucky to count you as my friends and I thank you for all your encouragement and support.

“It is through science that we prove, but through intuition that we discover”

Henri Poincare

Table of Contents **Page number**

List of Figures

A. Introduction

B. Aim, objectives and hypothesis

C. Models and Methods

D. TNFα, TNFR2 & ischaemic postconditioning

E. Cardioprotective agents as postconditioning mimetics

F. Ischaemic postconditioning, obesity & diabetes

List of Tables

F. ISCHAEMIC POSTCONDITIONING, OBESITY & DIABETES

Abbreviations

α	alpha
β	beta
δ	delta
γ	gamma
ADAM	a disintegrin and metalloprotease
ADP	adenosine diphosphate
AMI	acute myocardial infarction
ANOVA	analysis of variance
ATP	adenosine triphosphate
Bad	Bcl-2 associated death agonist
Bcl-2	B-cell lymphoma-2
Bcl-xl	B-cell lymphoma-xl
$CaCl_2$	calcium chloride
COX-2	cyclooxygenase-2
cGMP	cyclic guanosine monophosphate
cIAP2	cellular inhibitor of apoptosis protein 2
CK	creatine kinase
CVD	cardiovascular disease
DADLE	D-Ala2', D-Leu5'-enkephalin

DD	death domain
DED	death effector domain
EDTA	ethylene diamine tetra-acetic acid
ERK	extracellular signal-regulated kinases
ERK1/2	extracellular signal-regulated kinase 1/2
eNOS	endothelial nitric oxide synthase
FADD	fas-associated death domain
FAN	factor associated with neutral sphingomyelinase activation
FFA	free fatty acids
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
Gi	G inhibitory
GIK	glucose, insulin, potassium
GSK-3β	glycogen synthase kinase-3 beta
GTT	glucose tolerance test
H_2O_2	hydrogen peroxide
HCD	high carbohydrate diet
HDL	high density lipoprotein
HMGB-1	high-mobility group box-1

IL-1	interleukin-1
IL-6	interleukin-6
ip	intraperitoneal
IPC	ischaemic preconditioning
IPostC	ischaemic postconditioning
I/R	ischaemia/reperfusion
JAK	janus kinase
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LDL	low density lipoprotein
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase extracellular regulated kinase
MEKK	mitogen activated protein kinase extracellular regulated kinase kinase
mK_{ATP}	mitochondrial potassium-sensitive adenosine triphosphate channel
MPG	mercaptopropionylglycine
mPTP	mitochondrial transition pore
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate

NaH_2PO_4	sodium dihydrogen phosphate
ND	normal diet
NFκB	nuclear factor kappa B
NIDDM	non-insulin dependent diabetes mellitus
NO	nitric oxide
NOS	nitric oxide synthase
oxLDL	oxidised low density lipoprotein
PAR-2	proteinase activated receptor-2
PCI	percutaneous coronary intervention
PCTA	percutaneous transluminal coronary angioplasty
PI3-Kinase	phosphotidylinositol 3-kinase
PKB/Akt	protein kinase B
PKC	protein kinase C
p70s6K	p70-ribosomal s6 kinase
PKG	protein kinase G
RAGE	receptor for advanced glycation end products
RIP	receptor interacting protein
RIPC	remote ischaemic preconditioning
RISK	reperfusion injury salvage kinases

ROS	reactive oxygen species
sarcK_{ATP}	sarcolemmal potassium-sensitive adenosine triphosphate channel
SAFE	survivor activating factor enhancement
SODD	silencer of death domain
STAT	signal transducer and activator of transcription
STZ	Streptozotocin
TACE	TNFα converting enzyme
TLR	Toll-like receptors
TNFα	tumour necrosis factor alpha
TNFR1	tumour necrosis factor alpha receptor 1
TNFR2	tumour necrosis factor alpha receptor 2
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
TTC	2,3,5-triphenyltetrazolium chloride
VEGF	vascular endothelial growth factor
VDAC	voltage dependent anion channel

Abstract

Aims: Ischaemic postconditioning (IPostC) is a powerful protective mechanism that activates prosurvival intrinsic signalling cascades to limit reperfusion injury but the exact signalling pathway involved in this cardioprotective effect still remains unclear.

In ischaemic preconditioning, a powerful prosurvival path, known as the Survivor Activating Factor Enhancement (SAFE) pathway, has recently been discovered to protect against reperfusion injury. The SAFE pathway involves the activation of the cytokine, tumour necrosis factor alpha (TNFα) and the signal transducer and activator of transcription-3 (STAT-3) during ischaemic preconditioning. The aim of this thesis was to investigate whether ischaemic and pharmacological postconditioning can protect via activation of the SAFE pathway. In addition, we explored the influence of obesity and diabetes in the successful effect of ischaemic postconditioning, as these two pathologies are often associated with patients with myocardial infarction.

Methods: Isolated Langendorff perfused mouse hearts from TNF knockout ($TNF^{-/-}$), TNF receptor 1 or receptor 2 knockouts ($TNFR1^{-/-}$ or $TNFR2^{-/-}$), and cardiomyocyte-specific STAT-3 knockouts ($STAT\text{-}3^{-/-}$) and their respective wild types were subjected to a global ischaemia and were postconditioned with either short periods of ischaemia, TNFα (0.5 ng/ml), adenosine (100 μM), bradykinin (100 nM) or the specific delta opioid agonist, D-Ala2', D-Leu5'-enkephalin acetate salt (DADLE) (100 nM). Infarct size was measured using triphenyltetrazolium chloride staining technique and computerized planimetry. Western blot analysis of nuclear, cytosolic or mitochondrial proteins was performed to evaluate activation of STAT-3, Akt and extracellular signal-regulated kinase 1/2 (Erk1/2), during reperfusion.

To explore the influence of obesity and diabetes on IPostC-induced cardioprotection, TNF-WT and $TNF^{-/-}$ animals were fed with a high carbohydrate diet for 11 weeks to induce obesity. Diabetes was induced by administration of a single intraperitoneal injection of streptozotocin (180mg/kg).

Results: IPostC or pharmacological postconditioning with bradykinin or DADLE reduced infarct size in TNF-WT, $TNFR1^{-/-}$ and STAT-3-WT mice, but not in $TNF^{-/-}$, $TNFR2^{-/-}$ and $STAT\text{-}3^{-/-}$ mice. Adenosine reduced infarct size in TNF-WT, $TNF^{-/-}$, and STAT-3-WT but failed to protect $STAT\text{-}3^{-/-}$ mice. TNFα-postconditioning mimicked IPostC in TNF-WT and STAT-3-

WT but not in $TNF^{-/-}$ or $STAT\text{-}3^{-/-}$ hearts. Both IPostC and TNF-PostC increased phosphorylated STAT-3 in the nucleus, while Akt and Erk1/2 were only activated after an IPostC stimulus. Similarly, bradykinin and DADLE increased phosphorylated STAT-3 in the mitochondria.

IPostC protected hearts against ischaemia-reperfusion in obese or diabetic WT mice. Surprisingly, the protective effect of IPostC was partially restored in $TNF^{-/-}$ obese mice but the infarct-sparing effect was absent in diabetic $TNF^{-/-}$ mice.

Conclusion: In this thesis we have delineated a novel prosurvival pathway activated in IPostC to limit ischaemia-reperfusion injury. This pathway, known as the SAFE pathway, requires activation of TNFα and activates STAT-3 via TNFR2. Pharmacological postconditioning with bradykinin and opioids mimics IPostC via the SAFE pathway. Although adenosine does not require TNFα in pharmacological postconditioning, STAT-3 is still required, thus confirming the existence of multiple protective pathways in the heart to protect against reperfusion injury. In our experimental conditions, obesity and diabetes do not influence the outcome of ischaemic postconditioning but TNFα appears to play a deleterious role in obesity.
The existence of multiple alternate pathways in IPostC and pharmacological postconditioning at the time of reperfusion may have potential therapeutic application against ischaemia-reperfusion injury. Furthermore, we show that the protective effect of ischaemic postconditioning is maintained in a modestly obese and diabetic mouse model suggesting that, relative to the severity of the disorder, obese and diabetic patients should be considered for this therapy.

A. INTRODUCTION

Transporting blood throughout the body to each organ and tissue, throughout our lives, requires an efficient and healthy heart. As we grow older, the heart becomes progressively less efficient in maintaining a healthy circulatory system. However, even as our age increases, the heart, as is the case with any muscle, benefits from a healthy lifestyle, coupled with regular age-appropriate exercise. An individual who lives such a lifestyle could expect to live to be a "ripe old age". Unfortunately, with the modern world's fast pace of living and working, people are exposed to a number of risk factors which promote cardiovascular diseases resulting in a shortened lifespan as well as being a burden on the social system of a country. Risk factors such as hypertension, diabetes, elevated cholesterol levels, just to mention a few, all contribute eventually to a narrowing of the arteries, compromising the circulatory system. The flow of blood and therefore, the oxygen supply and essential nutrients, to the muscle cells of the heart, is decreased resulting in a "heart attack" or myocardial infarction.

The heart is a resilient organ and possesses intrinsic survival mechanisms in an attempt to protect itself. Recently, the heart has been shown to possess a remarkable ability to adapt to conditions of stress and this has been one of the focal points of intense research over the past 15 years in the hope that the underlying mechanisms may be harnessed for therapeutic development.

In this thesis we propose to explore the activation of a major intrinsic cytokine, tumour necrosis factor alpha (TNFα), as a possible tool to protect the heart against stressful conditions observed during myocardial infarction. This study will be performed in healthy animals or in obese/diabetic animals, two pathologies often triggering myocardial infarction.

1. Ischaemic Heart Disease

Ischaemic heart disease is characterized by a reduced blood supply (ischaemia) to the heart muscle, usually due to coronary artery disease (atherosclerosis of the coronary arteries).

In the adult human body there are 96,600 kilometers of arteries, veins and capillaries pulsating at 100,000 rhythmic, muscular heart beats per day. These arteries, veins and capillaries, together with the heart, make up the cardiovascular system. Ischaemic heart disease affects individuals in their prime of life, reducing their life expectancy and their contribution towards the economy of a country substantially and is thus perceived as a growing global burden. Ischaemic events can occur as a consequence of either a thrombosis (blood clot) or narrowing and hardening of the arteries as a result of plaque accumulation. Atherosclerotic plaques within coronary arteries cause rapid occlusion in coronary artery disease resulting in the heart suffering a loss of blood flow and oxygen supply with subsequent death of the cardiomyocytes, the muscle cells of the heart. During occlusion of

the artery or arteries supplying the myocardium, the demand for oxygen may exceed the supply and myocardial ischaemia develops. Rupture of a plaque may result in a secondary thrombus formation in a coronary artery. If left untreated, this form of coronary artery disease culminates in an acute reduction of blood supply to the myocardium and is characterized by the rapid development of myocardial necrosis (Jennings, 1991).

The number one cause of death worldwide is cardiovascular disease (CVD), with more people dying annually from coronary artery disease than from any other cause (WHO, 2009). Low and middle income countries seem to be more affected than developed countries with 82% of cardiovascular related deaths occurring in the lesser developed countries and almost equally in men and women. By 2030, it is predicted that almost 23.6 million people will die from coronary artery disease – mainly from ischaemic heart disease and stroke (WHO, 2009) and the leading cause of death in the industrialized world is already ischaemic heart disease. Ischaemic heart disease is projected to become the major worldwide cause of death and disability by 2020 – only a decade away!! (WHO, 1996).

"*As long as Africa remains impoverished, a major rise in coronary artery disease is unlikely*" – this was the general perception about ischaemic heart disease in Africa in 1997 as expressed by Walker et al (Walker, 1998). However, in 2006, the World Bank reported that Africa was making economic progress. Unfortunately, along with economic improvements, there is a rise in chronic diseases of lifestyle such as ischaemic heart disease, a consequence of epidemiological transition. Ischaemic heart disease is rapidly becoming a leading cause of death in sub-Saharan Africa and in 2008 it was ranked as the 8th leading cause of death in men and women of this region (Menash, 2008). The progression of ischaemic heart disease in the ranking is attributed largely to lifestyle and behavioural changes associated with urbanization of the region (Menash, 2008). Ischaemic heart disease is responsible for the greatest number of cardiovascular fatalities in males and the second largest number of cardiovascular related deaths in females, in South Africa, (Figure 1). More alarmingly, a report issued by the South African Medical Research Council in 2005, states that ischaemic heart disease was the leading cause of death in the total population of the Western Cape (one of the provinces of South Africa) in a single year (Bradshaw, 2005).

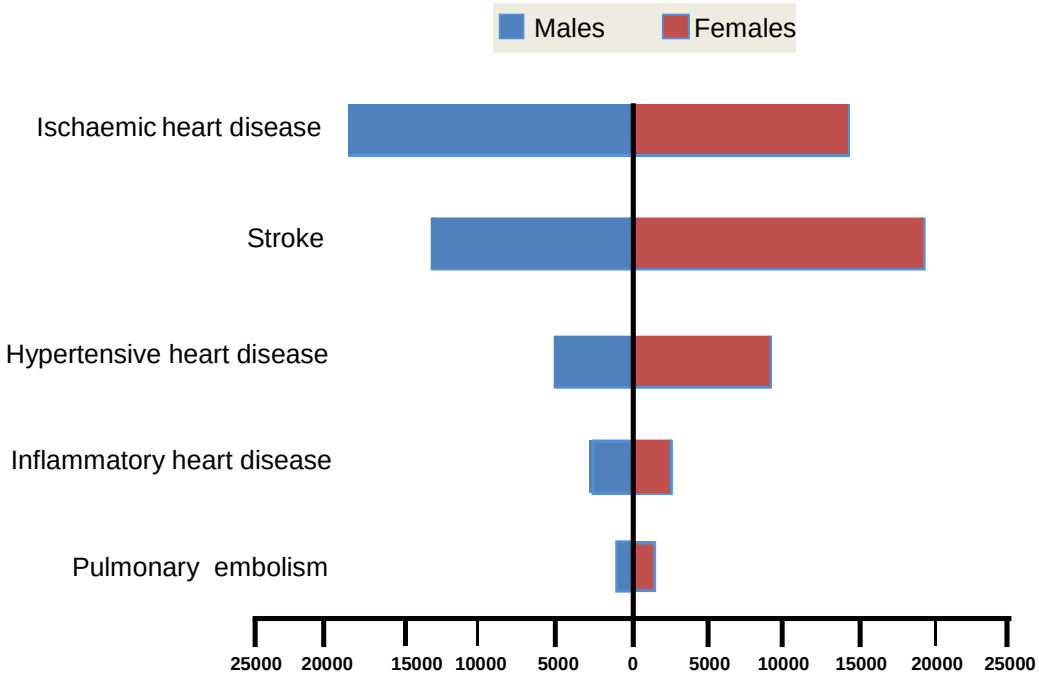


Fig 1: Ischaemic heart disease. Estimated to be the greatest cause of CVD mortality in 2000. (adapted from Norman, 2000)

1.1 Major risk factors for ischaemic heart disease

The major cardiovascular risk factors are as follows:

1.1.1 Atherosclerosis and dyslipidaemia

One of the main causes of coronary artery disease is atherosclerosis (Hansson, 2005). Atherosclerosis is an inflammatory disease in which the inflammatory system interacts with metabolic risk factors, triggering atherosclerotic plaques in the arterial tree. Myocardial infarction occurs when the atheromatous process prevents blood flow through the coronary artery and disruption of the plaque is considered dangerous as it releases harmful thrombotic material from the centre of the plaque into the circulation (Hansson, 2005). Dyslipidaemia is one of the causes of atherosclerosis, with low density lipoprotein cholesterol (LDL) contributing to the formation of plaques. Endothelial disruption and erosion also plays a role in plaque formation, contributing to ischaemic heart disease, (Figure 2).

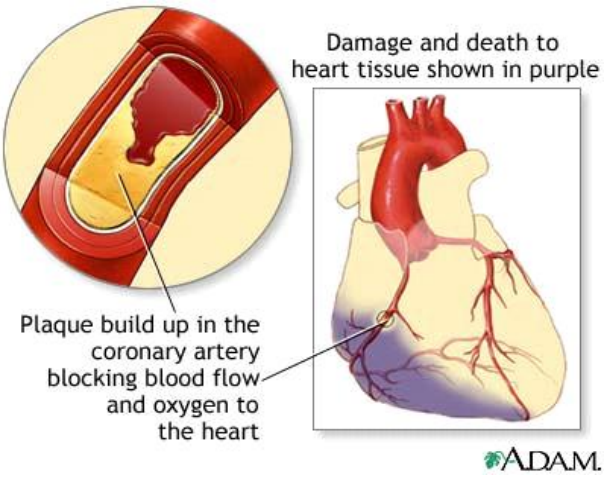


Fig 2: Plaque build up in coronary artery. Loss of blood flow and oxygen supply will eventually lead to morphological changes in the heart and ischaemic heart disease. (www.whatis heartdisease.us/images)

The cholesterol that accumulates in atheromatous lesions is derived primarily from plasma low density lipoprotein (LDL), which accounts for 60-75% of plasma cholesterol, giving LDL a powerful and direct relationship with coronary heart disease (Nestruck and Davignon, 1986). Oxidative stress and smoking are just two of the many factors which can contribute to damage of the endothelium. Consequently, excess production of oxidised LDL-cholesterol (oxLDL) occurs in the bloodstream. In an attempt to reduce the amount of oxLDL cholesterol to normal levels, macrophages engulf the oxLDL and in doing so become "foam cells", (Figure 3). However, if there is too much oxLDL-cholesterol present, the macrophages accumulate against the vessel wall in what will become an atherosclerotic plaque. Eventually this wall of plaque may either block the vessel completely or it may rupture, resulting in the release of pro-coagulant factors and the formation of a thrombosis (Libby, 2002). Both of these events inhibit the flow of blood to the heart and result in myocardial infarction and ischaemic heart disease, (Figure 4).

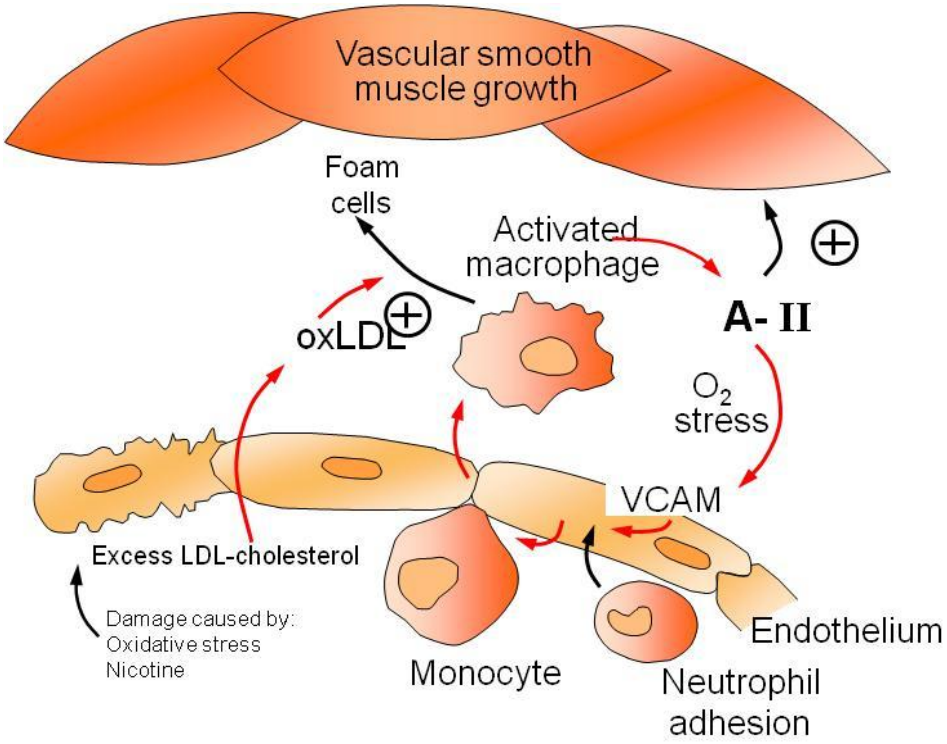


Fig 3: Formation of atherosclerotic plaque. Damaged endothelium favours the infiltration and oxidation of excess LDL-cholesterol. The oxidisised LDL-cholesterol (ox-LDL) is taken up by the macrophages in an attempt to keep plama cholesterol homeostasis. However, in the presence of excess ox-LDL the macrophages accumulate against the vessel wall and form an atherosclerotic plaque. A-II: angiotensin II; VCAM: vascular cell adhesion molecule, (adapted from Opie, 2004).

High density lipoprotein (HDL) cholesterol, also known as the 'good' cholesterol, helps to clear excess LDL-cholesterol from the blood back to the liver (a process known as "reverse cholesterol transport", thereby reducing the risk of atherosclerosis. Many studies have shown that there is an increased risk of ischaemic heart disease in individuals with low levels of HDL (Assmann et al., 1996; Curb et al., 2004; Gordon et al., 1989; Gordon et al., 1977; Sharrett et al., 2001; Turner et al., 1998). However, there is also a direct relationship between increased HDL-cholesterol and decreasing cardiovascular risk with epidemiological data indicating that for every increase of 0.03 mM/L of HDL-cholesterol, the risk for cardiovascular events decreases by 2-3% (Gordon et al., 1989).

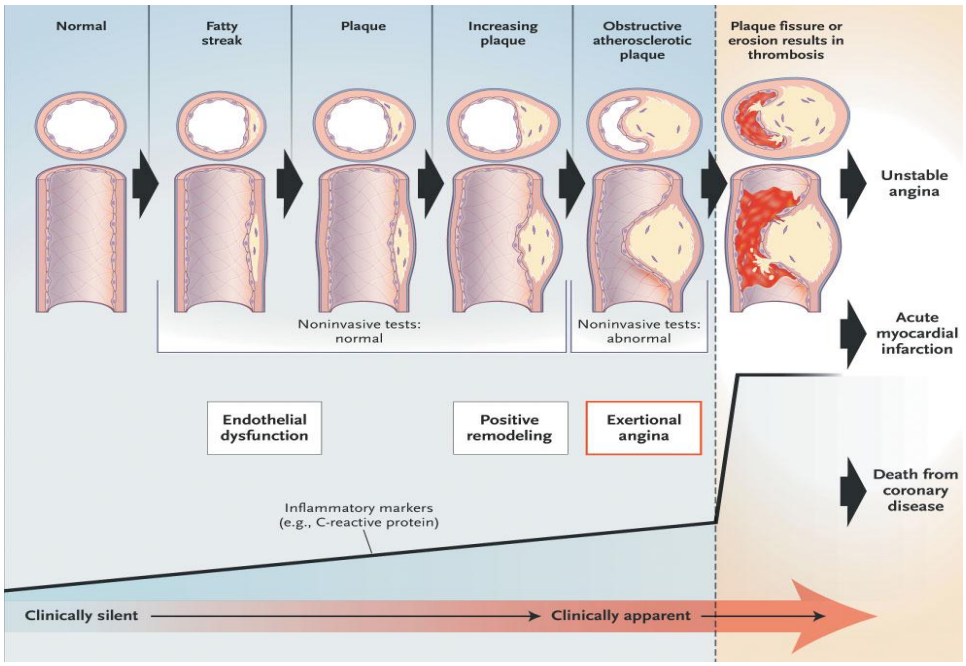


Fig 4: Typical progression of coronary atherosclerosis. At first, the atherosclerotic plaque tends to stay external to the lumen, which allows the coronary artery diameter to be maintained. As the plaque encroaches into the lumen, the diameter of the artery decreases until eventually the flow of blood is inhibited. Luminal narrowing of more than 65 to 75% may result in angina and myocardial infarction, (Abrams, 2005).

1.1.2 Obesity

Obesity has been defined by the World Health Organisation as a disease in which excess body fat has accumulated to such an extent that health may be adversely affected (WHO, 2000).

An imbalance between energy intake and expenditure results in excess energy being stored in fat cells which enlarge or increase in number, and this may eventually result in overweight and obesity. A healthy lifestyle does not necessarily mean a healthy heart if one carries excess weight, especially around the abdomen. Obesity is increasing, worldwide, at an alarming rate, and just 5 years ago, there was an estimated 1.3 billion people overweight or obese (WHO, 2005). Data from 14 years ago already revealed that this global trend towards overweight and obesity was being followed by South Africa with an disturbing number of citizens becoming overweight (South Africa Demographic Health Survey, 1996). Increasing rates of childhood and adolescent obesity are also a cause of great concern as once children have become overweight or obese, interventions to reverse these conditions are rarely successful (Pinhas-Hamiel and Zeitler, 2000). Current definitions designate overweight in adults as those having a body mass index

between 25.0 and 30.0 kg/m^2 and obesity as a BMI of greater than 30.0 kg/m^2 (Poirier et al., 2006). Distribution of body fat, or adipose tissue, appears to play a significant role in determining the presence or absence of metabolic dysfunctions that predispose to chronic diseases (Poirier, 2003). The skinfold test is another means of defining obesity by determining a person's body composition and body fat percentage. Metabolic syndrome, which consists of a cluster of diabetogenic and atherogenic abnormalities, correlates closely with CVDs and diabetes. Much has been written about the metabolic syndrome with some cardiologists favouring its existence and others questioning it. It appears that of all the parameters suggested to define metabolic syndrome, the one of most importance is that of the waist-circumference measurement (Alberti et al., 2005; Opie, 2006). A common finding in the obese state is a low-grade systemic inflammation with increased circulating levels of a number of cytokines, amongst which is tumour necrosis factor alpha (TNFα) (Bullo et al., 2003; Roytblat et al., 2000; Yudkin et al., 1999). Anti-inflammatory therapies targeting TNFα activity have indicated that blocking TNFα may interrupt the inflammatory cascade that occurs with abdominal obesity (Bernstein et al., 2006).

A large number of clinical problems are associated with being obese and these can be subdivided into two groups: those that are associated with excess adipose tissue and those that are linked to the metabolic effects of the increased adiposity (Bray, 2004). The diseases associated with the latter group include coronary heart disease, hypertension, type 2 diabetes mellitus and certain kinds of cancer (Garfinkel, 1985; Jooste et al., 1988; Lew, 1985; Manson et al., 1995; Pi-Sunyer, 1990; Pi-Sunyer, 1991; Steyn et al., 1990). Accumulation of abdominal fat increases the probability of cardiovascular disease and mortality (Donahue et al., 1987; Ducimetiere et al., 1986; Lapidus et al., 1984; Larsson et al., 1984). The metabolic effects of obesity result from the increased release of free fatty acids (FFA) and the production of adipokines which are secreted by several factors, including the cytokines TNFα and interleukin-6 (IL-6), hormones such as leptin and adiponectin and other adipokines such as resistin and angiotensinogen. These factors can impair insulin signalling and alter glucose homeostasis as well as lipid metabolism (Arner, 2003; Nieto-Vazquez et al., 2008). Obesity is therefore, now being considered as a chronic state of low-intensity inflammation, with one of the major health consequences being the increased risk of ischaemic heart disease (Larsson et al., 1981).

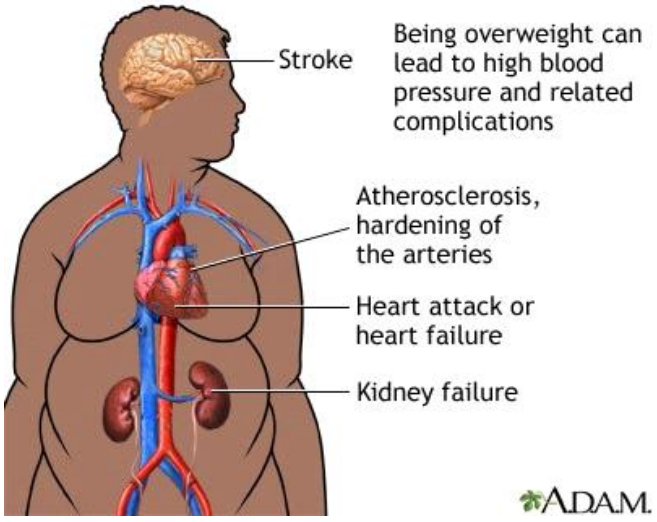


Fig 5: Cartoon illustrating possible complications arising from obesity. (www.clarion.org)

1.1.3 Diabetes

Type 1 diabete*s: the pancreatic beta cells of the body fail to produce insulin as the body's immune system has attacked and destroyed them. At present, this type of diabetes requires the afflicted person to inject insulin.*

Type 2 diabetes: *results from insulin resistance, a condition in which cells fail to use insulin properly and is sometimes combined with an absolute insulin deficiency. At first, the pancreas produces more insulin to compensate, but eventually it loses the ability to secrete insulin in response to meals. Being overweight and lack of exercise increase the risk of developing this type of diabetes.*

Diabetes and its associated complications have become a worldwide health problem. The prevalence of diabetes is rising, reaching epidemic proportions in some population groups. Type 2 diabetes is responsible for much of the increased occurrence of diabetes but there is also evidence that the incidence of type 1 diabetes is on the increase, although to a lesser extent than type 2 diabetes (Podar et al., 2001). In addition, impaired glucose tolerance is on the increase with about 197 million individuals worldwide having this disorder, predominantly as a result of obesity and the associated metabolic syndrome (Hossain, 2007). Abnormalities in both the action and secretion of insulin are responsible for the pathogenesis of diabetes. Insulin resistance is defined as the diminished ability of the cell to respond to the action of insulin with a subsequent

increase in lipolysis, release of glycerol and free fatty acids (FFA) into the circulation, ultimately leading to diabetes, (Figure 6).

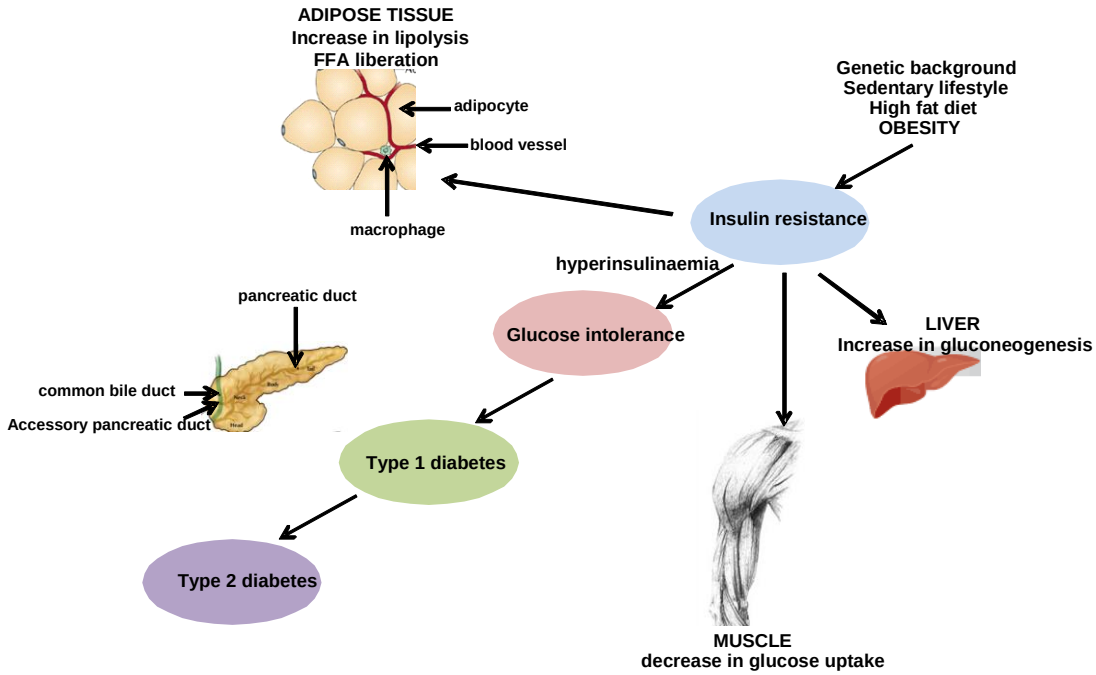


Fig 6: Schematic representing the different steps in the development of type 1 and type 2 diabetes, (adapted from Nieto-Vasquez, 2008).

The development of insulin resistance has been linked to both genetic and environmental factors, with obesity being an important contributor. Most studies thus far have focussed on type 2 diabetes. However, type 1 diabetes also has serious cardiovascular consequences which occur in a much younger age group (Orchard et al., 2006). The powerful effect of the duration of type 1 diabetes should be taken into consideration when regarding cardiovascular risk associated with this type of diabetes. In the intervening years between diagnosis and the patient's 3rd decade, 20 years or more have passed and a considerable amount of damage to the cardiovasculature may have occurred. The development of type 1 diabetes is shown in Figure 7:

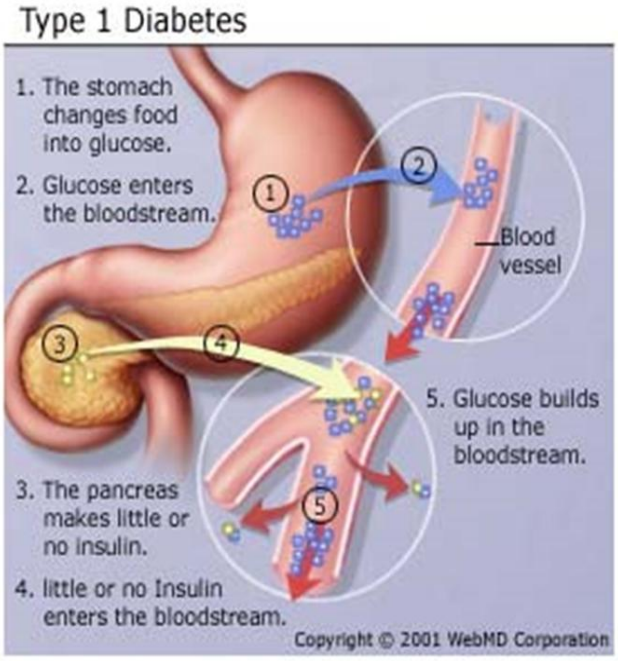


Fig 7: Development of type 1 diabetes: As production of insulin by the pancreatic cells is impaired, the level of glucose in the blood increases, leading to type 1 diabetes. (www.medicinenet.com)

Both type 1 and type 2 diabetic individuals are prone to developing ischaemic heart disease, including acute myocardial infarction and subsequent complications (Aguilar et al., 2004; Ramani et al., 1996; Stevens et al., 2004; Zairis et al., 2004). More than 50% of deaths in diabetic patients have been attributed to ischaemic heart disease (Kannel and McGee, 1979) and the mortality rate from acute myocardial infarction is almost doubled in diabetic individuals compared to non-diabetics (Abbott et al., 1988). Although it is well established that the long-term outcome of diabetic patients with ischaemic heart disease is worsened, studies have been contradictory as to whether the diabetic heart responds well or not to treatment in the event of an ischaemic episode. For instance, hearts from streptozotocin-induced diabetic rats were more resistant initially to ischaemia/reperfusion, but over a period of 8 weeks, this effect was reversed (Tosaki et al., 1996). This initial positive response to ischaemia-reperfusion was also seen in a type 2 diabetes model with an *in vivo* study (Liu et al., 1993). Indeed, in an alloxan-induced diabetic rabbit model, a decrease in infarct size was observed 8 weeks after the alloxan injection, compared to controls (Hadour et al., 1998).

The majority of clinical studies have however, demonstrated a worse outcome from acute myocardial infarction in patients who are already compromised by diabetes (Osario, 2010).

1.1.4 High blood pressure (hypertension)

Hypertension has been defined as when the arterial blood pressure rises above 140 mmHg for systolic pressure and 90 mmHg for diastolic pressure (Kaplan et al., 2010).

Elevated arterial blood pressure is the most common cause of enlargement of the left ventricle of the heart (Diamond et al., 2005; Ferdinandy, 2007; Massie and Shah, 1997; Prisant, 2005). Experimental evidence suggests that the hypertrophied myocardium is more susceptible to ischaemia-reperfusion damage than a normotensive heart (Attarian et al., 1981). However, no difference in infarct size between hypertensive and normotensive animals was demonstrated either *in vivo* in a rat heart infarct model (Ebrahim et al., 2007) or *in vitro* in a rat model of coronary occlusion (Speechly-Dick et al., 1994).

2.0 Reperfusion and reperfusion injury

Reperfusion is defined as the restoration of blood flow to an organ or tissue that has had its blood supply cut off. Reperfusion injury refers to the damage done to the organ or tissue caused when blood supply returns to the tissue after a period of ischaemia.

In the event of an acute myocardial infarction, rapid restoration of the blood flow to the ischaemic area is one of the best ways to salvage the heart. Provided that reperfusion occurs shortly after coronary artery occlusion, it is effective in reducing the infarct size thus limiting the damage inflicted by the myocardial infarct (Maroko et al., 1971). Despite the undoubted benefit of reperfusion of blood to the ischaemic tissue, reperfusion itself is not risk free. A cascade of adverse reactions can occur during reperfusion, (see review (Yellon and Downey, 2003)) which paradoxically results in an exacerbation of tissue injury known as "reperfusion injury".

Restoration of the blood flow in the clinical setting involves either thrombolytic agents or cardiac catheterization (angioplasty), in which a stent is inserted into the occluded vessel followed by gentle inflation of a balloon to rupture the plaque or clot which is causing the blockage.

The destructive role played by reperfusion has been the subject of much debate amongst clinicians and scientists (Ferrari and Hearse, 1997; Kloner, 1993; Ovize, 1997; Przyklenk, 1997; Yellon, 1992).

There is little doubt that unremitting ischaemia causes cell death but until recently, the predominant view was that if ischaemia itself was the culprit in cell death as a result of adenosine triphosphate (ATP) depletion and its numerous consequences, then reperfusion was

essential to restore ATP synthesis, thereby rescuing the uninjured cells or those which were reversibly injured.

Others, however, held the view that the death of cells irreversibly injured by ischaemia was merely accelerated by the restoration of blood flow. Yet another point of view was that reperfusion injury was caused by the sudden influx of blood releasing reactive oxygen species and other harmful substances. Agreement has, however, been reached that 4 forms of reperfusion injury exist (Ovize, 1997):

i) myocardial stunning,

ii) reperfusion arrythmias,

iii) vascular reperfusion injury and

iv) lethal reperfusion injury

Lethal reperfusion injury is a term used to describe myocardial cell death caused by reperfusion, and is the type of reperfusion injury addressed in this thesis, (Figure 8).

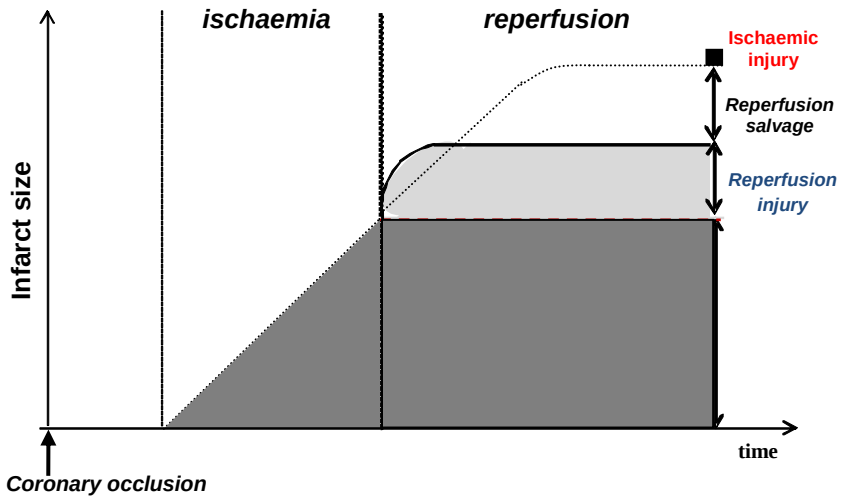


Fig 8: Lethal reperfusion injury. During ischaemia some of the cardiomyocytes are irreversibly injured (dark grey), whilst even more damage is done during the reperfusion period (light grey), (adapted from Thibault, 2007).

2.1 Mechanisms resulting in reperfusion injury

Reperfusion injury is associated with all the signs of necrotic cell death, such as release of intracellular proteins and major morphological changes which include swelling of the mitochondria. Studies over the past 10 years have revealed that mitochondria play a critical part in determining the fate of the cell after cellular stresses, (see review Kroemer et al., 2007). One of the critical components of mitochondrial response to stress is the activation of the mitochondrial permeability transition pore (mPTP) (Bernardi et al., 2006; Halestrap and Brenner, 2003). The mPTP is a non-specific, multimeric pore structure that bridges the outer and inner mitochondrial membranes. The true composition of the pore remains uncertain and is the subject of much debate. Cyclophilin-D is the only known component of the mPTP and is inhibited by cyclosporine-A (Crompton et al., 1988; Gomez et al., 2009). The inner mitochondrial membrane is impermeable to solutes while it remains closed under normal physiological conditions. During conditions of cell stress, with high matrix calcium ion accumulation, accompanied by depletion of adenine nucleotide, elevated inorganic phosphate and oxidative stress, the mPTP opens in the inner mitochondrial membrane, releasing cytochrome C and promoting cell death. These are exactly the conditions which occur during reperfusion after a period of ischaemia (Javadov et al., 2003). The mechanisms determining whether the pore opens or not during ischaemia-reperfusion have been extensively studied over the last 20 years with most investigators now being of the opinion that it is the conditions during early reperfusion which trigger the opening of the pore and that if the pore remains closed during these first early moments of reperfusion this will protect against cell death (Crompton et al., 1987; Di Lisa and Bernardi, 2006; Di Lisa et al., 2001; Duchen et al., 1993; Halestrap et al., 2004; Halestrap et al., 1998; Lemasters et al., 1997)

Griffiths et al, demonstrated that if the pore remains closed at the onset of reperfusion, a distinct reduction in infarct size is observed in experimental ischaemia-reperfusion (Griffiths and Halestrap, 1993). The past 5 years have seen the proposal of mPTP inhibition as being the ultimate cardioprotective target, with mounting evidence that it may be a crucial point of convergence for many of the cardioprotective signalling pathways. If this proves to be the case, administration of cyclosporine-A, a known mPTP antagonist, will be a simple and practical procedure, which could be delivered in the ambulance en route to the hospital and reduce the subsequent reperfusion injury which occurs with percutaneous coronary intervention (PCI) or with administration of a thrombolytic agent (Gomez et al., 2009).

In summary, this necrotic cell death, which has been termed "lethal reperfusion injury", is a major obstacle to reperfusion therapy without which the ischaemic tissue cannot be salvaged. In addition, it has been suggested that the greater the degree of the initial ischaemic insult, the more severe the reperfusion injury will be and that if the ischaemic insult lasts long enough, no recovery is possible (Vinten-Johansen et al., 2005). Reperfusion has thus been dubbed as a "double edged sword" and a reassessment of reperfusion injury and its mechanisms is clearly needed to maximise the benefits of reperfusion and limit infarct size.

3.0 Conditioning the heart to limit reperfusion injury

3.1 Ischaemic preconditioning

Ischaemic preconditioning is defined as brief periods of ischaemia followed by reperfusion which occur prior to the prolonged ischaemic insult.

While investigating adenosine triphosphate (ATP) levels in a canine model of the failing heart, Murry and Jennings serendipitously discovered a biological mechanism which harnessed the intrinsic ability of the heart to protect itself (Murry et al., 1986). By applying four brief episodes of alternating reperfusion and occlusion prior to the infarct-inducing episode of myocardial ischaemia, Murry was able to demonstrate an astonishing reduction of 75% in the infarct size of canine hearts (Murry et al., 1986). This type of cardioprotection became known as "ischaemic preconditioning" (IPC) and a flurry of activity followed with the reproducibility of IPC being proved in multiple experimental settings using various animal models (Kuzuya et al., 1993; Marber et al., 1993; Murry et al., 1986; Przyklenk et al., 1993).

Further studies revealed the existence of two "windows" of protection in IPC. The first window, termed as "classic preconditioning" occurs immediately after initiating IPC and lasts for up to 3 hours. The second window of protection, known as SWOP or delayed preconditioning, occurs 24 to 72 hours after the initial application of IPC and is thought to protect the heart to a lesser extent than classical IPC, (see review (Yellon and Downey, 2003)).

3.1.1 Mechanisms of IPC:

Following the discovery of IPC, intensive research to delineate the mechanism involved has been undertaken but the exact signalling pathway still remains unclear. The major signalling pathways which have been implicated in IPC are illustrated in the following schematic, (Figure 9) and discussed below:

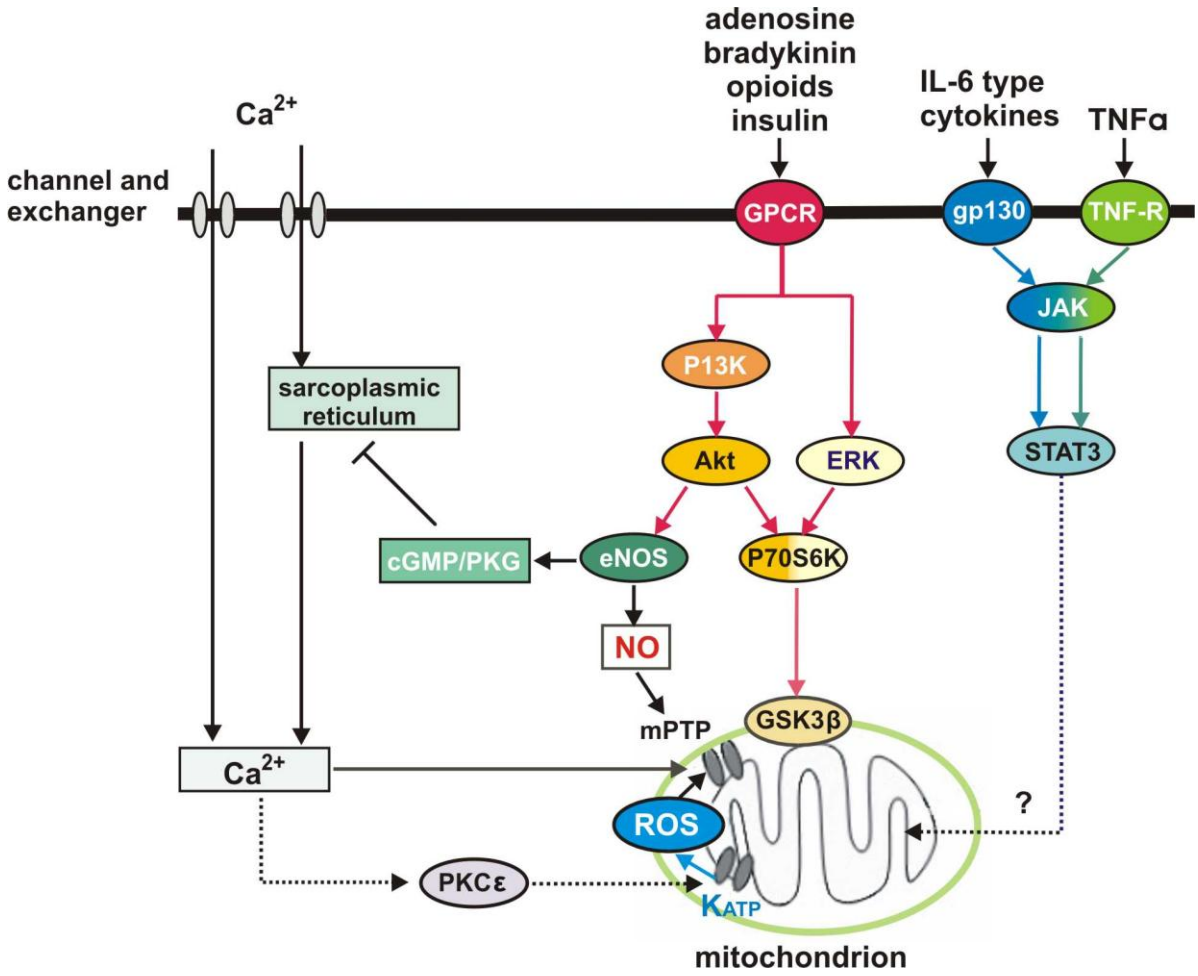


Fig 9: Diagram depicting suggested prosurvival signalling pathways involved in classic ischaemic preconditioning. IPC invokes the activation of signal transduction cascades by autocoid triggers and kinases. ROS targets the mK_{ATP} and the mPTP. Abbreviations as defined in text, (adapted from Ovize, et al, 2010).

3.1.2 Gi-protein coupled receptors (GPCR)

G-proteins are situated within the lipid bilayer of cells and they can move freely, thus allowing a large number of G-proteins to be activated by a single occupied receptor complex (Lubert, 1988). Gi-protein coupled receptors constitute a large and diverse family of proteins, the primary function of which is to transduce extracellular stimuli into intracellular signals.

Two types of G-proteins have been identified: the G stimulatory protein (Gs), which is cholera toxin sensitive, and the G-inhibitory protein (Gi) which is pertussis toxin sensitive. However, controversy surrounds the role of the Gi protein in cardioprotection. Thornton et al, used an *in vivo* rabbit model and Miura et al, used a canine model to investigate the involvement of the Gi-proteins in the protective effects of IPC. Both groups found that IPC-induced cardioprotection was conferred via the Gi-proteins (Miura et al., 1997; Thornton et al., 1993). In contrast, Liu and

Downey were unable to demonstrate a role of the Gi-proteins in IPC-induced protection in an open chest rat model of myocardial infarction (Liu and Downey, 1993).

G-coupled protein receptors activated by adenosine, bradykinin, and the opioid receptors will be further discussed under "Cardioprotective agents and ischaemic postconditioning" in Section E.

3.1.3 The RISK pathway

A powerful cardioprotective signalling pathway termed as the "Reperfusion Injury Salvage Kinase (RISK) pathway" consists of a group of prosurvival protein kinases, including phosphotidylinositol 3-kinase (PI3-kinase)/Akt and extracellular signal-regulated kinases, p44/p42 (Erk1/2) (Hausenloy and Yellon, 2004).

i) Phosphotidylinositol 3-kinase/Akt

The PI3-kinase/Akt signalling cascade is activated by the stimulation of a wide range of receptors, including those of growth factors and G-protein coupled receptors (Cross et al., 2000). In the setting of ischaemia-reperfusion, signalling through PI3-kinase has been demonstrated to confer protection against ischaemia-reperfusion injury by inhibiting apoptosis in cardiomyocytes subjected to hypoxia via the activation of Akt (Matsui et al., 1999).

In 2002 Mocanu et al, first demonstrated a link between ischaemic preconditioning, PI3-kinase /Akt and infarct limitation in the isolated perfused rat heart by individual infusion of the inhibitors, wortmannin and LY294002, during the preconditioning protocol (Mocanu et al., 2002).

ii) Extracellular signal-regulated kinase

The role of Erk1/2 has been extensively studied in the setting of cardioprotection. Erk 1 (p44) was not activated by global ischaemia in a perfused rat heart model but ischaemia-reperfusion increased the phosphorylation and kinase activity of both Erk1 and Erk2 (p42) in the nuclear fraction (Mizukami and Yoshida, 1997). In an *in vivo* rat model of ischaemia-reperfusion, activation of Erk1 was not triggered by ischaemia alone but was activated by 5 – 30 minutes of reperfusion (Omura et al., 1999).

Hausenloy and colleagues demonstrated that IPC resulted in the activation of both PI3-kinase/Akt and the Erk1/2 signal cascades at the time of reperfusion (Hausenloy et al., 2004b). They reported that inhibition of PI3-kinase at the time of reperfusion resulted in the activation of Erk1/2 which, in turn, activated p70-ribosomal s6 kinase (p70s6K). However, when Erk1/2 was inhibited, the PI3-kinase/Akt pathway was activated but p70s6K remained inactive, therefore

suggesting a cross talk between the kinases (Hausenloy et al., 2004b). During the preconditioning stimulus, Erk1/2 was phosphorylated and activated in the isolated rat heart and this was associated with a decrease in infarct size (Fryer et al., 2001). In contrast, Mocanu et al, found that infusion of PD98059, the Erk1/2 inhibitor, during the IPC stimulus of the isolated rat heart, did not block the protection afforded by preconditioning, therefore suggesting that Erk1/2 was not involved in the PI3-kinase–induced reduction in infarct size during IPC (Mocanu et al., 2002).

By 2007, the downstream targets of the RISK pathway included other cardioprotective kinases such as protein kinase C (PKC), protein kinase G (PKG), p70-ribosomal s6 kinase (p70s6K) and glycogen synthase kinase-3 beta (GSK-3β) (Hausenloy and Yellon, 2007b) (Figure 10).

3.1.4 Protein Kinase C

The first kinase to be examined in detail in ischaemia-reperfusion was PKC. Of the 11 isoforms of protein kinase C, PKC alpha (α), PKC delta (δ) and PKC epsilon (ε) have been implicated in IPC in the rat heart (Yoshida K et al., 1997), while PKCε and PKC gamma (γ) have been implicated in IPC in the rabbit heart and mouse heart (Gray et al., 2004; Ping et al., 1997). Enhanced membrane PKC activity in myocardial ischaemia was first demonstrated by Prasad et al, in 1992, using a protein kinase activity assay of the cytosol and membrane fractions of hearts which had been subjected to ischaemia or ischaemia-reperfusion (Prasad and Jones, 1992).

Wei et al, demonstrated in an *in vivo* rat ischaemia-reperfusion model, that when PKCβ inhibitors were administered for a period of 2 weeks prior to experimentation, protection was conferred against cardiac microvascular ischaemia-reperfusion injury, thus implicating the β isoform of PKC in cardioprotection (Wei et al., 2010).

Activation and subsequent translocation of PKC has been reported as necessary for the protective effects of IPC (Ping et al., 1997). Administration of inhibitors of PKC prior to and/or during IPC abolished the cardioprotective effect of IPC in most animal models (Pain et al., 2000; Yellon and Downey, 2003). Furthermore, IPC could be mimicked by administration of a PKC agonist prior to an I/R insult (Speechly-Dick et al., 1994). Other studies in which agonists of PKC were used in isolated rat hearts and in cell culture (cardiomyocytes) supported these findings (Gray et al., 1997). It has been suggested that the PI3-kinase/Akt pathway, nitric oxide (NO), the mitochondrial potassium-sensitive adenosine triphosphate channel (mK_{ATP}) channel and ROS are upstream of PKC activation in the preconditioned heart (Baines et al., 1997; Ping et al., 1999a; Tong et al., 2000; Wang., 1999). Hausenloy et al, however, speculated that PKC may contribute to the cardioprotective effect of IPC by the phosphorylation of PI3-kinase/Akt at the

time of reperfusion (Hausenloy et al., 2004b). Downstream targets of PKC include p38 mitogen activated protein kinase (p38 MAPK), c-Jun-NH_2-terminal kinases (JNK), and extracellular signal-regulated kinase 1/2 (Erk1/2) (Maulik et al., 1996; Ping, et al., 1999b; Ping, et al., 1999c).

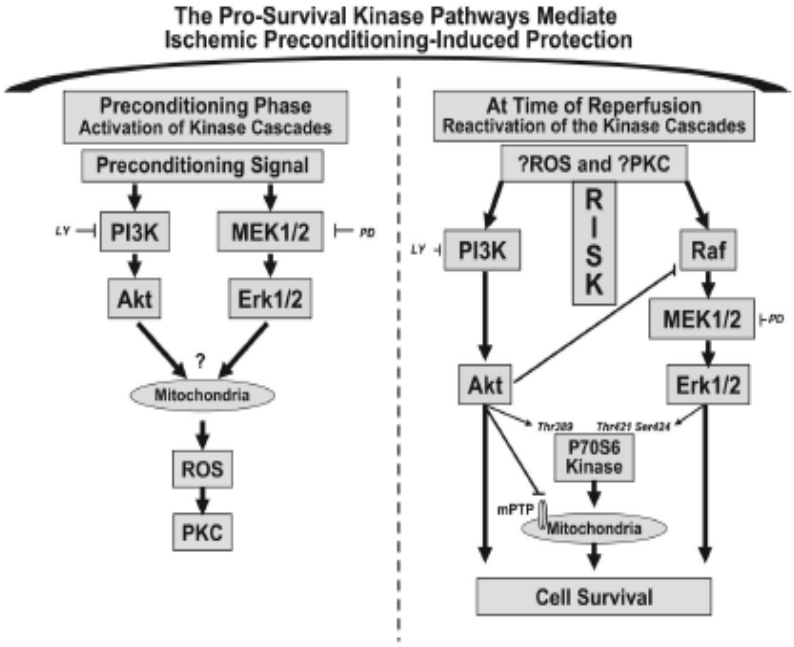


Fig 10: Hypothetical scheme outlining two phases of kinase cascade activation in response to IPC. During IPC, mitochondrial reactive oxygen species are released and PKC is activated. These events reactivate the PI3-kinase/Akt/p70S6K and Erk1/2/p70S6K cascades which comprise the reperfusion injury salvage kinase (RISK) pathway, at reperfusion. The RISK pathway mediates cellular survival through several possible mechanisms, which may include inhibition of mitochondrial permeability transition pore (mPTP) opening. MEK1/2: MAPK1/2. Further abbreviations as defined in the text. (Hausenloy, 2004)

3.1.5 Mitogen activated protein kinases and c-jun-NH_2-terminal kinases

Activation of the mitogen activated protein kinases (MAPK), p38 and c-Jun-NH2-terminal kinases (JNK) to confer protection at the time of reperfusion gave conflicting results, with some studies reporting cardioprotective roles of these kinases at the time of myocardial reperfusion (da Silva et al., 2004) and others suggesting a detrimental role (Sun et al., 2006). A study conducted in a conscious rabbit model demonstrated that IPC stimulation increased the activity of JNK and p38 MAPK (Ping et al., 1997). This increase, together with the protective effect of IPC, was abrogated by PKC inhibition (Ping et al., 1999d). Earlier studies in rabbit and rat hearts had reported an increased activation of p38MAPK following an IPC stimulus and also during the index ischaemia in isolated rat and rabbit hearts (Maulik et al., 1996; Maulik et al., 1998; Nakano et al., 2000; Weinbrenner et al., 1997). In contrast, an *in vivo* study in pigs demonstrated that the activation of p38 MAPK was unaltered (Behrends et al., 2000) and a later

study reported that in the *in vivo* canine heart, the activation of p38 MAPK was actually decreased (Sanada et al., 2002). Therefore, the exact role of p38MAPK in IPC still needs to be clarified.

3.1.6 Nitric oxide - cyclic guanosine monophosphate - protein kinase G signalling cascade

Many studies, in different animal models, have reported that inhibition of nitric oxide (NO) is deleterious in ischaemia-reperfusion (Gonon et al., 2000; Maulik et al., 1995; Ockaili et al., 1999; Rakhit et al., 2000; Xi and Kukreja, 2000). In these studies, different nitric oxide agonists were administered before initiation of ischaemia. The protection afforded by the activation of nitric oxide was abrogated when antagonists were administered during the ischaemic period and continued for the first few minutes of reperfusion. A study done in the isolated rat heart demonstrated a reduction in infarct size when the vasodilator, adrenomedullin, was given during early reperfusion and this effect was lost if nitric oxide synthase (NOS) was inhibited (Hamid and Baxter, 2005). Similar results were observed in the mouse heart, after administration of adrenomedullin prior to reperfusion (Hamid et al., 2007).

Evidence for the generation of NO and peroxynitrite during ischaemia-reperfusion has been provided by numerous studies (Csonka et al., 1999; Wang and Zweier, 1996; Weselcouch et al., 1995; Yasmin et al., 1997), but no increase in tissue NO could be demonstrated during the preconditioning protocol itself (Csonka et al., 1999). In 2000 Lochner et al, using an isolated rat heart model, demonstrated a role for NO and cyclic guanosine monophosphate (cGMP) as a trigger in classic preconditioning (Lochner et al., 2000).

NO generation results in the activation of soluble guanyl cyclase and an accumulation of cyclic guanosine monophosphate (cGMP), with subsequent activation of protein kinase G, a cGMP-dependent protein kinase (Hausenloy and Yellon, 2006). PKG is considered to be an important mediator of cardioprotection in IPC, (see review (Burley et al., 2007)). Activation of PI3-kinase/Akt phosphorylates endothelial nitric oxide synthase (eNOS), producing NO (Cohen et al., 2000; Critz et al., 2005; Oldenburg et al., 2002) which has been implicated in the protection of endothelial cells (Dimmeler et al., 1999). NO, in turn, has been shown to protect by inhibiting the opening of the mPTP (Balakirev et al., 1997).

3.1.7 Reactive oxygen species

Free radicals are molecules or atoms with unpaired electrons in their outer shell. Free radicals that originate from oxygen are called reactive oxygen species (ROS) and are highly reactive.

ROS are produced by several enzymes and biochemical processes in the body. In the cardiovascular system the main sources of ROS are: a) Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, (b) the enzyme, xanthine oxidoreductase, (c) nitric oxide synthase (NOS) and, (d) the mitochondrial cytochromes (Donzelli et al., 2006; Grisham et al., 1999; Mason et al., 2000; Wink and Mitchell, 1998). However, in the myocardium itself, the mitochondria appear to be the main source of ROS production (Di Lisa et al., 2007).

Production of ROS during ischaemia has been shown in isolated cardiomyocytes and in isolated hearts (Becker et al., 1999; Kevin et al., 2003). A recent *in vivo* model of endothelial ischaemia-reperfusion injury in the arm demonstrated for the first time, in humans, that endothelial function after I/R injury is affected by ROS production (Loukogeorgakis et al., 2010). At the onset of reperfusion, a sudden burst of ROS occurs, which has been shown to contribute to myocardial injury and cardiomyocyte death (see review(Hausenloy, 2009)).

In an isolated rat heart model IPC reduced the excessive amount of ROS production at the onset of reperfusion, suggesting a deleterious role for the excess ROS in the early stages of reperfusion (Crestanello et al., 1996). In contrast, a study by Baines et al, suggested that ROS may also play a critical role in the signal transduction pathway underlying IPC. This group demonstrated that the antioxidant, N-2-mercaptopropionylglycine (MPG), blocked the infarct-sparing effect of IPC in both *in vitro* and *in vivo* rabbit heart experiments, therefore suggesting that the presence of ROS was necessary for protection (Baines et al., 1997). In fact, a moderate release of ROS following the preconditioning stimulus leads to a decrease of ROS production at the onset of reperfusion (see review (Hausenloy, 2009)).

3.1.8 The JAK-STAT pathway

The Janus Kinases (JAKs) are a family of tyrosine kinases associated with membrane receptors. Ligands such as TNFα, interleukin 6 (IL-6) or growth factors activate the membrane receptors enabling the transphosphorylation and activation of two adjacent juxtaposed JAKs which can then subsequently activate the signal transducer and activator of transcription-3 (STAT-3) during ischaemia-reperfusion (see review (Lecour, 2009a)). STAT-3 is then phosphorylated at the tyrosine level, enabling it to form homodimers or heterodimers that subsequently translocate to the nucleus, resulting in gene transcription, (Figure 11).

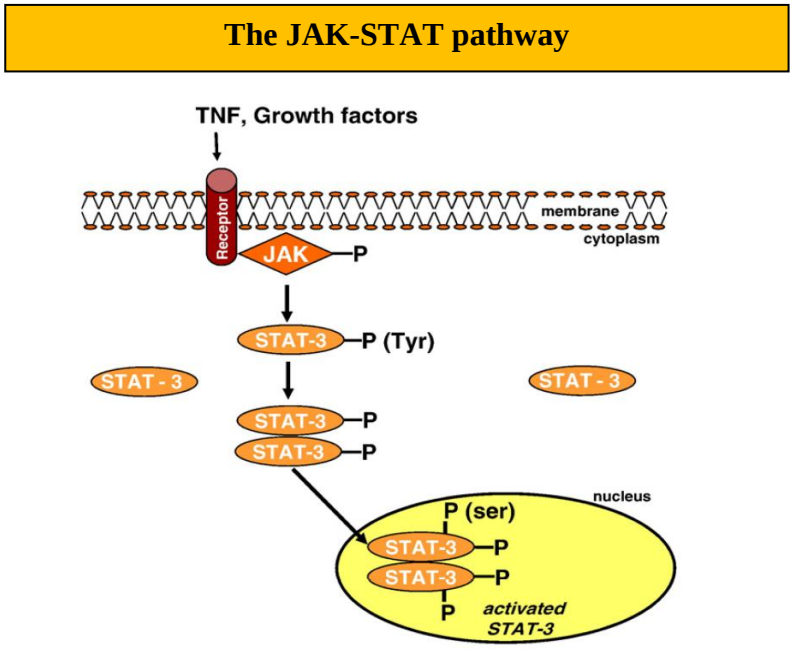


Fig 11: The JAK-STAT-3 signalling pathway. JAK2 is activated in response to a stimulus. Activated JAK 2 recruits STAT-3 from the cytoplasm and phosphorylates STAT-3 on a tyrosine residue. This phosphorylation allows STAT-3 to form homodimers and translocate to the nucleus where it upregulates transcription. STAT-3 is further phosphorylated on a serine residue to improve DNA interaction. JAK=Janus kinases, Ser=serine, Tyr=tyrosine, (Lecour, 2009).

The first study to implicate STAT-3 after acute myocardial infarction was performed in 2001 by Negoro and colleagues. In this experiment, the activation and increased phosphorylation of STAT-3 was observed for up to 24 hours after ligation of the left coronary artery in rats (Negoro et al., 2001) and the inhibitor of the JAK/STAT pathway, AG490, suppressed the phosphorylation of STAT-3 with a resulting increase in the number of apoptotic cells, implicating STAT-3 as a protective agent in ischaemia (Negoro et al., 2001).

Using an *in vivo* left coronary artery ligation model in mice, Bolli's group was the first to demonstrate the phosphorylation and translocation of STAT-3 to the nucleus in the delayed phase of ischaemic preconditioning (Xuan et al., 2001). In early ischaemic preconditioning, JAK2 and STAT-3 are activated following the IPC stimulus in isolated working rat hearts and this effect was almost completely abrogated with the inhibitor of JAK2, AG490 (Hattori et al., 2001). Furthermore, cardiac-specific STAT-3 knockout mice cannot be protected with an ischaemic preconditioning stimulus (Smith et al., 2004).

Confirmation of the role of STAT-3 in ischaemic preconditioning has also been shown with pharmacological protective agents such as cardiotrophin-1 (Brar et al., 2001), erythropoietin

(Nishihara et al., 2006; Rafiee et al., 2005) or cannabinoid agonists (Montecucco et al., 2009). Preconditioning mimetics such as TNFα (Suleman et al., 2008) and opioids (Gross et al., 2006; Gross et al., 2007) also activate STAT-3 during the trigger phase of preconditioning.

Several downstream effectors of the JAK/STAT pathway have been identified, including growth factors such as vascular endothelial growth factor (VEGF), pro survival proteins, proteins involved in cell proliferation such as Bcl-2, Bcl-xl, Mcl-1, Fas, cyclin D1, E1, p21 and other transcription factors (Amit-Vazina et al., 2005; Ikezoe et al., 2004; Liu et al., 2006; Marques et al., 2007; Torbenson et al., 2002; Zhang et al., 2007). In the context of ischaemic preconditioning, STAT-3 increases the anti-apoptotic gene, Bcl-2 and reduces the pro-apoptotic gene Bax (Hattori et al., 2001). Also, STAT-3 has been implicated in the mediation of preconditioning protection via the phosphorylation and therefore, the inactivation, of the pro-apoptotic factor Bad (Lecour et al., 2005b). Other possible targets of STAT-3 may be mitochondrial connexion 43, an important component of the signal transduction cascade of IPC-induced protection (Ozog et al., 2004; Schulz et al., 2007), Akt and GSK3-β (Gross et al., 2006; Juhaszova et al., 2004).

3.1.9 The SAFE pathway

An alternative and, possibly parallel, signalling pathway to the RISK path has recently been implicated in IPC. Lecour et al, have named this pathway the Survivor Activating Factor Enhancement (SAFE) pathway, (Figure 22) which includes the cytokine, tumour necrosis factor alpha (TNFα) and the transcription factor STAT-3 as triggers and mediators (Lecour, 2009a). This pathway will be discussed in further detail in the section 4.8 and in Section D.

3.1.10 Receptors for Advanced Glycation End Products (RAGE)

Advanced glycation end products (AGEs) are formed by the nonenzymatic glycation and oxidation of proteins and lipids. The signal transduction receptor for AGEs is known as RAGE.

A range of signal transduction cascades is activated by RAGE, including the MAPKs, PI3-kinase/Akt, JAK-STAT, NFκB, GSK-3β and production of ROS via NADPH oxidase (Aleshin et al., 2008; Bierhaus et al., 2001; Huang et al., 2001; Kim et al., 2008; Marsche and others, 2007; Shang et al., ; Wautier et al., 2001).

Multiple cell types express RAGE, including cardiomyocytes and ischaemia-reperfusion is a potent generator of AGEs. In a study using ischaemic rat hearts, expression of RAGE was

significantly upregulated and pretreatment of the rats, 3 days prior to experimentation, with soluble RAGE (which acts as a decoy to bind the ligands of the receptor thus blocking it) reduced ischaemic injury and improved functional recovery of the myocardium (Bucciarelli et al., 2006). Moreover, the same group demonstrated that isolated and perfused hearts from RAGE null mice subjected to ischaemia-reperfusion were strikingly protected against I/R damage (Bucciarelli et al., 2006). RAGE can also modulate hypoxia/reoxygenation injury in adult murine cardiomyocytes via JNK and GSK-3β signalling pathways (Shang et al.). High-Mobility Group Box-1 (HMGB-1), a nuclear factor released by necrotic cells, signals via RAGE. When mice were preconditioned with HMGB-1 in an ischaemia-reperfusion model, TNFα levels were decreased and lipopolysaccharide (LPS) tolerance was induced, suggesting that the RAGE receptor was involved in ischaemic preconditioning (Aneja et al., 2008).

3.1.11 Proteinase activated receptor-2

Proteinase activated receptors (PAR-2) are a recently described, novel family of seven transmembrane G-protein coupled receptors which are activated by protease cleavage (Cirino et al., 2000; Nystedt et al., 1994)

PAR-2 is expressed in vascular tissue and highly vascularized organs (Bohm et al., 1996; Nystedt et al., 1994; Nystedt et al., 1995; D'Andrea et al., 1998). The first evidence that PAR-2 was expressed in the heart and that it also contributes in the reduction of myocardial ischaemia-reperfusion injury was provided by Napoli et al, (Napoli et al., 2000). In Napoli's seminal study, an agonist of PAR-2 was added to the perfusate of the isolated heart 15 minutes into the stabilisation period and continued for 5 minutes into reperfusion. This study demonstrated that activation of PAR-2 resulted in an improved functional response associated to an improvement of metabolic parameters of the heart during experimental ischemia-reperfusion injury. Napoli and colleagues subsequently used an isolated perfused rat heart model to demonstrate that enhanced activation of PAR-2 during the IPC stimulus, with a PAR-2 activating peptide, improved the protective effect of ischaemic preconditioning (Napoli, 2002). A study conducted in rat neonatal ventricular cardiomyocytes, demonstrated that in the heart, PAR-2 stimulated p38 MAPK and ERK1/2, elevated calcium concentration and also activated JNK and Akt (Sabri et al., 2000). PAR-2 has also been implicated in vessel relaxation via the NO pathway (Cheung et al., 1998; Cicala et al., 1999; Emilsson et al., 1997) and endothelin-1 (Magazine et al., 1996) (Figure 12).

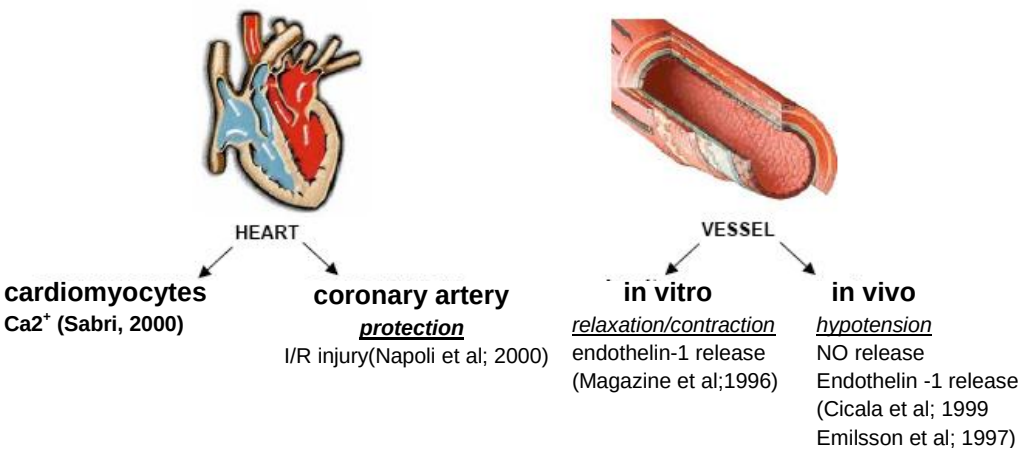


Fig 12: In vitro and in vivo effects of PAR-2 activation, (adapted from Bucci, 2005).

In addition, activation of PAR-2 significantly reduced the infarct size 24 hours after administration in a mouse model of acute myocardial ischaemia (Lim et al., 2007). The cardioprotective effect of PAR-2 agonists has been linked to PKC and Erk1/2 activation (Blackheart et al., 1996), increased TNFα levels in cardiac tissue (Bopassa et al., 2006a) and manganese superoxide dismutase activity (Camerer et al., 2000).

3.1.12 Glycogen synthase kinase-3 beta

GSK-3β is a serine/threonine kinase that was originally identified as an enzyme that phosphorylates and downregulates glycogen synthase, the rate limiting enzyme of glycogen metabolism (Rylatt et al., 1980). Subsequently, it has been shown that the role and importance of GSK-3β extends far beyond intermediary metabolism but the nomenclature remains in use.

Phosphorylation of GSK-3β results in its inactivation which has been reported to be antiapoptotic. Inhibition of GSK-3β plays an important role in the mediation of cardioprotection via phosphorylation by proteins such as the tyrosine kinases, Akt, PKC and activation of the mitochondrial ATP-sensitive potassium channel (mK_{ATP}) and the mPTP (Juhaszova et al., 2004). A study using a pressure overload hypertrophic rabbit heart model subjected to ischaemia-reperfusion, demonstrated that contractile function after ischaemia in hypertrophied hearts treated with a GSK-3β inhibitor was better preserved and that tolerance to ischaemia-reperfusion injury in the hypertrophied myocardium was improved in the presence of the inhibitor (Barillas et al., 2007). Similarly, inhibitors of GSK-3β added in Langendorff perfused rat hearts subjected

to a regional ischaemia-reperfusion insult at two different perfusion pressures showed an improved infarct size (Mozaffari et al, 2008).

Numerous studies have implicated inhibition of GSK-3β in the cardioprotective effects of IPC (Gross et al., 2004; Gross et al., 2006; Juhaszova et al., 2004; Tong et al., 2002). In one of these studies, evidence implicating the involvement of GSK-3β in the protective effect of IPC was provided with the use of two inhibitors of GSK-3β, lithium and SB216763, administered during the IPC protocol in an isolated rat heart model of ischaemia-reperfusion (Tong et al., 2002). From this study it was reported that phosphorylation and inactivation of GSK-3β occurred during IPC which would be consistent with a role for GSK-3β in preconditioning. In addition, this group linked PI3-kinase to the cardioprotective effect of phosphorylation of GSK-3β (Tong et al., 2002). In 2004, Juhaszova et al, suggested the inhibition of GSK-3β as a "master signalling switch" which leads to the convergence of the various prosurvival pathways to the putative end effector of protection, the mPTP, (Juhaszova et al., 2009) (Figure 13).

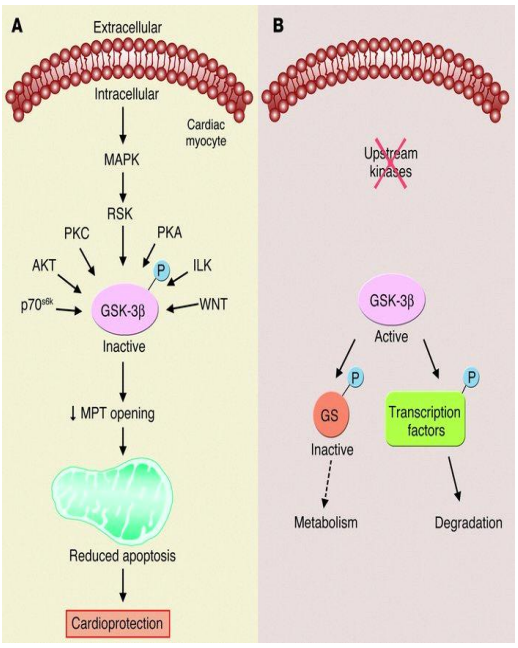


Fig 13: Scheme showing various kinases that phosphorylate and inacivate GSK-3β. In the absence of upstream kinases, unphosphorylated GSK-3β is active, phosphorylating and deactivating downstream targets such as glycogen synthase, or phosphorylating transcription factors and targeting them for degradation. GS = glycogen synthase; RSK = p90 ribosomal S6 kinase; ILK = integrin-linked kinase; WNT = homologues of Wingless; P = phosphorylation, (Murphy, 2004).

3.1.13 Role of the mitochondria, the mitochondrial ATP-sensitive potassium channel and the mitochondrial permeability transition pore in IPC

Mitochondria are spherical or rod shaped organelles found within the cytoplasm of eukaryotic cells and often referred to as the "powerhouse of the cell" since they act as the site for the production of adenosine triphosphate (ATP) which is vital as an energy source for several cellular processes.

Many prosurvival signalling cascades appear to converge to the mitochondria and given that the mitochondria are the "powerhouse" of the cell, this is no surprise. It is still a matter of debate as to how the prosurvival signals actually terminate at the mitochondria.

i) Mitochondrial Adenosine Triphosphate-sensitive potassium channel

Two potassium adenosine triphosphate-sensitive potassium channel subtypes coexist in the myocardium. One is located in the sarcolemmal membrane (sarcK_{ATP}) and the other in the inner mitochondrial membrane (mK_{ATP}). These channels allow coupling of membrane potential to cellular metabolic status and can be pharmacologically modulated by potassium channel openers and inhibitors.

Pharmacological agents which open the mK_{ATP} channel protect the ischaemic myocardium by direct protection of cardiac myocytes (Weselcouch et al., 1995). A study using an isolated canine gracilis muscle model demonstrated that when a mK_{ATP} opener was administered by direct infusion into the artery supplying the muscle, the infarct size was significantly reduced, therefore suggesting that mK_{ATP} activation is an important protective mechanism in this tissue (Weselcouch et al., 1995).

Garlid's group perfused isolated rat hearts with diazoxide, a potent opener of the putative mK_{ATP} channel, prior to ischaemia, demonstrating that opening of the channel was cardioprotective (Garlid et al., 1997). In addition, pharmacological activation of both PKC and MAPK which induce cardioprotection can be blocked with 5HD, a mK_{ATP} channel inhibitor, thus indicating that the channel may be downstream to these preconditioning mediators (Hopper et al., 2000; Yue et al., 2002). Other studies have demonstrated that PKC activates both the $sarcK_{ATP}$ and the mK_{ATP} channels (Hu et al., 1996; Sato T et al., 1998) but the actual role of these channels in IPC is controversial (Hanley P J., 2005). It should be noted that the mK_{ATP} channel has never been cloned and that much of the evidence obtained which has implicated the mK_{ATP} channel as playing a critical role in IPC was obtained using pharmacological inhibitors (Garlid et al., 1997; Liu et al., 1998b).

ii) Mitochondrial permeability transition pore

The mPTP is a large non-selective ion channel in the inner mitochondrial membrane. Mitochondrial permeability transition is an increase in the permeability of the mitochondrial membranes to molecules of less than 1500 Daltons in molecular weight. Mitochondrial permeability transition results from opening of mitochondrial permeability transition pores.

Under normal physiological conditions the mitochondrial inner membrane is impermeable to most molecules. During stressful conditions, such as ischaemia-reperfusion, the mPTP opens, allowing the transfer of molecules to the inner mitochondria (Hausenloy et al., 2004a) causing swelling and rupture of the outer mitochondrial membrane (Halestrap et al., 2004). Irreversible opening of this channel in response to the sudden return of flow of blood at reperfusion is considered to be responsible for cardiomyocyte death by uncoupling oxidative phosphorylation (Hausenloy, 2009).

Transient opening and closure of the mitochondrial permeability transition pore (mPTP) at the time of reperfusion has emerged as an important target for cardioprotection in IPC. In 2002, Hausenloy et al used an isolated rat heart model of IPC to demonstrate that an infusion of cyclosporine-A (CsA) at reperfusion, inhibited the opening of the mPTP, mimicking IPC-induced protection (Hausenloy et al., 2002). Subsequent studies have also implicated the closure of the pore at reperfusion in the protective effect of preconditioning (Hausenloy et al., 2004a; Javadov et al., 2003). Although the mechanism for this effect still needs to be elucidated, it may involve certain components of the RISK pathway such as Akt, Erk1/2 or GSK-3β (Abdallah et al., 2006; Bopassa et al., 2006b).

3.1.14 Applications of ischaemic preconditioning

A robust and reproducible protection is exerted by the phenomenon of IPC and this has been demonstrated in the heart of every animal species tested thus far e.g. rabbits, dogs, ferrets, pigs, guinea pigs, sheep, mice, chicken and man (see review (Lawson and Downey, 1993)). However, these experimental results, done mainly in healthy, young animals may not be reproducible in humans as comorbidities, such as diabetes, obesity and left ventricular hypertrophy often accompany ischaemic heart disease in humans and these may influence the outcome of IPC (Moolman et al., 1997; Tsang et al., 2005). Moreover, the need to intervene before the index ischaemic event limits the clinical application of IPC as an acute myocardial infarction (AMI) cannot yet be predicted. The application of IPC in the clinical arena is, therefore, limited to scenarios in which the index ischaemia can be reliably predicted, such as in patients undergoing

surgery with extracorpal circulation. Yellon et al, were the first to report that IPC induced by intermittent cross-clamping of the aorta in coronary artery bypass graft surgery preserved the patient's ATP levels during the subsequent global ischaemic period (Yellon et al., 1993). Brief episodes of ischaemia are experienced by many patients prior to an acute myocardial infarction and it is theoretically possible that this pre-infarct angina may precondition the heart. Hence, it has been demonstrated that the presence of angina before AMI is associated with a smaller infarct size (Kloner et al., 1995). The discovery of "remote ischaemic preconditioning" in which either an upper limb or the leg can be used to apply IPC (usually by repeated, intermittent blood pressure cuff inflations and deflations) has revived the interest in IPC. This non-invasive remote form of IPC, known as RIPC, has been used successfully in children undergoing corrective heart surgery (Cheung et al., 2006), patients undergoing coronary artery bypass grafts (Hausenloy et al., 2007; Venugopal et al., 2009) and in elective percutaneous coronary interventions (Hoole et al., 2009).

3.2 Ischaemic postconditioning

Ischaemic postconditioning has been defined as a series of brief mechanical interruptions of reperfusion applied at the onset of reperfusion (Zhao et al., 2003).

Ischaemic postconditioning (IPostC) was first proposed in 2003 by Vinten-Johansen's group as an attempt to offer an intervention which could be applied at the time of myocardial reperfusion to protect patients diagnosed with an acute myocardial infarction, (Figure 14).

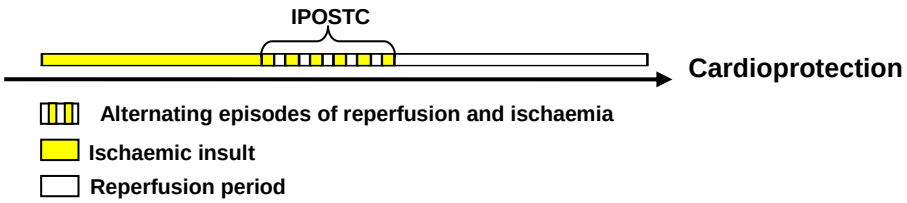


Fig 14: Schematic representation of ischaemic postconditioning. IPostC is initiated at the onset of reperfusion. The number and duration of the cycles depends upon the animal species.

As in IPC, many experiments have demonstrated that IPostC reduces infarct size in hearts of several different animal species such as: rabbit, mice, rats dogs, and pigs (Bopassa et al., 2006b; Darling et al., 2005; Heusch et al., 2006; Iliodromitis et al., 2006; Tsang et al., 2004; Yang et al., 2005; Zhao et al., 2003). The degree to which the heart is protected by ischaemic

postconditioning appears to be similar or slightly less than that obtained by ischaemic preconditioning (Fantinelli and Mosca, 2007; Halkos et al., 2004; Kin et al., 2004; Zhao et al., 2003) but the effect of IPostC is not enhanced by IPC (Halkos et al., 2004). As the clinical application of IPC was limited, the IPostC phenomenon has opened a novel era of research to limit reperfusion injury.

In their seminal paper, Zhao and colleagues, demonstrated that interruption of myocardial reperfusion with 3 x 30 second episodes of myocardial ischaemia and reperfusion in a canine model, resulted in a dramatic reduction in myocardial infarct size, comparable to that seen with IPC (Zhao et al., 2003). Thus, ischaemic postconditioning (IPostC) evolved as a method to reduce reperfusion injury. The protective effect of IPostC is clearly demonstrated in figure 15 which shows a section from a mouse heart subjected to IPostC compared to a heart which received no intervention.

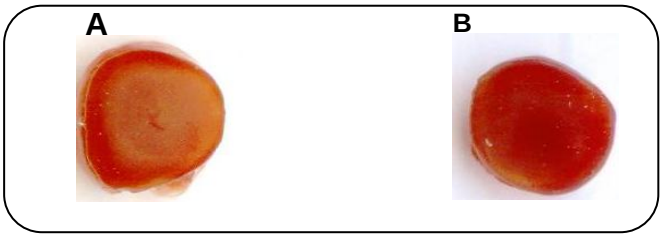


Fig 15: Ischaemic postconditioning reduces infarct size. A: heart subjected to ischaemia-reperfusion with no intervention. **B:** heart postconditioned with alternating cycles of reperfusion and ischaemia. Red area = healthy tissue; White area = infarcted tissue.

3.2.1 Mechanisms of IPostC: A remarkable assortment of molecular mechanisms, similar to those implicated in IPC, has been demonstrated to contribute to the protective effects of IPostC. A schematic showing some of the shared signalling cascades is shown in Figure 16.

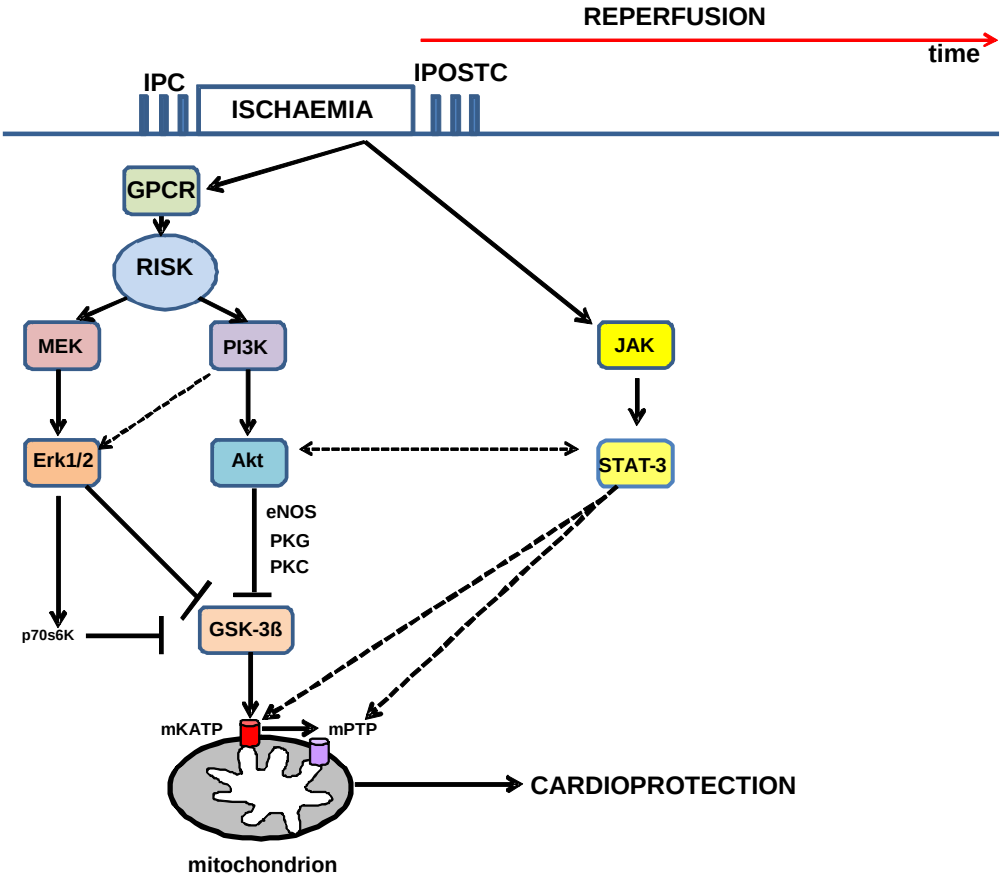


Fig 16: Hypothetical scheme outlining a few major signalling mechanisms common to IPC and IPostC. Abbreviations as defined in the text.

A number of signalling pathways which have been recruited by IPostC have been identified. These can be divided into the following:

3.2.2 Gi-protein coupled receptors

The human genome has been estimated to have an extremely large number of GPCRs and based on their sequences as well as on their known or suspected functions, there are estimated to be five or six major classes of GPCRs (Kroeze et al., 2003). Depending on the type of G protein to which a receptor is coupled, a variety of downstream signalling pathways can be activated (Marinissen and Gutkind, 2001; Neves et al., 2002).

The role of adenosine, bradykinin and opioids receptors will be discussed in section 3.3 related to pharmacological pre- and postconditioning.

3.2.3 The RISK pathway

In an isolated perfused rat heart, inhibition of PI3-kinase at reperfusion with wortmannin or LY290042 abolished the protective effect of IPostC (Tsang et al., 2004). Similarly, addition of PD98059, an inhibitor of Erk1/2 at the onset of reperfusion suggested that the protective benefits of IPostC were dependent on the activation of Erk1/2 (Yang et al., 2004b). Subsequent studies in both healthy and diseased animals as well as human atrial muscles have confirmed the role for Akt and Erk1/2 in IPostC (Feng et al., 2006; Sivaraman et al., 2007; Zhu et al., 2006). Potential distal signalling elements of the RISK pathway are suggested to be PKG, PKCε and GSK-3β (Burley and Baxter, 2009; Gomez et al., 2008) as already described in section 3.1.4.

However, a few recent experimental studies conducted in swine and rabbit models have failed to show components of the RISK pathway as mediators of IPostC-induced protection, therefore suggesting the existence of alternative pathways (Darling et al., 2005; Skyschally et al., 2009).

3.2.4 The JAK-STAT pathway

Phosphorylation of STAT-3 was first implicated in the cardioprotective effect of IPostC in 2006 by Lecour's group (Suleman et al., 2006). In isolated hearts, the protective effect of IPostC was abolished in the presence of the JAK2 inhibitor, AG490, and western blot analysis after 15 minutes of reperfusion revealed that IPostC decreased the phosphorylation of STAT-3 in the cytosol, therefore suggesting activation and translocation of STAT-3 to the nucleus after IPostC. The role of STAT-3 in IPostC was confirmed by Boengler et al in an *in vivo* STAT-3 knockout mouse model (Boengler et al., 2008). However, the evidence of interaction between the JAK/STAT pathway and the PI3-kinase pathway at the onset of reperfusion and during IPostC is inconclusive (Goodman et al., 2008) and the mechanism responsible for the activation of the JAK/STAT pathway in IPostC is unknown.

3.2.5 Nitric oxide-cGMP-PKG signalling cascade

The protective effects of IPostC have been demonstrated to be sensitive to pharmacological inhibition of the NO-cGMP-PKG pathway (Penna et al., 2006a; Yang et al., 2005). It is believed that, in response to IPostC, activated PKG opens the mK_{ATP} channel through PKCε (Costa et al., 2005) which is presumably activated via the Akt component of the RISK pathway and eNOS (Tsang et al., 2004).

3.2.6 ROS signalling

IPostC reduced the amount of harmful ROS generation at the onset of myocardial reperfusion (Zhao et al., 2003). ROS signalling in the first moments of reperfusion, however, appears necessary for the protective effect of IPostC (Penna et al., 2006b) as inhibitors of ROS given at the onset of reperfusion, abolished the protective effect of IPostC. At present, the role of ROS signalling during IPostC still remains unclear.

3.2.7 Receptor for advanced glycation end products

A current literature search revealed no articles on RAGE and IPostC but the receptors for advanced glycation end products have been involved in protection during ischaemia-reperfusion and seem to be implicated in IPC (Aneja et al., 2008; Bucciarelli et al., 2006). Therefore, it is appropriate to suggest that RAGE may play a role in IPostC.

3.2.8 Proteinase Activated Receptor-2

Ischaemia-reperfusion injury and angioplasty balloon catheter injury are reported to increase the expression of PAR-2 (Damiano et al., 1999; Napoli et al., 2000) and PAR-2 activation has been shown to be protective in other tissues, including brain, airways and myocardium undergoing ischaemia or lipopolysaccharide challenge (Jin et al., 2005; Morello et al., 2005; Napoli et al., 2000).

Although never studied in IPostC, Vinten-Johansen's group demonstrated firstly, that PAR-2 is present in the left ventricular tissue of rat heart, in neonatal rat cardiomyocytes and in H_9C_2 rat cardiac-derived cells and secondly, that a PAR-2 agonist administered 5 minutes before reperfusion in the isolated rat heart, salvaged the myocardium via an Erk1/2 pathway (Jiang et al., 2007). These data suggested that IPostC may protect via activation of PAR-2.

3.2.9 Glycogen synthase kinase-3 beta (GSK-3β)

Inactivation of GSK-3β is a well-established downstream target of the RISK pathway. As in IPC, inhibition of GSK-3β is believed to play an important role in IPostC, although experimental results have been somewhat contradictory. In an isolated rat heart model, Gross et al, first implicated inhibition of GSK-3β at the onset of reperfusion (Gross et al., 2004). Gomez et al, reported that inhibition of GSK-3β by postconditioning was required in transgenic mice to prevent the opening of the mPTP during reperfusion (Gomez et al., 2008). In contrast, Nishino et al, also using transgenic mice, found that inactivation of GSK-3β was not required to confer

protection in either IPC or IPostC (Nishino et al., 2008). Skyschally and colleagues postconditioned pigs in the presence of inhibitors of the RISK pathway and found that protection was still induced in the pigs although blockade of the RISK pathway resulted in a reduced GSK-3β phosphorylation and p70s6K (Skyschally et al., 2009).

However, as previously mentioned, GSK-3β could possibly be the central point where a number of protective signalling cascades converge and its cellular location close to the mPTP may be of significance in transferring prosurvival signals (Juhaszova et al., 2009).

3.2.10 Mitochondrial involvement in IPostC

Many of the signalling pathways transmitting the cardioprotective signal of IPostC appear to converge to the mitochondria. The heart is a rich source of mitochondria with approximately 40% of total cardiomyocyte volume consisting of mitochondria (Barth et al., 1992). Thus, mitochondria are both targets and sources of injury during cardiac ischaemia and reperfusion with both the putative mK_{ATP} channel and the mPTP being implicated as possible end effectors of the protective pathway (Gross and Gross, 2006; Gross et al., 2004; Hausenloy et al., 2002).

i) Mitochondrial potassium ATP channels

Pharmacological inhibition of the mK_{ATP} at the onset of reperfusion abrogated IPostC-induced protection (Donato et al., 2007; Yang et al., 2004b) but no direct studies have shown that IPostC can actually open this channel during reperfusion. A recent study by Garlid's group suggested that IPostC generates a signalosome which conveys the signal from the G-protein coupled receptors to the opening of the mK_{ATP} leading to the inhibition of the opening of the mPTP (Quinlan et al., 2008).

ii) Mitochondrial permeability transition pore

Several studies suggest a critical role of the mPTP in IPostC. Isolated rabbit heart mitochondria subjected to a standard IPostC protocol, are more resistant to calcium-induced opening of the mPTP, therefore suggesting that IPostC inhibits mPTP opening at reperfusion (Argaud et al., 2005). Bopassa et al, subsequently reported that inhibition of PI3-kinase/Akt with wortmannin or LY294002 at the onset of reperfusion abolishes the inhibitory effect on the pore's opening of IPostC, implicating IPostC in the mediation of pore inhibition via the activation of PIK-kinase/Akt (Bopassa et al., 2006a). Also, mice lacking cyclophilin-D, the only known component of the mPTP, cannot be protected by IPostC (Lim et al., 2007).

3.2.11 Clinical application of ischaemic postconditioning

Within 2 years following its initial description, IPostC proved to be effective in clinical studies. Several sequences of inflation/deflation of the angioplasty balloon applied after opening of the infarcted vessel, reduced infarct size, improved coronary blood flow and improved myocardial reperfusion in patients with acute myocardial infarction (Laskey, 2005; Ma et al., 2006; Staat et al., 2005; Yang et al., 2007). However, the application of IPostC in these studies was strictly restricted to patients undergoing primary percutaneous intervention (PCI) as the protocol was invasive.

Therefore, it is important to delineate the signalling pathways which are responsible for the cardioprotective effect of IPostC. Knowledge of these signalling cascades may pave the way for manipulation of pharmacological targets to achieve the same end result as IPostC. Cyclosporine-A, which prevents the opening of the mPTP at reperfusion has already been used in the clinical setting with a promising outcome. An important "proof of concept" study, provided evidence in humans that inhibition of the mPTP at reperfusion in patients with acute myocardial infarction is associated with reduction in infarct size (Piot et al., 2008).

3.3 Pharmacological pre- and postconditioning – *the next step in limiting infarct size*

Given the invasive nature of ischaemic pre and postconditioning and its limited use to elective PCI patients or patients undergoing cardiac surgery, pharmacological agents mimicking the protective effects of IPostC were sought and found. Included amongst the protective pharmacological agents were: adenosine, bradykinin and opioids.

3.3.1 Adenosine

Adenosine is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule moiety. It is a purine nucleoside endogenously expressed in the body and modulates many biological processes after binding to one of the adenosine receptors.

Increased release of adenosine has been reported during ischaemia and early reperfusion (Schrader et al., 1977). Adenosine, infused directly into the left descending coronary artery in a canine model reduced the deleterious effects of ischaemia by decreasing infarct size. This protective effect was attributed to the vasodilatory effects of adenosine (Engler, 1987; Olafsson et al., 1987; Takeo et al., 1988). Adenosine is one of three autacoids released by ischaemic tissue, the other two autacoids being bradykinin and opioids.

3.3.2 Adenosine receptors

There are 4 adenosine receptor subtypes, A_1, A_{2A}, A_{2B} and A_3 with all of the receptors being expressed in different cell types of the heart and blood vessels. Cardioprotection has been attributed mainly to the A_1 and A_3 receptors, (Black et al., 2002; Reichelt et al., 2005) with the A_3 receptor appearing to share a few similarities with the A_1 receptor, both having been associated with different kinase systems such as PKC, MAPK's, PI-3 kinase and Akt (Dana et al., 2000; Headrick et al., 2003; Mubagwa and Flameng, 2001). However, evidence also exists showing that the A_{2A} receptor is associated with cardioprotection (Norton et al., 1992; Xu et al., 2001). Adenosine and adenosine receptor agonists are the most widely studied cardioprotective ligands, but as yet, there is no definite agreement as to which receptor subtype contributes to cardioprotection during ischaemia or reperfusion phases.

3.3.3 Preconditioning with adenosine

The cardioprotective effect of IPC has been successfully mimicked by adenosine administration in various animal models tested, and in human atrial trabeculae (Cleveland et al., 1997; Liu et al., 1994; Liu et al., 1991). In a seminal study using *in vivo* rabbit hearts, Liu et al, infused two different non-selective adenosine antagonists directly before the IPC protocol was applied and each successfully blocked the IPC-induced protection (Liu et al., 1991). This data suggested that adenosine released during preconditioning stimulates the A_1 receptor. A few years later Liu et al, using a selective A_1 receptor antagonist and a selective A_3 receptor agonist, provided evidence demonstrating that the protection of IPC was not exclusively mediated by the A_1 receptor in heart but could also involve the A_3 receptor (Liu et al., 1994).

3.3.4 Postconditioning with adenosine

Downey's research group was the first to implicate adenosine in IPostC (Yang et al., 2005). In an isolated perfused rabbit heart, the presence of a non-selective A_1 receptor antagonist, an inhibitor of the PI3-kinase signalling cascade, wortmannin, and an inhibitor of guanyl cyclase abolished the infarct-sparing effect of IPostC and their data suggest that postconditioning-induced protection requires the adenosine A_1 receptor to activate PI3-kinase and guanyl cyclase (Yang et al., 2005). Similarly, Kin and colleagues used an isolated perfused mouse heart model to demonstrate that IPostC reduces the level of adenosine released during reperfusion (Kin et al., 2005a). This same group confirmed their data in an open chest rat model of myocardial infarction using A_1, A_2, or A_3 receptor antagonists, administered 5 minutes before reperfusion in the presence or absence of IPostC. Interestingly, IPostC in the rat protected the heart, reduced

the adenosine washout at 2 minutes of reperfusion and reduced infarct size and this effect was not abolished by the A_1 receptor antagonist. However, the A_2 and A_3 receptor antagonists did abrogate the protective effect of IPost, therefore suggesting that IPostC involved endogenous activation in the heart of the A_2 or A_3 receptor but not the A_1 receptor subtype (Kin et al., 2005a).

3.3.5 Proposed mechanisms in adenosine-induced cardioprotection

Contrary to the proposal by Cohen et al, suggesting that adenosine-induced protection was not dependent on opening of the mK_{ATP} (see review (Cohen and Downey, 2008)), intracoronary infusion with adenosine and dipyridamole (a drug which inhibits thrombus formation and also causes vasodilation) before occlusion in a canine model of IPC produced a significant reduction in infarct size which was abolished by pretreatment with glybenclamide, an ATP-dependent potassium channel antagonist. This suggested that activation of the A_1 receptors in the heart produces myocardial preconditioning via the opening of the K_{ATP} channels (Auchampach and Gross, 1993). The mechanism of protection may, therefore, be dependent on whether adenosine acts as a trigger or mediator of IPC.

PKCε has also been implicated as a mediator in adenosine's preconditioning signalling pathway (Cohen and Downey, 2008). Cohen et al, hypothesized that in postconditioning, adenosine activates PKCε which subsequently activates the A_{2B} receptor, Erk1/2, Akt and p70s6K, therefore inhibiting GSK-3β and preventing the opening of the mPTP (Cohen and Downey, 2008). PI3-kinase and guanyl cyclase are involved in adenosine-induced postconditioning (Yang et al., 2005) and other investigators have also implicated the components of the RISK pathway in adenosine-induced cardioprotection (Kis et al., 2006; Park et al., 2006a; Yang et al., 2004a).

3.3.6 Clinical application of Adenosine

The ability of adenosine to limit reperfusion injury has been tested in two clinical trials involving patients with AMI. In the AMISTAD-I trial, patients undergoing fibrinolysis were randomised to receive either adenosine (70μg/kg/min for 3 hours) or a normal saline infusion within 6 hours of infarction onset. Infarct size was reduced by 33%, relative to the area at risk, ($p<0.085$). However, the percentage reduction in infarct size appeared to depend upon which area of the heart was infarcted. In addition, adenosine showed no significant effects on secondary endpoints and clinical outcomes such as death, stroke, congestive heart failure, re-infarction and cardiogenic shock (Mahaffey et al., 1999).

A second, larger AMISTAD trial (AMISTAD-II) sought to assess two doses of adenosine infusion, 50 and 70 µg/kg/min given for 3 hours, with treatment starting 15 min before the start of fibrinolysis or PCI, but still within 6 hours after infarction onset. Both doses reduced infarct size with the higher dose achieving a 59% reduction compared to 15% reduction with the lower dose. Despite the reduction in infarct size, no difference was observed in the survival rate between placebo-treated or adenosine-treated patients (Ross et al., 2005). The inconsistencies in the results of the two AMISTAD trials lead to major reservations about the reliability of infarct size quantification in clinical studies and also cast doubt about the protective effect of adenosine and selective adenosine receptor agonists at reperfusion in experimental studies (see review (Burley and Baxter, 2009)).

3.3.7 Bradykinin

Bradykinin is a nonapeptide kinin formed from a plasma protein, high-molecular-weight (HMW) kininogen, by the action of kallikrein; it is a very powerful endothelium-dependent vasodilator that helps to enlarge or open blood vessels. This lowers blood pressure, allowing the blood to flow more smoothly throughout the body. In addition, bradykinin constricts smooth muscle and stimulates pain receptors.

Similar to adenosine, bradykinin is elevated during and after an ischaemic insult (Baumgarten et al., 1993; Pan et al., 2000; Schulz et al., 1998).

3.3.8 Bradykinin receptors

Cardiomyocytes have two bradykinin receptors, a constitutive B_2 receptor and an inducible B_1 receptor (Bhoola et al., 1992; Marceau et al., 1998). Induction of the B_1 receptor in the left ventricle of rats occurs 6 hours after reperfusion, and increases up to 4 fold after 24 hours (Tschope et al., 2000; Tschope et al., 1999). Bradykinin was first associated with IPostC when two different pharmacological antagonists of the bradykinin 2 receptor abolished the protective effect of IPostC in the isolated rat heart (Penna et al., 2007).

3.3.9 Preconditioning with bradykinin

The cardioprotective effect of bradykinin has been demonstrated in various animal models such as mouse, rat, rabbit and pig hearts (Bell and Yellon, 2002; Penna et al., 2007; Penna et al., 2008; Tobe et al., 1991; Yang et al., 2004a).

Using B_2 receptor knockout mice, or a rat model deficient in high-molecular-weight (HMW) kininogen, Yang et al, demonstrated that IPC fails to protect against ischaemia-reperfusion,

therefore suggesting an important role for the B_2 receptor in IPC (Yang et al., 1997). The exact role of the B_1 receptor in cardioprotection is unknown although knockout of this receptor in female mice had no effect on remodelling after a myocardial infarction (Xu et al., 2005). Bradykinin can also mimic IPC in the rabbit heart. Exogenous bradykinin protected to a similar extent compared with IPC and the B_2 receptor blocker completely abrogated the protection (Goto et al., 1995). In addition, bradykinin can mimic the effects of IPC in humans undergoing percutaneous transluminal coronary angioplasty (Leesar et al., 1999). However, it is unknown whether bradykinin receptor inhibitors can abrogate the effect of IPC (Gross and Gross, 2006).

3.3.10 Postconditioning with bradykinin

Using an isolated rat heart model of postconditioning, Penna et al, demonstrated that intermittent infusion of bradykinin at the beginning of reperfusion stimulates a postconditioning-like cardioprotection (Penna et al., 2007). The bradykinin-induced protection was abrogated in the presence of a B_2 receptor antagonist, implicating this receptor in the protective effect. Similarly, isolated hearts from mice in which the bradykinin B_2 receptor, the bradykinin B_1 receptor or the adenosine A_1 receptor had been knocked out, showed that the protective effect of IPostC was partially attenuated in the B_1 receptor knockout but was abrogated in the absence of the B_2 receptor and the adenosine A_1 receptor (Liu et al., 2008). Using three distinctive strains of gene knockout mice, this study provided the first conclusive evidence showing IPostC-induced cardioprotection could be triggered by activation of multiple types of cell membrane receptors, which include adenosine A_1 and bradykinin B_1 and B_2 receptors.

3.3.11 Proposed mechanisms in bradykinin-induced cardioprotection

Bradykinin, given at reperfusion protects via PI3-kinase/Akt and Erk1/2 pathway and by opening the mitochondrial ATP-sensitive potassium channels with the generation of reactive oxygen species that act as second messengers to activate PKC (see review (Critz et al., 2005)). Studies in rat hearts with intermittent application of bradykinin at reperfusion, have demonstrated a sizeable reduction in infarct size with activation of NO, cGMP, PKG, mK_{ATP} opening, and ROS production being implicated in the protective pathway (Oldenburg et al., 2004).

An interesting study by Penna and colleagues in the isolated rat heart, reported the novel finding that postconditioning required endogenous activation of cyclooxygenase (COX) and release of prostacyclin GI_2 (PGI_2) and that bradykinin, given as a postconditioning mimetic, also triggered the COX activation and induced PGI_2 formation during the late phase of reperfusion (Penna et

al., 2008). This data suggested that ischaemic postconditioning and intermittent bradykinin postconditioning were not solely dependent on the activation of the RISK pathway but that it also depended on the late release of PGI2 via activation of COX (Penna et al., 2008).

Quinlan and co-workers proposed another mechanism in which bradykinin interacted with its B_2 receptor, inducing the formation of a signalosome (a vesicular caveolar signalling platform). This would then phosphorylate an unknown receptor on the outer mitochondrial membrane. As the terminal kinase of bradykinin is protein kinase G (PKG), this would phosphorylate the unknown receptor at a serine/threonine residue. The signal would then be transmitted to activate protein kinase epsilon$_1$ (PKCε_1) on the inner mitochondrial membrane. PKCε_1 is situated close to the mitochondrial K_{ATP} channel and causes this to open with a subsequent increase in hydrogen peroxide (H_2O_2) production. H_2O_2 then activates a second PKCε - PKCε_2, which would then inhibit the opening of the mitochondrial permeability transition pore (mPTP) thereby leading to cardioprotection (Quinlan et al., 2008). However, the "signalosome" hypothesis remains a working hypothesis and requires further validation.

3.3.12 Clinical application of bradykinin

In a small clinical trial, thirty patients were randomised to receive an intracoronary infusion of bradykinin or normal saline for 10 minutes prior to percutaneous transluminal coronary angioplasty (PTCA). The purpose of this study was to see if bradykinin could reproduce the protective effect of IPC. Electrocardiogram (ECG), chest pain score and regional wall motion during inflation of the balloon were used to evaluate the effect of bradykinin infusion. Bradykinin-treated patients fared significantly better than the control subjects suggesting that intracoronary infusion of bradykinin before PTCA renders the myocardium relatively resistant to subsequent ischaemia. The extent of this bradykinin-induced protection was comparable to that afforded by IPC in control subjects. In addition, no adverse effects of the bradykinin infusion were reported, suggesting that bradykinin could be used in selected patients undergoing PTCA to reduce ischaemia-reperfusion injury (Leesar et al., 1999).

3.3.13 Opioids

Opioids are endogenous autacoids, released by ischaemic tissues. They are pain-attenuating peptides that occur naturally in the brain.

Endogenous opioid peptides are synthesised and released into the circulatory system after myocardial infarction. In the rat, the ventricular tissue was shown to have much higher levels of

preproenkephalin (an opioid) compared to the other organs, indicating that the heart may have a very significant endogenous opioid system (Romano et al., 2004).

3.3.14 Opioid receptors

Opioids exert their numerous pharmacological functions after fixation to their opioid receptors. Three opioid receptor subtypes have been cloned. In adult ventricular cardiomyocytes only two receptor subtypes have been reported, the kappa (κ) and the delta (δ) (Krumins et al., 1985; Ventura et al., 1989; Wittert et al., 1996; Zhang et al., 1996). However, the presence of the mu (μ) receptor is also suggested in human atrial trabeculae (Bell et al., 2000). There appears to be a converse relationship between the opioid receptors and the β-adregenic pathway, in that when opioid receptors are inhibited, cardiac responsiveness to β-adregenic stimulation is increased (Pepe et al., 1997). Conversely, when agonists of β-adregenic receptors are used, the number and affinity of opioid receptors is increased (Pepe et al., 1997) which indicates a feedback mechanisms between these two systems. The protective effect of IPostC has recently been linked to the endogenous activation of the opioid receptors in a series of experiments by Zatta and colleagues (Zatta et al., 2008).

3.3.15 Preconditioning with opioids

Opioid release from ischaemic tissues appears to be involved in ischaemic preconditioning as well as in remote ischaemic preconditioning (Sadat, 2009). In a rat model, morphine, given as a preconditioning stimulus, decreased the infarct size in a similar manner to that of IPC and glibenclamide blocked the cardioprotection, implicating the involvement of the mK_{ATP} channel in this protective effect (Schultz et al., 1996). Other studies in the isolated rat heart model have demonstrated the ability of morphine to deliver the same degree of cardioprotection when administered just prior to reperfusion as it does when given prior to ischaemia (Gross et al., 2004). The protective effect of IPC in the canine heart and rat hearts can be mimicked by non-selective opioid agonists, selective κ-receptor agonists, or selective agonists for the δ-receptor, while μ specific agonists failed to induce protection (Headrick et al., 2003; Schultz et al., 1997; Schultz et al., 1996; Wang, 2001). Alternatively, opioid antagonists have been demonstrated to abrogate or partially abrogate the infarct-sparing effect of IPC (Chien and Van Winkle, 1996; Schultz et al., 1996; Wang, 2001).

3.3.16 Possible mechanisms in opioid-induced preconditioning

Preliminary evidence suggests that opioid preconditioning is mediated via endogenous opioid receptors, since the protective effects are inhibited by both naloxone, an opioid receptor antagonist, and methiodide, a peripheral acting naloxone derivative (Gross and Gross, 2006).

Isolated rat and mouse models of ischaemia-reperfusion were unable to be protected with a selective κ-opioid agonist in the presence of wortmannin, a PI3-kinase/Akt inhibitor and 5-HD, a blocker of the mK_{ATP} channel (given prior to reperfusion) thus implicating the activation of PI3-kinase/Akt and the mK_{ATP} channel in opioid preconditioning (Peart et al., 2008). Downey et al, have reported that opioid-induced protection occurs through a complex pathway which includes PI3-kinase/Akt, NOS, guanyl cyclase, PKG, opening of the mK_{ATP} and activation of PKC by redox signalling (see review (Downey et al., 2007)).

3.3.17 Postconditioning with opioids

Morphine can also protect the heart during reperfusion as shown in a study by Chen et al, in which morphine was given for the first 10 minutes of reperfusion in isolated rat hearts (Chen et al., 2008). This group demonstrated that morphine can mimic ischaemic postconditioning via activation of the κ-opioid receptor and the mK_{ATP} channel and they suggest that the δ-opioid receptor is not involved (Chen et al., 2008).

3.3.18 Possible mechanisms in opioid-induced cardioprotection

Morphine, one of the major opioids, appears to only exert a beneficial effect when administered during the ischaemic index or during early reperfusion (Gross, 2003). In contrast, the selective δ-opioid agonist, DADLE, mimicked the effects of ischaemic preconditioning in human atrial trabeculae (Tomai et al., 1999). A recent study in isolated rat hearts has provided evidence that opioids signal via the JAK/STAT pathway, with JAK2 mediating Akt activation, GSK-3β and the inhibition of the mPTP (Gross et al., 2006). Another study in which morphine was administered just prior to reperfusion in the isolated rat heart, demonstrated a protective effect of morphine via inhibition of GSK-3β (Gross et al., 2004). In 2009, Jinkun et al, reported that morphine prevented opening of the mPTP through a signalling pathway involving NO/cGMP/PKG/Zn^{2+}/GSK-3β in cardiomyocytes (Jinkun et al., 2009). In addition, an elegant experiment using human atrial trabeculae obtained from patients undergoing coronary artery bypass surgery revealed that the δ opioid receptor agonist, DADLE, appeared to protect human muscle from simulated ischaemia via the opening of the mK_{ATP} (Bell et al., 2000).

3.3.19 Clinical applications of the opioids

A very recent study in humans has reported a combined protective effect of morphine and remote IPostC when treatment was initiated directly following PCI in patients with acute myocardial infarction (Rentoukas et al., 2010).

4.0 Tumour necrosis factor alpha (TNFα)

The principle of an antihumoral response of the innate immune system in vivo was already recognised in 1893 by the physician, William B Coley. His inspiration came from the observation that when his cancer patients had an infection, they were more likely to recover from the cancer. Injecting his patients with a concoction containing the bacteria, Streptococcus pyrogenes, reduced the tumours to a better extent when the patient developed a full-blown infection accompanied by a raging fever (Coley, 1893). This work was, however, not well documented and when Coley died in 1936, his treatment method died with him, having been replaced by radiation therapy. It is only in 1975 that Carswell and colleagues identified an endotoxin-induced serum factor that caused necrosis of tumours and they named this endotoxin "tumour necrosis factor alpha (TNFα)" (Carswell, 1975). Subsequently, a protein, named cachectin, was isolated from endotoxin-treated cells and was so named because it was presumed to play a role in the molecular basis of cachexia. Later on, cloning of TNFα and the protein, cachectin, confirmed that they were identical (Beutler et al., 1985). This soluble, multifaceted cytokine is produced upon activation of the innate immune system, mainly in macrophages but subsequent studies have shown that many other cells including cardiomyocytes, also express TNFα (Benigni et al., 1996; Frangogiannis et al., 1998). Two isoforms of TNF exist, TNFα and TNFβ. TNFα is first expressed as a transmembrane protein and subsequent studies have provided evidence that membrane TNFα is converted to a soluble homotrimeric form by proteolytic cleavage, a process known as ectodomain shedding, allowing this cytokine to have more widespread effects (Beutler et al., 1985).

Studies in various culture systems have reported ADAM17 (**a d**isintegrin **a**nd **m**etalloproteinase 17) as a sheddase of TNFα and its receptors (Black et al., 1997; Moss et al., 1997; Peschon et al., 1998; Reddy et al., 2000). In 2007, Bell et al demonstrated that ADAM17 plays a primary role in TNFα, tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2) shedding. However, deficiency of ADAM17 did not abrogate the shedding, suggesting that ADAM17 facilitates the shedding but is not a crucial element for shedding to occur (Bell et al., 2007). ADAM17 is also known as TACE (TNF alpha converting enzyme). An important determinant of shedding activity is thought to be the localisation of ADAM17 to a perinuclear compartment which raises the possibility that the intracellular environment may also be implicated in ADAM17-mediated ectodomain shedding (Peiretti, 2003).

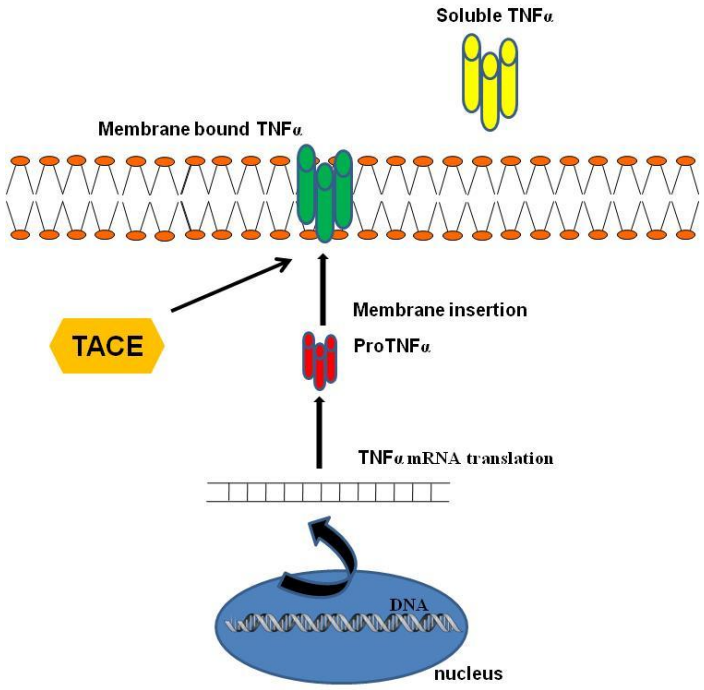


Fig 17: Production and release of TNFα: TNFα is first synthesised as a transmembrane proTNFα, allowing TNFα to exist in its membrane bound form. TACE then cleaves ProTNFα, releasing the soluble form of TNFα. TACE=TNFα converting enzyme; mRNA=messenger ribonucleic acid, (adapted from Meldrum, 1998).

4.1 TNFα receptors

Once released from the cell, TNFα docks with one of two TNFα receptors – a 55 kDa high affinity receptor, known as TNFR1 and a 75 kDa low affinity receptor, TNFR2 (Beutler, 1994; Vassilli, 1992). TNF ligand is believed to achieve all its different cellular and pathophysiological

effects by its binding to either the TNFR1 or TNFR2 receptor subtype. Like the ligand, the two receptors can undergo ectodomain shedding, generating soluble, functional receptors.

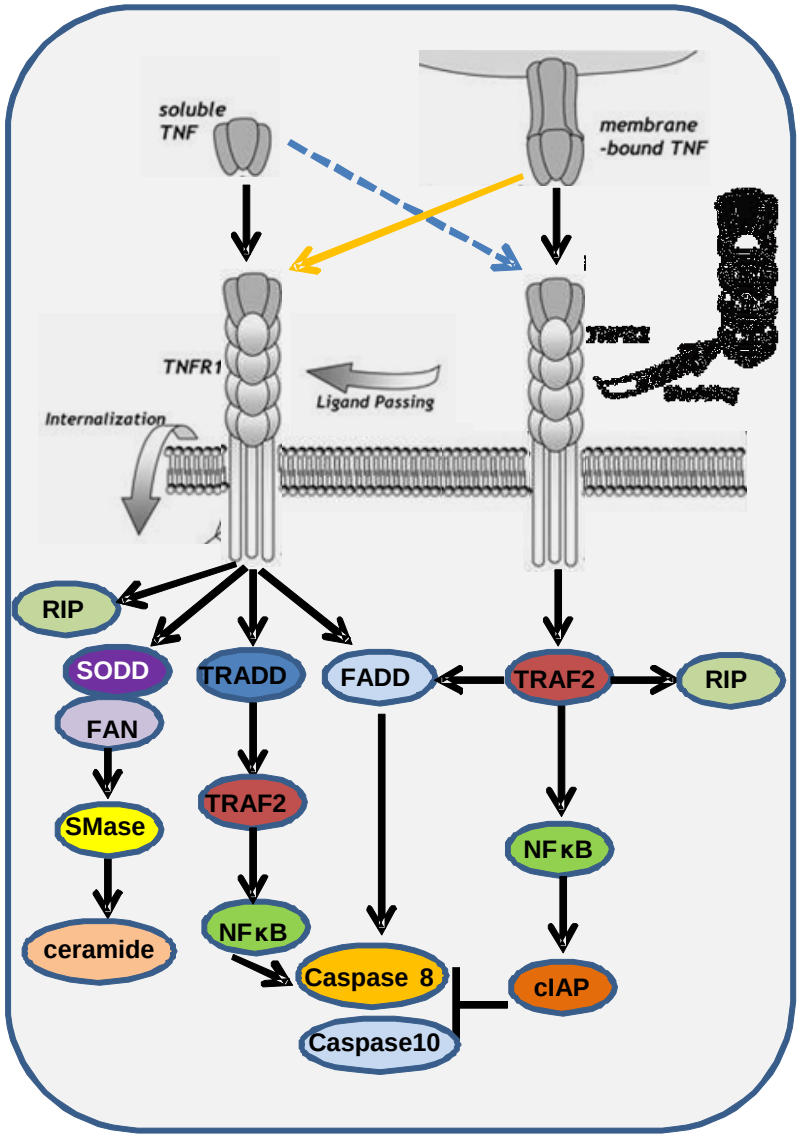


Fig 18: Simplified diagram of some of the signalling pathways modulated by TNFR1 and TNFR2 subtypes, (adapted from MacEwan, 2002**).**

TNFR1 contains a death domain (DD) motif which is critical in the death-inducing activity of this receptor (Tartaglia et al., 1993). A number of associating proteins and related molecules also contain a DD and signalling for cell death appears to be the function of these proteins and molecules. The silencer of death domain (SODD) is one such molecule and it binds to the DD in TNFR1 preventing the binding of other DD proteins (Jiang et al., 1999). When TNFR1 is stimulated by TNFα, the SODD dissociates from the DD and this allows access to the DD for

other activator proteins. An important element in the activation of neutral sphingomyelinase is an adaptor protein called **f**actor **a**ssociated with **n**eutral sphingomyelinase activation (FAN) (Adam et al., 1996; Adamklages, 1998). This adaptor protein is responsible for the generation of smaller sphingolipid molecules containing ceramide which play a key role in signalling (Kolesnick and Kronke, 1998). Both TNFR1 and TNFR-associated death domain (TRADD) are found at the perinuclear-Golgi complex but TRADD only associates with TNFR1 once at the plasma membrane (Jones et al., 1999; Ledgerwood et al., 1999). It then recruits tumour necrosis factor receptor-associated factor (TRAF2) to TNFR1 (Hsu et al., 1995). TRAF2, in turn, recruits nuclear factor kappa B (NFκB) (Vince et al., 2009). However, in addition to promoting NFκB signalling, TRAF2 also has anti-apoptotic signalling roles, with the ring domain of TRAF2 being required for protection against TNF-induced apoptosis (Vince et al., 2009).

In addition, TRADD recruits the downstream signalling adaptor molecules, Fas-associated death domain (FADD) and RIP (receptor interacting protein). The role of RIP remains uncertain at this point. FADD contains a death effector domain (DED) that interacts with caspase 8 and a number of other molecules that regulate cell death mechanisms (Muzio et al., 1996) (Figure 18).

TNFR2 does not have a DD motif but still recruits adaptor proteins including TRAF2. The structural and functional characterization of TNFR2 has not, to date, been studied in depth. However, the membrane-bound form of TNFα is suggested to be a potent activator of TNFR2, in contrast to the soluble form, which has a greater affinity for TNFR1 (Grell et al., 1995). In the immune system, TNFα would activate its receptors by cell – cell interactions. How the binding characteristics of membrane-bound TNFα at TNFR1 and TNFR2 compare to soluble TNFα still remains to be elucidated. Minor changes in the structure of the TNFα sequence can lead to major changes in its binding characteristics and therefore determine to which receptor it will bind (Lewis et al., 1991). Lewis et al, demonstrated that TNFR1 and TNFR2 are equally activated by murine TNFα, whereas human TNFα only stimulates TNFR1 and has no effect on TNFR2 in the mouse (Lewis et al., 1991). It has been suggested that most of the *in vivo* effects of TNFα may be mediated by membrane-bound TNFα with both receptors being activated, rather than the soluble form which would activate TNFR1 more robustly than it does TNFR2 (MacEwan, 2002). If this is so, than the physiological role of TNFR2 may be more important than first thought. TRAF2 has a direct affinity for TNFR2. Under certain conditions, FADD and RIP are also thought to be able to bind indirectly to TNFR2 via TRAF2. TRAF2 can also interact with NFκB which controls the transcription of a gene called cellular inhibitor of apoptosis protein 2 (c-IAP2). cIAP2 binds to TRAF2 thereby inhibiting the activation of caspases 8 and 10 and

subsequent apoptosis (MacEwan, 2002). This could provide a platform for the beneficial effects of TNFα (Figure 18).

4.2 TNFα and the heart

In physiological conditions, the primary role of TNF is the regulation of immune cells. As TNFα is involved in cellular differentiation, growth and apoptosis (Sack et al., 2000), it is relevant to consider whether this pluripotent cytokine is involved in normal cardiac development. The cardiac phenotype of the TNFα–deficient mice has only minor differences in cardiac mass compared with wildtype littermate controls (Smith et al., 2001) and cardiac studies in mice deficient in either of the TNF receptors showed no discernable congenital cardiac defects (Kurrelmeyer et al., 2000). These data, taken together, imply that normal cardiac morphogenesis does not require TNFα signalling, or that alternate signalling pathways compensate for lack of TNFα.

TNFα concentration is low in healthy hearts, where it is located mainly in endothelium and resident mast cells (Schulz, 2008). The discovery in the 1990s that TNFα is expressed in all nucleated cell types of the myocardium, including the cardiomyocyte itself, renewed interest in TNFα and its role in cardiac pathophysiological states such as myocardial infarction and heart failure (Levine, 1990; Herskowitz, 1995; Dorge, 2002).

Myocardial TNFα is expressed in direct response to many different forms of cardiac injury, including acute myocardial infarction (Mann, 2003; Yokoyama, 1997; Maury and Teppo, 1989). Elevated plasma levels of TNFα have also been reported in several studies conducted in heart failure patients (see review (Batista et al., 2009)). Transgenic mice, designed to overexpress cardiac TNFα have highlighted the maladaptive effects of TNFα in the heart. While wildtype counterparts remained healthy, the mice overexpressing TNFα developed myocarditis (Kubota et al., 1997).

Both human and rat cardiomyocytes possess the two TNFα cell surface receptors, TNFR1 and TNFR2. (Krown, 1995; Torre-Amione *et al*., 1995). Recently, the existence of a mitochondrial binding protein for TNFα has been proposed, therefore suggesting that TNFα may be delivered directly from the cell surface to the mitochondria independently of TNFR1 and TNFR2 (Busquets, 2003; Ledgerwood, 1998).

4.3 TNFα and ischaemia reperfusion

Many studies have documented myocardial TNFα production during acute myocardial ischaemia with or without reperfusion (Irwin et al., 1999; Meldrum et al., 1998a; Meldrum et al., 1998b).

Preformed TNFα is released within minutes from the resident mast cells and macrophages during myocardial ischaemia, but with persistent ischaemia, TNFα also originates from the cardiomyocytes (Dorge et al., 2002) and is also one of the multi-potent cytokines known to be released by ischaemia-reperfusion (Sharma and Das, 1997). TNFα levels have also been demonstrated to be increased at the onset of reperfusion in an ischaemia-reperfusion model (Cain et al., 1998) and other studies revealed that in adenosine mediated cardioprotection this upregulation of TNFα was prevented (Cain et al., 1998; Meldrum et al., 1997).

Initially, this cytokine was regarded as only having deleterious effects, contributing to myocardial dysfunction and cardiomyocyte death in ischaemia-reperfusion injury (Giroir, 1994; Latini, 1994; Levine, 1990). In a study conducted in mice, Maekawa et al, demonstrated that once reperfusion was instituted, a deficiency in TNFα signalling resulted in smaller infarct sizes (Maekawa et al., 2000). Confirmation of this putative detrimental effect of TNFα was forthcoming in a study conducted in wildtype mice using an identical ischaemia-reperfusion protocol in the presence of anti-TNF antibodies (Belosjorow et al., 2003; Gurevitch et al., 1997; Maekawa et al., 2002).

In contrast to the detrimental effects of TNFα, it was reported that cultured cardiomyocytes are protected against hypoxic injury when exogenous TNFα is added (Nakano et al, 1998) while larger myocardial infarct sizes were observed in mice deficient in both TNF receptors compared to their wildtype littermate counterparts following an acute myocardial infarction (Kurrelmeyer et al., 2000). Both these studies implicated TNFα in cardioprotection. A plausible explanation for these opposing roles of TNFα may be the existence of an intrinsic cell survival programme in response to ischaemia which is abrogated by the inflammatory response at reperfusion.

The concept, therefore, that selective, rather than total, inhibition of TNFα may be more feasible (Abraham et al., 1995) was further explored in 2002 by Sack, who proposed that a low acute concentration of TNFα would be protective whereas a high and sustained concentration would be maladaptive (Sack, 2002).

In humans, TNFR1 has been implicated in the initiation of the negative inotropic effects of TNFα in adult cardiomyocytes (Torre-Amione et al., 1995). In addition, Monden et al, demonstrated that TNFα was toxic via TNFR1 but was protective via TNFR2 in a murine model of myocardial infarction (Monden et al., 2007). Although administration of either a soluble TNF receptor or an anti-TNFα antibody abrogates the development of heart failure in experimental animals, paradoxically, the use of anti-cytokines in attempts to neutralize TNFα has not proved successful in patients with chronic heart failure (Anker and Coats, 2002; Mann, 2002).

Higuchi et al, bred TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice with cardiac-specific overexpression of TNFα and found that the absence of TNFR2 exacerbates heart failure and reduces survival whereas the removal of TNFR1 blunted heart failure and improved survival, therefore suggesting that TNFR2 signalling played a protective role in the pathogenesis of heart failure (Higuchi et al., 2004). Additionally, deletion of TNFR1 and TNFR2 in mice subjected to myocardial infarction demonstrated that inhibition of TNFR1-mediated pathways attenuated ventricular dysfunction and improved survival with a downregulation of other proinflammatory cytokines, whereas inhibition of TNFR2 was deleterious, exacerbating ventricular dysfunction accompanied by upregulation of TNFα and other proinflammatory cytokines (Monden et al., 2007).

Thus, it is appropriate to suggest that the role played by TNFα in ischaemia-reperfusion may be dependent on which TNF receptor is activated and the concentration of TNFα as well as the time it is present in the system (Deuchar et al., 2007; Flaherty et al., 2008; Lecour et al., 2002; Monden et al., 2007; Schulz, 2008). The function of TNFα in ischaemia-reperfusion remains controversial, with this pleiotropic cytokine's ability to act in both maladaptive and adaptive roles (Figure 19).

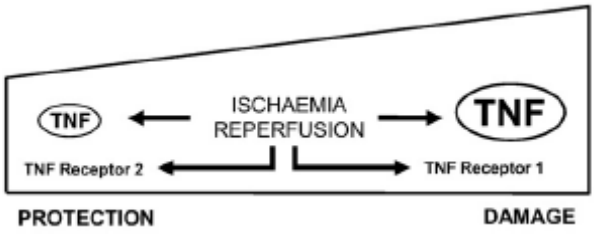


Fig 19: TNFα in myocardial ischaemia-reperfusion: protective versus damaging effects, (Lecour, 2009).

4.4 TNFα and ischaemic preconditioning

Persuasive evidence exists for a role of TNFα in ischaemic preconditioning. In 2002, Smith et al, used mice in which endogenous TNFα production was knocked out to demonstrate that the lack of TNFα precluded the protective effect of IPC. However, pharmacological preconditioning with adenosine or diazoxide was still able to reduce infarct size (Smith et al., 2002). Subsequent studies also demonstrated a lack of protection in mice in which TNFα had been knocked out (Ichikawa et al., 2004; Labruto et al., 2005). Pre-administration of TNFα, 24 hours prior to TNFα preconditioning, rescued the cardioprotective effect in TNF knockout mice, suggesting that the signalling effector system downstream of TNFα may be downregulated in TNFα

deficient mice (Smith et al., 2002). Transient low concentrations of TNFα given as a preconditioning mimetic prior to the ischaemic insult protected the heart against ischaemia-reperfusion in the isolated rat heart model (Lecour et al., 2005a; Lecour et al., 2002). In rats, mice or rabbits, classic ischaemic preconditioning was associated with increased levels of pre-ischaemic TNFα (Belosjorow et al., 1999; Smith et al., 2002) but a decreased level of endogenous TNFα was released during the ischaemia-reperfusion injury phase (Belosjorow et al., 1999; Kimura et al., 2006). Interestingly, preconditioning-induced cardioprotection with exogenous TNFα can only be achieved with a washout period prior to the sustained ischaemia, therefore suggesting that the time of TNFα production is another factor to be considered (Lecour et al., 2002).

4.5 TNFα and ischaemic postconditioning

Anaesthetized rats subjected to coronary artery ligation demonstrated that IPostC reduced levels of plasma TNFα during the reperfusion phase in a manner similar to that of IPC (Kin, 2008). However, it is presently unknown whether TNFα is involved in the protective effect of ischaemic postconditioning and the main focus of this thesis was to delineate the role of TNFα as a postconditioning mimetic.

4.6 Proposed mechanisms in TNFα-induced cardioprotection

In a manner similar to IPC, TNFα-induced preconditioning activated the sphingolipid pathway, PKC and the mK_{ATP} channel (Lecour et al., 2002). The inhibitors of these various pathways have been demonstrated to abolish the protection afforded with TNFα when given during the preconditioning stimulus (Lecour et al., 2002). In addition, the free radical scavenger, mercaptopropionyl glycine (MPG) also blocked the infarct-sparing effect of TNFα, implicating ROS in the prosurvival signalling cascade (Lacerda et al., 2006; Lecour et al., 2005a). One of the triggers for IPC is myocardial p38 MAPK which can be activated by TNFα. However, Tanno et al, demonstrated that TNFα-induced protection in the murine heart is independent to p38 MAPK activation (Tanno et al., 2003). Critical targets for downstream protection are activation of the mPTP (Gao et al., 2005), the Ca^{2+} activated potassium channel (Gao et al., 2005) and the phosphorylation of NFκB (Somers et al., 2005).

4.7 TNFα-induced cardioprotection is independent of the RISK pathway

Phosphorylation and activation of PI3-kinase and Erk1/2 at the time of reperfusion in both ischaemic pre-and postconditioning has been shown to protect against reperfusion injury as part

of the RISK pathway (see review (Hausenloy et al., 2005)). However, strong evidence is emerging in the literature in support of the hypothesis that protection against reperfusion injury does not always require activation of the RISK pathway. The protective effect of TNFα in isolated rat hearts could not be abolished by the Erk1/2 inhibitor, PD98059, given during the preconditioning stimulus (Lecour, 2005b;). Similarly, administration of wortmannin, an inhibitor of PI3-kinase/Akt during the preconditioning stimulus did not abrogate the protective effect of TNFα in a study conducted in isolated cardiomyocytes and the isolated rat heart (Suleman, 2008).

4.8 TNFα initiates the SAFE pathway via JAK/STAT-3 signalling

Cardiac-specific STAT-3 knockout mice could not be protected by the preconditioning mimetics, adenosine, diazoxide and TNFα administered before the index ischaemia (Smith, 2004). A study by Lecour et al, provided evidence that both TNFα and early IPC phosphorylated STAT-3 in the early phase of reperfusion in the isolated rat heart model and that AG490 administered at the time of reperfusion, abrogated both IPC and TNFα-induced protection, thus linking TNFα and STAT-3 in a prosurvival signalling pathway (Lecour et al., 2005b). In addition, AG490, the JAK/STAT antagonist, administered to isolated hearts from STAT-$3^{-/-}$ mice before the preconditioning stimulus, was shown to prevent phosphorylation of Akt (Suleman, 2008).

The activation of STAT-3 was demonstrated to co-incide with the activation of the RISK pathway during ischaemic preconditioning (Lecour et al., 2005b) but interestingly, TNFα-induced protection failed to activate the RISK pathway. This, therefore, implied that the JAK/STAT pathway may be activated independently of the RISK pathway.

The role of TNFα and its cognate receptors has not been extensively studied in the postconditioning setting although there is now a growing body of evidence implicating the signalling of the innate immune response to the events underlying myocardial adaptation to ischaemia (Valeur and Valen, 2009). However, it should be emphasized that there is very little known regarding TNFα and its effects during the first few critical minutes of reperfusion. Many studies have examined the effect of TNFα when given just before the ischaemic insult (preconditioning), but to date, no studies could be found in the literature which investigated the protective role of exogenous TNFα at reperfusion or the possible protective role of the TNFα/JAK/STAT signalling cascade in postconditioning. Only one study, to date, has reported that serum and myocardium levels of TNFα were demonstrated to be decreased at reperfusion in a rat model, resulting in protection for the heart by reducing apoptosis and preventing excess

ROS production (Kin, 2008), thus implicating a low concentration of TNFα in the protective effect of postconditioning. A schematic depicting the activation of the RISK and SAFE pathways following ischaemia-reperfusion is shown in figure 20.

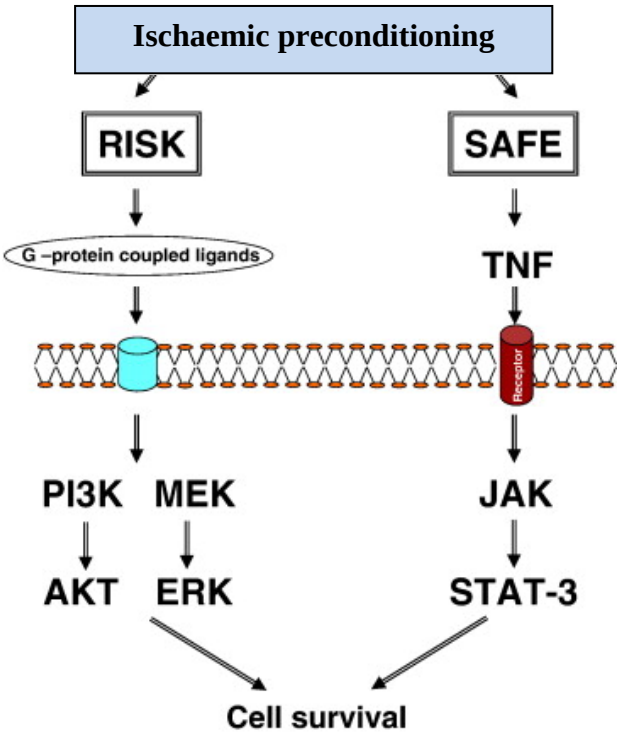


Fig 20: Activation of the RISK and SAFE pathways following ischaemia-reperfusion. Ischaemic preconditioning increases TNFα levels resulting in TNFα binding to its cell surface receptors activating the JAK/STAT pathway as part of the SAFE pathway. Similarly, activation of PI3-kinase and MEK activate Akt and Erk1/2 respectively, as part of the RISK pathway. Both pathways enhance cardioprotection against ischaemia-reperfusion. Abbreviations as defined in text, (adapted from Lecour, 2009).

4.9 TNFα, obesity and diabetes

TNFα is a multifaceted cytokine that exerts potent pro-inflammatory effects in metabolic and inflammatory disorders such as obesity and insulin resistance. An important fact is that obesity does not occur in isolation from other metabolic disorders. Chronic activation of the innate immune system is a feature of obesity which is associated with a low grade inflammation of white adipose tissue (WAT). Subsequently, this can lead to insulin resistance, impaired glucose tolerance and even diabetes (Bastard et al., 2006). The first detectable defect in type 2 diabetes is insulin resistance when insulin levels are greatly increased while glucose levels remain normal (Hotamisligil et al., 1995).

TNFα down-regulates insulin-stimulated glucose uptake by its effect on glucose transporter 4, insulin receptor autophosphorylation and insulin receptor substrate-1. TNFα is a powerful autocrine and paracrine regulator of adipose tissue. It can inhibit insulin action and insulin receptor signalling and this is thought to be mainly mediated by TNFR1 (Kanety et al., 1996; Liu et al., 1998a; Peraldi and Spiegelman, 1997). Investigators have also examined whether the TNF receptors play a role in the development of glucose homeostasis. These studies concluded that the receptors did not play a role in the induction of insulin resistance associated with obesity, but that the presence of both receptors may be required for normal glucose homeostasis (Schreyer et al., 1998). However, TNFα appears to have no significant effect on the development of dietary obesity, as the development of marked obesity was noted in mice which lack endogenous TNFα ($TNF^{-/-}$) and were fed a high fat, high caloric diet (Uysal et al., 1997). These mice remained highly insulin sensitive compared to their wild type counterparts. Furthermore, the insulin concentrations in obese $TNF^{-/-}$ mice were significantly lower than those of the obese TNF-WT and they remained at the same level as that of lean mice on a normal diet (Uysal et al., 1997). These results demonstrated that insulin resistance associated with dietary obesity is reduced in the absence of TNFα. Interestingly, genetic depletion of TNFα from macrophages in wild type mice does not protect these mice from developing obesity-related insulin resistance (De Taeye et al., 2007).

TNFα has also been linked to the development of type 1 diabetes (Yang et al., 1994). Treatment with non-toxic doses of TNFα in neonatal non-obese diabetic mice for 21 – 24 days resulted in the onset of type 1 diabetes being accelerated, whereas inhibition of TNFα for the same time period resulted in a complete absence of diabetes (Yang et al., 1994).

Interestingly, the cardioprotective effect of ischaemic preconditioning has been shown to be abolished in human diabetic patients (Ishihara et al., 2001). Experimental animal models have also indicated that diabetes abolishes the ability of IPC and pharmacological agents to reduce infarct size, but the mechanisms involved in this failure are unclear (Kersten et al., 2001; Kersten et al., 2000; Nawata et al., 2002; Paulson, 1997; Tosaki et al., 1996).

Cachexia, the wasting disorder of muscle and fat tissues, has previously been associated with TNFα expression, (Beutler and Cerami, 1989; Oliff et al., 1987; Torti et al., 1985) which is in contradiction to the possible link between TNFα and obesity. One explanation is the circulating levels of TNFα. These exceed 200 ng/ml in cases of cachexia whereas, in models of obesity-diabetes the circulating levels are very low, in the range of 10-200 pg/ml when detectable (Hotamisligil, 1993). Another explanation is that obesity is often associated with other hormonal abnormalities which may alter the body's response to TNFα. It has even been suggested that the

site of expression could determine the metabolic response to TNFα (Tracey, 1992). More investigative studies will be needed to elucidate how one molecule can have such diverse effects on a vast array of different pathophysiological states.

Obesity, diabetes and metabolic syndrome all alter the production of adipokines, such as leptin and TNFα. Hotamisligil and colleagues demonstrated that in human obesity, the expression of TNFα in adipose tissue was significantly elevated and that this increase in TNFα was strongly correlated with the level of hyperinsulinaemia (Hotamisligil et al., 1995).

4.10 TNFα and leptin

Leptin is an adipocyte secreted hormone involved in energy regulation and endocrine and immune function. It plays a role in innate and acquired immunity.

Both the structure of leptin and of its receptor suggests that leptin can be classified as a cytokine Known as the "appetite suppressing hormone", there is a strong correlation between serum leptin and TNFα levels (Margalet, 2003). Additionally, there is a strong association between circulating leptin levels and body mass index (BMI) as well as fat mass in obesity with TNFα upregulating leptin production and leptin also modulating the production of TNFα (Bastard et al., 2006). As a function of its cytokine-like structure, therefore, leptin could exert peripheral biological effects (Almanza-Perez et al., 2008).

It has been demonstrated that leptin expression is increased with administration of TNFα in rodents (Grunfeld, 1996; Sarraf, 1997). In a study conducted in humans, a positive and independent correlation between circulating levels of soluble TNFR1 and leptin was demonstrated, therefore suggesting an association between TNFα and leptin in humans similar to that seen in rodents where TNFα increases leptin expression and circulating leptin levels (Mantzoros, 1997). This possible effect of an activated TNFα system on blood leptin levels in humans could have a considerable impact on human physiology.

It has been previously reported that leptin protects the isolated mouse myocardium against ischaemia-reperfusion injury via direct action on the heart (Smith, 2006) and it is known that the JAK/STAT pathway mediates many of the metabolic actions of leptin (Frubeck, 2006). Recent evidence from isolated rat hearts demonstrated a reduction in infarct size when leptin was given at reperfusion. This cardioprotective effect was abrogated by co-administration of the JAK/STAT inhibitor, AG490, therefore implicating the JAK/STAT pathway in leptin-induced cardioprotection (Smith, 2010).

B. AIM, HYPOTHESIS AND OBJECTIVES

1.0 Aim

Ischaemic postconditioning, whereby alternating cycles of reperfusion and ischaemia are applied at the onset of reperfusion, is a powerful tool to reduce infarct size and preserve cardiac function. Delineating the exact mechanism involved in this phenomenon may lead to the development of novel therapies against the burden of ischaemic heart disease. A number of different signal transduction pathways have been reported to underlie the cardioprotective effect of IPostC. One of first signalling cascades to be linked to IPostC was the Reperfusion Injury Salvage Kinase (RISK) pathway which implicated the prosurvival kinases, phosphotidylinositol 3-kinase (PI3-kinase)-Akt and p44/p42 extracellular signal-regulated kinases (Erk1/2) in the cardioprotective effect of IPostC. The activation of these prosurvival kinases at the immediate onset of reperfusion by a diverse variety of agents such as adenosine, bradykinin and the opioids has been shown to reduce myocardial infarct size in the region of 40-50%. However, the mechanism by which these survival pathways confer cardioprotection is poorly understood.

TNFα is a cytokine implicated in inflammation, growth and apoptosis but previous work in our laboratory has demonstrated that TNFα can also protect against ischaemia-reperfusion in a dose and time dependent manner.

Therefore, the aim of the present study was to explore the role of TNFα in ischaemic and pharmacological postconditioning-induced cardioprotection.

1.1 Hypothesis and Objectives

We hypothesized that activation of TNFα is required in both IPostC and pharmacological postconditioning to confer cardioprotection and that this protective effect is mediated via the activation of TNFR2 and the transcription factor STAT-3 (Signal Transducer and Activator of Transcription). Furthermore, we investigated ischaemic postconditioning in obese and diabetic animals, two major risk factors for ischaemic heart disease that are associated with elevated levels of circulating TNFα (figure 21).

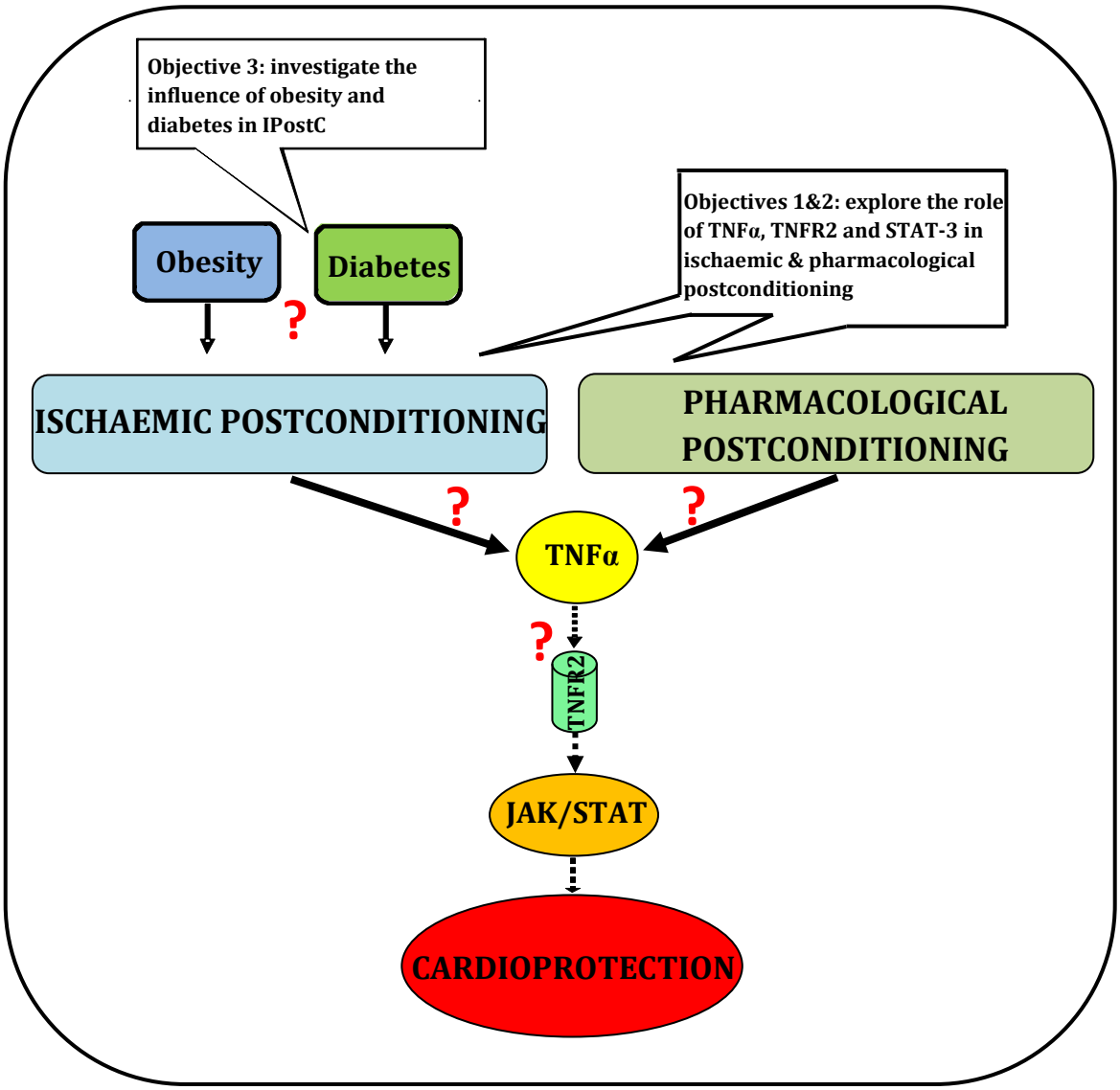


Fig 21: Schematic representation of hypothesis and objectives in ischaemic and pharmacological postconditioning. Ischaemic postconditioning activates TNFα which then binds to its TNFR2 with subsequent activation of the JAKs. This results in the dimerization of STAT-3 and its translocation to the nucleus, with the mPTP of the mitochondria being a putative end effector**.** Abbreviations as defined in the text.

To fulfil this aim, the following objectives will be pursued:

1) *To investigate the role of TNFα and its receptors in IPostC-induced cardioprotection.*
   To achieve this objective we will use TNF deficient ($TNF^{-/-}$), TNFR1 deficient ($TNFR1^{-/-}$), TNFR2 deficient ($TNFR2^{-/-}$), cardiac-specific STAT-3 deficient ($STAT\text{-}3^{-/-}$) mice and their respective wild types. Isolated hearts will be postconditioned following an ischaemia-reperfusion insult. Should TNFα prove to be necessary in IPostC-induced cardioprotection, we would then use inhibitors of various pathways and the western blot technique to explore possible downstream targets of TNFα such as GSK-3β, RAGE, PAR-2, Akt, Erk and STAT-3.

2) *To investigate the role of TNFα in pharmacological postconditioning.*
   To achieve this objective we will use TNF deficient ($TNF^{-/-}$), TNFR1 deficient ($TNFR1^{-/-}$), TNFR2 deficient ($TNFR2^{-/-}$) and cardiac-specific STAT-3 deficient mice ($STAT\text{-}3^{-/-}$) and their respective wild types. Isolated hearts will be subjected to an ischaemia-reperfusion insult and the well-known cardioprotective agents, adenosine, bradykinin and opioids will be used as pharmacological postconditioning mimetics. Should TNFα prove to be necessary in pharmacologically-induced cardioprotection, the western blot technique will be used to explore possible downstream targets such as STAT-3.

3) *To investigate the effect of obesity and diabetes in IPostC–induced cardioprotection.*
   For the obesity study, the mice will be fed a high carbohydrate diet from weaning for a total of 11 weeks. Thereafter, the isolated hearts of TNF-WT or $TNF^{-/-}$ mice will be subjected to IPostC and infarct size will be used as the endpoint. To explore the role of diabetes in IPostC, streptozotocin will be administered to the mice to initiate onset of diabetes. At day 5 and at day 10 post streptozotocin, the isolated hearts will be subjected to IPostC and infarct size used as the endpoint. Various parameters such as body weight, heart to body weight ratio, blood glucose, plasma leptin and plasma insulin will be examined in the obese and diabetic mice.

C. MODELS & METHODS

1.0 Genetically modified animals

The best animal models to achieve our objectives were whole body TNF-deficient mice which produce no endogenous TNFα and TNF receptor1 and 2-deficient mice. A third mouse model, cardiac-specific STAT-3 deficient mice were used to determine whether the JAK/STAT pathway was involved in the protective signalling.

In this thesis all animal studies performed were approved by the Animal Research Ethics Committee of the University of Cape Town, and followed the recommendations laid down in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85 {23}, revised 1996).

1.1 Mouse Models to investigate the role of TNFα in ischaemic postconditioning

Homozygous TNF-deficient ($TNF^{-/-}$) mice, TNF receptor 1-deficient mice ($TNFR1^{-/-}$) and TNF receptor 2-deficient mice ($TNFR2^{-/-}$) and their respective wildtype, Black 6 x 129S (TNF-WT) were a generous gift from Dr Muazzam Jacobs and Professor Bernhard Ryffel, Department of Immunology, University of Cape Town.

Cardiomyocyte-specific STAT-3-deficient ($STAT\text{-}3^{-/-}$) mice from C57 Black 6 background were created in our facilities by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre recombinase mice, as previously described (Smith et al., 2004). All mice were 12-14 weeks of age.

1.1.2 Genotyping of Mouse Models

To distinguish wildtype from $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$ and $STAT\text{-}3^{-/-}$ mice, tail cuts were performed at weaning (3 weeks of age) and genomic DNA extracted from the respective tails. Genotyping was performed by means of the polymerase chain reaction (PCR).

i) DNA Extraction

Approximately 1 cm of a candidate mouse tail was removed from the mouse and digested by incubating at 55˚C overnight in 400 µl tail digestion buffer (0.5M Tris {pH 8}, 0.1M EDTA, 1% SDS) with 1 mg/ml proteinase K (Roche, Mannheim) added to the buffer immediately prior to use. The following day, 400 µl of Phenol:Chloroform:Alcohol (25:24:1)(Sigma, USA) mixture was added to each digested tail and the samples were gently inverted to mix. Cellular debris was removed by centrifugation at 13,500 rpm (Heraeus MK202, Germany) for 10 minutes

at room temperature. The bottom layer containing cellular debris was carefully removed with sterile barrier tips taking care not to disturb the interface containing contaminating proteins. 200 µl of chloroform (Sigma, USA) was added to the remaining upper layer containing the required DNA to remove traces of phenol and the tube was again gently inverted to mix. The digests were centrifuged again at 13,500 rpm for 5 minutes. Two separate phases again occurred and the bottom phase was removed and discarded while the upper phase is once more centrifuged at 13,000 rpm for 10 minutes, forcing the remaining debris into a pellet at the bottom of the tube. The liquid phase was then transferred to a new sterile 1.5 ml eppendorff tube and 50 µl of 7.5 M ammonium acetate (Merck) and 1 ml 100% ethanol added to each tube. The tubes were gently inverted to mix and the DNA, which appears as a white, stringy precipitate, was transferred to a clean eppendorff tube containing 500 µl of 70% ethanol to hydrate the DNA. The samples were then centrifuged at 13,000 rpm for 10 minutes and the ethanol carefully decanted to prevent loss of the DNA pellet. DNA pellets were air dried until ethanol had completely evaporated, then 50 – 75 µl of ultra pure RNAse free water was added to dissolve the DNA. Thereafter samples were stored at -20˚C until PCR analysis.

ii) Polymerase Chain Reaction (PCR)

Targeted deletion of the TNFα gene has been previously described (Marino et al., 1997). Genotyping was carried out, at random intervals of breeding, to confirm the genetic background of each species of mouse model and, in the case of $TNF^{-/-}$, $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice, involved testing as to whether the respective mouse's TNFα, TNFR1 or TNFR2 gene was intact or had been disrupted by the insertion of a phosphoglycerate kinase-neomycin expression cassette within the gene. Genotyping of the STAT-3 mice had to be performed at each stage of the breeding process as breeding of $STAT\text{-}3^{-/-}$ mice gave rise to both the wild type and the STAT-3 deficient mice. Therefore, these mice had to be tested to see whether or not they were harbouring the Cre recombinase insertion as well as whether or not they were floxed.

iii) Sequences and PCR for $TNF^{-/-}$, $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mouse models

a) $TNF^{-/-}$: Sense primer: **5'-CTA AAT GAA CTG CAG GAC GA-3'**

Antisense primer: **5'-ATA CTT TCT CGG CAG GAG CA-3**

Neomycin: Sense primer: **5' CTG AAT GAA CTG CAG GAC GA 3'**

Antisense primer: **5' ATA CTT TCT CGG CAG GAG CA 3'**

The polymerase chain reactions were performed in 50 µl volumes made up as follows:

10xMagnesium free buffer:	10 µl
4mM $MgCl_2$:	8 µl
100µM dNTP's:	1.6 µl
Primer (TNF or Neo):	0.5 µl
β actin 3':	0.5 µl
β actin 5':	0.5 µl
H_2O:	26.9 µl
Taq:	0.5 µl
DNA sample:	1.5 µl
	50 .0 µl

PCR Conditions:

TNF:

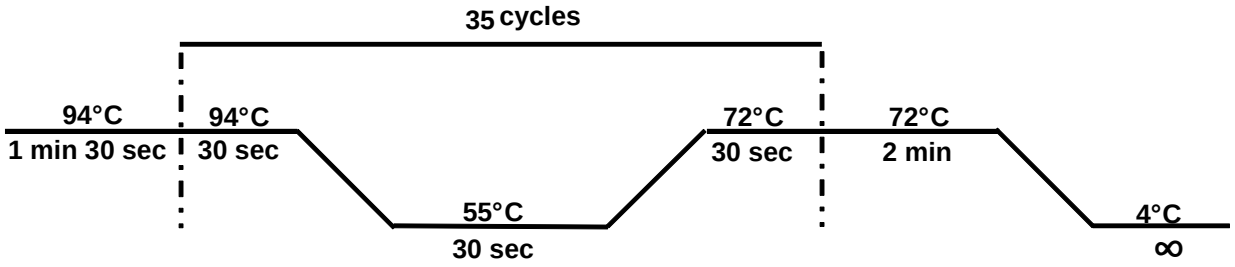
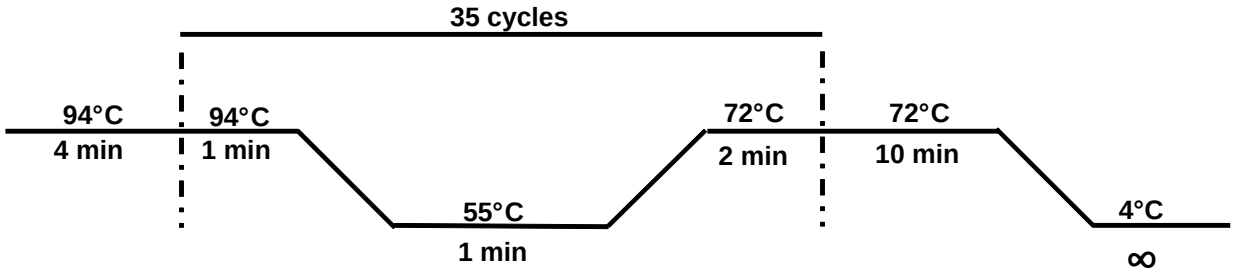


Fig 22: PCR conditions for TNF genotyping

Neo



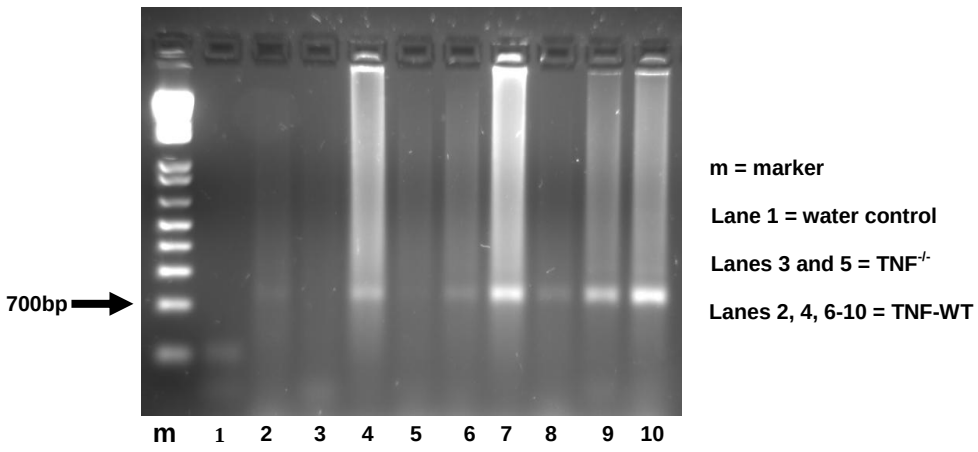


Fig 23: UV gel analysis of extracted mouse genomic DNA for the targeted disruption of the TNFα gene

b) TNFR1$^{-/-}$: TNFR1 - 4938: **5' AGA AAT CTC AG ACA ATT CTC TGC**

TNFR1 – 2883: **5' CTC TCT TGT GAT CAG CAC TG**

TNFR1 – Neo: **5' TCC CGC TTC AGT GAC AAC GTC**

The polymerase chain reactions were performed in 50 µl volumes made up as follows:

10xMagnesium free buffer:	5 µl
25 mM $MgCl_2$:	3 µl
10 mM dNTP's:	1 µl
Primers: $TNFR_1$ 2883	1 µl
$TNFR_1$ 4938	1 µl
$TNFR_1$ Neo 34	1 µl
H_2O:	35.875 µl
Taq:	0.125 µl
DNA sample:	2 µl
	50 µl

PCR Conditions

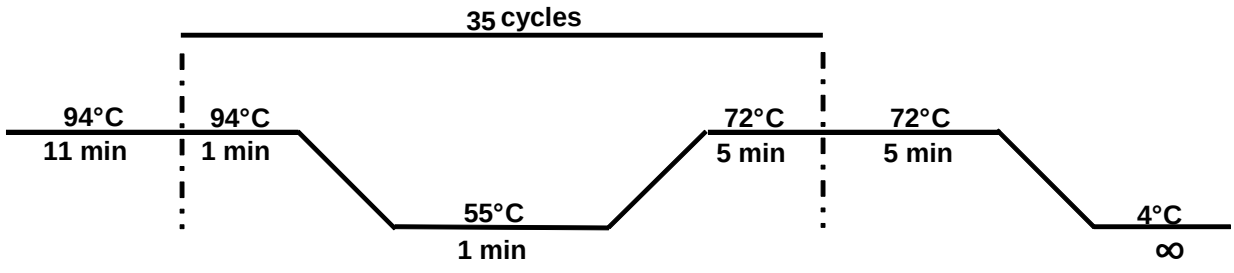


Fig 24: PCR conditions for TNFR1$^{-/-}$

c) TNFR2$^{-/-}$: IMR0338: **5'-CCT CTC ATG CTG TCC CGG AAT**

IMR0339: **5'-AGC TCC AGG CAC AAG GGC GGG**

IMR0340: **5' CGG TTC TTT TTG TCG AGA C**

IMR0341: **5' ATC CTC GCC GTC GGG CAT GC**

The polymerase chain reactions were performed in 50 µl volumes made up as follows:

10xMagnesium free buffer:	5 µl
25 mM $MgCl_2$:	3 µl
10 mM dNTP's:	1 µl
Primers: IMR_{338}	1 µl
IMR_{339}	1 µl
IMR_{340}	1 µl
IMR_{341}	1 µl
H_2O:	35.875 µl
Taq:	0.125 µl
DNA sample:	2 µl
	50 µl

PCR Conditions:

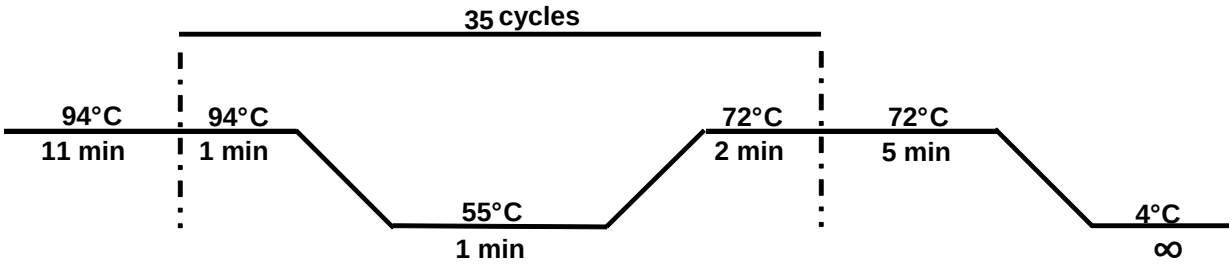


Fig 25: PCR conditions for TNFR2$^{-/-}$

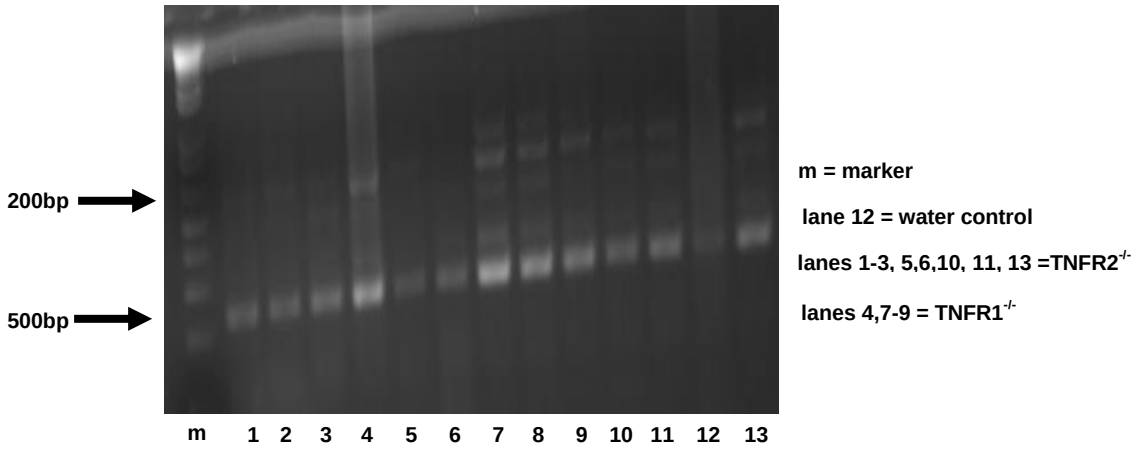


Fig 26: UV gel analysis of extracted mouse genomic DNA for the targeted disruption of the TNFR gene

iv) Sequences and PCR for STAT-3$^{-/-}$ mouse model

To determine whether the mice were Cre positive the following sequences were used:

Cre **Sense primer:** **5' GTT CGC AAG AAC CTG ATG GAC A 3'**

Cre **Antisense primer:** **5' CTA GAG CCT GTT TTG CAC GTT C 3'**

The mice were also genotyped for the MLC 2V gene as a positive control for the presence of DNA. For this, the following sequences were used:

MLC 2V Sense primer: **5' GGC AAC CCT CAG ACA CCA T 3'**

MLC 2V Antisense primer: 5' TGT GGA GGC TCT GGA TCA GGA C 3'

The polymerase chain reactions were performed in 50 µl volumes made up as follows:

10xMagnesium free buffer: 10 µl

4mM $MgCl_2$: 8 µl

100µM dNTP's: 1.6 µl

Primer: 0.5 µl

β actin 3': 0.5 µl

β actin 5':	0.5 µl
H_2O:	26.9 µl
Taq:	0.5 µl
DNA sample:	1.5 µl
	50 µl

PCR Conditions:

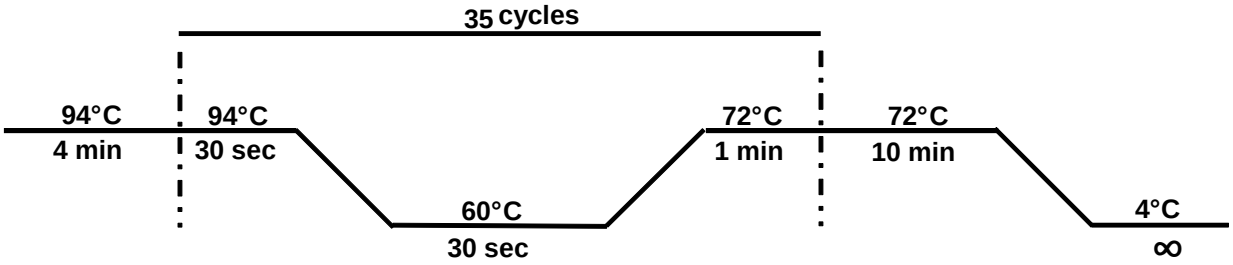


Fig 27: PCR conditions for STAT-3$^{-/-}$ mice.

To establish whether STAT-3 was floxed, one set of primers, yielding PCR fragments of differing lengths, were used. A 250 bp fragment is seen on the gel if the STAT-3 is floxed whereas a 200 bp fragment is visualized on the gel if the STAT-3 is not floxed. If the mouse being tested is heterozygous, then a double band is seen. STAT-3 floxed or non-floxed DNA was amplified with the following primers:

STAT-3 primer (a) 5'CCT GAA CAA GTT CAT CTG TGT GAC 3'

STAT-3 primer (b) 5' CAC ACA AGC CAT ACT CTG GTC TCC 3'

The polymerase chain reactions were performed in 50 µl volumes made up as follows:

10xMagnesium free buffer:	5 µl
4mM $MgCl_2$:	4 µl
100µM dNTP's:	0,8 µl

Primer:	0.25 µl
β actin 3':	0.25 µl
β actin 5':	0.25 µl
H_2O:	13.2 µl
Taq:	0.25 µl
DNA sample:	1.0 µl
	25 µl

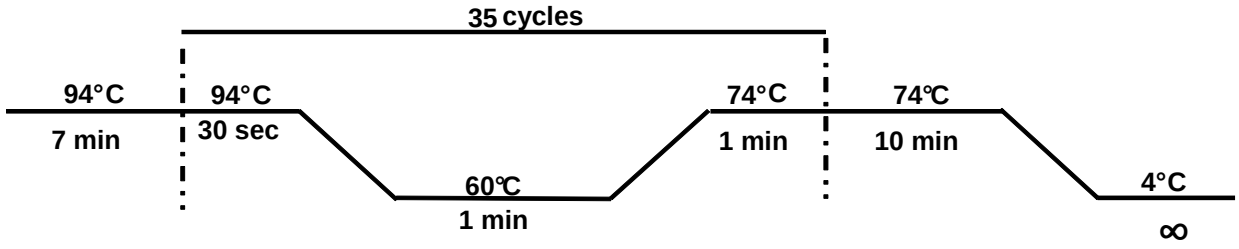


Fig 28: PCR conditions for STAT-3 floxed mice.

PCR Conditions:

1.1.3 Agarose Gel Electrophoresis

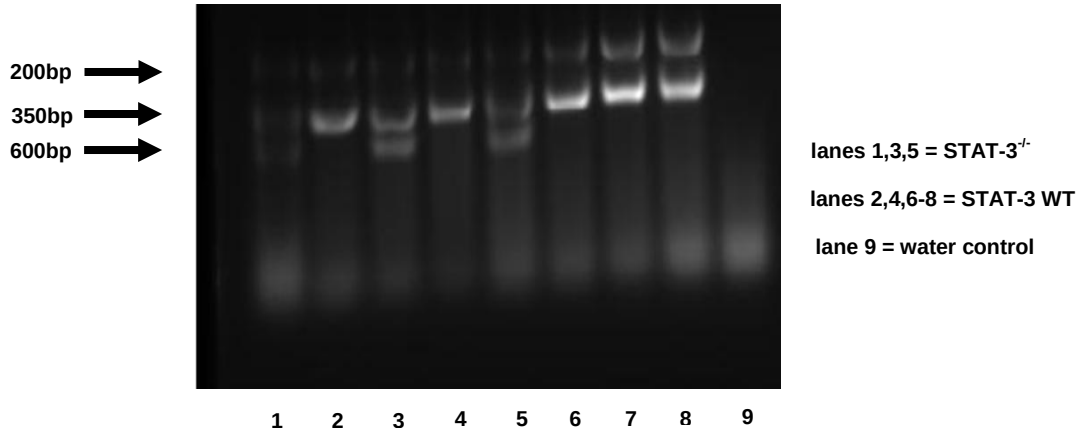


Fig 29: UV gel analysis of genomic mouse DNA showing Cre positive band (350bp) as well as the control Myosin Light Chain 2V (MLC2V) band at 600bp.

A 2% agarose gel was used to ensure optimal separation between bands. Two grams of agarose (Whitehead Scientific, RSA) was made up in 100 ml of 1 x Tris Acetate electrophoresis buffer (TAE) (0.04 M Tris-Acetate, 0.001 M EDTA) and heated in a microwave oven at 70% power until completely dissolved. Once dissolved, 1 µl ethidium bromide was added per 100 ml gel in order to visualize the amplified DNA fragment under ultra violet light. 1 x TAE buffer was added to the gel tank once the gel had set and before removal of the combs in order to prevent the wells being damaged. Subsequently, 10 µl of each PCR reaction was mixed with 2 µl gel loading buffer (0.25% Bromophenol Blue-Xylene Cyanole Dye Solution, Sigma) and carefully loaded into the wells. Simultaneously, 4 µl of a 100 bp DNA ladder (Roche, Germany) was run as a marker to verify the size of the PCR products. The gel was run at 90 V for 30 – 40 minutes and the bands were then visualized on a transilluminator (UviTec, USA). PCR results were recorded using the UviTec Geldoc apparatus with UVIband software v.97 (Cambridge, UK).

2.0 Isolated mouse heart model

Adult male $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$, cardiomyocyte-specific $STAT\text{-}3^{-/-}$ mice and their respective WTs were anaesthetized (pentabarbitone, 60 mg/kg ip) and heparinized (25 IU ip) to prevent the blood clotting. Once an adequate level of anaesthesia had been achieved (tested by lack of pedal reflex) the chest was opened and the sternum and attached costal cartilage excised to give adequate access to the mediastinum. Hearts were rapidly excised (Smith et al., 2002) and arrested in ice cold modified Krebs-Henseleit buffer (118 mM NaCl; 24 mM $NaHCO_3$; 4 mM KCl; 1 mM NaH_2PO_4; 2.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 0.5 mM di-sodium EDTA; 10 mM glucose; gassed with 95% O_2/5% CO_2 at 37˚C). The aorta was cannulated and the heart then mounted on the Langendorff system. The heart was fastened by means of a 4-0 silk suture (on a 13mm curved atraumatic needle) through the apex onto a rigid lightweight lexan coupling rod to a force displacement transducer (Grass Technologies, FT02C, Mass. USA). Diastolic resting tension was adjusted to 2 g and hearts were paced at 560 beats per minute while being retrogradely perfused with modified Krebs-Henseleit buffer at a constant pressure of 110 cm H_2O (Smith et al., 2002) (see Figure 30). The developed force was recorded on a Powerlab Labchart (ADI Instruments, Australia) and coronary flow rate was measured by timed collection. These parameters were utilized as measures of cardiac function for all groups of hearts used in the protocols. Temperature was measured by placement of a fine thermocouple wire (Physitemp, NJ, USA) into the right atrium and monitored on a Digitron 2600T temperature sensor (Torquay, UK), (figure 30). Isolated hearts were subjected to a 25 minute stabilization period followed by

35 minutes global ischaemia (obtained by shutting off the flow of buffer to the heart from the aortic cannula by means of a stopcock) and a 45 minute reperfusion period (Smith et al., 2002). Coronary flow, heart rate and developed force are used to determine the viability of an isolated heart. A minimum flow of 1.5 ml/min and maximum flow of 5.0 ml/min are acceptable for coronary flow, the acceptable heart rate is between 460bpm-600bpm and the developed force is accepted when ≥ 4.

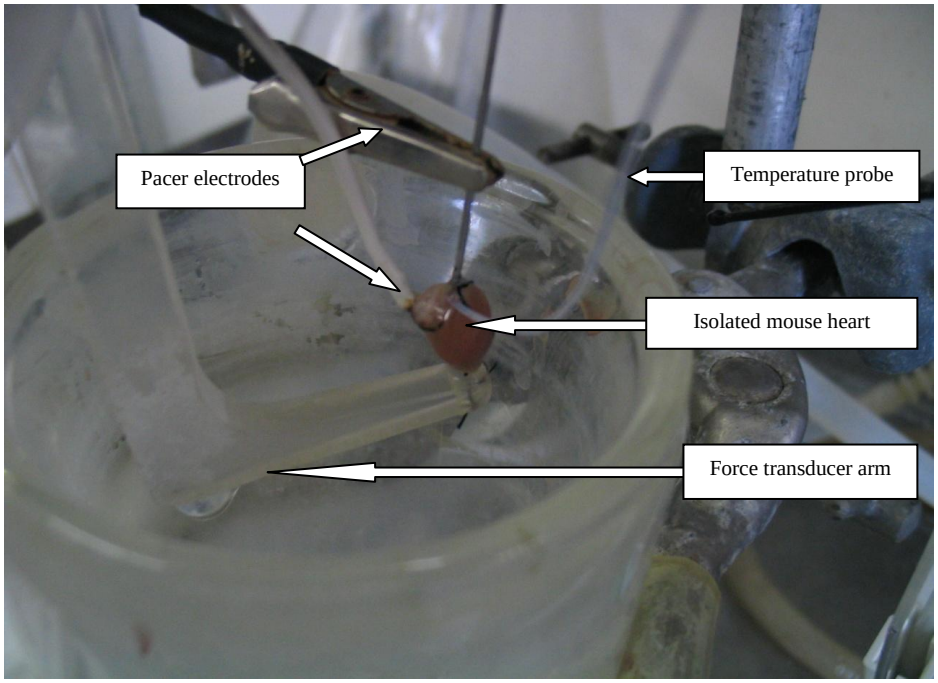


Fig 30: Mouse heart mounted on Langendorff apparatus. A cannula is inserted into aorta for retrograde perfusion. The pacer electrodes are used to pace the heart to 560 bpm and the heart pulls against a force of 2g.

2.1 Ischaemic and TNFα postconditioning protocol

The Langendorff retrograde perfusion technique was used to assess infarct size and to collect tissue for western blot analysis, where appropriate, from the $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$, $STAT\text{-}3^{-/-}$ and their respective wildtypes. The isolated hearts were subjected to IPostC at the onset of reperfusion by initiating six alternating cycles of 10 seconds reperfusion, 10 seconds ischaemia. In a separate group, pharmacological postconditioning with TNFα was initiated by six alternating cycles of 10 seconds reperfusion without TNFα, 10 seconds reperfusion with 0.5 ng/ml TNFα (TNF-PostC), figure 31. To assess whether the mounting of hearts itself had an effect, a time-matched control group was subjected to the full length of the protocol on the Langendorff apparatus with no ischaemic period or IPostC.

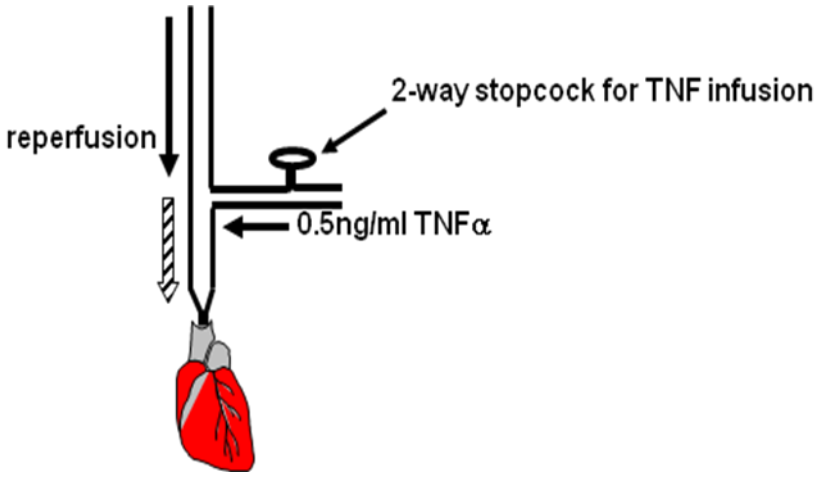


Figure 31: Protocol of pharmacological postconditioning with TNFα. Reperfusion is allowed for 10 sec, after which the stopcock is opened for 10 sec to allow the flow of 5 ng/ml TNFα to mix with the reperfusion flow, giving a final concentration of 0.5 ng/ml entering the heart. The stopcock is then closed and reperfusion with Krebs-Henseleit buffer alone takes place for 10 sec, after which the cycle is repeated a further 5 times.

2.2 Infarct size assessment

At the end of each experimental protocol, hearts were removed from the perfusion rig and infarct size assessed by triphenyltetrazolium chloride (TTC) staining as described previously (Sumeray et al., 2000). Briefly, the heart was placed in TTC stain (1% in phosphate buffer, pH7.4) for 1 minute at 37˚C after which it was frozen for 24 hours to allow for easy slicing. 1mm slices, perpendicular to the apex, were then cut. Each slice includes both right and left ventricle plus septum. These slices were then compressed between thin glass plates 0.5mm apart and the sections scanned, and infarct size assessed using computerized planimetry (Planimetry+, Boreal Software, Norway), (figure 32).

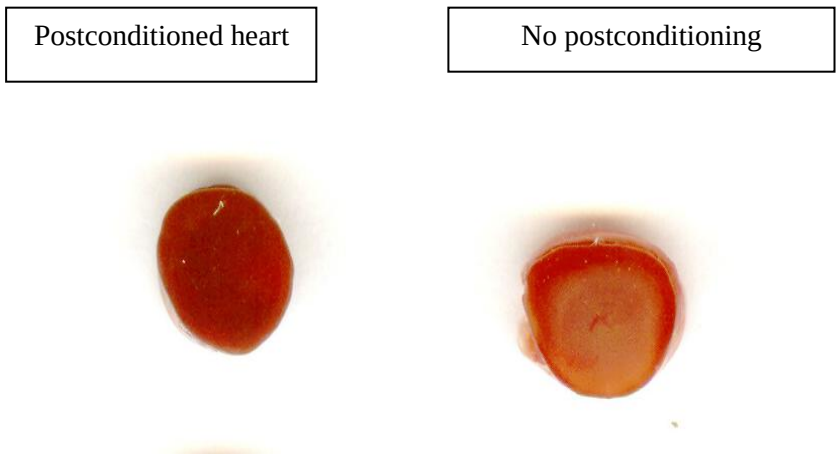


Fig 32: TTC staining for infarct size evaluation. TTC reacts with the NADPH in live tissue to give a brick red colour. There is no reaction in the infarct tissue, which remains pale and allows infarct size determination by planimetric analysis.

3.0 Nuclear and cytosolic protein extraction (adapted from Williams and Ford, 2001)

Nuclear and cytosolic proteins were extracted from the hearts by homogenization of the tissue in lysis buffer as follows:

Solution A (30ml)	**µl**	**Final Concentration**
1M Hepes, pH**7.9**	600	20 mM
2M $MgCl_2$	37.5	2.5 mM
500mM EDTA	6	100 µM
100mM β-glycerophosphate	6000	20 mM
Triton X-100 (100%)	15	0.5%
100mM DTT	1500	500 µM
100mM $NaVO_4$	30	100 µM
EDTA-free protease inhibitor	1200	-

100mM PMSF	300	1 mM
5M NaCl	450	75 mM
dH_2O	19862	-

Solution B (10ml)	**µl**	**Final Concentration**
1M Hepes	200	20 mM
2M $MgCl_2$	12.5	2.5 mM
500mM EDTA	2	100 µM
100mM β-glycerophosphate	2000	20 mM
Triton X-100 (100%)	100	1%
100mM DTT	1500	500 µM
100mM $NaVO_4$	10	100 µM
EDTA-free protease inhibitor	400	-
100mM PMSF	100	1 mM
5M NaCl	150	75 mM
dH_2O	6526	-

Frozen hearts were wrapped in aluminium foil, pulverized under liquid nitrogen then transferred into 900 µl of lysis buffer (Solution A). After homogenizing twice by Polytron, at setting 4, for 5 sec each time, the suspension was centrifuged at 10000 g (12000 rpm) for 5 min at 4˚C. The supernatant, corresponding to the cytosolic fraction, was carefully removed and placed in a fresh tube. The remaining pellet was resuspended in 300 – 500 µl of Solution B, transferred into a

clean Eppendorf tube and centrifuged for a further 30 min at 10000 g (12000 rpm) at 4˚C. The supernatant, corresponding to the nuclear fraction was removed into a clean tube.

3.1 Mitochondrial and cytosolic protein extraction (adapted from Tal M Lewin, 2008.) Mitochondrial and cytosolic proteins were extracted from the hearts by homogenization of the tissue in lysis buffer as follows:

Lysis buffer A	Incubation Buffer
100 mM Tris-HCl – pH 7.4	250 mM Sucrose
250 mM Sucrose	25 mM Tris
1 mM DTT	8.5 mM KH_2PO_4
1 mM EDTA	

Low ionic strength sample buffer (2X)

400 µl 10% SDS

400 µl glycerol

40 µl mercaptoethanol

1.16 ml 5 mM Tris (pH 6.8)

Bromophenol blue

Frozen hearts were minced finely with a pair of scissors in 1ml of lysis buffer. After homogenisation in a Dounce homogeniser, the suspension was centrifuged at 600 g for 5 min at 4°C. The supernatant was placed in a fresh tube and further centrifuged at 10 300 g (11,500 rpm) for 10 min. The resulting supernatant corresponds to the cytosolic fraction. The pellet was resuspended in 40 µl mitochondrial incubation buffer. After protein determination, an equal volume of low ionising buffer was added to the mitochondrial and cytosolic fractions.

3.2 Lowry Protein determination

Duplicate 5 µl aliquots of nuclear, cytosolic or mitochondrial sample were added to 995 µl of sterile distilled water (1:200 dilution) followed by addition of 1ml of the following CTC reagent.

i) ***CTC reagent: Soln A –*** Na_2CO3 – 20g/100ml dd H2O

Soln B - $CuSO_4.5H_2O$ - 0.2 g
K_2 tartrate - 0.4 g } 100 ml dd H20

Add **Soln A** to **Soln B** with constant stirring to prevent precipitation.

ii) SDS

10% SDS made by diluting a 20% solution

iii) NaOH

0.4 g/200ml dd H2O

Equal volumes of all 3 reagents were mixed immediately prior to use.

The samples were immediately mixed with the CTC reagent and incubated for 10 minutes at room temperature. 500 µl of Folin's Reagent, diluted 1:5 with double distilled water (Merck, Germany) was then added with continuous mixing, as the colour complex to be assayed forms immediately. The samples were incubated at room temperature for a further 30 minutes and optical density was read at 750 nm on a Varian 130 dual beam spectrophotometer (Peterson, 1977).

A standard curve was prepared using bovine serum albumin (Sigma, Germany) diluted to provide a concentration range from 10 – 200 µg/ml and the range was assayed in the same manner as the samples.

3.3 Polyacrylamide Gel Electrophoresis (PAGE)

100 µg of nuclear, mitochondrial or cytosolic protein was loaded per lane and run on a 10% (w/v) acrylamide/bisacrylamide gel at 120V for approximately 90 - 120 minutes. 7 µl of peqGold Protein Marker IV (PEQLAB Ltd, UK) was run simultaneously to identify the size of the candidate peptides. The gel was transferred overnight (0.02 amps at 4˚C) onto an Amersham PVDF membrane (Hybond, Amersham, UK). Thereafter, the membrane was fixed in methanol

for 5 minutes, air-dried and stained with Ponceau reagent (Sigma P7170, St Louis, MO) to quantitate and evaluate appropriate transfer of proteins from gel to membrane. The membrane was placed between two acetate sheets and scanned for densiometric analysis, using the UVIBand software package.

Subsequently, the Ponceau stain was rinsed off with three 5 minute washes in Tris buffered saline with 0.1% Tween (TBS-T). The membrane was then blocked at room temperature in 5% fat-free milk powder made up in TBS-T. After 2 hours the membrane was vigorously hand washed in TBS-T with 3 cycles of 5 minutes. The membrane was then probed overnight at 4˚C with one of the primary antibodies followed by 2 hours incubation with the appropriate secondary antibody.

3.4 Western blot analysis

Control or postconditioned mouse hearts were subjected to a 20 min stabilization period followed by 35 min of global ischemia and 15 min of reperfusion. The hearts were then snap-frozen in liquid nitrogen before being stored at -80ºC until protein extraction was performed. Nuclear and cytosolic proteins were extracted from the hearts by homogenization of the tissue in a lysis buffer, as previously described (Lecour et al., 2005b; Sack et al., 1997). Phosphorylated states of Akt, Erk1/2, STAT-3 (Phospho-STAT-3, tyr705), glycogen synthase kinase-3 beta (phospho-GSK-3β, ser 9) as well as total levels of Akt, Erk1/2, STAT-3, GSK-3β, RAGE, PAR-2 and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were analysed by sodium dodecyl sulphate-polyacrylamide gel immunoelectrophoresis (SDS-PAGE) using antibodies from Cell Signaling Technologies. Equal loading was verified with GAPDH. Levels of phosphorylated proteins were normalized, where appropriate, to their total protein levels done in the same samples and in the same conditions but on a separate membrane. Normalization is merely the ratio of phosphorylated protein to total protein, multiplied by 100. Relative densitometry was determined with the use of computerized software package (UVI Soft, UVI Band, UVI Tech, Cambridge, UK). A minimum of 6 hearts was used per group.

3.4.1 Primary and Secondary Antibodies

i: Total STAT

Primary antibody: STAT-3 Antibody rabbit polyclonal IgG 1:4000 in 5% (w/v) fat-free milk

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:3000 in 5% (weight/volume {w/v}) fat free milk

ii: Phosphorylated STAT

Primary antibody: PhosphoPlus ^{R} STAT-3 (Tyr705) rabbit polyclonal IgG 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:3000 in 5% (w/v) fat free milk

iii): Akt

Primary antibody: Akt Antibody rabbit polyclonal IgG 1:1000 in TBS-T

Secondary antibody: Anti-rabbit (2 hours at room temperature): 1:4000 in 2.5% (w/v) fat free milk

iv: Phosphorylated Akt

Primary antibody: Phospho-Akt (Ser473) Mouse mAB - 1:1000 in TBS-T.

Secondary antibody (2 hours at room temperature): Anti-mouse 1:4000 in 2.5% (w/v) fat free milk

v: Glycogen Synthase Kinase-3 Beta (GSK-3β)

Primary antibody: GSK-3β Rabbit mAb 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:4000 in TBS-T

vi: Phosphorylated Glycogen Synthase Kinase-3 Beta (pGSK-3β)

Primary antibody: Phospho- GSK-3β (Ser9) 1:3000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:3000 in TBS-T

vii: p44/42 MAPK (Erk1/2)

Primary antibody: p44/42 (Erk1/2) 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:4000 in 2.5% (w/v) fat free milk

viii: Phospho-p44/42 MAPK (Erk1/2)

Primary antibody: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:4000 in 2.5% (w/v) fat free milk

ix: Receptor for Advanced Glycation End Products (RAGE)

Primary antibody: Anti-mouse RAGE Antibody 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-goat 1:4000 in 2.5% (w/v) fat free milk

x: Protease-Activated Receptor -2 (PAR-2)

Primary antibody: PAR-2 (SAM11): sc-13504 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-mouse 1:4000 in 2.5% (w/v) fat free milk

xi: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Primary antibody: GAPDH (FL-335) 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Goat Anti-rabbit 1:3000 in TBS-T

For mitochondrial and cytosolic fractions, the following antibodies were used:

i: Total STAT

Primary antibody: STAT-3 Antibody rabbit polyclonal IgG 1:4000 in 5% (w/v) fat-free milk

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:3000 in 5% (weight/volume {w/v}) fat free milk

ii: Phosphorylated STAT

Primary antibody: phospho STAT-3 (Ser 727) rabbit polyclonal IgG 1:1000 in TBS-T

Secondary antibody (2 hours at room temperature): Anti-rabbit 1:4000 in 2.5% (w/v) fat free milk

iii: VDAC

Primary antibody: VDAC goat polyclonal IgG 1:3000 in TBS-T (1 hour at room temperature).

Secondary antibody: Anti-goat 1:4000 in 2.5% (w/v) fat free milk (30 minutes at room temperature).

All antibodies were from Cell Signaling Technology, USA with the exception of RAGE (RD Systems,USA), PAR-2 and GAPDH (both from Santa Cruz Biotechnology, USA). Relative densitometry was determined with the use of computerized software package (UVI Soft, UVI Band, UVI Tech, Cambridge, UK). A minimum of 6 hearts was used per group.

D. TNFα, TNFR2 & ISCHAEMIC POSTCONDITIONING

Part of the work in this section has been published in ***Cardiovasc Res, (2009) 84, 201-208***
"Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway"

Part of the experiments in this section have been performed in conjunction with Sarin Somers, registered as a PhD student at the University of Cape Town

Introduction

Following acute coronary occlusion, early reperfusion is the current optimal way to rescue the heart. Although essential, reperfusion is associated with cellular damage by activation of deleterious signalling cascades, as early as in the first seconds of reperfusion (Braunwald and Kloner, 1985). Attempts in the management of reperfusion injury have recently entered the clinical arena (Piot et al., 2008; Staat et al., 2005; Yellon and Hausenloy, 2007). IPostC is still often thought to protect only via activation of the RISK pathway (Reperfusion Injury Salvage Kinases) which includes activation of the prosurvival kinases Akt and Erk1/2 at the time of reperfusion (Hausenloy et al., 2005; Kin, 2008; Tsang et al., 2004). However, recent studies demonstrated that protection with ischaemic postconditioning can occur independently of the activation of the RISK pathway, therefore confirming the existence of multiple protective pathways (Heusch et al., 2008; Montecucco et al., 2009 Schwartz, 2006; Przyklenk et al., 2008; Skyschally et al., 2009).

The activation of the SAFE pathway, which includes the cytokine TNFα, activates the transcription factor STAT-3 and is now recognized as a "RISK-free" pathway that can confer protection in ischaemic preconditioning (Lecour, 2009a; Lecour, 2009b; Lecour et al., 2002; Lecour et al., 2005b; Suleman et al., 2008). However, the role of TNFα in the SAFE pathway during ischaemic postconditioning has not yet been elucidated.

It is known that TNFα is activated during the course of reperfusion, contributing to cardiac dysfunction and apoptosis (Belosjorow et al., 1999; Maekawa et al., 2002; Mann, 2002). Although ischaemic preconditioning is associated with an increase in TNFα during the preconditioning stimulus, its protective effect is partly attributed to a reduction in myocardial TNFα levels at the time of reperfusion (Kin, 2008). However, TNFα can have adaptive effects in relation to its concentration (with low concentrations being cardioprotective and high concentrations being harmful (Kurrelmeyer et al., 2000; Lecour *et al* 2005a; Lecour *et al*., 2005b; Lecour *et al*., 2002) and to which of its two receptors it binds (binding to TNF receptor 1 is suggested to have an adverse effect while binding to its receptor 2 is suggested to be cardioprotective) (Monden et al., 2007; Schulz, 2008; Schulz and Heusch, 2009).

Therefore, our aim was to explore whether TNFα-signalling, through the activation of its receptor 2 (TNFR2), was involved in the protective effect of IPostC and whether this signalling mechanism activated the SAFE pathway rather than the RISK pathway, to confer protection. We

also wished to explore whether GSK-3β, RAGE and PAR-2 were part of the signalling cascade in ischaemic postconditioning. We used an isolated heart model from wild type mice (TNF-WT) and 3 different transgenic mice from the same background, TNF knockout ($TNF^{-/-}$), TNF receptor 1 knockout ($TNFR1^{-/-}$) and TNF receptor 2 knockout ($TNFR2^{-/-}$) to demonstrate that binding of TNFα to its receptor 2 during ischemic postconditioning is indeed required for the protection. Pharmacological protein kinase inhibitors and mice which are deficient in the transcription factor, STAT-3 in their cardiomyocytes were used to demonstrate that, in the context of ischemic postconditioning, TNFα confers its cardioprotective effect via the activation of the transcription factor STAT-3 rather than the protein kinases Akt and Erk1/2.

Furthermore, using western blot analysis, we explored whether phosphorylation of GSK-3β, RAGE or PAR-2 may act as downstream targets of the SAFE (TNF/JAK/STAT) pathway. A schematic of our proposed hypothesis follows in figure 33.

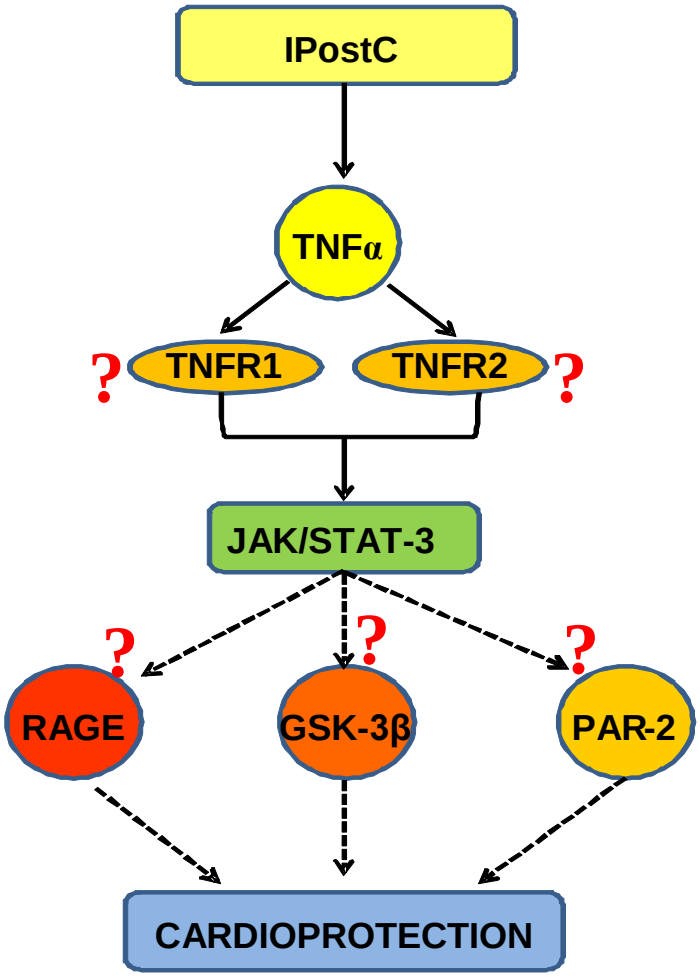


Fig 33: Schematic of proposed hypothesis. Does TNFα initiate cardioprotection during ischaemic postconditioning via the SAFE pathway? Which receptor is involved? Are RAGE, GSK-3β and PAR-2 involved in TNFα-induced protection? Abbreviations as described in text.

2.0 Methods

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health {NIH Publication No. 85 (23), revised1996}. All procedures were approved by the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town.

Homozygous TNF deficient mice ($TNF^{-/-}$), TNF receptor 1 deficient mice ($TNFR1^{-/-}$) or TNF receptor 2 deficient mice ($TNFR2^{-/-}$) and their respective wildtype, Black6x129S (TNF-WT) were used. All these mice were a generous gift from Dr Muazzam Jacobs and Prof Bernhard Ryffel, Department of Immunology, Faculty of Health Sciences, University of Cape Town. Cardiomyocyte-specific STAT-3 deficient mice ($STAT\text{-}3^{-/-}$) from C57Black6 background were created in our facilities by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre recombinase mice, as previously described (Smith et al., 2004). All mice were 12-14 weeks of age (weighing 25-30g).

2.1 Perfusion of isolated mouse hearts

Hearts from adult male $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$, cardiomyocyte-specific $STAT\text{-}3^{-/-}$ mice and their respective wild types were excised rapidly and perfused retrogradely using the Langendorff apparatus. Isolated hearts were subjected to a 20 minute (min) stabilization period followed by 35 min global ischemia and a 45 min reperfusion period as previously described (Smith et al., 2002)] (see Section C for protocol). At the onset of reperfusion, IPostC was initiated with 6 alternating cycles of 10 seconds reperfusion, 10 seconds ischaemia. In a separate group, pharmacological postconditioning with TNFα was initiated with 6 alternating cycles of 10 seconds reperfusion without TNFα, 10 seconds reperfusion with 0.5 ng/ml TNFα (TNF-PostC), (see Figure 31 in Section C). A time-matched control was used to assess infarct size to determine whether the technique itself had damaged the heart. Infarct size by TTC staining demonstrated that the technique had no adverse effect.

To determine whether STAT-3, Akt or Erk1/2 were involved, AG490, an inhibitor of the JAK/STAT pathway (100 nmol/L), wortmannin (wort), an inhibitor of the phosphotidylinositol-3 kinase (PI-3 Kinase)/Akt pathway (100 nmol/L) or PD98059 (PD), an inhibitor of the Mek/Erk1/2 pathway (100 nmol/L) were given during the first 15 min of reperfusion. To confirm the role of the TNF receptors, specific antibodies to TNFR1 or TNFR2 (3 mg/L) were

given to TNF-WT mice during the ischemic postconditioning protocol, (Figure 34). The concentration of the antibodies corresponded to the ND_{50} of the antibodies, as given by the manufacturer. A minimum of 6 hearts were used in all groups. Infarct size was evaluated by triphenyltetrazolium chloride (TTC) staining and assessed using computerized planimetry as described in Section C.

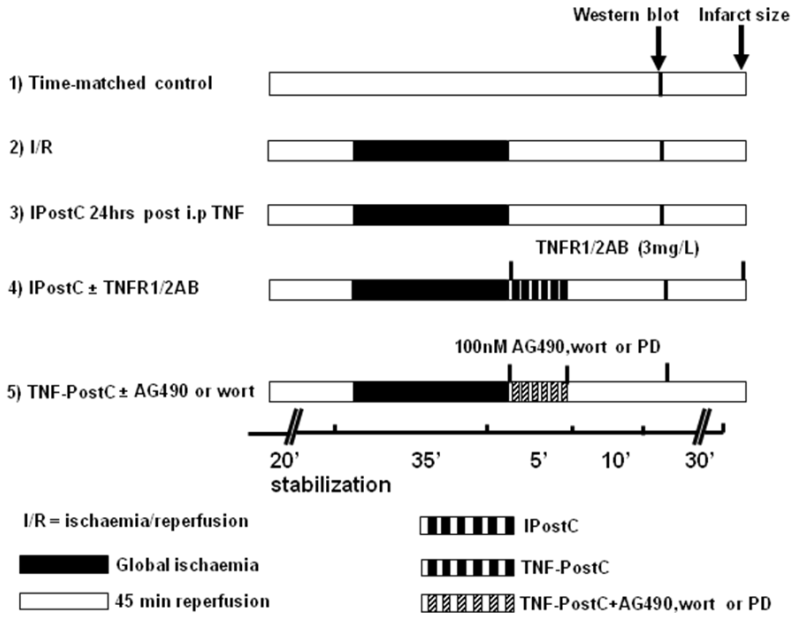


Fig 34: Schematic of experimental protocol. Hearts were removed for Western blot analysis at the timepoint indicated, in a separate experiment. TNF receptors antibodies were added throughout the reperfusion protocol. Inhibitors of PI-3 kinase, JAK/STAT and Erk1/2 were added during the postconditioning protocol as indicated. PD= PD98059; wort = wortmannin; AB = antibody; All other abbreviations as defined in text.

2.2 Ischaemic postconditioning 24 hours after administration of TNFα to $TNF^{-/-}$ mice

In a separate set of experiments, 24 hours prior to performing the ischaemic postconditioning protocol, each $TNF^{-/-}$ mouse was given a single ip injection of 0.5 ng TNFα. Thereafter, the isolated hearts underwent the identical ischaemic postconditioning protocol as described in the previous section.

2.3 TNFα release from heart tissue

Two minutes after the onset of postconditioning the hearts were removed from the cannula by cutting off the atria. They were then placed into an eppendorff tube and immediately immersed in liquid N_2, to snap freeze. Once frozen, the tubes were stored at -80°C until analysis. The

frozen hearts were wrapped in aluminium foil and pulverized under liquid nitrogen. The pulverized tissue was placed in 1.5 ml ice-cold phosphate buffered saline (PBS) and the suspension was then ultrasonically homogenised (Virtis, Virsonic; USA) at 50W for 10-20 seconds. Homogenates were centrifuged at 14000 rpm for 20 min at 4°C and the supernatant was collected for TNFα measurement by Elisa (kit) as per manufacturer's instructions.

2.4 Chemicals and Pharmacological Agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemicals, Germany. Murine TNFα was obtained from PeproTech Inc., Israel; TNF Receptor monoclonal antibodies were obtained from R&D Systems, U.S.A.

2.5 Statistical Analysis

Data are presented as mean ± SEM. Comparisons between multiple groups were performed by One-way ANOVA followed by Tukey post hoc test or Bonferroni multiple comparison test. The unpaired t test was used for TNFα release from heart tissue (Graph Pad Instat). $p<0.05$ was considered statistically significant.

3.0 Results

3.1 Postconditioning with TNFα

➢ TNFα is required in IPostC

To determine whether TNFα is required in IPostC mediated cardioprotection, we compared infarct size (calculated as a percentage of total ventricular volume) in TNF-WT versus $TNF^{-/-}$ mice following the IPostC protocol of 6 x 10 sec cycles of alternating reperfusion and ischaemia, (see Figure 34). IPostC in TNF-WT mice reduced infarct size from 52 ± 3% in the ischaemic control group (I/R) to 18 ± 2% for IPostC ($p<0.05$; Figure 35). IPostC with 6 x 10 sec cycles in the $TNF^{-/-}$ hearts failed to reduce the infarct size (37 ± 3%, p=ns versus $TNF^{-/-}$ I/R group. Similar data was obtained with TNF-PostC, (Figure 35).

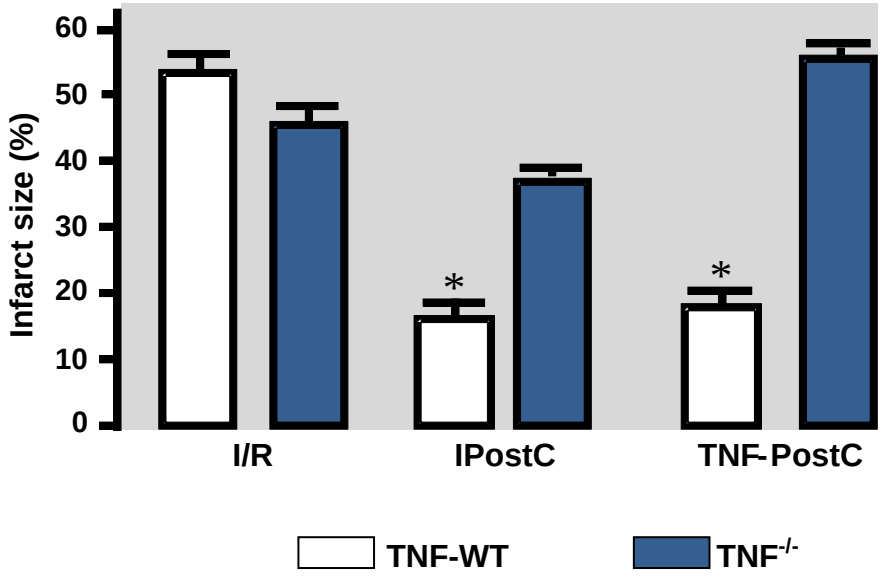


Fig 35: TNFα is required in, and can also mimic, IPostC. 6 x 10 sec cycles of IPostC reduced the infarct size in TNF-WT but not TNF-/- mice, n ≥ 6 per group, *p<0.05 vs respective I/R. Similarly, the infarct size in WT was reduced by 6 x 10 sec cycles of TNF-PostC but this failed to reduce the infarct size in TNF-/- mice, n ≥ 6 per group, p = ns. Abbreviations are as described in the text.

- **TNFR1$^{-/-}$ but not TNFR2$^{-/-}$ mice can be postconditioned**

To investigate the role of the TNF receptors in ischaemic postconditioning, we measured infarct size in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice subjected to both IPostC and TNF-PostC. Ischaemic control hearts from TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice had an infarct size of 42 ± 3 % and 42 ± 2%, respectively, which was similar to data obtained in TNF-WT mice**.** IPostC protocol significantly reduced the infarct size in TNFR1$^{-/-}$ mice (15 ± 1%, n=6; $p<0.05$ versus TNFR1$^{-/-}$ ischaemia-reperfusion control) but failed to protect the heart in TNFR2$^{-/-}$ mice (38 ± 3%, p=ns, versus TNFR2$^{-/-}$ I/R control group). Similarly, TNF-PostC decreased the infarct size to 22 ± 2% in TNFR1$^{-/-}$ hearts, ($p<0.05$ vs its TNFR1$^{-/-}$ I/R control group) but failed to protect the TNFR2$^{-/-}$ hearts (p=ns, versus TNFR2$^{-/-}$ I/R control group), (Figure 36A).

We confirmed these results by using specific neutralizing antibodies to each receptor during the ischaemic postconditioning protocol in TNF-WT mice. Ischaemic-reperfusion control hearts had an infarct size of 49 ± 2%. Perfusion of the TNFR1 antibody during the IPostC protocol did not affect the protective effect of postconditioning (19 ± 2%, $p<0.05$ versus ischaemic-reperfusion control group). In contrast, the protective effect of IPostC was lost in the presence of TNFR2 antibody (53 ± 1%, p=ns, versus ischaemic-reperfusion control group), (Figure 36B).

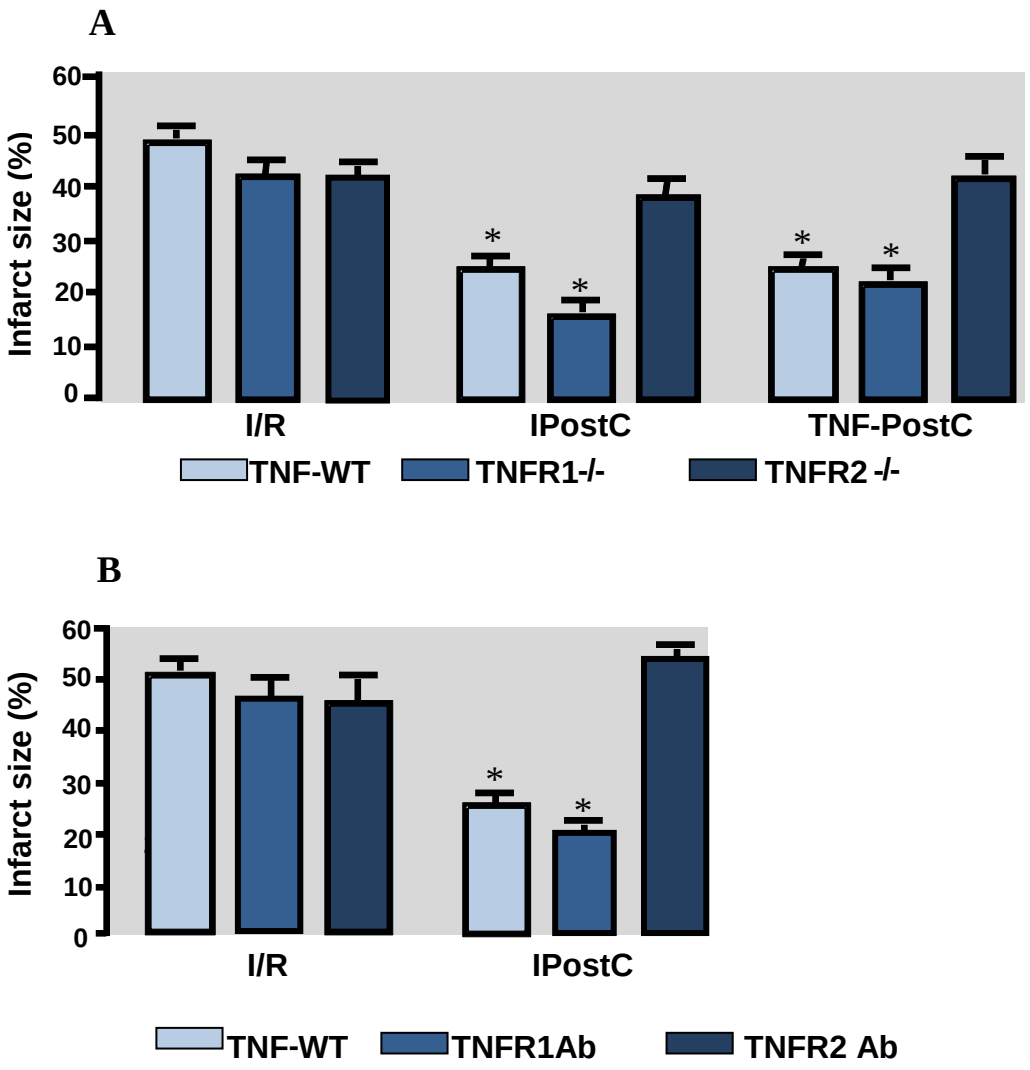


Fig 36: TNFR1$^{-/-}$ but not TNFR2$^{-/-}$ mice can be postconditioned. (A) 6 x 10 sec cycles of IPostC reduced infarct size in TNFR1$^{-/-}$ mice but not in TNFR2$^{-/-}$ mice, n = 6 per group. **(B)** Addition of TNFR2 antibody (Ab) but not TNFR1 Ab, abolished the infarct sparing effect of IPostC in TNF-WT hearts, *p<0.05 vs respective I/R; n = 6 per group; Abbreviations as described in the text.

Administration of TNFα 24 hours prior to ischaemia-reperfusion, restores IPostC cardioprotection in TNF$^{-/-}$ mice

A single ip injection of TNFα (0.5 ng/ml) 24 hours prior to I/R did not alter the infarct size in TNF$^{-/-}$ hearts compared to TNF-WT controls. However, the protective effect of IPostC was restored, significantly decreasing infarct size in TNF$^{-/-}$ hearts from 45 ± 2% to 16 ± 2%, p<0.001, (Figure 37).

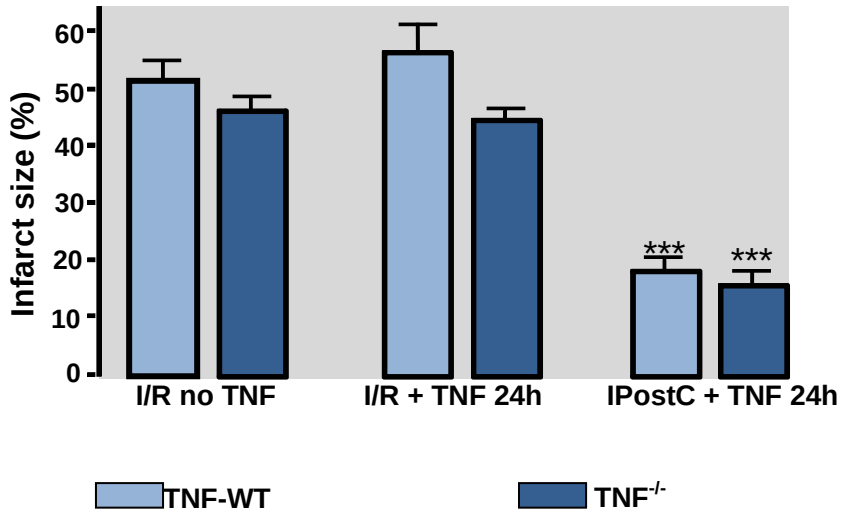


Fig 37: Restoration of protective effect in TNF$^{-/-}$ hearts. 24 hours after administration of a single ip injection of 0.5 ng TNFα, protection is restored in the isolated TNF$^{-/-}$ heart, ***p<0.001 vs I/R+TNF 24h

- **Inhibition of STAT-3 but not Akt or Erk abrogates the protective effect of pharmacological postconditioning with TNFα**

To further investigate the mechanism responsible for the cardioprotective effect of TNFα, we measured infarct size in hearts perfused with an inhibitor of the JAK/STAT pathway (AG490), an inhibitor of the PI-3 kinase/Akt pathway (wortmannin) or an inhibitor of Mek/Erk1/2 (PD 98059), given during the first 15 min of reperfusion in IPostC or TNF-PostC hearts. The inhibitors did not affect the infarct size in the ischaemic control group. Addition of wortmannin (wort) or PD98059 did not alter the protective effect of TNF-PostC (19 ± 2% and 20 ± 1% respectively, $p<0.05$, versus 49 ± 2% in the ischaemia-reperfusion control group). Similarly, PD98059 did not affect the protective effect of IPostC (29±2%, $p<0.05$ versus I/R+PD;) but wortmannin completely abrogated the effect of IPostC (43±1%, p=ns versus 44±0.5% for I/R+ wort). Inhibition of the JAK/STAT pathway with AG490 during the IPostC or TNF-PostC protocols, abolished the infarct sparing effect of both postconditioning protocols (47±2% for IPostC; p=ns versus 47±3% for I/R and 51 ± 2% for TPostC, p=ns, versus 47±3% for ischaemia-reperfusion control group), (Figure 38).

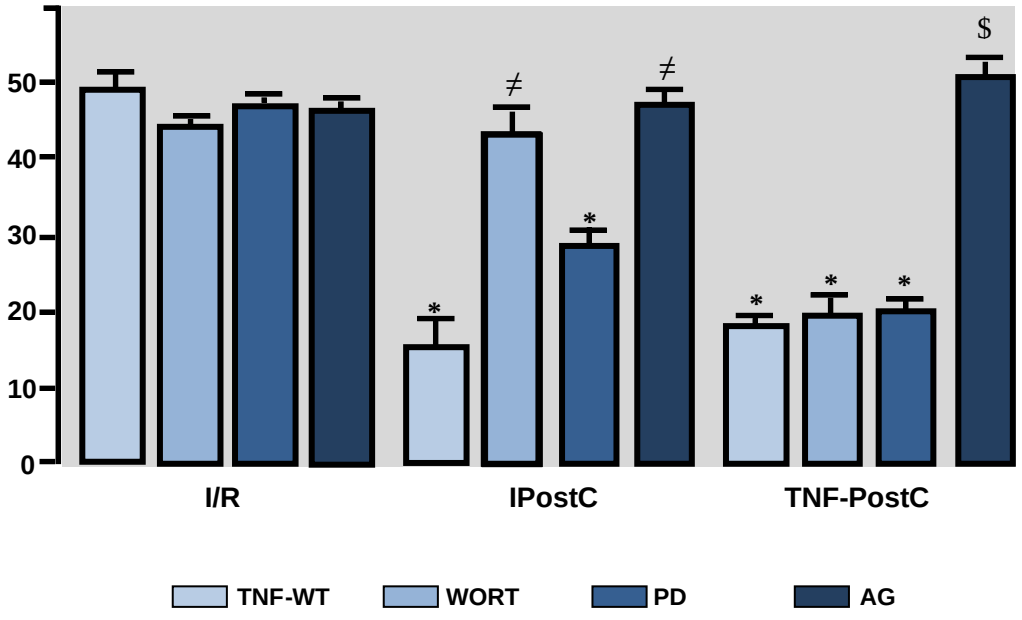


Fig 38: AG 490 abrogates TNF-PostC protection in TNF-WT mice. Inhibiting the activation of STAT-3 (by AG 490) during early reperfusion abrogates the protective effect of TNF-PostC in wild type mice. However, administration of wortmannin (a PI-3K inhibitor) or PD98059 (an inhibitor of Erk1/2) during early reperfusion did not abolish the postconditioning effect of TNFα; n = 6 per group *$p<0.05$ vs I/R alone; ≠$p<0.05$ vs IPostC alone and \$$p<0.001$ vs TNF-PostC alone. Abbreviations as described in text.

- **TNFα failed to mimic ischemic postconditioning in STAT-3$^{-/-}$ mice**
  60

The infarct size in STAT-3-WT mice compared to the ischaemic control group was significantly decreased by pharmacological postconditioning with 6 x alternating cycles of 10 sec reperfusion without TNFα and 10 sec reperfusion with 0.5 ng/L TNFα initiated at the onset of reperfusion (20 ± 1% for TNF-PostC versus 52 ± 2% in I/R, $p<0.05$; Figure 39). In contrast, the STAT-3$^{-/-}$ mice could not be postconditioned with TNFα (44 ± 3% in I/R versus 47 ± 1% in TNF-PostC, p = ns), (Figure 39).

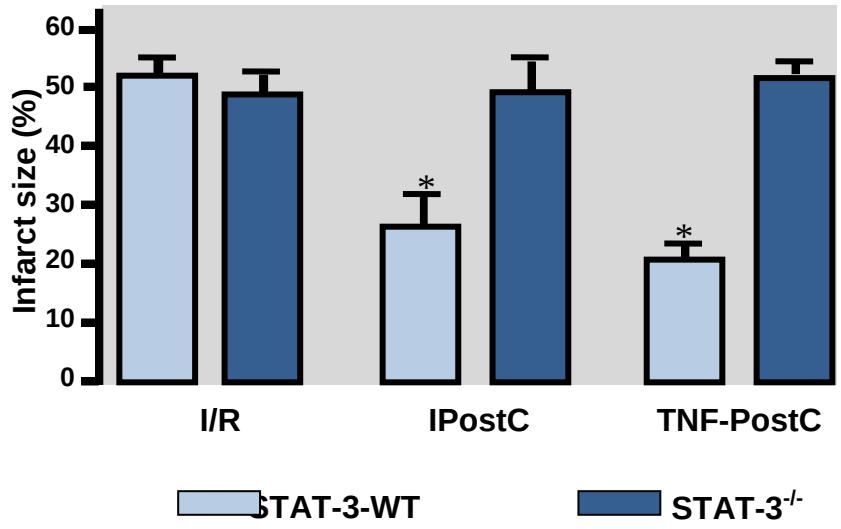


Fig 39: TNFα can mimic IPostC in STAT-3-WT but not STAT-3$^{-/-}$ mice. 6 x 10 sec cycles of IPostC reduced the infarct size in STAT-3-WT but not STAT-3$^{-/-}$ mice, n ≥ 6 per group. Similarly, the infarct size in STAT-3-WT was reduced by 6 x 10 sec cycles of TNF-PostC but this failed to reduce the infarct size in STAT-3$^{-/-}$ mice, n ≥ 6 per group, *p<0.05 vs I/R. Abbreviations are as described in the text.

➢ **TNFα release from heart tissue after IPostC**

I/R reduced TNFα levels from 33.2 ± 4.4 pg/100mg tissue to 21.7 ± 1.9 pg/100mg tissue, $p<0.05$ IPostC restored the TNFα levels to 36.7 ± 3.2 pg/100mg tissue, versus I/R, $p<0.05$.

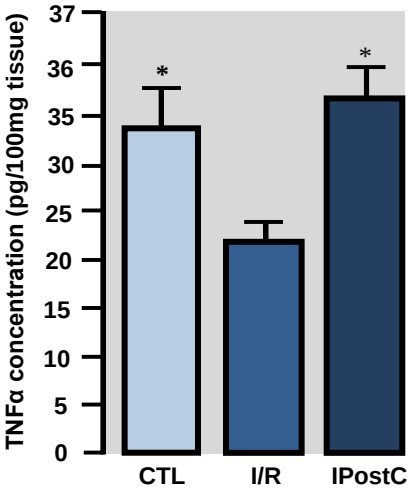


Fig 40: TNFα release by IPostC. The TNFα released from untreated hearts was 33.2 ± 4.4 pg/100mg tissue. IPostC restored TNFα release to 36.7 ± 3.2 pg/100 mg tissue whereas I/R significantly decreased the amount of TNF α released to 21.7 ± 1.9 pg/100mg tissue, *$p<0.05$ versus I/R.

3.2 Western blot results

➢ **Levels of STAT-3, Akt, Erk1/2 and GSK-3β in wild type and TNF$^{-/-}$ mice in nuclear and cytosolic fractions**

a) Nuclear fraction

We examined the levels of STAT-3, Akt, Erk1/2 and GSK-3β during the first 15 min of reperfusion with no ischaemic episode (time-matched control) and after either IPostC or TNF-PostC. An increase in phosphorylated STAT-3 (in arbitrary units [AU] from 18 ± 2 for I/R to 54 ± 6, $p<0.05$), GSK-3β (from 5 ± 0.4 for I/R to 15 ± 1, $p<0.05$) and Erk1/2 (from 9 ± 1 for I/R to 26 ± 2) occurred in the nuclear fraction after TNF-PostC in TNF-WT hearts. In contrast, Akt phosphorylation was decreased after TNF-PostC from 18 ± 2 for I/R to 6 ± 1, $p<0.05$. In TNF-

WT hearts subjected to IPostC, an increase in phosphorylation of STAT-3 (from 18 ± 2 for I/R to 46 ± 5, $p<0.05$) and GSK-3β (from 5 ± 0.4 for I/R to 32 ± 2, $p<0.05$) was observed but the Akt level was unchanged. In $TNF^{-/-}$ mice subjected to IPostC, a modest increase in phosphorylated STAT-3 occurred compared to the I/R group, but no increase in GSK-3β was observed in the nuclear fraction after the postconditioning stimulus compared with the I/R group. Of note, phosphorylation of both Akt and Erk1/2 was significantly increased in the nuclear fraction by IPostC compared with I/R in $TNF^{-/-}$ mice, (Figures 41a & 41b).

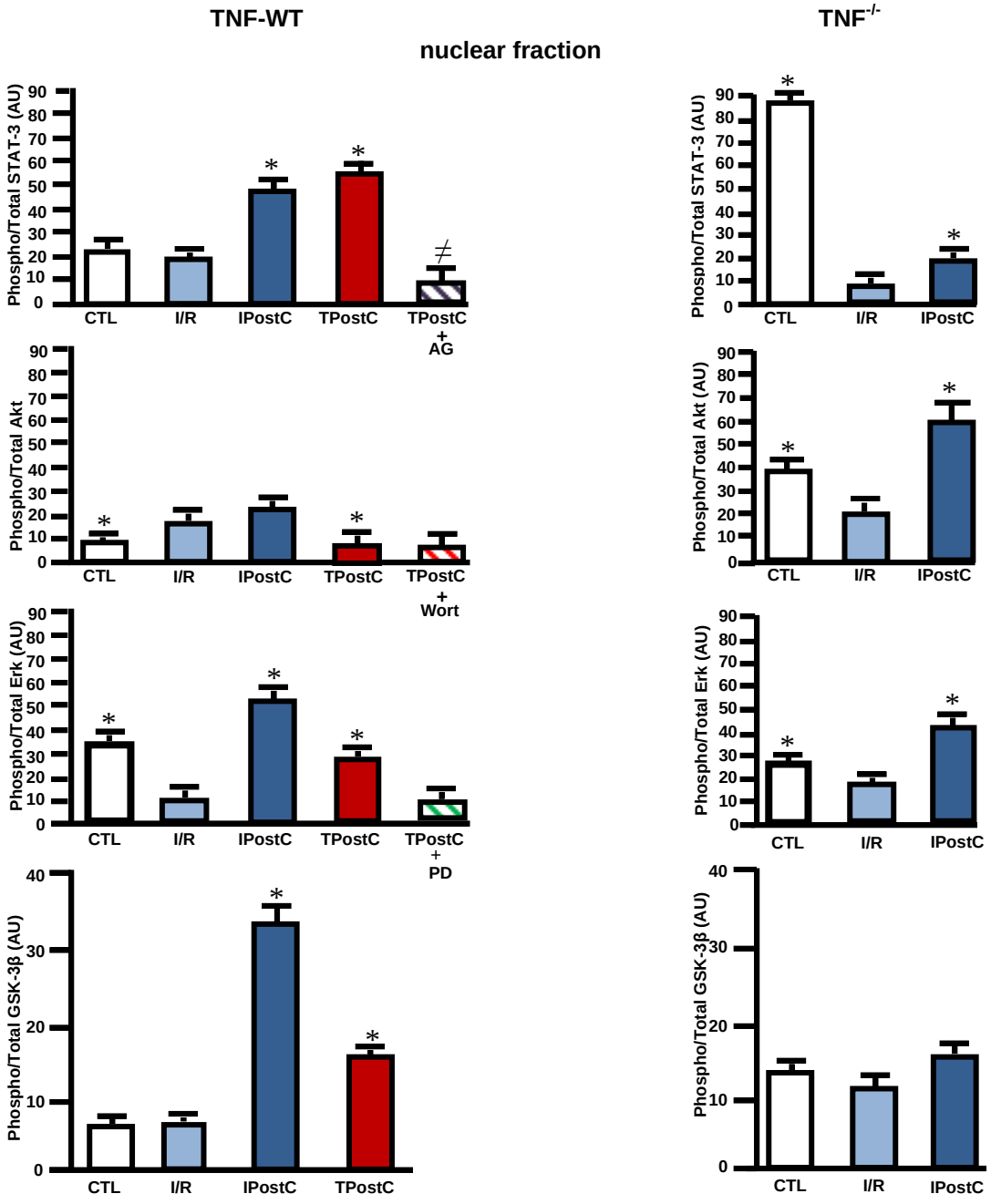


Fig 41a: Phosphorylation of STAT-3, Akt, Erk1/2 and GSK-3β in nuclear fraction. Bar graphs of data derived from western blots of the nuclear fraction demonstrating phosphorylation of STAT-3, Akt and Erk1/2 after 15 minutes of reperfusion in isolated TNF-WT and TNF$^{-/-}$ hearts; *p<0.05 versus I/R; ≠p<0.05 vs TPostC. Specific bands were quantified by densitometry and expressed in AU. Equal loading was confirmed with GAPDH Abbreviations as described in the text.

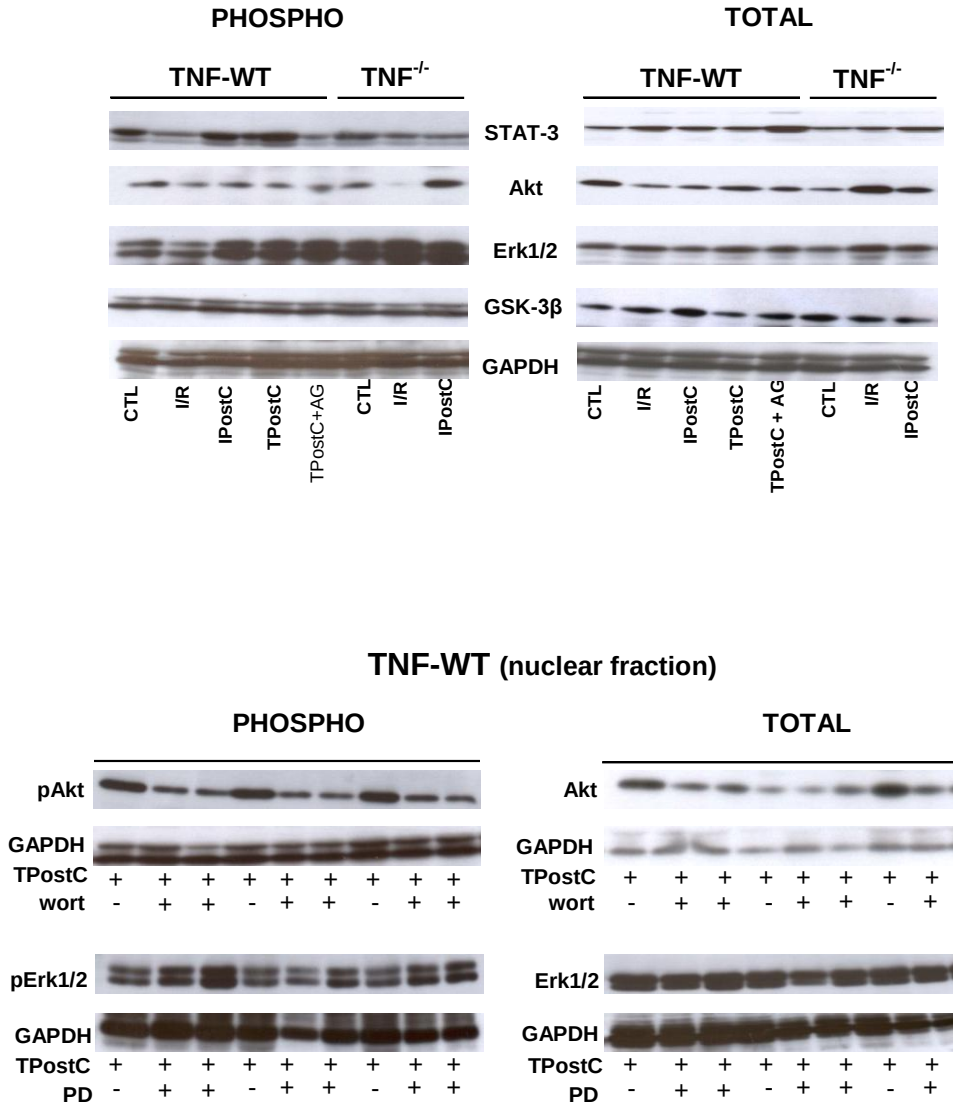


Fig 41b: Representative western blots of STAT-3, Akt, Erk1/2 and GSK-3β in nuclear fraction. Representative western blots of the nuclear fraction demonstrating phosphorylation of STAT-3, Akt GSK-3β and Erk1/2 after 15 minutes of reperfusion in isolated TNF-WT and $TNF^{-/-}$ hearts; $p<0.05$ versus I/R. Specific bands were quantified by densitometry and expressed in AU. Equal loading was confirmed with GAPDH Abbreviations as described in the text.

b) Cytosolic fraction

In the cytosolic fraction, I/R increased phosphorylation of STAT-3 and decreased phosphorylation of Erk1/2 and Akt in wild type animals. IPostC reduced phosphorylated STAT-3 but increased the phosphorylation of both Akt and Erk1/2 in the cytosol compared to the I/R group. Pharmacological postconditioning with TNFα increased phosphorylation of Erk1/2 while phosphorylation levels of STAT-3 remained unchanged in the cytosol.

In the $TNF^{-/-}$ mice, we were surprised to observe an increase of phosphorylation in both STAT-3 and Akt in the cytosol associated with a decrease in phosphorylated Erk1/2 following an IPostC stimulus, (Figure 42a & 42b).

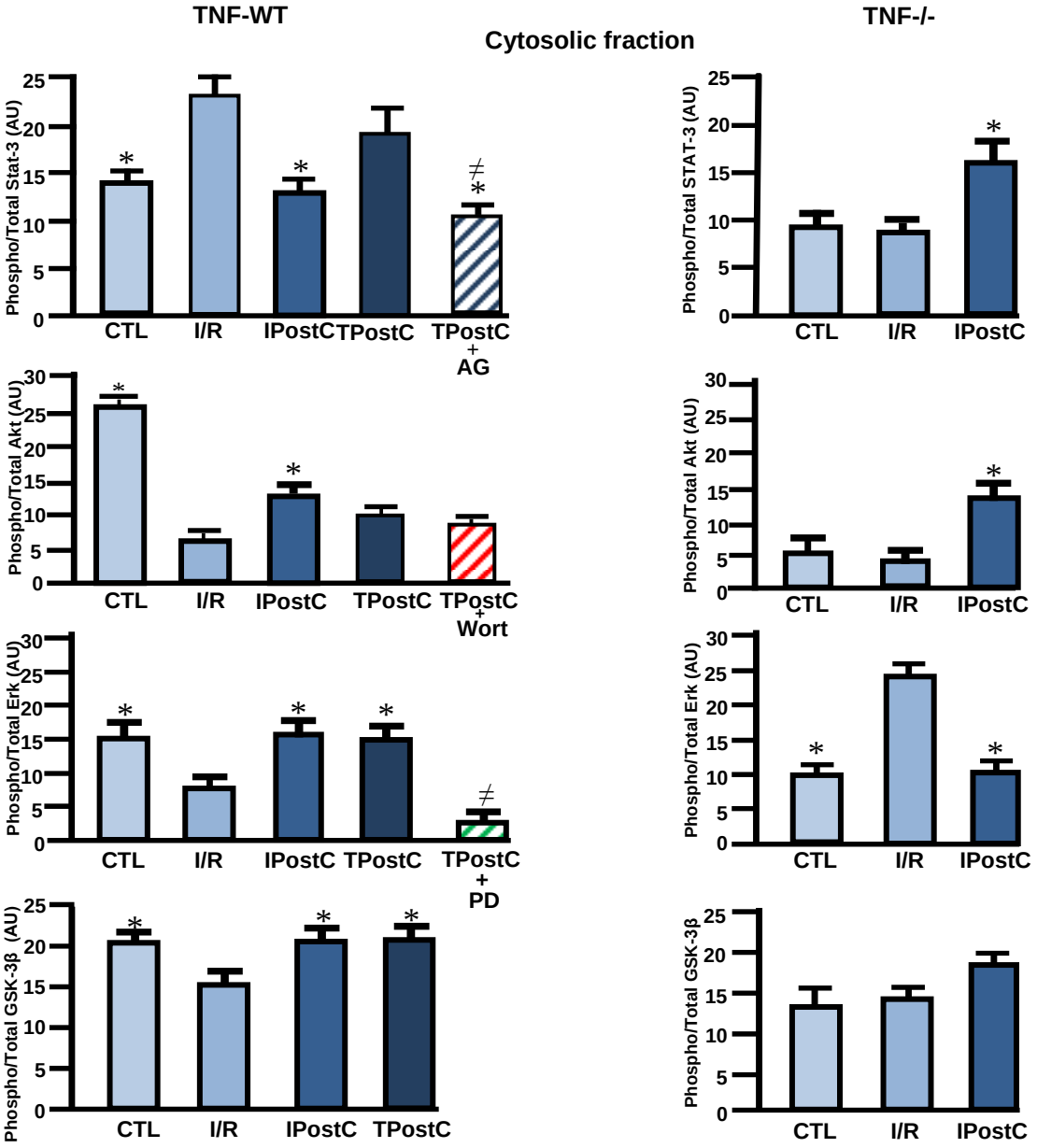


Fig 42a: Phosphorylation of STAT-3, Akt, Erk1/2 and GSK-3β in cytosolic fraction. Bar graphs of data derived from western blots of the cytosolic fraction demonstrating phosphorylation of STAT-3, Akt and Erk1/2 after 15 minutes of reperfusion in isolated TNF-WT and $TNF^{-/-}$ hearts; *p<0.05 versus I/R; ≠p<0.05 vs TPostC; n=6. Specific bands were quantified by densitometry and expressed in AU. Equal loading was confirmed with GAPDH. Abbreviations as described in the text.

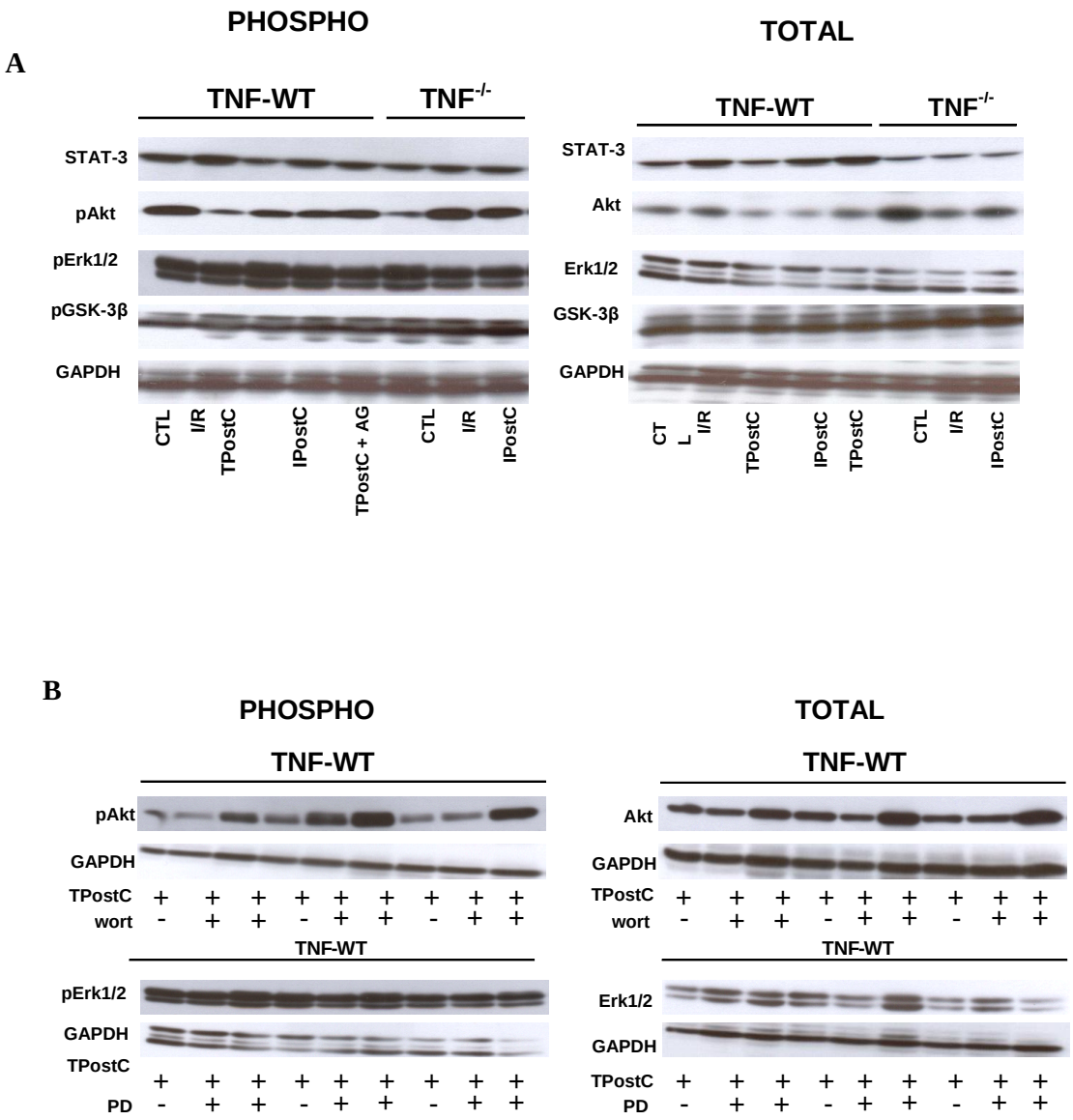


Fig 42b: Representative western blots of STAT-3, Akt, Erk1/2 and GSK-3β in cytosolic fraction. Representative western blots of the cytosolic fraction demonstrating phosphorylation of STAT-3, Akt and Erk1/2 after 15 minutes of reperfusion in isolated TNF-WT and TNF$^{-/-}$ hearts; $p<0.05$ versus I/R. Specific bands were quantified by densitometry and and expressed in AU. Equal loading was confirmed with GAPDH Abbreviations as described in the text.

- **RAGE in TNF-WT and TNF$^{-/-}$- mice**

  a) Nuclear fraction

We examined the expression of total RAGE in nuclear fractions extracted from TNF-WT and TNF$^{-/-}$ hearts, during the first 15 min of reperfusion after either IPostC or TNF-PostC. In TNF-WT hearts, IPostC decreased the expression of RAGE significantly in the nuclear fraction from 142.8 ± 9.6 AU in control hearts to 41.0 ± 4.5 AU in IPostC; $p<0.05$. TPostC, however, restored the level of RAGE to a similar level seen in the controls, (p=ns). TNF$^{-/-}$ hearts showed no significant difference in RAGE expression in relation to the treatment, (Figure 43).

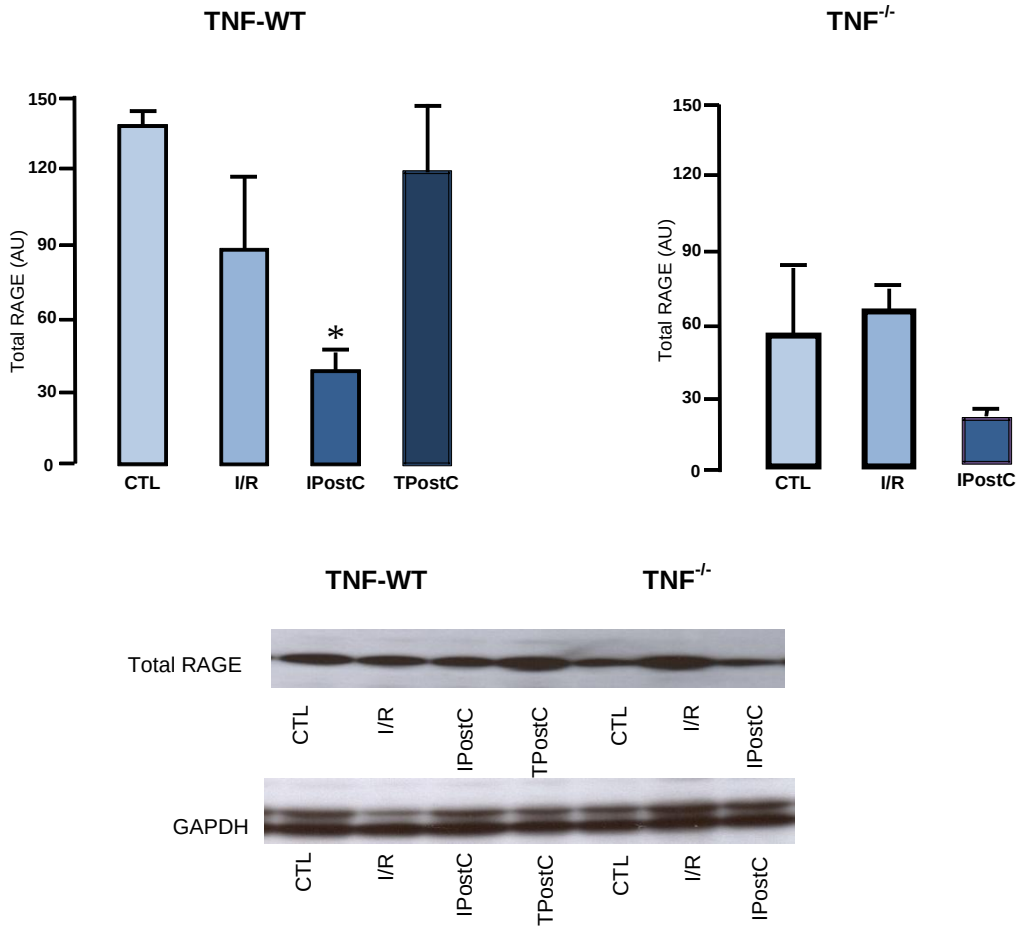


Fig 43: Expression of Total RAGE in nuclear fraction. Bar graphs of data derived from western blots and representative western blots demonstrating total RAGE expression after 15 minutes of reperfusion in isolated TNF-WT and TNF-/- hearts; *p<0.05 versus respective CTL; n =6. Specific bands were quantified by densiometry and expressed in AU. Equal loading was confirmed with GAPDH. Abbreviations as described in text.

b) Cytosolic fraction

In the cytosolic fraction of TNF-WT hearts, RAGE expression after IPostC and TNF-PostC was significantly decreased versus the control group, from 530 ± 10 AU to 267 ± 100 AU, $p<0.05$ and 116 ± 20 AU, $p<0.01$, respectively. No significant differences were observed amongst the groups in $TNF^{-/-}$ mice, p=ns. However, the I/R group of $TNF^{-/-}$ hearts expressed significantly less RAGE compared with the I/R group of TNF-WT, ($p<0.01$), (Figure 44).

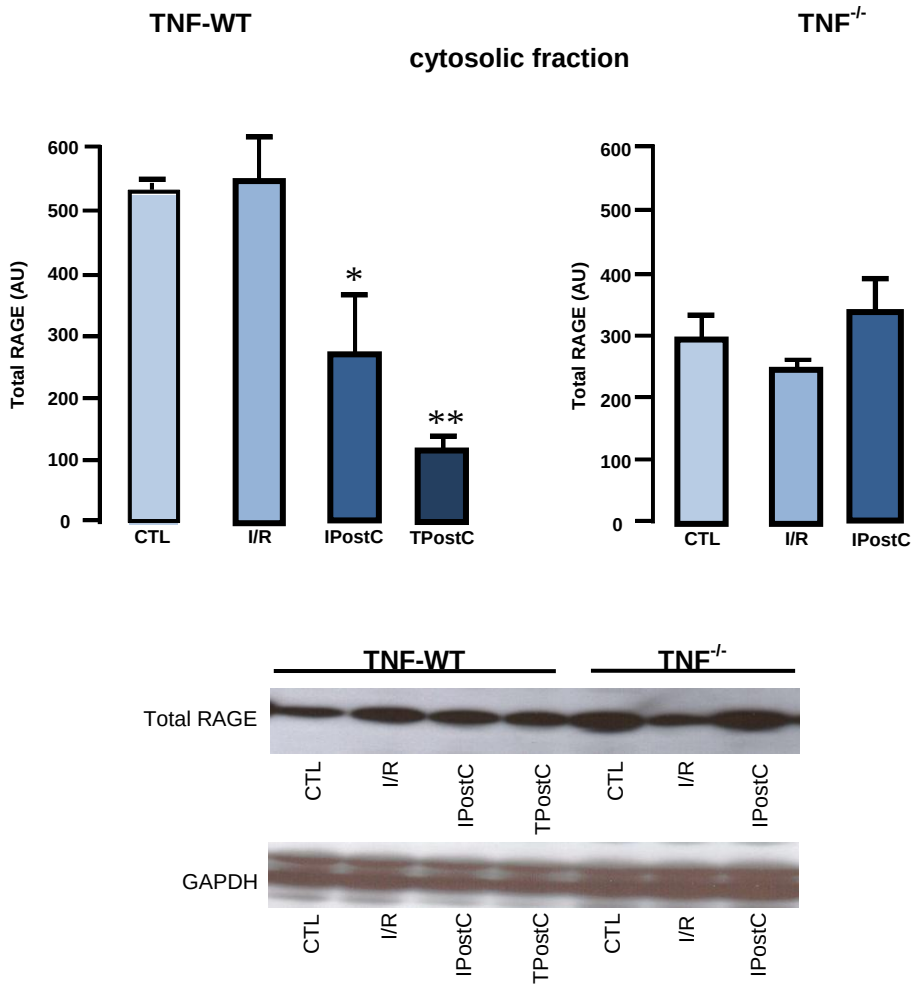


Fig 44: Expression of Total RAGE in cytosolic fraction. Bar graphs and representative western blots demonstrating total RAGE expression after 15 minutes of reperfusion in isolated TNF-WT and TNF-/- hearts; *p<0.05 versus TNF-WT I/R, **p<0.01 versus TNF-WT I/R. Specific bands were quantified by densitometry and expressed in AU. Abbreviations as described in text

➢ **Expression of Total PAR-2 in TNF-WT and TNF$^{-/-}$ mice**

We examined the expression of PAR-2 in total protein extracted from TNF-WT and TNF$^{-/-}$ hearts, during the first 15 min of reperfusion after either IPostC or TNF-PostC. In TNF-WT hearts, both I/R and IPostC decreased the expression of PAR-2 significantly to 76 ± 7 AU and 70 ± 12AU respectively, when compared to control hearts (172 ± 21 AU) which underwent no interference, ($p<0.05$). TPostC, however, restored the level of PAR-2 to a similar level as that of the controls, (p=ns). TNF$^{-/-}$ control hearts had a significant decrease in PAR-2 expression (63 ± 5 AU) compared to hearts from TNF-WT control group (172 ± 21AU), ($p<0.01$). TNF$^{-/-}$ I/R group significantly increased the PAR-2 expression to 160 ± 8 AU versus their control group, ($p<0.05$), figures 45 & figure 46.

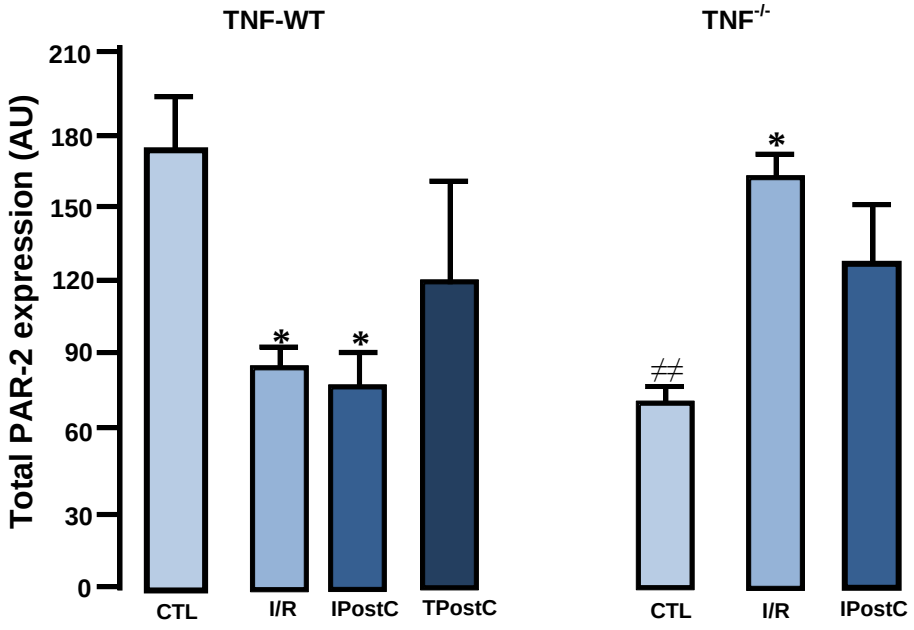


Fig 45: Expression of Total PAR-2. Bar graphs of data derived from western blots demonstrating total PAR-2 expression after 15 minutes of reperfusion in isolated TNF-WT and TNF$^{-/-}$ hearts; *$p<0.05$ versus respective CTL. CTL hearts from TNF$^{-/-}$ had significantly decreased PAR-2 expression versus CTL hearts from TNF-WT, ##$p<0.01$. Specific bands were quantified by densitometry and all groups were corrected to GAPDH densities and expressed in AU. Abbreviations as described in the text.

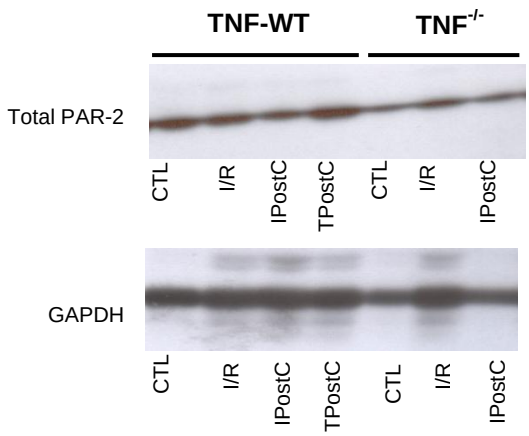


Fig 46: Representative western blots of Total PAR-2 expression. Representative western blots, demonstrating Total PAR-2 expression after 15 minutes of reperfusion in isolated TNF-WT and TNF-/- hearts; $p<0.05$ versus CTL. Specific bands were quantified by densitometry and all groups were corrected to GAPDH densities and expressed in AU. Abbreviations as described in the text.

4.0 Discussion

Using the different animal models, our data show, for the first time and possibly counter intuitively, that endogenous or exogenous TNFα, at the time of reperfusion, initiates a novel and RISK-free protective pathway in ischaemic postconditioning. In brief, six episodes of IPostC, performed by alternating cycles of reperfusion and ischaemia in TNF deficient mice, failed to protect against ischaemia-reperfusion damage. The activation of TNFR2 is implicated in the postconditioning protective effect as $TNFR1^{-/-}$, but not $TNFR2^{-/-}$ mice, could be postconditioned. The protection of IPostC was reduced in the presence of wortmannin, PD98059 and AG490, while postconditioning with TNFα could only be abrogated by AG490. Furthermore, the $STAT\text{-}3^{-/-}$ hearts could not be postconditioned. Our data demonstrate that protection with ischaemic postconditioning can be achieved via the activation of TNFα, its binding to TNFR2 and subsequent activation of STAT-3, key components of the prosurvival SAFE pathway.

4.1 Cardioprotective role of TNFα

Previously, we have consistently demonstrated, in both *in vitro* and *in vivo* experiments, that exogenous TNFα could confer a cardioprotective effect against reperfusion injury in a time and concentration dependent manner (see review (Lecour, 2009b)). Our group has shown that tissue levels of TNFα in the *in vivo* rat heart were significantly increased in the area at risk and that various doses of exogenous TNFα administered prior to the ischaemic protocol could be used as a preconditioning stimulus (Deuchar et al., 2007). Furthermore, a recent *in vivo* rat study demonstrated that the protection with IPostC is concomitant with decreased plasma levels of TNFα at reperfusion (Kin, 2008). Our present data demonstrate that TNFα, given immediately at the onset of reperfusion, confers protection in the isolated heart. This is in apparent contradiction to previous reports from isolated rat hearts, suggesting that the release of TNFα, as part of the inflammatory response during the reperfusion phase, contributes to the development of myocardial infarction, contractile dysfunction and apoptosis (Belosjorow et al., 1999; Kupatt et al., 1999). However, the recent demonstration of a biphasic kinetic pattern of TNFα release in a murine reperfusion model suggests that protection is conferred by the limited release of TNFα during the first few minutes of reperfusion (2-10min) (Reil et al., 2007). This limited amount of TNFα is then washed out and replaced with a more robust release of TNFα in the later stages of reperfusion. This subsequent increase in TNFα levels is thought to be responsible for its detrimental effects (Reil et al., 2007). In our present study we show, for the first time, in an

isolated murine heart model, that during either IPostC or TNF-PostC, the binding of TNFα to TNFR2 at the early stage of reperfusion is crucial for protection. Therefore, a possible explanation of our potentially controversial findings is that the synergetic limited early release/addition of TNFα, together with its binding to TNFR2, at the onset of reperfusion, may limit further harmful production of TNFα during the later stages of reperfusion.

Using $TNF^{-/-}$ mice in our experiments, we were surprised to observe that TNFα postconditioning in these mice failed to protect the heart. In a previous study, our research group demonstrated that the administration of TNFα to TNF knockout mice, 24 hours prior to experimentation rescued the protective effect of ischaemic preconditioning in these mice (Smith et al., 2002). These data suggest that in TNF knockout mice, the signalling pathways downstream of TNFα may be downregulated. Similar data were obtained in our postconditioning setting in the $TNF^{-/-}$ mice. In these mice the protective effect of TNF-PostC could be restored following administration of 0.5 ng TNFα, ip 24 hours prior to the experiments. Further functional studies are necessary to evaluate the TNF receptor densities and receptor-ligand interaction to fully explain this ability to rescue the protective effect in $TNF^{-/-}$ hearts.

4.2 TNFα initiates the activation of a prosurvival pathway independent of the RISK pathway

An established protective pathway, known as the RISK pathway and involving the activation of protein kinases Akt and Erk1/2 in early reperfusion, has been described in both pre- and postconditioning (Hausenloy et al., 2005; Tsang et al., 2004).

A recent study done in pig hearts reported an activation of the kinases involved in the RISK pathway following an ischemic postconditioning stimulus (Skyschally et al., 2009). However, the inhibition of these kinases did not alter the protective effect, therefore questioning the essential role of the RISK kinases in their model (Skyschally et al., 2009). Similarly, our present study, performed on a mouse model, demonstrates that pharmacological postconditioning with TNFα is independent of the RISK pathway, as neither PD98059 nor wortmannin abolished the protective effect of TNF-PostC. In contrast, the protective effect of both IPostC and pharmacological postconditioning with TNFα was abolished in the presence of AG490, the JAK/STAT pathway inhibitor. In addition, neither ischaemic nor pharmacological postconditioning with TNFα could protect in STAT-3 deficient mice.

Our new data show the existence of an alternative protective pathway during the reperfusion period of IPostC. This pathway, previously described in ischaemic preconditioning, and involving the activation of both TNFα and STAT-3, is now recognized as the SAFE pathway (Lecour, 2009a; Lecour, 2009b). Our present data strongly support the hypothesis that the recent studies describing a RISK-independent protective effect of ischaemic postconditioning may in fact activate the SAFE pathway (Przyklenk et al., 2008; Schwartz and Lagranha, 2006; Skyschally et al., 2009).

Current and past studies, done in our laboratory, suggest that inhibiting either the SAFE or RISK pathway is enough to abolish the infarct sparing effect of ischaemic pre or postconditioning (Fuglesteg et al., 2008; Lecour et al., 2002; Suleman et al., 2008). Attempts to delineate a possible crosstalk between the two pathways reveal a complex interaction. Hence, in the presence of wortmannin during the ischaemic preconditioning stimulus, phosphorylation of STAT-3 was inhibited (Suleman et al., 2008). Similarly, Akt failed to be phosphorylated in STAT-3 knockout mice subjected to ischaemic preconditioning (Suleman et al., 2008). In contrast, a substantial increase of phosphorylated Akt is observed in $TNF^{-/-}$ mice subjected to ischaemic postconditioning (data from this study). Further studies are needed to characterize the crosstalk between the SAFE and RISK pathways in both ischaemic pre and postconditioning.

4.3 Possible downstream targets of SAFE pathway

Inactivation of GSK-3β is a well established downstream target of the protective pathway (Gross et al., 2006; Hausenloy and Yellon, 2007a; Juhaszova et al., 2004; Juhaszova et al., 2009). Our present work aimed to delineate whether GSK-3β could be a common target for the SAFE and RISK pathways. In our model, both ischaemic postconditioning and pharmacological postconditioning with TNFα increased the phosphorylation of GSK-3β in WT mice. In contrast, we were surprised to observe that the absence of an infarct sparing effect in $TNF^{-/-}$ mice was associated with a similar level of Phospho/Total GSK-3β as that of wild type mice subjected to the TNF-PostC protocol. Although the data from this study support the proposal suggesting that inactivation of GSK-3β is not an essential link in the cardioprotective effect of ischaemic postconditioning, this still remains controversial (Juhaszova et al., 2009; Nishino et al., 2008). Further studies using GSK-3β genetically modified mice will be required to confirm whether GSK-3β is, or is not, an effective downstream target of the TNFα/JAK/STAT-3 pathway.

In contrast to previous studies (Napoli et al., 2000; Napoli, 2002) the expression of RAGE was not increased by I/R in our TNF-WT model, although it showed a slight increase in $TNF^{-/-}$ mice. In addition, we observed that IPostC gave a significant reduction in RAGE expression whereas with TNF-PostC the expression returned to baseline levels. $TNF^{-/-}$ mice also exhibited this significant decrease in RAGE during IPostC, suggesting that RAGE is not a downstream target of the SAFE pathway.

PAR-2 levels were decreased significantly by I/R and IPostC but were restored by TNF-PostC. In $TNF^{-/-}$ mice I/R increased PAR-2 expression significantly. Although there was also an increase in PAR-2 expression after IPostC this was not considered significant. Our data suggest that PAR-2 is not a downstream target of the SAFE pathway.

Additional possible downstream targets of the SAFE pathway include phosphorylation of the pro-apoptotic target Bad (Deuchar et al., 2007; Lecour et al., 2005b) and the inactivation of NFκB (Kin, 2008; Nishinakamura et al., 2007). TRAF2 has also been recently implicated in the action of TNFα on NFκB by promoting the binding of TNFα to TNFR2 and discouraging the binding to TNFR1, thereby preventing TNFα-induced apoptosis (Vince et al., 2009). The inhibition of the mitochondrial permeability transition pore opening (Gao et al., 2004) is likely to be the common point at which the SAFE and RISK pathways converge.

4.4 Other alternative pathways operative in postconditioning

The presence of multiple protective pathways has already been suggested in ischaemic preconditioning (Jin et al., 2004; Lecour et al., 2005b) and it is likely that the SAFE and RISK pathways are activated in parallel with other paths in ischaemic postconditioning. Hence, sphingolipids have been proposed as members of a downstream activated path in ischaemic postconditioning (Jin et al., 2008; Vessey et al., 2008). Sphingosine-1-phosphate can activate Akt, Erk1/2 and STAT-3 in the heart (Frias et al., 2009; Radeff-Huang et al., 2004; Vessey et al., 2008). While TNFα protects via the activation of the sphingolipids, (Heusch et al., 2008) and preliminary data in our laboratory suggest that sphingosine-1-phosphate mimics ischaemic preconditioning via the activation of STAT-3 (Kelly, 2008), the existence of sphingosine-1-phosphate as an alternative path to the SAFE or RISK pathway still needs to be confirmed.

In summary, our studies have consistently shown that activation of the SAFE pathway, as represented by the activity of TNFα and STAT-3 is required in both ischaemic pre- and

postconditioning (Deuchar et al., 2007, Baxter, 2003#242 ; Lecour et al., 2002; Lecour et al., 2005b; Smith et al., 2002; Suleman et al., 2008). The specific involvement of TNFR2 in our studies suggests that this protective pathway is linked to this receptor. Overall, these results further emphasize the importance of RISK-independent pathways in cardiac protection at the time of reperfusion, which may have potential therapeutic application in the mitigation of ischaemic-reperfusion injury. A schematic of the SAFE pathway in postconditioning, based on the data from the present study, is shown in figure 47.

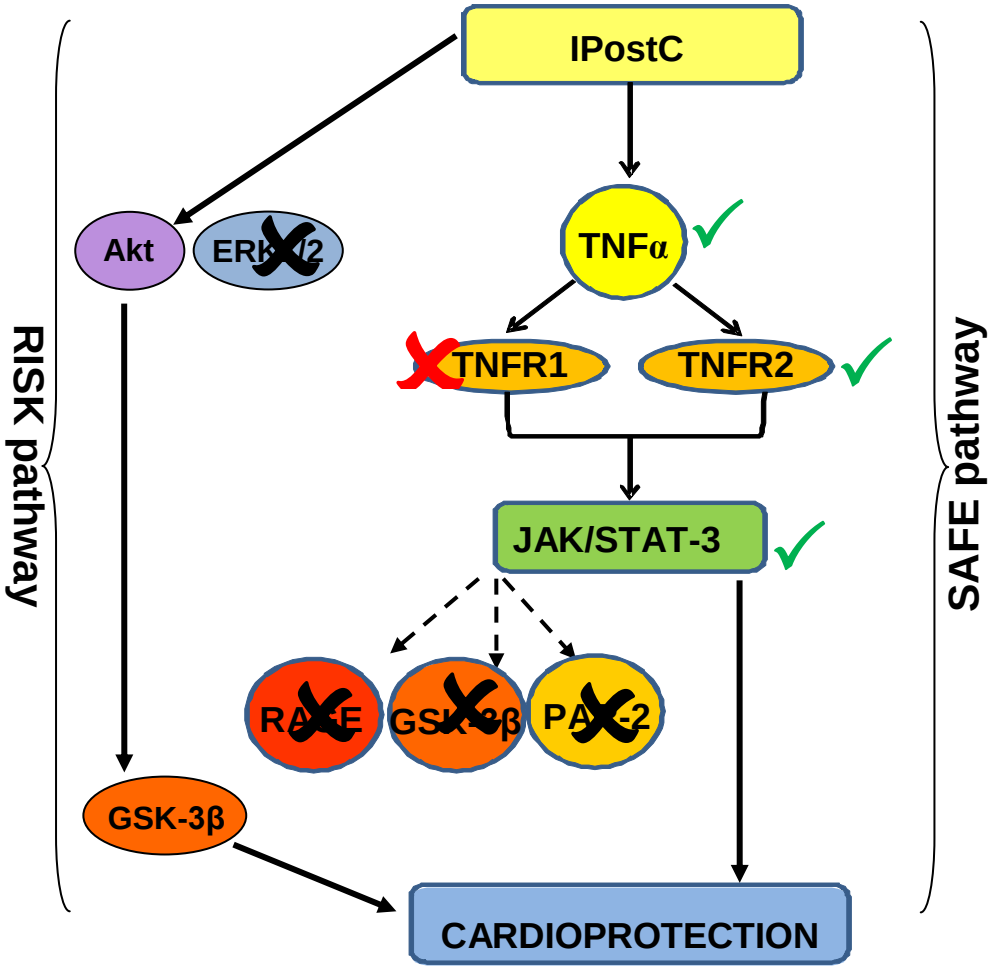


Fig 47: Schematic of SAFE pathway. TNFα activates JAK/STAT-3 via TNFR2 to confer cardioprotection. RAGE, GSK-3β and PAR-2 are not downstream signalling targets of STAT-3 in the SAFE pathway. The RISK pathway signals through Akt and GSK-3β.

E. CARDIOPROTECTIVE AGENTS AS POSTCONDITIONING MIMETICS

Part of the experiments in this section have been performed in conjunction with Sarin Somers, registered as a PhD student at the University of Cape Town

1.0 Introduction

Ischaemic postconditioning with intermittent episodes of ischaemia and reperfusion at the onset of reperfusion requires precise and timed applications that are impractical to apply in a clinical setting. Pharmacological agents such as bradykinin, opioids and adenosine can be substituted as preconditioning mimetics to reduce infarct size following an ischaemia-reperfusion insult but their role as postconditioning mimetics has been poorly studied (Cohen, 2001; Gross, 2006; Downey, 2007). These three mediators are known to protect via the activation of the RISK pathway (Bell, 2003; Riksen, 2004; Fryer, 2002) and several studies conducted in a preconditioning setting suggest that divergent pro-survival signalling pathways may be activated by these components. Hence, bradykinin and opioids, but not adenosine, require the activation of the mK_{ATP} channel and free radicals to confer cardioprotection in IPC (Cohen, 2001).

In the previous section, our data demonstrate that activation of the prosurvival SAFE pathway, with TNFα and STAT-3, acts as an alternative signalling pathway to confer cardioprotection in IPostC. The role of TNFα as a downstream signalling target of bradykinin, opioids and adenosine has never been investigated.

Therefore, the purpose of our study is to explore whether adenosine, bradykinin and opioids can mimic IPostC in an isolated mouse heart model. In addition, we will use $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$ and cardiomyocyte specific STAT-3 deficient mice ($STAT\text{-}3^{-/-}$) to demonstrate that this pharmacological postconditioning effect can be attributed, at least in part, to the activation of the prosurvival SAFE pathway initiated by TNFα and STAT-3. A recent study suggested the possibility that STAT-3 can translocate to the mitochondria after its phosphorylation in the cytosol (at the serine site) (Wegrzyn, 2009). As mitochondria are considered as the end target for the protection afforded with postconditioning (Heusch, 2010), we will use western blot techniques to investigate the possible activation and translocation of STAT-3 to the mitochondria following a pharmacological postconditioning stimulus.

Our aim, therefore, was to firstly delineate whether adenosine, bradykinin and opioids could mimic IPostC in an isolated mouse heart model and secondly, to explore whether this postconditioning effect could be attributed, at least in part, to the activation of the SAFE pathway via translocation of STAT-3 to the cardiac mitochondria, (Figure 48).

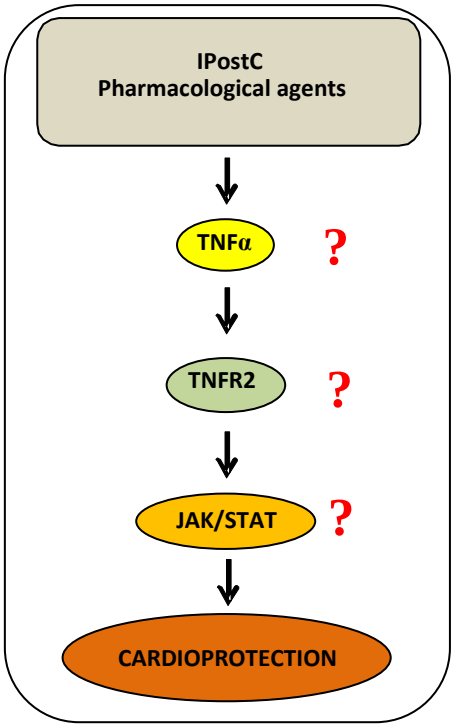


Fig 48: Schematic of proposed hypothesis. Pharmacological agents activate TNFα via TNFR2 and initiate the phosphorylation of JAK/STAT and subsequent cardioprotection.

2.0 Methods

2.1 Perfusion of mouse hearts

All animal studies performed were approved by the Animal Research Ethics Committee of the University of Cape Town, and followed the recommendations laid down in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85 {23}, revised 1996). Six mice were used per experimental group for each genotype of mouse.

Isolated hearts of adult male $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$ and $STAT\text{-}3^{-/-}$ mice and their respective WTs were subjected to the identical retrograde perfusion technique as described in Section C. Postconditioning with each of the well known cardioprotective agents as postconditioning mimetics was performed in the same manner as we have described in Section D, with 6 x 10 second alternating cycles of reperfusion and reperfusion together with each of the drugs in turn as outlined in the following figure 49.

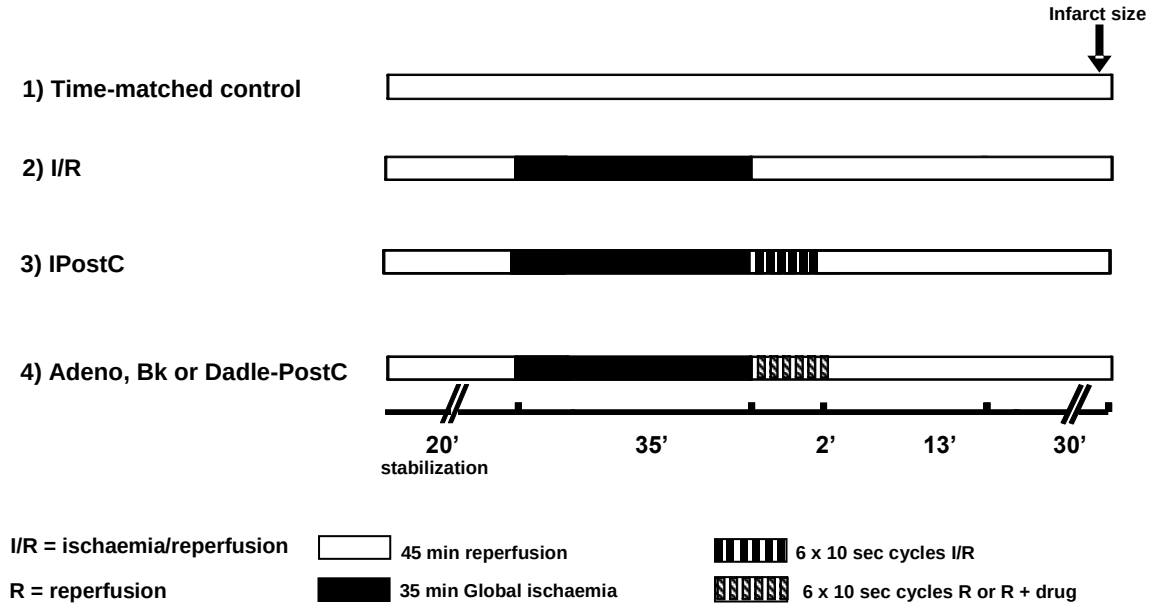


Fig 49: Schematic of Langendorff experimental model. Isolated hearts were subjected to a stabilization period of 20 min followed by 35 min global ischaemia and a 45 min reperfusion period. At onset of reperfusion, ischaemic postconditioning (IPostC) was initiated with 6 alternating cycles of 10 sec reperfusion, 10 seconds ischaemia. In a separate group, postconditioning with 100 µM adenosine (Adeno-PostC), 100 nM Bradykinin (Bk-PostC) or 100 nM DADLE (D-PostC) was initiated with 6 alternating cycles of 10 sec reperfusion with no drug, 10 seconds reperfusion with the drug of choice.

2.1.2 Infarct size assessment

At the end of each experimental protocol, hearts were removed from the perfusion apparatus and infarct size assessed by triphenyltetrazolium chloride (TTC) staining and planimetry as described previously in Section C.

2.2 Postconditioning with classic cardioprotective agents

Adenosine (100 μM), bradykinin (100 nM), and 100 nM of the delta opioid agonist, D-Ala 2', D-Leu 5'- enkephalin acetate salt (DADLE) were used as postconditioning mimetics in hearts from $TNF^{-/-}$, $TNFR1^{-/-}$, $TNFR2^{-/-}$ and $STAT\text{-}3^{-/-}$ mice and their respective wild types. Six alternating cycles of 10 seconds reperfusion, 10 seconds reperfusion with pharmacological agent was initiated at onset of reperfusion (Figure 49). A minimum of 6 hearts was used in all groups.

2.3 Western blot analysis

Control or murine hearts postconditioned with bradykinin, adenosine or DADLE were subjected to a 20 min stabilization period followed by 35 min of global ischemia and 15 min of reperfusion. The hearts were then snap-frozen in liquid nitrogen before being stored at -80ºC until protein extraction was performed. To determine whether the activation of the prosurvival signalling cascade initiated the translocation of activated STAT-3 from the cytosol to the mitochondria we extracted mitochondrial and cytosolic proteins from the hearts by homogenization of the tissue in a lysis buffer, as previously described in Section C. Total and phosphorylated states of STAT-3 (Phospho-STAT-3, ser 737), were analysed by sodium dodecyl sulphate-polyacrylamide gel immunoelectrophoresis (SDS-PAGE) using antibodies from Cell Signaling Technologies and Santa Cruz Biotechnology, Inc. USA.. Equal loading for the mitochondrial fraction was verified with an antibody for the voltage dependent anion channel (VDAC). GAPDH was used as a loading control for the cytosolic fraction. The ratio of phosphorylated STAT-3 translocated from the cytosolic fraction to the mitochondrial fraction was calculated. Relative densitometry was determined with the use of computerized software package (ImageJ analysis and processing in Java). A minimum of 6 hearts was used per group.

2.4 Chemicals and Pharmacological Agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemicals, Germany.

2.5 Statistical Analysis

Data are presented as mean ± SEM. Comparisons between multiple groups were performed by 1-way ANOVA followed by Student-Newman-Keuls Multiple Comparison Test (Graph Pad Instat). $p<0.05$ was considered statistically significant.

3.0 Results

3.1 Postconditioning with classic cardioprotective agents

In TNF-WT mice, ischaemic postconditioning (IPostC) reduced infarct size from 48 ± 1% in the ischaemic control group (I/R) to 16 ± 1% in IPostC, ($p<0.001$). Bradykinin postconditioning (Bk-PostC), DADLE postconditioning (D-PostC) and Adenosine postconditioning (Adeno-PostC) reduced the infarct size in a similar manner to TNF-WT hearts, ($p<0.001$versus I/R). In contrast, (IPostC), Bk-PostC and D-PostC failed to protect hearts from $TNF^{-/-}$ mice, (p=ns), whereas adenosine protected the TNF-deficient hearts against ischaemia-reperfusion injury, decreasing infarct size from 45 ± 1% in I/R to 19 ± 1% in Adeno-PostC, ($p<0.001$), figure 50.

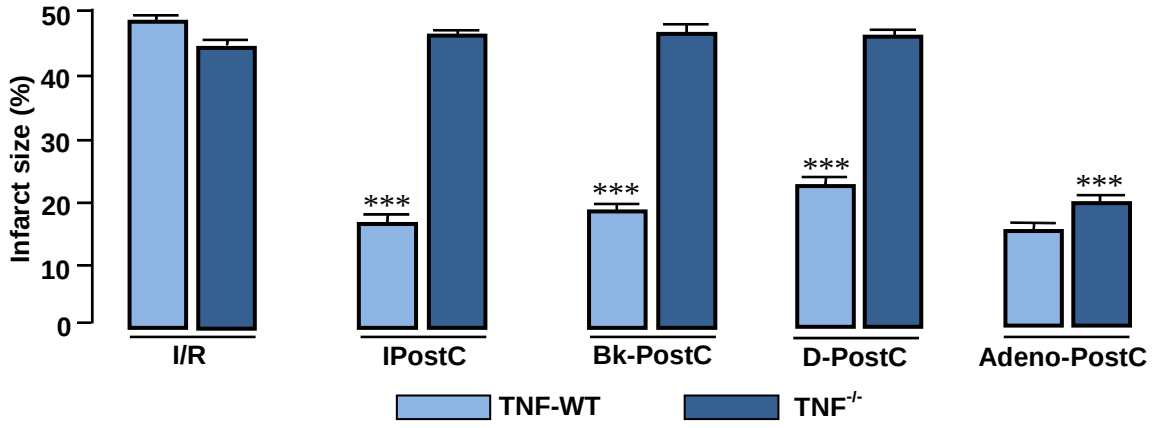


Fig 50: Adenosine protects $TNF^{-/-}$ mice. IPostC decreased infarct size in TNF-WT from 48 ± 1% to 16 ±1% versus I/R. Similarly, Bk-PostC, D-PostC and Adeno-PostC reduced infarct size in TNF-WT hearts to 19 ± 1%, 24 ± 1% and 15 ± 1%, respectively. Postconditioning with adenosine protected $TNF^{-/-}$ hearts against ischaemia-reperfusion injury, reducing infarct size from 45 ± 1% to 19 ± 1%, ***$p<0.001$ vs respective I/R control. In contrast, IPostC, Bk-PostC and D-PostC failed to protect hearts from $TNF^{-/-}$ mice, p=ns. n=6 per group. IPostC=ischaemic postconditioning; Bk-PostC=bradykinin postconditioning; D-PostC=DADLE postconditioning; Adeno-PostC=adenosine postconditioning.

3.2 Pharmacological postconditioning with bradykinin and DADLE protects TNFR1$^{-/-}$ but not TNFR2$^{-/-}$ mice

Infarct size measured after I/R in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ were similar to WT mice. IPostC reduced infarct size in TNF-WT and TNFR1$^{-/-}$ hearts to 19 ± 1% and 20 ± 1%, respectively versus their respective I/R controls, (***p<0.001). In contrast, TNFR2$^{-/-}$ hearts failed to be protected, (p=ns versus I/R). Postconditioning with 100 nM bradykinin (Bk-PostC) and 100 nM DADLE (D-PostC) significantly decreased infarct size in TNFR1$^{-/-}$ hearts from 50 ± 1% in I/R to 21 ± 1% and 20 ± 1%, respectively, (***p<0.001). However, TNFR2$^{-/-}$ hearts could not be protected by either pharmacological agent, (p=ns versus I/R), figure 51.

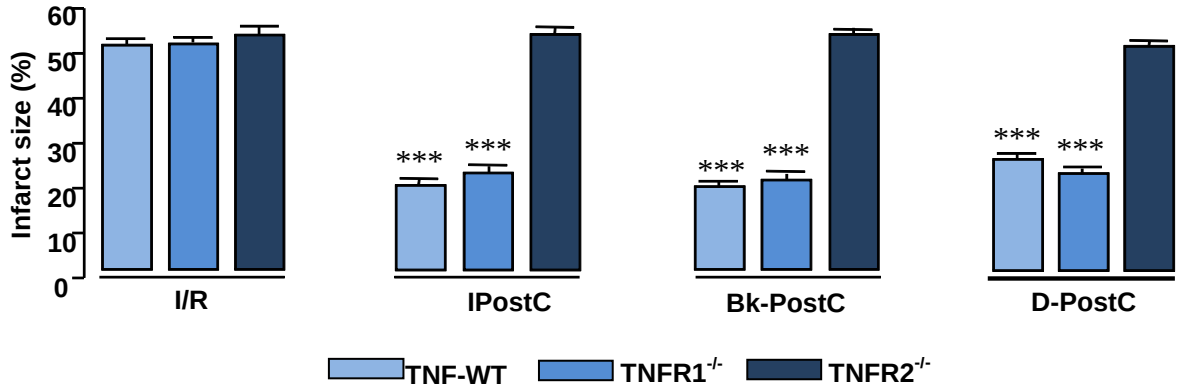


Fig 51: Bradykinin and DADLE postconditioning protect TNFR1$^{-/-}$ but not TNFR2$^{-/-}$: IPostC significantly decreased infarct size in TNF-WT and TNFR1$^{-/-}$ hearts vs their respective I/R controls. Similarly, Bk-PostC and D-PostC decreased infarct size in TNFR1$^{-/-}$, (***p<0.001 versus respective I/R controls). In contrast, hearts from TNFR2$^{-/-}$ mice failed to be protected, (p=ns); n=6 per group. IPostC=ischaemic postconditioning; Bk-PostC=bradykinin postconditioning; D-PostC=DADLE postconditioning.

3.3 Bradykinin, DADLE and Adenosine fail to protect STAT-3$^{-/-}$ hearts

IPostC in STAT-3-WT mice reduced infarct size from 48 ± 2% in the ischaemic control group (I/R) to 23 ± 5% for IPostC, ($p<0.001$). Bk-PostC, D-PostC and Adeno-PostC reduced the infarct size in a similar manner to STAT-3-WT hearts, from 48 ± 2% in I/R to 18 ± 3%, 18 ± 2% and 17 ± 2%, respectively, ($p<0.001$versus I/R). In contrast, STAT-3$^{-/-}$ mice failed to be protected by the pharmacological agents, (p=ns).

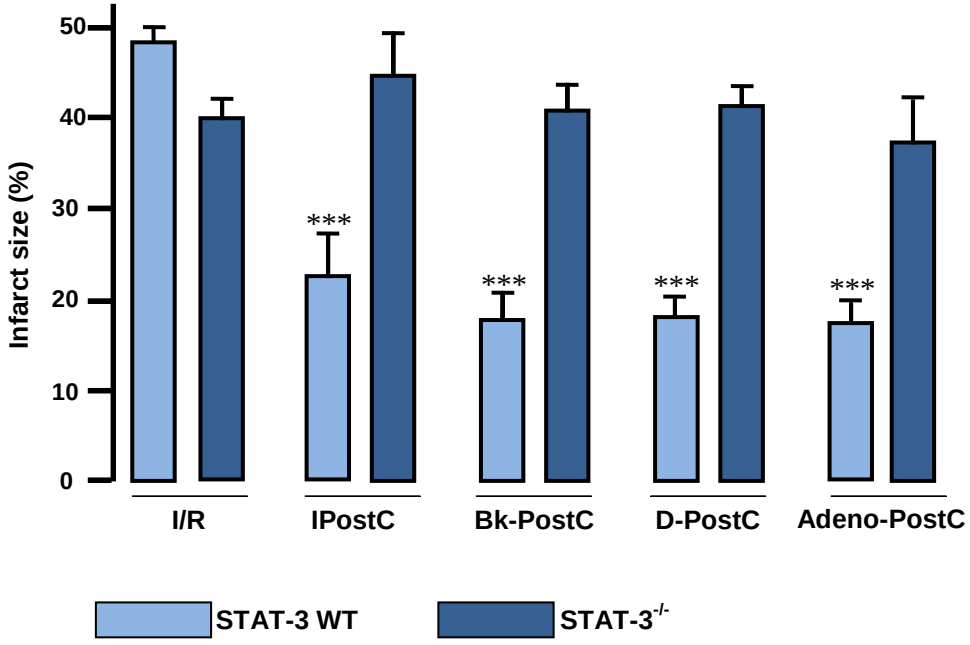


Fig 52: Pharmacological postconditioning in STAT-3-WT and STAT-3$^{-/-}$ mice. IPostC protected STAT-3-WT from I/R, ***p<0.001. Similarly, Bk-PostC, D-PostC and Adeno-PostC reduced infarct size in STAT-3-WT hearts (***p<0.001 versus I/R). In contrast, none of the postconditioning protocols protected the STAT-3$^{-/-}$ hearts against ischaemia-reperfusion injury, p=ns; n=6 per group; IPostC=ischaemic postconditioning; Bk-PostC=bradykinin postconditioning;D-PostC=DADLEpostconditioning;Adeno-PostC=adenosine postconditioning.

3.4 Western blot analysis

➢ Levels of pSTAT-3 in mitochondrial and cytosolic fractions of TNF-WT and $TNF^{-/-}$

We examined the translocation of phosphorylated STAT-3 from the cytosolic fraction to the mitochondrial fraction after pharmacological postconditioning. An increase in the ratio of mitochondrial pSTAT-3 to cytosolic pSTAT-3 (expressed in arbitrary units) was observed with Bk-PostC and D-PostC from 156 ± 4 in I/R to 220 ± 2 and 280 ± 4, respectively, ($p<0.05$). There was no significant translocation of pSTAT-3 observed when adenosine was used as a postconditioning mimetic, (p=ns), figure 53. A similar trend was observed in $TNF^{-/-}$ mice. VDAC was used as evidence for the mitochondrial fractions.

a) Mitochondrial and cytosolic fractions

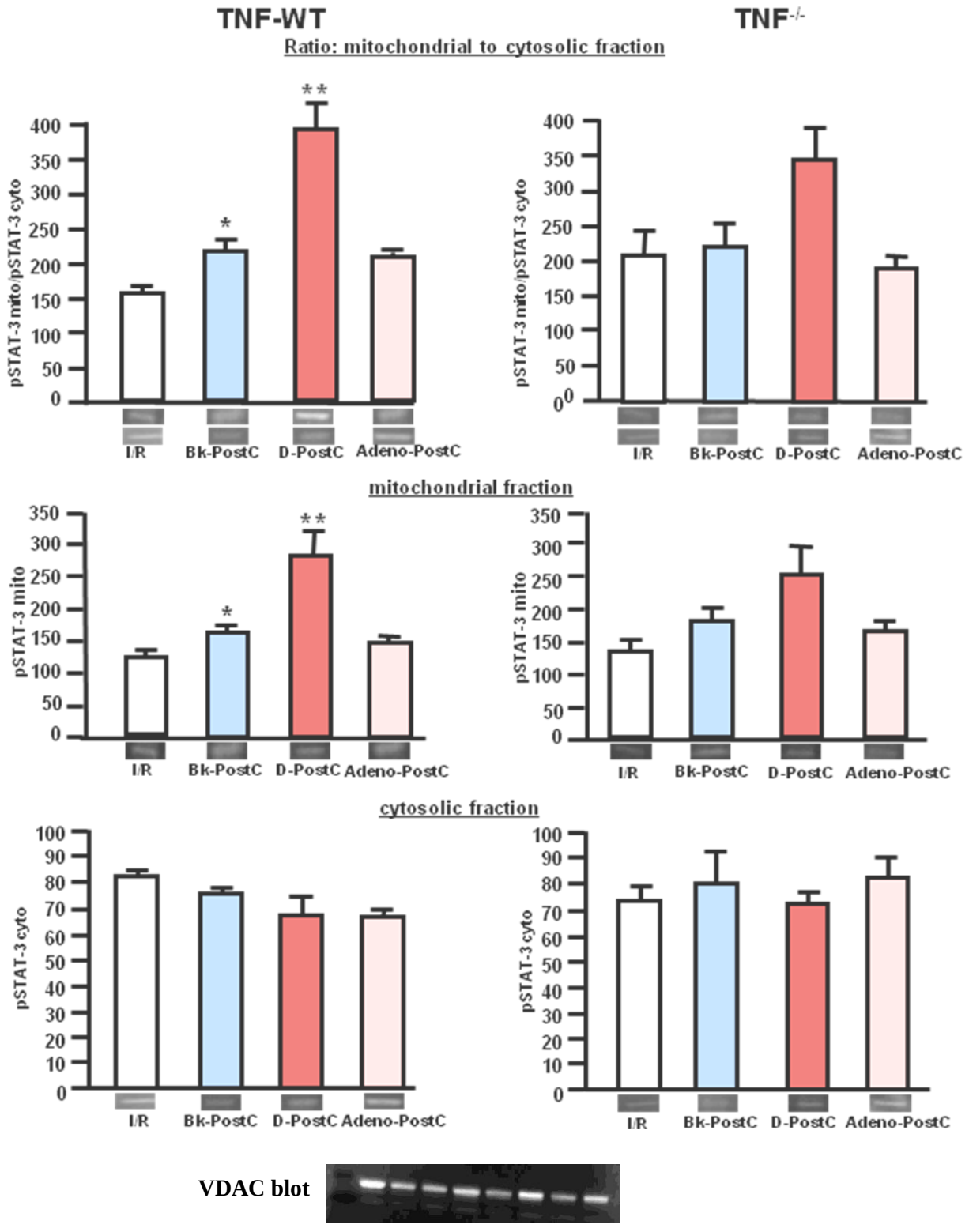


Fig 53: Phosphorylation and translocation to mitochondria of STAT-3. Bar graphs and representative western blots of mitochondrial and cytosolic fractions demonstrating the translocation of pSTAT-3, *p<0.05 vs I/R; **p<0.01 vs I/R. The VDAC blot is evidence of the mitochondrial fraction. n=4. IPostC=ischaemic postconditioning; Bk-PostC=bradykinin postconditioning; D-PostC=DADLE postconditioning; Adeno-PostC=adenosine postconditioning.

4.0 Discussion

The novel data in this section show that bradykinin and opioids, given as true postconditioning mimetics, can mimic the cardioprotective effects of IPostC via the activation TNFα binding to TNFR2, and leading to subsequent activation of JAK/STAT-3, major components of the recently discovered SAFE pathway. Adenosine, however does not require TNFα to mimic IPostC, but activates JAK/STAT-3 to confer protection. In brief, six alternating episodes of reperfusion and reperfusion with bradykinin or DADLE in the $TNF^{-/-}$ mice failed to reduce the infarct size, whereas the protective effect was still evident when adenosine was used as a postconditioning mimetic. Postconditioning with bradykinin or DADLE protected against reperfusion injury in the TNFR1 deficient, but not in TNFR2 deficient mice. In addition, $STAT\text{-}3^{-/-}$ mice failed to be protected against ischaemia-reperfusion by bradykinin, DADLE or adenosine. Surprisingly, $TNF^{-/-}$ hearts were still protected with Adeno-PostC, therefore suggesting adenosine-induced cardioprotection may occur through partial but not total activation of the SAFE pathway.

4.1 Cardioprotective role of adenosine

Endogenous adenosine is released during ischaemia and reperfusion with blood and interstitial concentrations being elevated after ischaemic insults. In emergency situations adenosine plays an important role in the cardiovascular system. For several decades, adenosine has been widely used for treatment of supraventricular arrythmias. The potential of adenosine therapy for myocardial infarction has been shown in clinical trials and it has also exhibited cardioprotective effects in preclinical models of myocardial stunning and infarction (Claeys et al., 2004; Kloner et al., 2006; Marzilli et al., 2000; Quintana et al., 2003; Ross et al., 2005). However, adenosine has a short half life in the plasma and has the unwanted side effect of lowering the blood pressure. Great progress is being made in the development of agonists and antagonists which can mimic the beneficial effect of the drug without the deleterious side effects. One such agonist is GP531, a potent second generation adenosine-regulating agent, which has a better half life than adenosine with none of the harmful side effects, such as the lowering of blood pressure (Hale and Kloner, 2010).

In contrast with our data, Penna et al failed to protect the isolated rat heart when adenosine (10μM or 30μM) was given as a postconditioning stimulus (5 cycles of 10seconds) at the onset of reperfusion (Penna et al, 2009). These data suggest that adenosine may protect as a postconditioning mimetic in a time and concentration dependent manner.

Delineating the mechanisms involved in adenosine-induced cardioprotection would certainly contribute to the development of agents which would encompass the beneficial effects of adenosine without presenting its deleterious side-effects. In the present study, we found that $TNF^{-/-}$ mice but not cardiomyocyte specific $STAT\text{-}3^{-/-}$ mice could still be protected with an adenosine postconditioning stimulus. Our data suggest that STAT-3 was not activated as a downstream target of TNFα. This is in accordance with previous work conducted in our laboratory whereby adenosine, given as a preconditioning stimulus, was able to protect against ischaemia-reperfusion in $TNF^{-/-}$ mice but failed to protect $STAT\text{-}3^{-/-}$ mice (Smith, 2002; Smith 2005). In fact, STAT-3 activation with adenosine may occur via Erk1/2 activation (Frias, 2009) or PKC activation. Hence, in a late preconditioning setting, Bolli's group demonstrated that activation of PKC contributed, at least in part, to the phosphorylation of STAT-3 (Xuan, 2005; Xuan, 2007) (figure 54).

Adenosine is one of the most widely studied endogenous signalling molecules in cardioprotection, but results found in the literature have not always proved to be consistent. Further investigation into the exact signalling mechanisms involved in ischaemia-reperfusion injury is required.

4.2 Bradykinin and opioids mimic IPostC via the SAFE pathway

Bradykinin is released in significant amounts from the ischaemic myocardium to activate protective effects and plays a role in assuaging reperfusion injury.

There is almost complete agreement that B_2 receptor activation is required for bradykinin-induced protection since icatibant, a selective and specific B_2 receptor antagonist, abolishes bradykinin-induced protection in most models (see review, (Baxter and Ebrahim, 2002)). IPostC-induced cardioprotection in isolated rat hearts was abrogated in the presence of selective inhibitors of the bradykinin B_2 receptor during intermittent bradykinin administration, therefore confirming targeting of the B_2 receptors during early reperfusion is responsible for the protective effects of bradykinin (Penna et al., 2007). The signalling mechanisms downstream of bradykinin receptors involved in the acute protective actions of bradykinin are, however, poorly understood.

Similarly, an ischaemic insult to the heart results in the synthesis and release of endogenous opioid peptides which have also been documented in postconditioning-induced cardioprotection (Kin et al., 2005b). IPostC-mediated protection in rat hearts has been reported to be abrogated by a selective antagonist of the opioid δ-receptor as well as a selective antagonist of the κ-receptor (Wang et al., 2007) suggesting that the protective effect of IPostC may be mediated by the δ and κ opioid receptors.

In our present study, intermittent bradykinin or opioid (DADLE) postconditioning at reperfusion required TNFα activation via TNFR2 to confer cardioprotection. Our novel data confirm the protective effect of bradykinin and DADLE as ischaemic postconditioning mimetics and we show for the first time that bradykinin and opioids activate TNFα via TNFR2. To our knowledge, opioids have not been given intermittently as postconditioning mimetics, but only towards the end of ischaemia and/or during reperfusion. Both bradykinin and opioids are known to activate the RISK pathway when given at reperfusion (Gross and Gross, 2006; Penna et al., 2007). Our present data show that both agents can activate the SAFE pathway as a RISK-free signalling mechanism, therefore confirming the existence of multiple prosurvival pathways.

4.3 STAT-3 in mitochondria

STAT-3 is a transcription factor but it is unlikely to act as one as the effects seen in our experimental conditions occur too rapidly. A new role for serine STAT-3 by its translocation to the mitochondria was suggested by Wegrzyn et al, (Wegrzyn *et al*., 2009). This group provided evidence that STAT-3 is required for optimal function of the mitochondrial electron transport chain, which may permit STAT-3 to facilitate responses to cellular homeostasis. Our western blot analysis demonstrates that postconditioning with bradykinin or DADLE increases the ratio of phosphorylated STAT-3 in the mitochondrial fraction to that of the STAT-3 in the cytosolic fraction, therefore suggesting the involvement of mitochondrial STAT-3 in the signalling pathway of these cardioprotective agents. However, although adenosine protection required STAT-3 signalling, it did not stimulate its translocation to the mitochondria, therefore suggesting an additional mechanism of action for STAT-3 which may be linked to its activation in the cytosol (phosphorylation at the serine site) or translocation to the nucleus.

In conclusion, our data provide new evidence that bradykinin and opioids as postconditioning mimetics activate STAT-3 via TNFα and TNFR2 to confer cardioprotection (SAFE pathway). Considering the low levels of STAT-3 found in the mitochondria (Wegrzyn *et al*, 2009), it is appropriate to suggest that the role of STAT-3 in the signalling cascade may be attributed to a "kinase-like" activity. Overall, the existence of multiple alternate pathways in ischaemic and pharmacological postconditioning at the time of reperfusion may have potential therapeutic application against ischaemia-reperfusion injury. However, further studies will be required to investigate the exact prosurvival role of STAT-3 within the mitochondria

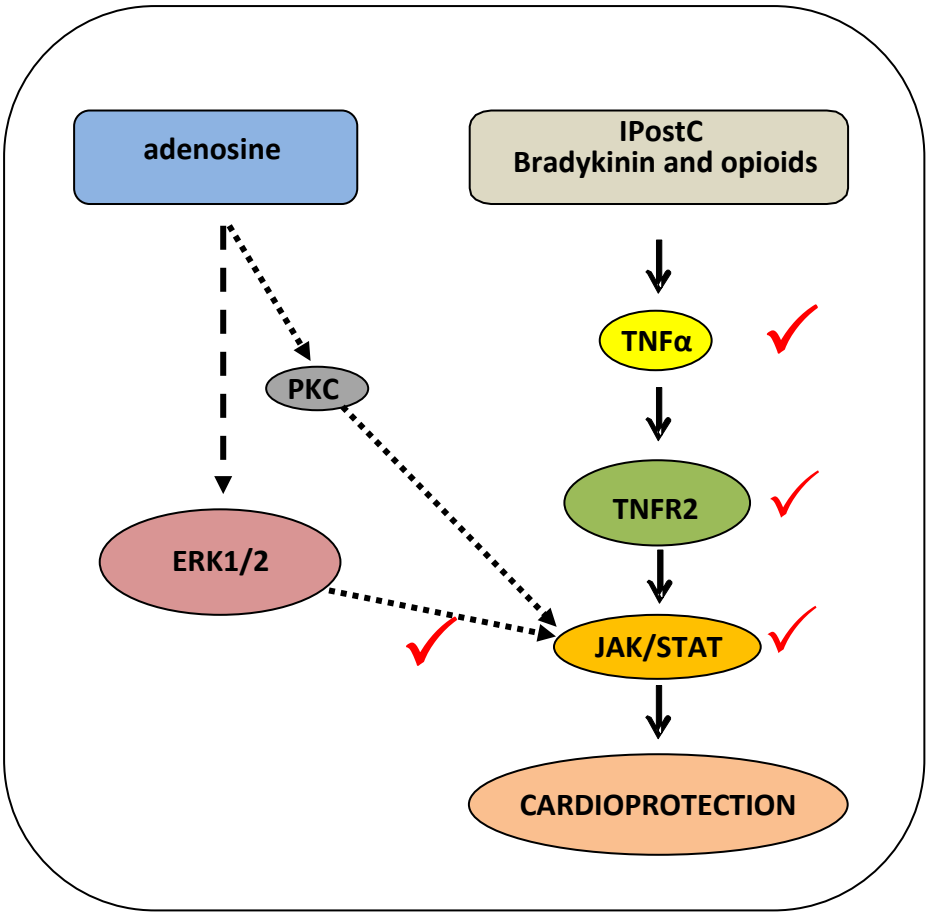


Fig 54: Schematic of our recent data. Activation of TNFα via TNFR2 by bradykinin and DADLE initiates the phosphorylation of JAK/STAT and subsequent cardioprotection. Adenosine makes partial use of the SAFE pathway via STAT-3 signalling but does not require TNFα.

F. ISCHAEMIC POSTCONDITIONING, OBESITY & DIABETES

1.0 Introduction

There is an increasing awareness that both obesity and diabetes are not only growing epidemics but also pose a serious risk factor for cardiovascular disease, in particular myocardial infarction. Prolonged exposure to environmental factors such as high fat or high carbohydrate diets and physical inactivity leads to the development of overweight and resultant obesity and diabetes (MacDonald, 1998).

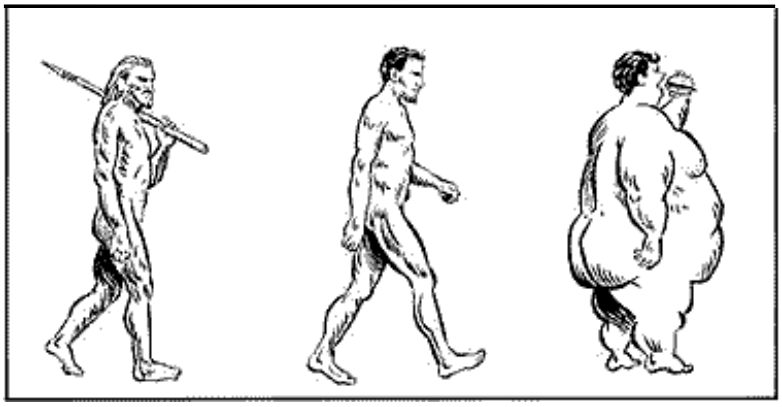


Fig 55: The evolution of man to obesity and diabetes. Fast food and lack of exercise can lead to obesity and diabetes, (adapted from www.evolutionminute.com).

Interestingly, obesity can also be considered as an inflammatory disorder, with TNFα overexpressed in adipose and muscle tissue of obese humans (Hotamisligil et al., 1995; Kern et al., 1995; Krogh-Madsen et al., 2006; Saghizadeh et al., 1996). Nearly all animal models of obesity and insulin resistance seem to produce higher levels of TNFα mRNA and protein compared to their lean counterparts (Hamann et al., 1995; Hofmann et al., 1994; Hotamisligil et al., 1994a; Hotamisligil et al., 1996). A model of obesity in TNFα-deficient mice suggested that the absence of TNFα limits the development of dietary obesity and these mice had significantly improved insulin sensitivity in both diet-induced obesity and in the ob/ob mouse model compared to their wildtype counterparts (Uysal et al., 1997). These data suggested that TNFα facilitates the development of insulin resistance associated with obesity and diabetes and it is further suggested that this effect is mediated via TNFR1 (Uysal et al., 1998).

Animal studies suggest that the ischaemic tolerance is attenuated with obesity, cholesterol and elevated glucose levels (Aasum et al., 2008; Ferdinandy., 2007). In Zucker diabetic fatty and lean Goto-Kakizaki type 2 diabetic rats, ischaemic preconditioning failed to protect the isolated hearts from ischaemia-reperfusion injury (Kristiansen et al., 2004). Similarly, Zucker obese rats subjected to ischaemia-reperfusion failed to be protected with pharmacological preconditioning (Katakam et al., 2007). The outcome of postconditioning in obese and diabetic patients is

uncertain as the recent application of ischaemic postconditioning in clinical studies has excluded patients who were diabetic. Therefore, the aim of our study was to evaluate:

1) whether obesity or diabetes would alter the protective outcome of ischaemic postconditioning and
2) whether TNFα may alter the cardiovascular risk of obese or diabetic mice subjected to ischaemia-reperfusion and postconditioning, (Figure 56).

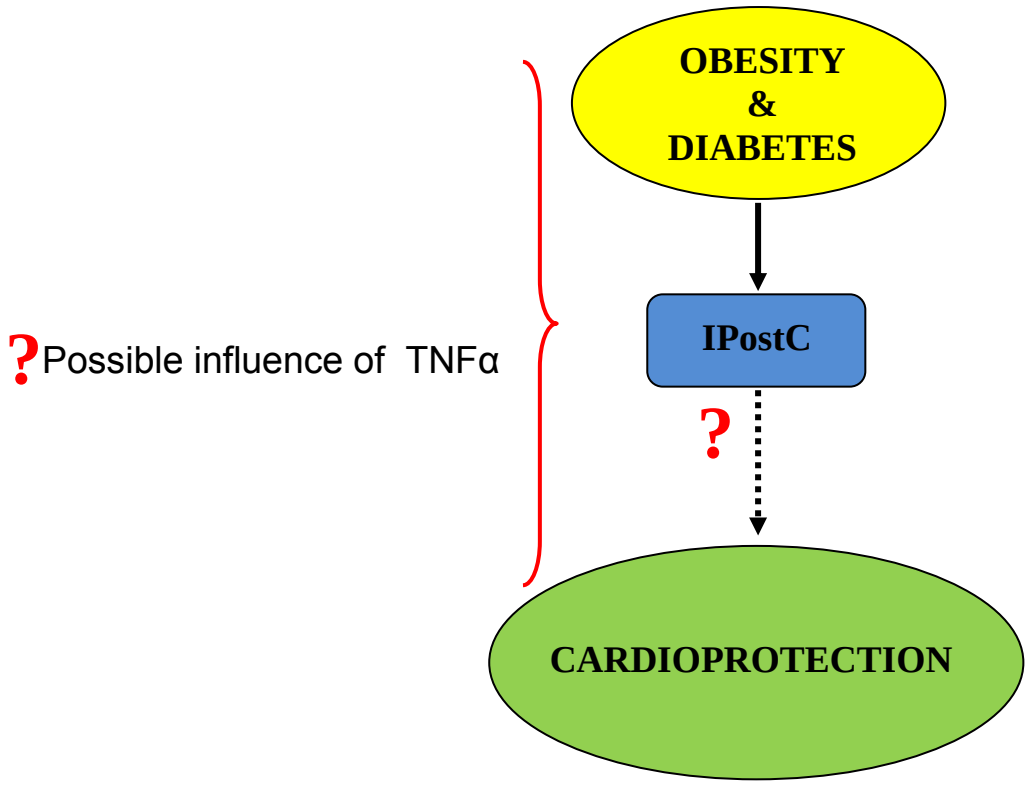


Fig 56: Influence of obesity and diabetes on the outcome of ischaemic postconditioning: does TNFα contribute to this effect?

To investigate our aims we will use TNF-WT and $TNF^{-/-}$ mice.

1) A high carbohydrate diet will be fed to the mice for a total of 11 weeks, commencing upon weaning (at 3 weeks of age) to create an obese murine model.
2) Diabetes will be induced with a single streptozotocin injection administered intraperitoneally.
3) Isolated mouse hearts from the obese and diabetic animals will be subjected to ischaemia-reperfusion and an ischaemic postconditioning protocol as outlined in Section C.

4) Physiological parameters, such as glucose tolerance test, blood glucose, plasma insulin and plasma leptin will be analysed.

2.0 Methods

2.1 All animal studies performed were approved by the Animal Research Ethics Committee of the University of Cape Town, and followed the recommendations laid down in the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85 {23}, revised 1996). The total number of animals used in this study was 24.

2.2 High carbohydrate diet (HCD) mouse model

For an obese murine model, male mice were removed from the animal unit at weaning (3 weeks of age) and placed in our facility. TNF-WT and $TNF^{-/-}$ were separated into 2 groups each. One group of mice received a normal diet (ND) of mouse chow and the other group received a diet containing elevated carbohydrates and fats that resembles a Western-type diet (HCD), for 11 weeks, (figure 57). The high carbohydrate diet compared to normal chow, provided energy in the form of carbohydrate, protein and fats, as illustrated in the table below (Aasum et al., 2008; du Toit et al., 2008).

	Carbohydrates (%)	Protein (%)	Fats (%)
Normal diet (ND)	60	30	10
High carbohydrate diet (HCD)	69	17	14

Table 1: Energy provided by high carbohydrate diet (HCD) versus normal chow (ND).

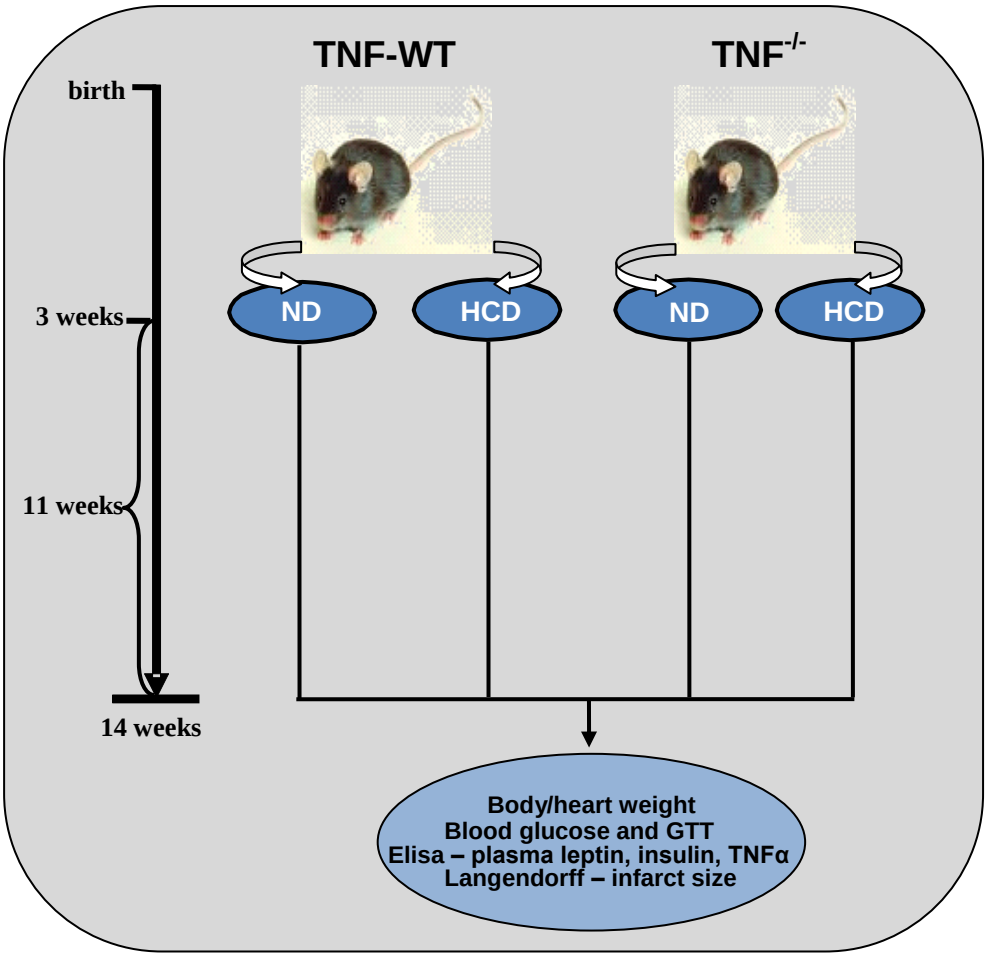


Fig 57: Creation of obese mouse model in TNF-WT and TNF$^{-/-}$ mice.
ND = normal mouse chow; HCD = high carbohydrate diet.

2.3 Streptozotocin diabetic mouse model (STZ)

Experimental diabetes type 1 was induced in a total of 10 mice per group by a single ip injection of 180 mg streptozotocin (STZ)/kg body weight, dissolved in 0.1 M citrate buffer (Daniel et al., 2007). Non-diabetic control animals were treated with solvent (citrate buffer) alone. Standard mouse chow and tap water were provided ad libitum for all groups. At the end of each time period, only STZ-treated mice with a blood glucose level greater than 16 mmol/l were considered as diabetic (normal blood glucose levels in mice range from 3.4 to 9.7 mmol/l). Of the 10 STZ-treated TNF-WT mice, 7 met the criteria, 2 did not achieve sufficiently high glucose levels, and 1 died shortly after receiving the STZ. No death occurred in the 10 non-diabetic control animals in each group.

2.4 Blood glucose and glucose tolerance test

Blood glucose and the glucose tolerance test (GTT) were done at 14 weeks. Mice were fed normal chow or HCD as described in Table 1. Prior to performing the blood glucose tolerance test, the mice were fasted for 7 hours, but had free access to drinking water. A 20% solution (w/v) of glucose was made up in sterile distilled water. Each mouse was then sedated with a mixture of ketamine (75mg/kg) and medetomidine (0,5mg/kg) given intraperitoneally. Sufficient depth of anaesthesia was determined by pedal reflex. Body weight was recorded for each mouse, a tail cut was done and blood glucose determined in mmol/L by means of a blood glucose monitor (Accu-Chek Active; Roche Diagnostics, Mannheim, Germany) as per the manufacturer's instructions. The Accu-Chek Active allows very small volumes of blood (1-2 μl) to be accurately assessed. A bolus of glucose was given ip (15 mg/kg) and the time of the injection noted. Thereafter, a blood glucose measurement was performed every 30 min post injection, until a decrease in the glucose level was observed (120 to 150 min).

2.5 Perfusion of mouse hearts

The HCD fed mice (14 weeks of age), the STZ-treated mice (5 and 10 days post STZ treatment) and their respective controls were anaesthetized (pentabarbitone, 60 mg/kg ip) and heparinized (25 IU ip) to prevent the blood clotting. Once an adequate level of anaesthesia had been achieved (tested by lack of pedal reflex), body weight was recorded and the hearts were isolated and perfused retrogradely as described in Section C. At the same time, blood was taken from the thoracic cavity of each mouse and placed in a chilled heparinized tube for plasma collection.

2.6 Ischaemic postconditioning

HCD fed mice and STZ-treated mice were subjected to the ischaemic postconditioning (IPostC) protocol which consisted of six alternating cycles of 10 seconds reperfusion, 10 seconds ischaemia, commencing at the onset of reperfusion as described in detail in Section C.

2.7 Ratio of heart weight to body weight

At the end of the perfusion protocol, each heart was carefully dried and weighed after staining with TTC. The heart weight to body weight ratio for each mouse was then calculated.

2.8 Insulin levels

The blood samples were centrifuged at 5000 rpm for 5 min at 4°C and the plasma was removed and frozen until required. Quantitative determination of baseline insulin levels were evaluated using the Ultra Sensitive Mouse Insulin Elisa Kit (Crystal Chem Inc; USA) as per the manufacturer's instructions.

2.9 Leptin levels

For analysis of leptin levels, blood samples were centrifuged at 5000 rpm for 5 min at 4°C and the plasma was removed and frozen until required. Quantitative determination of baseline leptin levels were evaluated using the Ultra Sensitive Mouse Leptin Elisa Kit (Crystal Chem Inc; USA) as per the manufacturer's instructions.

2.10 Chemicals and Pharmacological Agents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemicals, Germany.

2.11 Statistical Analysis

Data are presented as mean ± SEM. Comparisons between multiple groups were performed by 1-way ANOVA followed by Tukey post hoc test or Bonferroni multiple comparison test (GraphPad Instat). Two-way ANOVA followed by Bonferroni multiple comparison test (GraphPad Prism) was performed where species or diet differed. $p<0.05$ was considered to be statistically significant.

3.0 Results

3.1 Effect of high carbohydrate diet on physiological parameters in TNF-WT mice

HCD increased the body weight significantly in the TNF-WT mice from 29.0 ± 1.0 grams to 32 ± 0.7 grams, ($p<0.05$ vs ND; figure 58A), but the heart weight to body weight ratio remained unchanged between ND and HCD fed mice, (p=ns, figure 58B). Although HCD did not affect baseline blood glucose levels, (p=ns, figure 58C), it improved glucose tolerance in the WT animals , ($p<0.05$ vs ND, figure 58D), and increased plasma insulin and leptin levels to 0.64 ± 0.06 ng/ml from 0.46 ± 0.03 ng/ml in ND, ($p<0.05$), and to 10.6 ± 0.9 ng/ml from 4.5 ± 0.4 ng/ml in ND, ($p<0.001$, respectively, figure 58E and figure 58F).

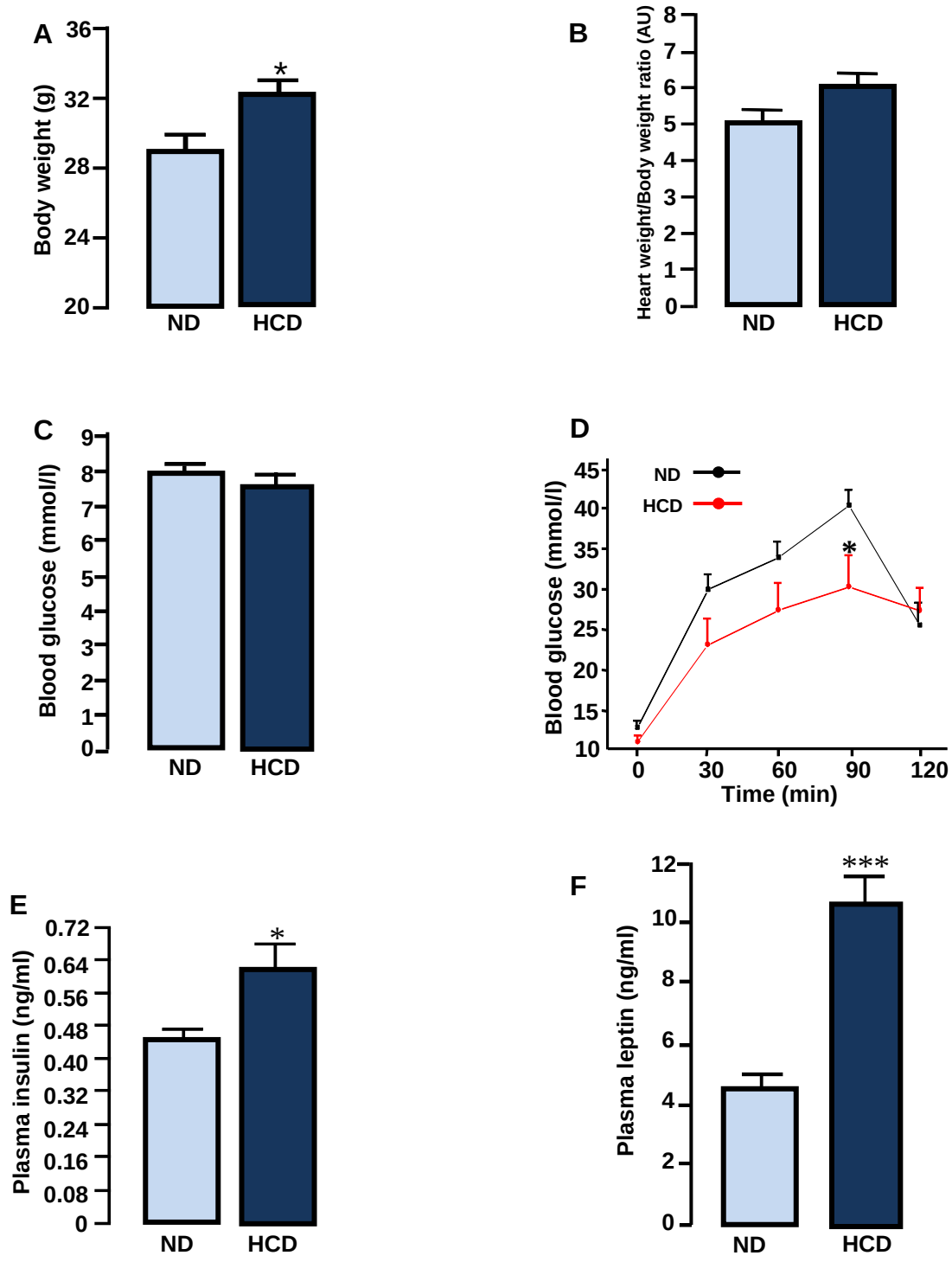


Fig 58: Effects of HCD on physiological parameters in TNF-WT mice. Body weight, plasma insulin and plasma leptin levels were significantly increased by HCD, but no significant difference was observed in heart to body weight ratio or blood glucose levels, p=ns; *p<0.05; ***p<0.001 vs ND; n≥6 for all groups.

3.2 Effect of high carbohydrate diet on IPostC-induced cardioprotection in TNF-WT hearts

To determine whether obesity can affect the outcome of IPostC, the isolated hearts of mice fed with either a normal diet or a HCD were subjected to the IPostC protocol with 6 x 10 second cycles of alternating reperfusion and ischaemia. HCD mice subjected to I/R presented a similar infarct size compared to mice fed with ND (p=ns). Furthermore, IPostC conferred protection to a similar extent in HCD mice compared with ND mice versus their respective ischaemia reperfusion control groups (p<0.001), (Figure 59).

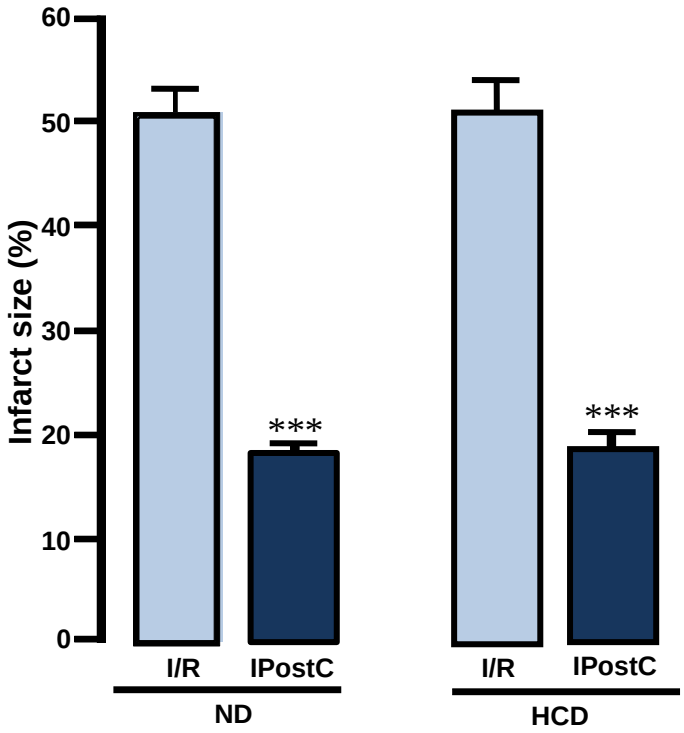


Fig 59: Effect of HCD on IPostC-induced cardioprotection in isolated TNF-WT hearts. IPostC in TNF-WT (ND) reduced infarct size from 50 ± 2% in the ischaemic control group (I/R) to 18 ± 0.5% for IPostC. The infarct size in TNF-WT (HCD) was decreased to 18.5 ± 1.6% versus 50 ± 2% for the I/R group, ***p<0.001 vs I/R; n≥6.

3.3 Effect of high carbohydrate diet on physiological parameters in $TNF^{-/-}$ mice

Mice in which endogenous TNFα is not produced were used to investigate the role of TNFα in obesity. $TNF^{-/-}$ mice were fed either the normal diet or the HCD and physiological parameters were assessed 11 weeks later. Similar to TNF-WT mice, body weight was increased with HCD from 26 ± 0.4 grams to 31.5 ± 0.5 grams, ($p<0.05$ versus ND, figure 60A). However, $TNF^{-/-}$ mice on the HCD had a significantly lower heart weight to body weight ratio than their ND counterparts, ($p<0.05$ versus ND, figure 60B). Baseline blood glucose and tolerance of HCD-fed TNF-deficient mice to glucose remained unchanged by HCD, (p=ns; figure 60C and 60D). Plasma insulin levels were increased significantly by HCD from 0.41 ± 0.02 ng/ml to 0.57 ± 0.01 ng/ml, ($p<0.05$ versus ND, figure 60E). Similarly, the plasma leptin levels were significantly increased by the HCD from1.3 ± 0.08 ng/ml to 11.2 ± 0.8 ng/ml, ($p<0.05$ versus ND, figure 60F).

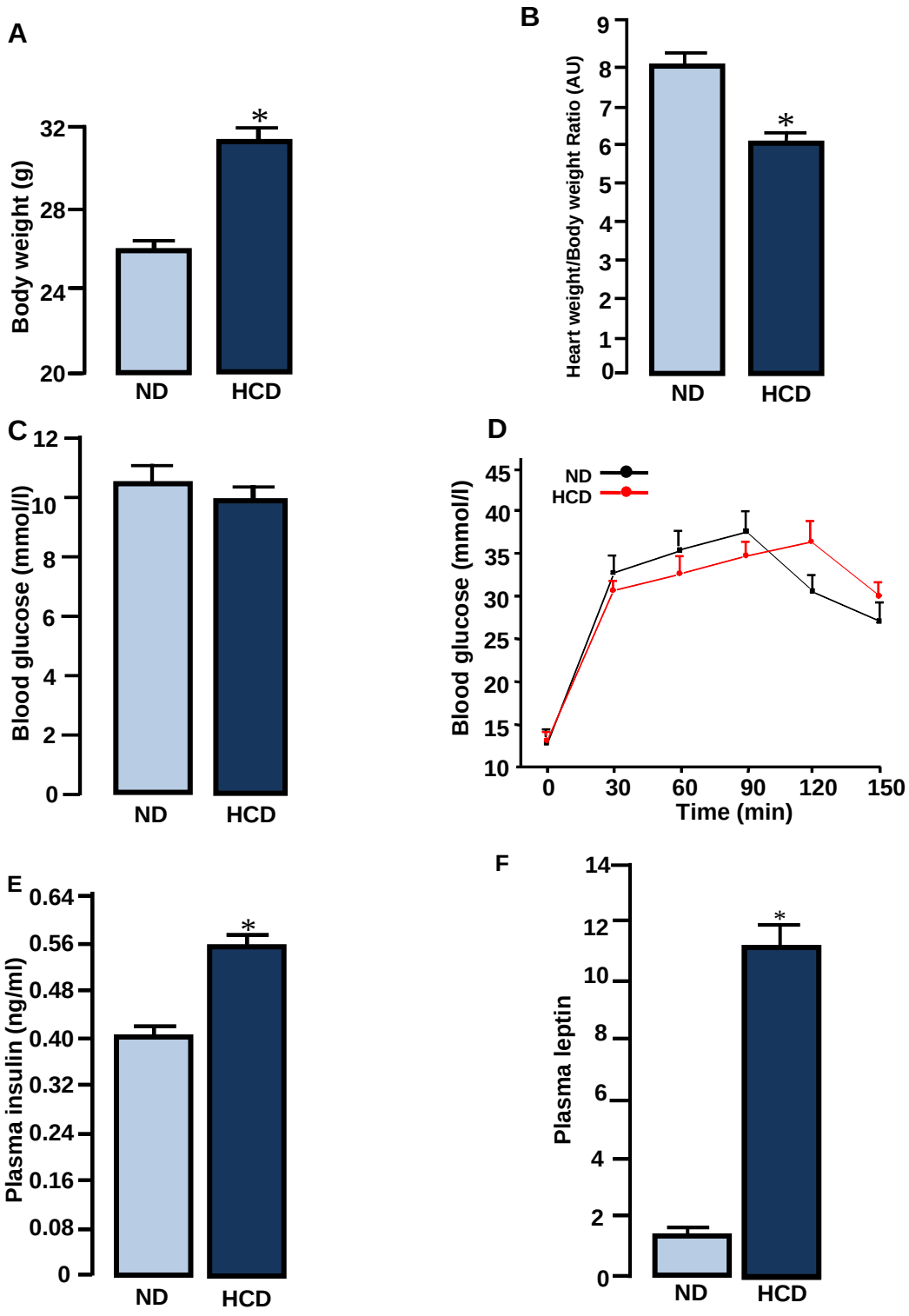


Fig 60: Effects of HCD on physiological parameters of TNF$^{-/-}$ mice. Body weight, plasma insulin and plasma leptin levels were significantly increased by HCD, but blood glucose levels remained unchanged, p=ns. However, heart weight to body weight ratio was partially decreased by the HCD, *p<0.05 vs ND; n=6 for all groups.

3.4 Effect of high carbohydrate diet on IPostC-induced cardioprotection of TNF$^{-/-}$ hearts

To determine whether absence of TNFα in obesity can affect the outcome of IPostC , the isolated hearts of TNF$^{-/-}$ mice fed with either a normal diet or a HCD were subjected to the IPostC protocol as described in Section C. HCD mice subjected to I/R presented a similar infarct size compared to mice fed with ND (p=ns). Surprisingly, the hearts from TNF$^{-/-}$ mice fed with HCD demonstrated a partial reduction in infarct size versus the I/R control, ($p<0.05$; figure 61).

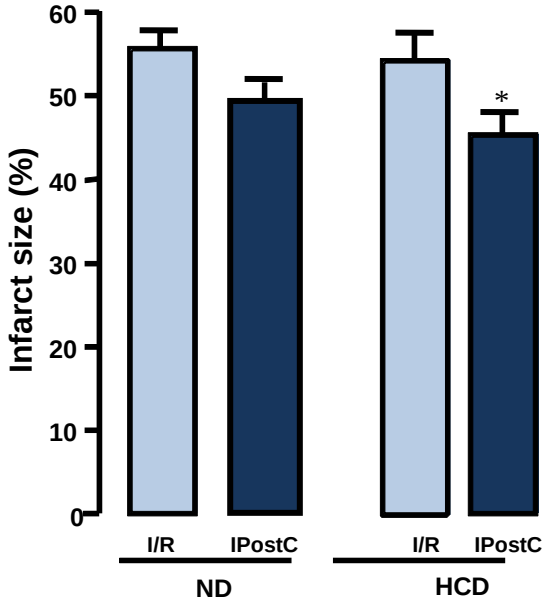


Fig 61: HCD restores cardioprotection in TNF$^{-/-}$ mice. IPostC reduced infarct size in the HCD fed mice from 56 ± 1.8% to 45 ± 4.2% whereas mice fed the normal diet could not be protected, *$p<0.05$ vs I/R; $n\geq6$.

3.5 Effects of diabetes on physiological parameters in TNF-WT mice

To create a type 1 diabetic model, TNF-WT mice were given a single intraperitoneal injection of streptozotocin (180 mg/kg body weight). Physiological parameters and experiments were performed either 5 days or 10 days post STZ administration. TNF-WT had a significant decrease in body weight 5 days post treatment, from 29.7 ± 1.3 grams to 21.0 ± 2.6 grams ($p<0.001$ versus no STZ). However, the weight was restored by day 10, (p=ns versus no STZ; figure 62A). There was no change in the heart weight to body weight ratio (p=ns; figure 62B). Streptozotocin injection increased baseline blood glucose at day 5 and 10, ($p<0.01$ vs no STZ; figure 62C), but decreased plasma insulin from 1.02 ± 0.2 ng/ml to 0.53 ± 0.14 ng/ml (5 days post STZ) and to 0.36 ± 0.02 ng/ml (10 days post STZ), ($p<0.05$ vs no STZ-treatment; figure 62D). Similar results for glucose and insulin after streptozotocin treatment have been reported (Nakamura et al., 2006). Leptin levels were also reduced by STZ at day 5 and 10 after injection from 4.5 ± 0.7 ng/ml to 0.6 ± 0.2 ng/ml and 0.23 ± 0.04 ng/ml, respectively, ($p<0.001$ vs no STZ treatment; figure 62E)

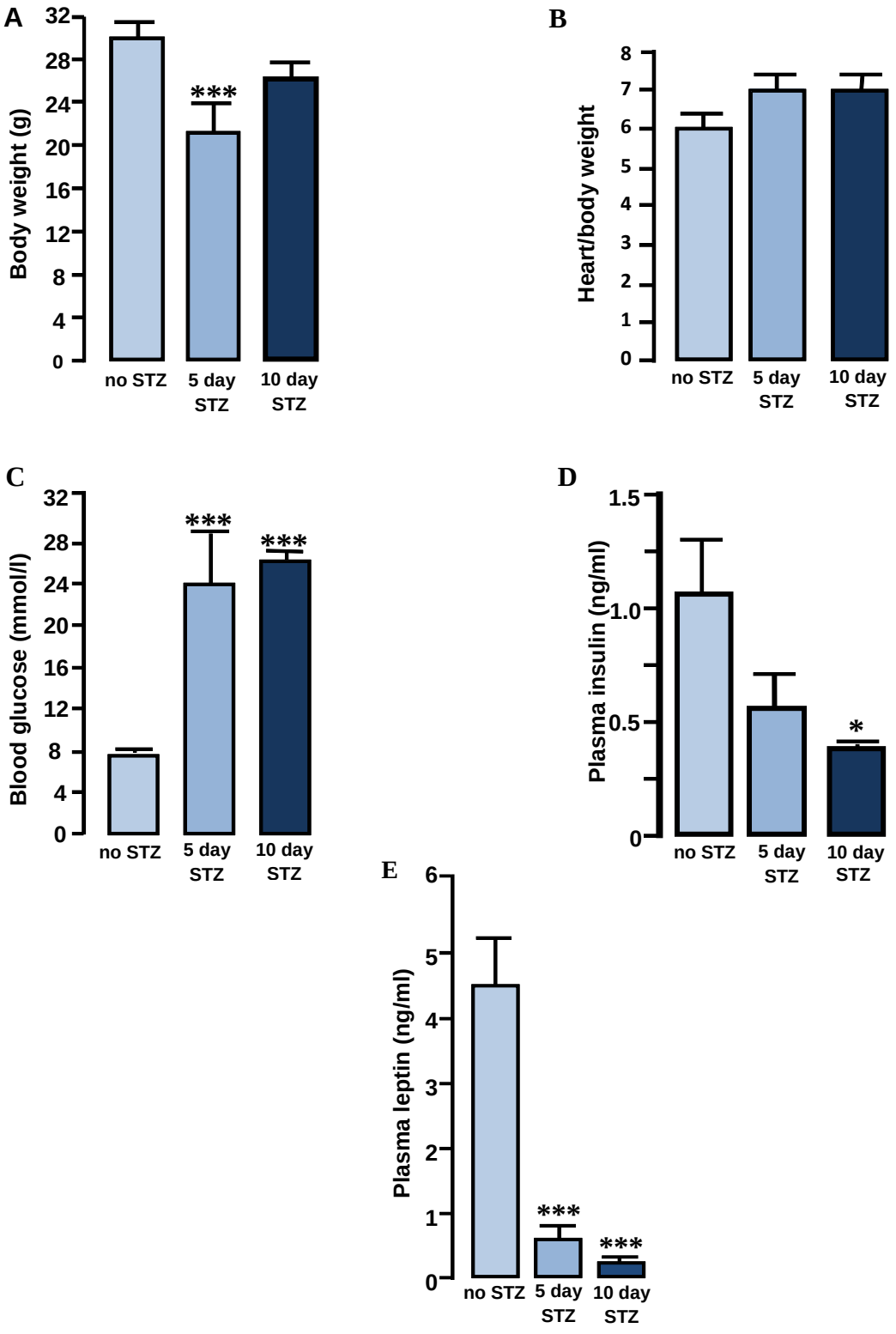


Fig 62: Effects of diabetes on physiological parameters in STZ-treated TNF-WT mice. Body weight was decreased 5 days post STZ treatment, ***p<0.001 vs untreated controls and returned to normal at day 10, p=ns. Heart weight to body weight ratio remained unchanged at both time points, p=ns. Insulin and leptin levels were both decreased, respectively. STZ increased baseline blood glucose at days 5 and 10, *p<0.05 and ***p<0.01, vs no STZ,

3.6 Effect of diabetes on IPostC-induced cardioprotection in TNF-WT hearts

Isolated hearts from TNF-WT were subjected to the IPostC protocol. STZ-treated TNF-WT mice subjected to I/R showed a similar infarct size to the untreated I/R control, at both time points, ($p<0.001$ vs I/R). STZ-treatment did not affect the cardioprotective effect of IPostC after 5 or 10 days versus the untreated animals, ($p<0.001$)

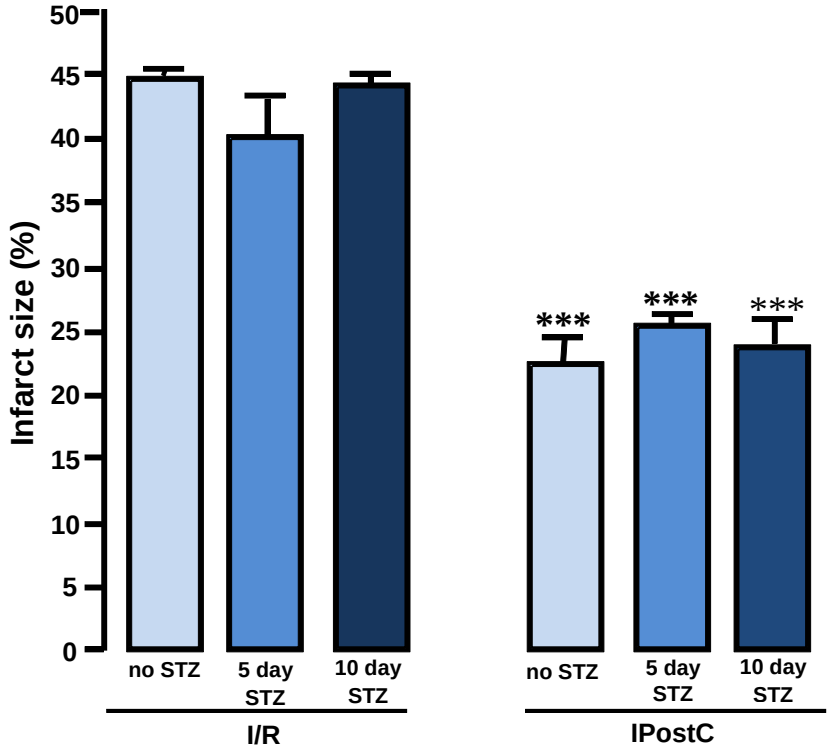


Fig 63: IPostC protects diabetic TNF-WT hearts. Infarct size was significantly decreased by IPostC in untreated animals, ***$p<0.001$ vs I/R. Similarly, IPostC reduced infarct size from 40 ± 3% to 25.3 ± 0.5% at 5 days and from 44 ± 1% to 23.6 ± 1.8% at 10 days in the STZ-treated mice, ***$p<0.001$ vs respective I/R controls; n=6.

3.7 Effect of diabetes on physiological parameters in TNF$^{-/-}$ mice

To investigate whether TNFα plays a role in type 1 diabetes, TNFα-deficient mice were injected intraperitoneally with a single dose of streptozotocin (180 mg/kg body weight). Similar to TNF-WT mice, STZ administration had no effect on body weight, 5 or 10 days after STZ treatment (p=ns, figure 64A). However, the heart weight to body weight ratio was significantly increased by the STZ after 10days, ($p<0.01$ vs untreated, figure 64B). As expected, STZ increased baseline blood glucose at both time points ($p<0.001$ vs untreated, figure 64C). Although no significant difference was seen in plasma insulin levels at 5 days post STZ treatment, there was a significant increase 10 days after treatment, from 0.32 ± 0.1 ng/ml to 1.06 ±0.3 ng/ml, ($p<0.01$ versus untreated control, figure 64D). The diabetic TNF$^{-/-}$ animals also demonstrated significantly elevated levels of plasma leptin at 5 days post STZ administration, from 2.3 ± 0.1 ng/ml to 3.0 ± 0.4 ($p<0.001$ versus untreated control), but the levels were drastically reduced in the 10 day post treatment group to 0.3 ± 0.08 ng/ml, ($p<0.05$ versus untreated control group, figure 64E)

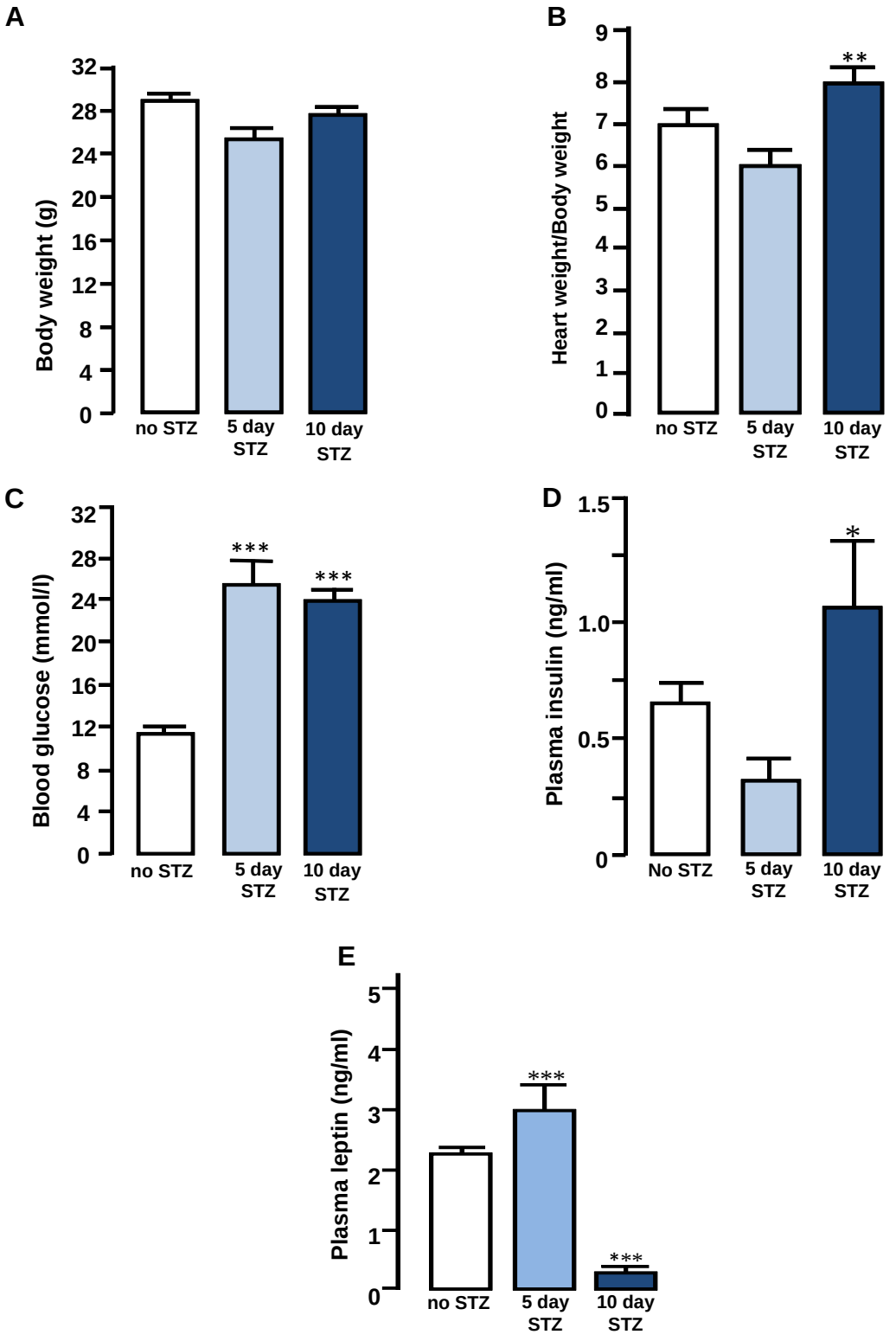


Fig 64: Effects of diabetes on physiological parameters in STZ- treated TNF$^{-/-}$ mice. Body weight remained unchanged by STZ-treatment. STZ significantly increased the heart to body weight ratio and plasma insulin levels after 10 days. Blood glucose was increased at both timepoints. Plasma leptin was significantly increased at day 5 but decreased below baseline at day 10, *p<0.05, **p<0.01, ***p<0.001vs no STZ; n=6.

3.8 Effect of diabetes on IPostC-induced cardioprotection in TNF$^{-/-}$ hearts

Hearts were isolated from the two groups of STZ-treated mice at the appropriate time point. The hearts were subjected to the IPostC protocol of 6 x 10 sec cycles of alternating reperfusion and ischaemia. IPostC could not protect the hearts of STZ-treated animals, (p=ns vs I/R).

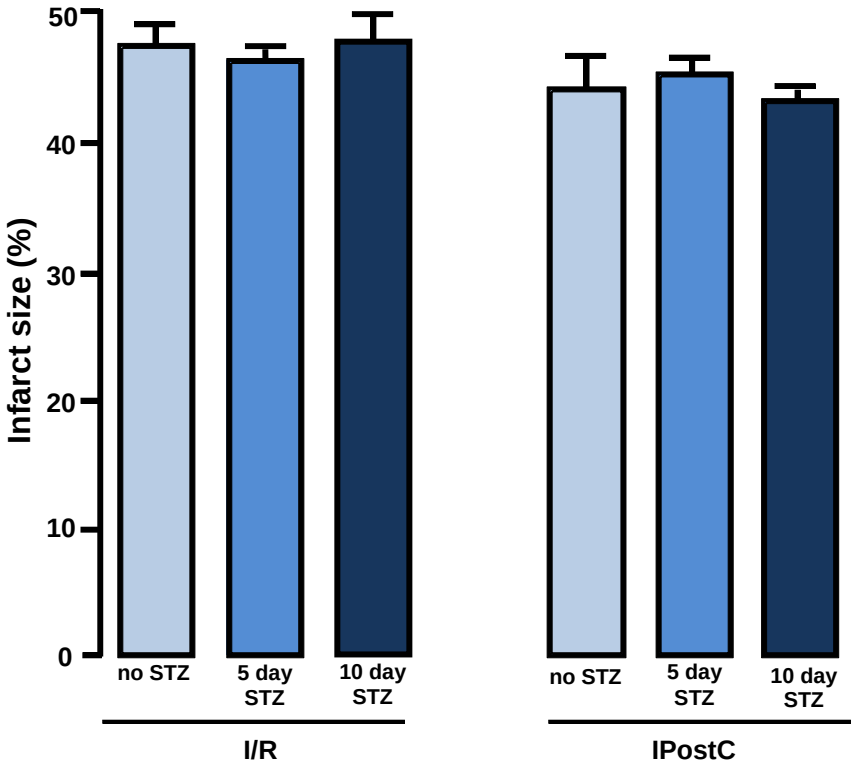


Fig 65: STZ-induced diabetes in TNF$^{-/-}$ mice abrogates the cardioprotective effect of IPostC. Ischaemic postconditioning failed to protect hearts from TNF$^{-/-}$ mice after both 5 and 10 day STZ-treatment, p=ns, n=6.

4.0 Discussion

As most of the patients hospitalized with myocardial infarction are obese or diabetic, it is critical to investigate whether IPostC can be considered as a successful therapy for these patients. Ischaemic heart disease accounts for more than 50% of deaths in diabetic patients (Kannel and McGee, 1979), therefore it is important to explore the cardioprotective signalling mechanisms involved in diabetes associated with ischaemia-reperfusion injury.

Our data revealed that 11 weeks of a high carbohydrate diet or the administration of a single intraperitoneal injection of streptozotocin resulted in a modest model of obesity or diabetes as demonstrated by changes in body weight, blood glucose levels, plasma insulin and plasma leptin levels. IPostC-induced cardioprotection was evident in the TNF-WT with either obesity or streptozotocin-induced diabetes, whereas in the absence of TNFα no protection occurred with the IPostC stimulus in the diabetic mice. Suprisingly, partial restoration of the cardioprotective effect was seen in the $TNF^{-/-}$ mice fed with the HCD.

4.1 Obesity/diabetes and susceptibility to ischaemia-reperfusion

The HCD used in our study is of a similar composition to the conventional Western-type diet of humans and was chosen to represent a moderately obese phenotype (Aasum et al., 2008), unlike the more severe obese models of either ob/ob mice or the db/db mice which are either leptin deficient or have no leptin receptors or the rat models with severe obesity or diabetes. Despite the possible benefits of a low carbohydrate diet, the possibility exists that there are potential adverse effects in those with ischaemic heart disease. A diet low in carbohydrates as commonly used to reduce weight, has been demonstrated to affect myocardial energy substrates, insulin signalling and increased myocardial injury following ischaemia-reperfusion injury in the isolated rat heart (Wang et al., 2008).

Obesity is associated with hyperinsulinaemia which markedly modulates the extent to which myocardial injury occurs during ischaemia-reperfusion (Jonassen, 2000; Jonassen, 2001; Zhang, 2004). Therefore, it is plausible to suggest that in obesity, the impact of high levels of circulating insulin on myocardial injury during ischaemia and reperfusion could over-shadow myocardial susceptibility to ischaemia-reperfusion injury.

Diabetes is associated with an increased mortality and reduced myocardial tolerance to ischaemia (Aronson, 1997; Aasum, 2003) and myocardial ischaemic injury has been shown *in vivo* in animal and human studies to be aggravated by diabetes and insulin resistance (Huang, 2009). However, a wide range of results, ranging from no change, increased or decreased

sensitivity to ischaemia with or without reperfusion injury has been demonstrated in several experimental studies conducted in diabetic animal models (Forrat, 1993; Liu, 1993; Vogel, 1988; Ferdinandy, 2007; Boudina, 2007).

In our obese and diabetic models, the detrimental effect of ischaemia-reperfusion on infarct size was unchanged in the TNF-WT and the $TNF^{-/-}$ mice.

4.2 Obesity/diabetes susceptibility to IPC and IPostC

A study demonstrating the absence of protection in the heart after an IPC stimulus was conducted using rat models which have severe forms of diabetes and obesity (Zucker diabetic fatty rats and the Goto-Kakizaki type 2 diabetic rat) (Kristiansen et al., 2004). Other scientists have reported attenuation of IPC-induced protection in the diabetic hearts of rats and dogs (Tosaki, 1996; Kersten, 2000; Ravingerova, 2000). In contrast, several experimental studies have reported that the streptozotocin-induced diabetic heart is protected by IPC (Liu, 1993; Tatsumi et al, 1998; Thirunavukkarasu, 2007).

The majority of experimental investigations on postconditioning-induced cardioprotection have been done using healthy, young animals and there is a scarcity of studies exploring the role of IPostC in the presence of associated risk factors such as obesity and diabetes. Our data demonstrate that obesity, induced by a high carbohydrate diet or streptozotocin-induced diabetes did not affect the cardioprotective effect of IPostC in the wildtype animals.

Failure of IPostC to limit infarct size was reported from a study conducted in ob/ob mice, which are leptin deficient (Bouhidel et al., 2008). However, a limitation of this study was the lack of leptin in this mouse strain. In contrast to Bouhidel's study, we found that in our model of modest obesity infarct size was decreased and this was accompanied by an increase in plasma insulin and leptin levels, suggesting a cardioprotective role for these two hormones in obesity.

Postconditioning in the diabetic animal model has also not been thoroughly studied but data from experiments conducted in Goto-Kakizaki rats suggested that to protect the diabetic myocardium, the IPC stimulus needed to be increased in order to achieve the necessary threshold for cardioprotection (Hausenloy, 2006b; Tsang, 2005). A very recent study in a murine model of streptozotocin-induced diabetes (using a similar dose to ours) reported a loss of efficacy in IPostC-induced cardioprotection (Przyklenk, 2010). Possible explanations for the contradiction between this study's findings and the findings of our study are: 1) the insulin levels of the mice in Przyklenk's study were significantly lower (0.18 ± 0.08 ng/ml) than the insulin levels found in our diabetic mice (0.36 ± 0.02 ng/ml); 2) the difference in mouse species; and 3) the number of

I/R cycles performed to postcondition the heart. The disparity, therefore, in IPC and IPostC experimental studies conducted in obese and diabetic animals may possibly be due to a number of factors such as: the type of animal model, the choice of experimental protocol, the type of diabetes and the model to induce obesity or diabetes.

4.3 Role of TNFα in obese and diabetic animals

TNFα production is markedly increased in muscle and adipose tissue in obese humans and rodent models of obesity-diabetes, compared with tissues of lean individuals. Several studies have demonstrated that TNFα plays a role in mediating insulin resistance as a result of obesity (Hotamisligil, 1993; Hotamisligil, 1994a; Hotamisligil, 1994b; Kern, 1995; Hotamisligil, 1995; Saghizadeh, 1996). Weight loss or treatment with pioglitizone, an insulin-sensitizing agent, reduces TNFα levels (Hotamisligil, 1993; Hotamisligil, 1995; Kern, 1995a; Kern, 1995b; Hofman, 1994). Three factors which contribute to the control of body weight have been linked to TNFα: 1) the intake of food, 2) expenditure of energy and 3) storage of energy. Administration of TNFα in a rat model resulted in reduced food intake (Rothwell, 1993) and also inhibited gastric emptying, leading to a feeling of satiation, most likely due to activation of leptin (Arbós et al., 1992; Fargeas et al., 1993). Neutralization of TNFα by intravenous administration of a soluble TNF receptor-immunoglobulin G chimeric protein provided a significant improvement in insulin sensitivity in fatty rats (Hotamisligil, 1993; Hotamisligil, 1994a), but treatment of humans with non-insulin-dependent diabetes mellitus with a specific TNFα antibody had no effect on insulin sensitivity (Ofei, 1996). Although TNFα has been proposed as a link between obesity and insulin resistance (Hotamisligil, 2003), in our study the baseline blood glucose was unchanged by HCD in the TNF-WT and $TNF^{-/-}$ mice but plasma insulin levels were increased, therefore suggesting the development of insulin resistance, even in the absence of TNFα.

Surprisingly, in our study the HCD partially restored the protective effect of IPostC in the TNF-deficient mice, therefore suggesting that absence of TNFα in obesity may be of benefit to the heart. In our modestly obese $TNF^{-/-}$ mice, which could be partially protected by IPostC, the plasma leptin level was significantly elevated compared to the animals kept on the normal diet where IPostC-induced protection was abrogated, therefore suggesting that this adipokine may be implicated in a compensatory mechanism. The increased plasma leptin levels observed in our $TNF^{-/-}$ mice correlate with an increase in body weight and the same correlation was found in mice fed a high fat diet (Ahren, 1999).

In contrast to our obese $TNF^{-/-}$ mice, our STZ-induced diabetic TNF-WT model had significantly decreased leptin levels and the protective effect of IPostC was abolished.

It has recently been reported that the tissue preserving actions of leptin are influenced by obesity (Dixon et al., 2009). Dixon's group showed that leptin decreased the infarct size in Wistar and Zucker lean rats, which have functional leptin receptors, but the cardioprotection was lost in the Zucker obese rats in which the leptin receptors are non-functional. This data provided evidence suggesting that the tissue preserving actions of leptin are influenced by the severe obesity seen in Zucker obese rats. Thus, the degree of obesity as well as the presence or absence of TNFα may be of importance in determining the protective effects of leptin.

In experimental conditions, using a TNFα infusion to induce insulin resistance requires concentrations of TNFα which far exceed the physiological range usually observed in insulin resistant rodent models (Altomonte et al., 2003). Rodent data and human data therefore, do not always coincide and the role of TNFα in insulin sensitivity is far from being straightforward.

The presence of TNFα in obesity has been reported to contribute towards the development of cardiac hypertrophy in cardiomyocytes (Yokoyama, 1997). In support of this hypothesis, our data demonstrate a decrease in the heart to body weight ratio in TNF-deficient mice fed with a diet high in carbohydrates.

Interestingly, when TNF receptor deficient mice were fed a high fat diet the data suggested that the two TNF receptors work in concert to protect against diabetes (Schreyer et al., 1998). In this study TNFRI knock out mice were protected against diet-induced obesity implicating TNFα and TNFR2 in protection against obesity. Our data support this study as in our $TNF^{-/-}$ mice fed with a HCD, the body weight increased by 21% whereas in comparison, the body weight of TNF-WT increased by only 11%, therefore suggesting that absence of TNFα leads to an increase in weight and would thus, influence the development of obesity. It would be of interest to repeat our experiments in our $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ animals to further delineate the role of TNFα receptors in our model.

In conclusion, our data demonstrate that the cardioprotective effect of IPostC was unaltered in a high carbohydrate diet model of obesity and streptozotocin-induced obesity. Whilst TNFα is necessary for IPostC-induced cardioprotection, maintenance of glucose homeostasis and in the control of appetite to prevent obesity it can also lead to cardiac hypertrophy. In our obesity model, absence of TNFα partially restores cardioprotection, while in our diabetic model, the absence of TNFα abrogated the protective effect of IPostC, illustrating the bidirectional effect of TNFα and the fact that the role of TNFα in obesity and diabetes related ischaemic heart disease remains a complex one. Further studies measuring the levels of TNFα or using TNF antibodies

will be required to better understand the protective/deleterious role of TNFα in these pathologies.

A schematic illustrating our present data on the influence of obesity and diabetes on the outcome of IPostC is shown in figure 66.

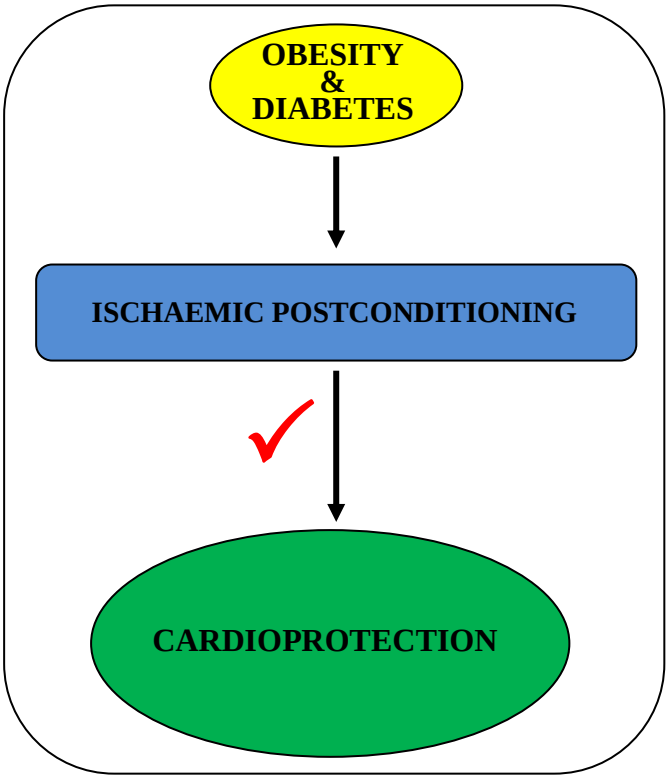


Fig 66: Schematic illustrating the effect of obesity and diabetes on the outcome of IPostC. Diabetic and modestly obese mice can be protected against ischaemia-reperfusion by ischaemic postconditioning

G. FINAL DISCUSSION - CONCLUSION

1.0 Concluding remarks

The discovery of IPostC in the last decade has opened a novel era for therapeutic targets against reperfusion injury. In IPostC, the alternating cycles of reperfusion and ischaemia are applied at the onset of reperfusion, making this a more applicable treatment for patients with myocardial infarction, either in the ambulance or on admission to hospital. However, delivering these alternating cycles requires precise and timed application which is difficult and could substantially differ with the pathophysiology of the individual. Therefore, pharmacological postconditioning, where a drug, agonist or antagonist could be administered at the onset of reperfusion would seem to be a better alternative in the quest to attenuate the injury caused by reperfusion therapy

To further understand the signalling mechanisms of cardiac postconditioning, we aimed to explore the role of TNFα in ischaemic postconditioning as well as pharmacological postconditioning. As obesity and diabetes are now considered to be burden diseases, we also wished to investigate the influence of obesity and diabetes on the protective effect of ischaemic postconditioning and explore the role of TNFα in obesity/diabetes related ischaemic heart disease.

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

1. IPostC confers cardioprotection in the murine model of I/R via the SAFE pathway which involves the activation of TNFα, TNFR2 and the subsequent triggering of the JAK/STAT-3 protective signalling cascade.
2. TNFα mimics IPostC independently of the RISK pathway.
3. Bradykinin and opioids mimic IPostC via the SAFE pathway and therefore require the activation of TNFα and STAT-3 for protection. Bradykinin and opioids possibly protect via translocation of STAT-3 to the mitochondria. In contrast, adenosine protects via STAT-3 but independently of TNFα.
4. Our data confirm the existence of multiple parallel prosurvival signalling pathways in IPostC, (Figure 67).
5. Cardioprotection can still be conferred by ischaemic postconditioning in modestly obese mice or in streptozotocin-induced diabetic mice.
6. Obese TNF knockout mice could be partially protected with IPostC, therefore suggesting a compensatory protective mechanism in $TNF^{-/-}$ mice.

Our data suggest that obese and type 1 diabetic individuals may still benefit from IPostC, relative to the severity of the disease. The existence of multiple alternate pathways in IPostC and pharmacological postconditioning at the time of reperfusion may provide novel therapeutic applications against ischaemia-reperfusion injury in the clinical setting.

In summary, our data provide evidence for the alternative protective prosurvival SAFE pathway in both ischaemic and pharmacological postconditioning, involving activation of TNFα, TNFR2 and STAT-3.

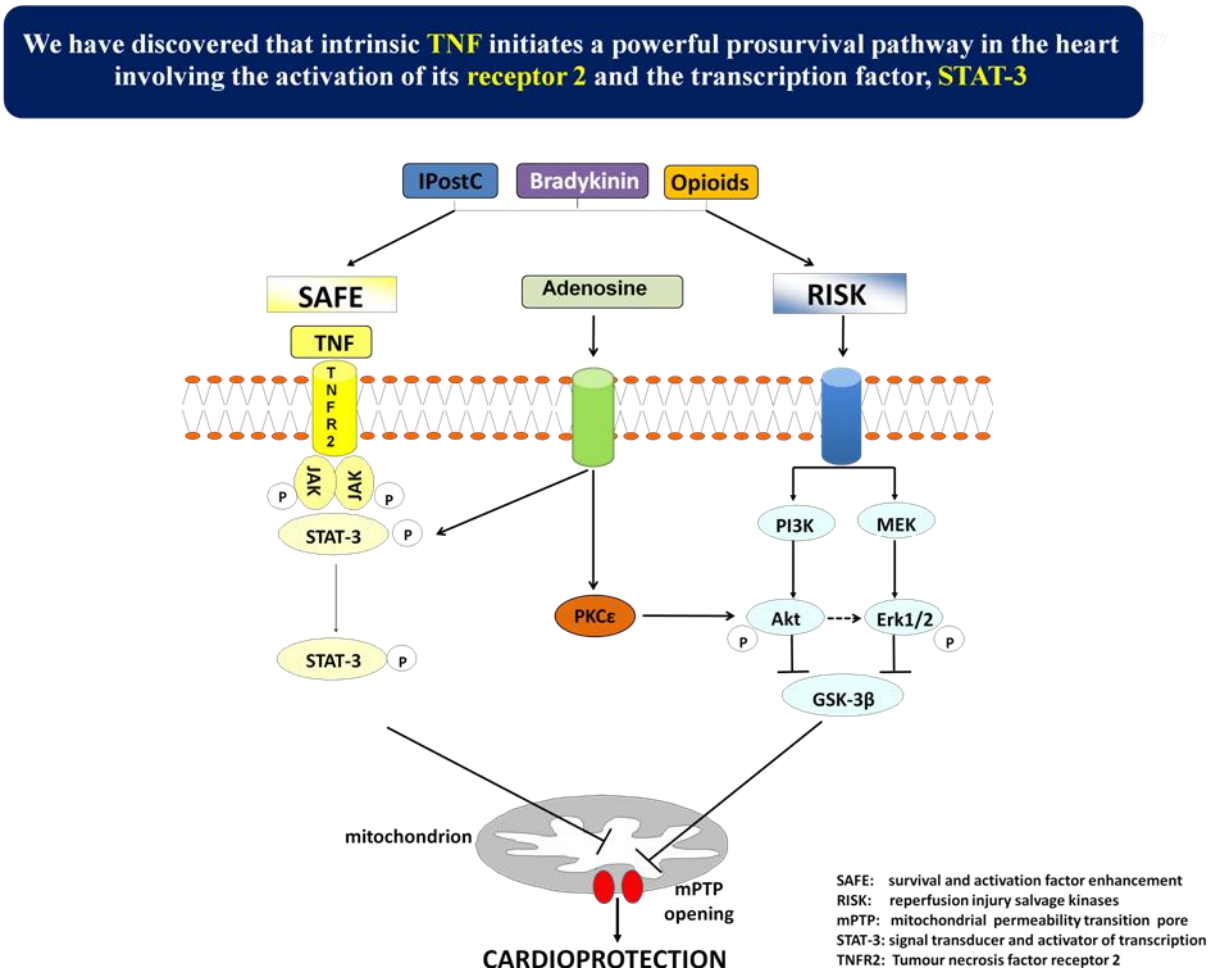


Fig 67: Proposed signalling scheme of the novel SAFE pathway and the known RISK pathway in ischaemic and pharmacological postconditioning-induced cardioprotection.

2.0 Study Limitations

The use of an isolated heart system in this thesis does not take into account the intracoronary activation of leucocytes which may result in a detrimental effect of TNFα in the setting of myocardial ischaemia. Our data will need to be validated *in vivo*. Kinetic analysis of blood and cardiac TNFα levels following ischaemic postconditioning would facilitate the understanding of the exact role of TNFα as a cardioprotective agent against reperfusion injury. However, previous data exploring the protective effect of TNFα have demonstrated a good correlation between *in vitro* and *in vivo* preconditioning experiments, therefore suggesting that the circulating leucocytes do not play a major role (Deuchar et al., 2007; Lecour et al., 2005a). Alternatively, the cellular origin of the cardioprotective TNFα may reside in cardiomyocytes, and other cells such as resident cardiac mast cells and macrophages (Dorge et al., 2002; Frangogiannis et al., 1998)

Another limitation to the study lies in the use of non-specific inhibitors, such as wortmannin and AG490. Wortmannin inhibits the PI-3 kinase signaling cascade and not Akt directly. AG490 blocks JAK2, which is upstream of STAT-3. Therefore, the use of such inhibitors can only indicate that a particular signaling cascade is blocked, but cannot pinpoint the exact downstream protein kinase involved. Further studies, either with siRNA techniques or genetically modified mice, such as Akt null mice and GSK-3β knockout mice, may give greater specificity to the findings of the present study.

The high carbohydrate diet was given to our mice from 3 weeks of age (at weaning) for a period of 11 weeks only as we wished to keep the age of the mice consistent with the 12-14 weeks we had used in our previous experiments. Therefore, the increase in weight in our mice was modest and all the manifestations of obesity may not have yet developed. Future work should include a group in which the HCD is given for a total of 16 weeks to explore the role of severe obesity on the cardioprotective effect of IPostC, bearing in mind that an increase in age generally affects the outcome of postconditioning adversely.

Our diabetic model was created with streptozotocin which induces type 1 diabetes. Future experiments should be done with a type 2 diabetic mouse model to confirm the data observed in our present model.

3.0 Future directions

The cellular origin of TNFα is mainly in the macrophages and resident mast cells (Frangogiannis et al., 1998), but cardiomyocytes also produce TNFα under prolonged ischaemic conditions (Dorge et al., 2002). Delineation of the cellular source of TNFα production in the context of myocardial infarction may be of clinical value. For this purpose we are in the process of generating a cardiomyocyte-specific $TNF^{-/-}$ mouse, using the Cre-Lox P technology. The Cre-Lox P system of genetic targeting requires two separate mouse lines, a homozygous TNF floxed mouse and an organ specific heterozygous Cre recombinase-expressing mouse. These two mice are crossed to produce the cellular specific $TNF^{-/-}$ mouse. Using the same Cre-Lox P technology, we are also in the process of breeding macrophage-neutrophil TNFα-deficient mice. Together with the cardiomyocyte specific $TNF^{-/-}$ this should provide us with a better insight into cellular origin of the cardioprotective TNFα.

The mechanisms triggering the activation of TNFα in the SAFE pathway remain uncertain. Toll-like receptors (TLR's) play a role in the innate immune system to activate TNFα. Recent data exploring the impact of the toll-like receptors 2 or 4 in ischaemia-reperfusion, have not been conclusive as to whether TLR2 or TLR4 are protective or deleterious (Abarbanell et al., 2010; Arslan et al., 2010; Dong et al., 2010). Using our TNF-WT and $TNF^{-/-}$ mice we aim to investigate the possibility that TLR's contribute to the activation of TNFα in the initiation of the prosurvival SAFE pathway.

H. PUBLICATIONS ARISING FROM THIS WORK

Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. **Lydia Lacerda,** Sarin Somers, Lionel H Opie and Sandrine Lecour. *Cardiovasc Res* (2009) 84, 201-208.

I. PREVIOUS PUBLICATIONS

1: Ischemic and Pharmacological Preconditioning in Girardi Cells and C2C12Myotubes Induce Mitochondrial Uncoupling. Jan Minners, **Lydia Lacerda,** Joy McCarthy, James J. Meiring, Derek M. Yellon and Michael N. Sack *Circ Res:* 2001(89): 787-792

2: TNFα-induced cytoprotection requires production of free radicals within mitochondria in C_2C_{12} myotubes. **Lydia Lacerda**, Robert M. Smith, Lionel Opie, Sandrine Lecour. *Life Sciences; 2006* : 2194-2201

3: Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. Robert M. Smith, Naushaad Suleman, **Lydia Lacerda,** Lionel H. Opie, Shizuo Akira, Kenneth R. Chien, Michael N. Sack. *Cardiovasc Res:* 2004 (63); 611-616

4: Pharmacological Preconditioning With Tumor Necrosis Factorα Activates Signal Transducer and Activator ofTranscription-3 at Reperfusion Without Involving Classic Prosurvival Kinases (Akt and ExtracellularSignal–Regulated Kinase).Sandrine Lecour, PharmD, PhD; Naushaad Suleman, MS; Graeme A. Deuchar, PhD; Sarin Somers, BSc; **Lydia Lacerda, BSc;** Barbara Huisamen, PhD; Lionel H. Opie, MD, PhD. *Circulation:* 2005; 3911-3918

6: Diazoxide-induced respiratory inhibition-a putative mitochondrial K_{ATP} channel independent mechanisms of pharmacological preconditioning. Jan Minners, **Lydia Lacerda,** Derek M. Yellon, Lionel H. Opie, Christopher J. McLeod, and Michael N. Sack. *Mol Cell Biochem:* 2007; 11-18

7: Signal Transducer and Activator of transcription factor 3 is involved in the cardioprotective signalling pathway activated by insulin therapy at reperfusion. Britt N.Fuglesteg, Naushaad Suleman, Crina Tiron,Tambuzai Kanhema, **Lydia Lacerda,** Thomas V. Andreasen, Michael N. Sack, Anne K. Jonassen, Ole D. Mjøs, Lionel H. Opie, Sandrine Lecour. *Basic Res Cardiol:* 2008; 444-453

8. TNFα protects cardiac mitochondria independently of its cell surface receptors. **Lydia Lacerda,** Joy McCarthy, Shazia F.K. Mungly, Edward G Lynn, Michael N. Sack, Lionel Opie, Sandrine Lecour. *Basic Res Cardiol (article in press) DOI 10.1007/s00395-010-0113-4*

J. PEER REVIEWED, PUBLISHED CONFERENCE PROCEEDINGS:

2007: "Ischaemic postconditioning requires TNF activation to confer protection". **Lydia Lacerda,** Lionel Opie and Sandrine Lecour. International Society for Heart Research; Italy; *published in J Mol Cell Cardiol, 2007; Vol 42, S184.*

2007: "Critical role of age, strain and number of cycles in ischemic postconditioning" International Society for Cardiovascular Research; Italy; Sarin Somers, **Lydia Lacerda,** Naushaad Suleman, Lionel Opie, Sandrine Lecour. International Society for Heart Research; Italy; *published in J Mol Cell Cardiol, 2007; Vol 42, S186.*

2008: "TNF alpha can mimic ischaemic postconditioning". **Lydia Lacerda**, Lionel Opie, Sandrine Lecour. International Society for Heart Research; Greece; *published in J Mol Cell Cardiol; 2008; Vol 44(4); 718 (abstract No.17).*

2008: "TNFα signalling – a critical role in ischemic postconditioning?" **Lydia Lacerda.** 9th Annual conference of the SA Heart association, South Africa; published in *SA Heart, Spring, 2008; Vol 5, No.4; 217.*

2009: "Pharmacological postconditioning with TNF alpha protects via the SAFE pathway rather than the RISK". **L. Lacerda,** S. Somers, L.H. Opie, S. Lecour. European Society of Cardiology- Heart Failure Conference-ISHR section; France; *published in European J Heart Failure, 2010; Vol 12(1).*

2009: "Innate immunity and protection against reperfusion injury via the SAFE pathway". 10th Annual Congress of SA Heart Association, South Africa; **Lydia Lacerda,** Sarin Somers, Lionel Opie and Sandrine Lecour; *published in SA Heart, Spring, 2009; Vol 6, No.4; p 272.*

2010: Bradykinin, insulin and opioids mimic ischaemic postconditioning via the SAFE pathway. **Lydia Lacerda,** Sarin Somers, Lionel Opie and Sandrine Lecour. Frontiers in Cardiovascular Biology, Berlin, Germany, 2010 – *published in Cardiovasc Res, Vol 87; Supplement 1, 15th July 2010.*

2010: Does obesity and/or diabetes influence the outcome of ischaemic postconditioning? **Lydia Lacerda,** Lionel Opie and Sandrine Lecour. 11th Annual Congress of SA Heart Association, South Africa; *published in SA Heart, Winter 2010; Vol 7, No.3; p 208.*

2010: HDL protects against lethal reperfusion injury via the SAFE pathway. Miguel Frias, Sarin Somers, **Lydia Lacerda,** Richard James and Sandrine Lecour. 11th Annual Congress of SA Heart Association, South Africa; *published in SA Heart, Winter 2010; Vol 7, No.3; p 202.*

K. CONFERENCE OUTPUTS

Ischaemic postconditioning requires TNF activation to confer protection. **Lydia Lacerda,** Lionel Opie and Sandrine Lecour. Physiology Society of Southern Africa, Muldersdrift, Gauteng, South Africa, 2007.

TNFα can mimic ischaemic postconditioning. **L. Lacerda,** L.H. Opie and S. Lecour. International Society for Heart Research, Athens, Greece 2008

Activation of TNFα signalling at onset of reperfusion protects the heart. **L. Lacerda,** L.H. Opie and S. Lecour. South African Medical Research Council Research Day, Parow, South Africa 2008

TNFα signalling – a critical role in ischaemic postconditioning? **L. Lacerda,** L.H. Opie and S. Lecour. South African Heart Conference, Wild Coast Sun, South Africa 2008

Pharmacological postconditioning with TNFα protects via the SAFE pathway rather than the RISK pathway. **Lydia Lacerda,** Sarin Somers, Lionel Opie and Sandrine Lecour. International Society for Heart Research – ESC meeting, Nice, France 2009

Pharmacological postconditioning with TNF alpha protects via the SAFE pathway rather than the RISK pathway. **Lydia Lacerda,** Sarin Somers, Lionel Opie and Sandrine Lecour. Physiological Society of Southern Africa, Stellenbosch, South Africa, 2009

Innate immunity and protection against reperfusion injury via the SAFE pathway. **Lydia Lacerda,** Sarin Somers, Lionel Opie and Sandrine Lecour. South African Heart Conference, Sun City, Johannesburg, South Africa. 2009.

Adenosine, bradykinin, insulin and the opioids can mimic ischaemic postconditioning: Role of the cytokine, tumour necrosis factor alpha (TNFα). **Lydia Lacerda,** Lionel Opie and Sandrine Lecour. Astrazeneca Conference, Tygerberg, South Africa, 2010

Bradykinin, insulin and opioids mimic ischaemic postconditioning via the SAFE pathway. **Lydia Lacerda,** Lionel Opie and Sandrine Lecour. Frontiers in Cardiovascular Biology, Berlin, Germany, 2010

Does obesity and/or diabetes influence the outcome of ischaemic postconditioning? **Lydia Lacerda,** Lionel Opie and Sandrine Lecour. 11th Annual Congress of SA Heart Association, South Africa, 2010

HDL protects against lethal reperfusion injury via the SAFE pathway. Miguel Frias, Sarin Somers, **Lydia Lacerda,** Richard James and Sandrine Lecour. 11th Annual Congress of SA Heart Association, South Africa, 2010

REFERENCES

Aasum, E., Khalid, A. M., Gudbrandsen, O. A., How, O. J., Berge, R. K., and Larsen, T. S. (2008). Fenofibrate modulates cardiac and hepatic metabolism and increases ischemic tolerance in diet-induced obese mice. J Mol Cell Cardiol *44*, 201-209.

Aasum, E., Hafstad, A.D., Severson, D.L., and Larsen, T.S. (2003) Age-dependent changes in metabolism, contractile function, and ischemic severity in hearts from db/db mice. Diabetes *52*, 434-441.

Abarbanell, A. M., Wang, Y., Herrmann, J. L., Weil, B. R., Poynter, J. A., Manukyan, M. C., and Meldrum, D. R. (2010). Toll-like receptor 2 mediates mesenchymal stem cell-associated myocardial recovery and VEGF production following acute ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol *298*, H1529-1536.

Abbott, R. D., Donahue, R. P., Kannel, W. B., and Wilson, P. W. (1988). The impact of diabetes on survival following myocardial infarction in men vs women. The Framingham Study. Jama *260*, 3456-3460.

Abbud, Z., Shindler DM., Wilson AC., and Kostis, J. B. (1995). Effect of diabetes mellitus on short- and long-term mortality rates of patients with acute myocardial infarction: a statewide study. Myocardial Infarction Data Acquisition System Study Group. Am Heart J *130*, 51-58.

Abdallah, Y., Gkatzoflia, A., Gligorievski, D., *et al.* (2006). Insulin protects cardiomyocytes against reoxygenation-induced hypercontracture by a survival pathway targeting SR Ca2+ storage. Cardiovasc Res *70*, 346-353.

Abraham, E., Wunderink, R., Silverman, H., Perl, T. M., Nasraway, S., Levy, H., Bone, R., Wenzel, R. P., Balk, R., Allred, R., *et al*. (1995). Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group. Jama *273*, 934-941.

Adam, D., Wiegmann, K., Adam-Klages, S., Ruff, A., and Kronke, M. (1996). A novel cytoplasmic domain of the p55 tumor necrosis factor receptor initiates the neutral sphingomyelinase pathway. J Biol Chem *271*, 14617-14622.

Adamklages, S., Schwandner, R., Adam, D., Kreder, D., Bernardo, K., Kronke, M. (1998). Distinct adapter proteins mediate acid versus neutral sphingomyelinase activation through the p55 receptor for tumor necrosis factor. J Leukoc Biol *63*, 678.

Aguilar, D., Solomon, S. D., Kober, L., Rouleau, J. L., Skali, H., McMurray, J. J., Francis, G. S., Henis, M., O'Connor, C. M., Diaz, R., *et al.* (2004). Newly diagnosed and previously known diabetes mellitus and 1-year outcomes of acute myocardial infarction: the VALsartan In Acute myocardial iNfarcTion (VALIANT) trial. Circulation *110*, 1572-1578.

Ahren, B. (1999). Plasma leptin and insulin in C57BI/6J mice on a high-fat diet: relation to subsequent changes in body weight. Acta Physiol Scand *165*, 233-240.

Alberti, K. G., Zimmet, P., and Shaw, J. (2005). The metabolic syndrome--a new worldwide definition. Lancet *366*, 1059-1062.

Aleshin, A., Ananthakrishnan, R., Li, Q., Rosario, R., Lu, Y., Qu, W., Song, F., Bakr, S., Szabolcs, M., D'Agati, V., *et al.* (2008). RAGE modulates myocardial injury consequent to LAD infarction via impact on JNK and STAT signaling in a murine model. Am J Physiol Heart Circ Physiol *294*, H1823-1832.

Almanza-Perez, J. C., Blancas-Flores, G., Garcia-Macedo, R., Alarcon-Aguilar, F. J., and Cruz, M. (2008). Leptin and its association with obesity and type 2 diabetes. Gac Med Mex *144*, 535-542.

Altomonte, J., Harbaran, S., Richter, A., and Dong, H. (2003). Fat depot-specific expression of adiponectin is impaired in Zucker fatty rats. Metabolism *52*, 958-963.

Amit-Vazina, M., Shishodia, S., Harris, D., Van, Q., Wang, M., Weber, D., Alexanian, R., Talpaz, M., Aggarwal, B. B., and Estrov, Z. (2005). Atiprimod blocks STAT3 phosphorylation and induces apoptosis in multiple myeloma cells. Br J Cancer *93*, 70-80.

Aneja, R. K., Tsung, A., Sjodin, H., Gefter, J. V., Delude, R. L., Billiar, T. R., and Fink, M. P. (2008). Preconditioning with high mobility group box 1 (HMGB1) induces lipopolysaccharide (LPS) tolerance. J Leukoc Biol *84*, 1326-1334.

Anker, S. D., and Coats, A. J. (2002). How to RECOVER from RENAISSANCE? The significance of the results of RECOVER, RENAISSANCE, RENEWAL and ATTACH. Int J Cardiol *86*, 123-130.

Arbós, J., López-Soriano, F.J., Carbó, N., and Argilés, J. M. (1992). Effects of tumour necrosis factor-alpha (cachectin) on glucose metabolism in the rat. Intestinal absorption and isolated enterocyte metabolism. Mol Cell Biochem *112*, 53-59.

Argaud, L., Gateau-Roesch, O., Muntean, D., Chalabreysse, L., Loufouat, J., Robert, D., and Ovize, M. (2005). Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. J Mol Cell Cardiol *38*, 367-374.

Arner, P. (2003). The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. Trends Endocrinol Metab *14*, 137-145.

Aronson, D., Rayfield, E.J., and Chesebro, J.H. (1997). Mechanisms determining course and outcome of diabetic patients who have had acute myocardial infarction. Ann Intern Med *126*, 296-306.

Arslan, F., Smeets, M. B., O'Neill, L. A., Keogh, B., McGuirk, P., Timmers, L., Tersteeg, C., Hoefer, I. E., Doevendans, P. A., Pasterkamp, G., and de Kleijn, D. P.(2010). Myocardial ischemia/reperfusion injury is mediated by leukocytic toll-like receptor-2 and reduced by systemic administration of a novel anti-toll-like receptor-2 antibody. Circulation *121*, 80-90.

Assmann, G., Schulte, H., von Eckardstein, A., and Huang, Y. (1996). High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. Atherosclerosis *124 Suppl*, S11-20.

Attarian, D. E., Jones, R. N., Currie, W. D., Hill, R. C., Sink, J. D., Olsen, C. O., Chitwood, W. R., Jr., and Wechsler, A. S. (1981). Characteristics of chronic left ventricular hypertrophy induced by subcoronary valvular aortic stenosis. II. Response to ischemia. J Thorac Cardiovasc Surg *81*, 389-395.

Auchampach, J. A., and Gross, G. J. (1993). Adenosine A1 receptors, KATP channels, and ischemic preconditioning in dogs. Am J Physiol *264*, H1327-1336.

Baines, C. P., Goto, M., and Downey, J. M. (1997). Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. J Mol Cell Cardiol *29*, 207-216.

Balakirev, M., Khramtsov, V. V., and Zimmer, G. (1997). Modulation of the mitochondrial permeability transition by nitric oxide. Eur J Biochem *246*, 710-718.

Barbash, G., White, H.D., Modan, M., and Van de Werf, F. (1993). Significance of diabetes mellitus in patients with acute myocardial infarction receiving thrombolytic therapy. Investigators of the International Tissue Plasminogen Activator/Streptokinase Mortality Trial. J Am Coll Cardiol *22*, 707-713.

Barillas, R., Friehs, I., Cao-Danh, H., Martinez, J.F., and del Nido P.J. (2007). Inhibition of glycogen synthase kinase-3beta improves tolerance to ischemia in hypertrophied hearts. Ann Thorac Surg *84*, 126-133.

Barth, E., Stammler, G., Speiser, B., and Schaper, J. (1992). Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. J Mol Cell Cardiol *24*, 669-681.

Basaran, Y., Basaran, M. M., Babacan, K. F., Ener, B., Okay, T., Gok, H., and Ozdemir, M. (1993). Serum tumor necrosis factor levels in acute myocardial infarction and unstable angina pectoris. Angiology *44*, 332-337.

Bastard, J. P., Maachi, M., Lagathu, C., Kim, M. J., Caron, M., Vidal, H., Capeau, J., and Feve, B. (2006). Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw *17*, 4-12.

Batista, M. L., Lopes, R. D., Seelaender, M. C., and Lopes, A. C. (2009). Anti-inflammatory effect of physical training in heart failure: role of TNF-alpha and IL-10. Arq Bras Cardiol *93*, 643-651, 692-700.

Baumgarten, C. R., Linz, W., Kunkel, G., Scholkens, B. A., and Wiemer, G. (1993). Ramiprilat increases bradykinin outflow from isolated hearts of rat. Br J Pharmacol *108*, 293-295.

Baxter, G. F., and Ebrahim, Z. (2002). Role of bradykinin in preconditioning and protection of the ischaemic myocardium. Br J Pharmacol *135*, 843-854.

Becker, L. B., vanden Hoek, T. L., Shao, Z. H., Li, C. Q., and Schumacker, P. T. (1999). Generation of superoxide in cardiomyocytes during ischemia before reperfusion. Am J Physiol *277*, H2240-2246.

Behrends, M., Schulz, R., Post, H., Alexandrov, A., Belosjorow, S., Michel, M. C., and Heusch, G. (2000). Inconsistent relation of MAPK activation to infarct size reduction by ischemic preconditioning in pigs. Am J Physiol Heart Circ Physiol *279*, H1111-1119.

Bell, J. H., Herrera, A. H., Li, Y., and Walcheck, B. (2007). Role of ADAM17 in the ectodomain shedding of TNF-alpha and its receptors by neutrophils and macrophages. J Leukoc Biol *82*, 173-176.

Bell, R. M., and Yellon, D. M. (2002). Bradykinin limits infarction when administered as an adjunct to reperfusion in mouse heart: the role of PI3K, Akt and eNOS. J Mol Cell Cardiol *35*, 185-193.

Bell, S. P., Sack, M. N., Patel, A., Opie, L. H., and Yellon, D. M. (2000). Delta opioid receptor stimulation mimics ischemic preconditioning in human heart muscle. J Am Coll Cardiol *36*, 2296-2302.

Belosjorow, S., Bolle, I., Duschin, A., Heusch, G., and Schulz, R. (2003). TNF-alpha antibodies are as effective as ischemic preconditioning in reducing infarct size in rabbits. Am J Physiol Heart Circ Physiol *284*, H927-930.

Belosjorow, S., Schulz, R., Dorge, H., Schade, F. U., and Heusch, G. (1999). Endotoxin and ischemic preconditioning: TNF-alpha concentration and myocardial infarct development in rabbits. Am J Physiol *277*, H2470-2475.

Benigni, F., Sacco, D., Pennica, D., and Ghezzi, P. (1996). Cardiotrophin-1 Inhibits Tumor Necrosis Factor Production in the Heart and Serum of Lipopolysaccharide-Treated Mice and in vitro in Mouse Blood Cells. Am J Pathol *149*, 1847-1850.

Bennet, P. H. (1990). Epidemiology of diabetes mellitus (New York, Elsevier Science Publishing Co Inc).

Bernardi, P., Krauskopf, A., Basso, E., Petronilli, V., Blachly-Dyson, E., Di Lisa, F., and Forte, M. A. (2006). The mitochondrial permeability transition from in vitro artifact to disease target. Febs J *273*, 2077-2099.

Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., Georgiev, I., Schober, A., Leng, L., *et al.* (2007). MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med *13*, 587-596.

Bernstein, L. E., Berry, J., Kim, S., Canavan, B., and Grinspoon, S. K. (2006). Effects of etanercept in patients with the metabolic syndrome. Arch Intern Med *166*, 902-908.

Beutler, B., and Cerami, A. (1989). The biology of cachectin/TNF--a primary mediator of the host response. Annu Rev Immunol *7*, 625-655.

Beutler, B., Greenwald, D., Hulmes, J. D., Chang, M., Pan, Y. C., Mathison, J., Ulevitch, R., and Cerami, A. (1985). Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature *316*, 552-554.

Beutler, B., van Huffel C (1994). Unraveling function in the TNF ligand and receptor families. Science *264*, 667-668.

Bhoola, K. D., Elson, C. J., and Dieppe, P. A. (1992). Kinins--key mediators in inflammatory arthritis? Br J Rheumatol *31*, 509-518.

Bierhaus, A., Schiekofe, S., Schwaninge, M., Andrassy, M., Humbert. P., Chen, J., Hong, M., Luthe, T., Henle, T., Kloting, I., *et al.* (2001). Diabetes-associated sustained activation of the transcription factor NFκB. Diabetes *50*, 2792-2809.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., *et al.* (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature *385*, 729-733.

Black, R. G., Jr., Guo, Y., Ge, Z. D., Murphree, S. S., Prabhu, S. D., Jones, W. K., Bolli, R., and Auchampach, J. A. (2002). Gene dosage-dependent effects of cardiac-specific overexpression of the A3 adenosine receptor. Circ Res *91*, 165-172.

Blackheart, B. D., Emilsson, K., Nguyen, D., and others (1996). Ligand cross-reactivity within the proteinase -activated receptor family. J Biol Chem *271*, 16466-16471.

Boengler, K., Buechert, A., Heinen, Y., Roeskes, C., Hilfiker-Kleiner, D., Heusch, G., and Schulz, R. (2008). Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. Circ Res *102*, 131-135.

Bohm, S. K., Kong, W. Y., Bromme, D., *et al.* (1996). Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochem J *314*, 1009-1016.

Bopassa, J. C., Ferrera, R., Gateau-Roesch, O., Couture-Lepetit, E., and Ovize, M. (2006a). PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. Cardiovasc Res *69*, 178-185.

Bopassa, J. C., Vandroux, D., Ovize, M., and Ferrera, R. (2006b). Controlled reperfusion after hypothermic heart preservation inhibits mitochondrial permeability transition-pore opening and enhances functional recovery. Am J Physiol Heart Circ Physiol *291*, H2265-2271.

Boudina, S; Abel, E.D. (2007). Diabetic cardiomyopathy revisited. Circulation *115*, 3213-3223.

Bouhidel, O., Pons, S., Souktani, R., Zini, R., Berdeaux, A., and Ghaleh, B. (2008). Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice. Am J Physiol Heart Circ Physiol *295*, H1580-1586.

Bradshaw, D. (2005). What do we know about the burden of cardiovascular disease in South Africa? Cardiovascular J S Afr *16*, 140-141.

Brar, B., Stephanou, A., Liao, Z., O'Leary, RM., Pennica, D., Yellon, DM., and Latchman, D. (2001). Cardiotrophin-1 can protect cardiac myocytes from injury when added both prior to simulated ischaemia and at reoxygenation.Cardiovasc Res *51*, 265-274.

Braunwald, E., Sobel, B. E. (1969). Treatment of paroxysmal supraventricular trachycardia by electrical stimulation of the carotid-sinus nerves. N Engl J Med 281 *16, 885-887.*

Braunwald, E., and Kloner, R. A. (1985). Myocardial reperfusion: a double-edged sword? J Clin Invest *76*, 1713-1719.

Bray, G. A. (2004). Medical consequences of obesity. J Clin Endocrinol Metab *89*, 2583-2589.

Bryant, D., Becker, L., Richardson, J., Shelton, J., Franco, F., Peshock, R., Thompson, M., and Giroir, B. (1998). Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor-alpha. Circulation *97*, 1375-1381.

Bucciarelli, L. G., Kaneko, M., Ananthakrishnan, R., Harja, E., Lee, L. K., Hwang, Y. C., Lerner, S., Bakr, S., Li, Q., Lu, Y., *et al.* (2006). Receptor for advanced-glycation end products: key modulator of myocardial ischemic injury. Circulation *113*, 1226-1234.

Bullo, M., Garcia-Lorda, P., Megias, I., and Salas-Salvado, J. (2003). Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. Obes Res *11*, 525-531.

Burley, D. S., and Baxter, G. F. (2007). B-type natriuretic peptide at early reperfusion limits infarct size in the rat isolated heart. Basic Res Cardiol *102*, 529-541.

Burley, D. S., and Baxter, G.F. (2009). Pharmacological targets revealed by myocardial postconditioning. Curr Opin Pharmacol *9*, 177-188.

Burley, D. S., Ferdinandy P, and Baxter, G. F. (2007). Cyclic GMP and protein kinase-G in myocardial ischaemia-reperfusion: opportunities and obstacles for survival signalling. Br J Pharmacol *152*, 855-869.

Cain, B. S., Meldrum, D. R., Dinarello, C. A., Meng, X., Banerjee, A., and Harken, A. H. (1998). Adenosine reduces cardiac TNF-alpha production and human myocardial injury following ischemia-reperfusion. J Surg Res *76*, 117-123.

Camerer, E., Huang, W., and Coughlin, S. R. (2000). Tissue factor- and factor X-dependent activation of protease activated receptor 2 by factor VIIa. Proc Natl Acad USA *97*, 5255-5260.

Carswell, E. A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA *72*, 3666-3670.

Chan, J. M., Rimm, E. B., Colditz, G. A., Stampfer, M. J., Willett, W.C. (1994). Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care *17*, 961-969.

Chen, Z., Li, T., and Zhang, B. (2008). Morphine postconditioning protects against reperfusion injury in the isolated rat hearts. J Surg Res *145*, 287-294.

Cheung, M. M., Kharbanda, R. K., Konstantinov, I. E., Shimizu, M., Frndova, H., Li, J., Holtby, H. M., Cox, P. N., Smallhorn, J. F., Van Arsdell, G. S., and Redington, A. N. (2006). Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. J Am Coll Cardiol *47*, 2277-2282.

Cheung, W. M., Andrade-Gordon, P., and Damiano, B. P. (1998). Receptor activating peptides distinguish thrombin receptor (PAR-1) and protease-activated receptor 2 (PAR-2) mediated hemodynamic response in vivo. Can J Physiol *76*, 16-25.

Chien, G. L., and Van Winkle, D. M. (1996). Naloxone blockade of myocardial ischemic preconditioning is stereoselective. J Mol Cell Cardiol *28*, 1895-1900.

Cicala, C., Pinto, A., Bucci, M., Sorrentino, R., Walker, B., Harriot, P., Cruchley, A., Kapas, S., Howella, G. L,, and **Cirino, G.** (1999). Protease activated receptor-2 involvement in hypotension in normal and endotoxemic rats in vivo. Circulation *99*, 2590-2597.

Cirino, G., Napoli, C., Bucci, M., and Cicala, C. (2000). Inflammation-coagulation network: are serine protease receptors the knot? Trends Pharmacol Sci *21*, 170-172.

Claeys, M. J., Bosmans, J., De Ceuninck, M., Beunis, A., Vergauwen, W., Vorlat, A., and Vrints, C. J. (2004). Effect of intracoronary adenosine infusion during coronary intervention on myocardial reperfusion injury in patients with acute myocardial infarction. Am J Cardiol *94*, 9-13.

Cleveland, J. C., Jr., Meldrum, D. R., Rowland, R. T., Banerjee, A., and Harken, A. H. (1997). Adenosine preconditioning of human myocardium is dependent upon the ATP-sensitive K+ channel. J Mol Cell Cardiol *29*, 175-182.

Cohen, M. V., Baines, C. P, and Downey, J. M. (2000). Ischemic preconditioning: from adenosine receptor to K_{ATP} channel. Annu Rev Physiol *62*, 79-109.

Cohen, M. V., and Downey, J. M. (2008). Adenosine: trigger and mediator of cardioprotection. Basic Res Cardiol *103*, 203-215.

Cohen, M. V., Yang, X. M., Liu, G. S., Heusch, G., and Downey, J. M. (2001). Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels. Circ Res *89*, 273-278.

Colditz, G. A., Willett, W. C., Rotnitsky, A., Manson, J. E. (1995). Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med *122*, 481-486.

Coley, W. (1893). The Treatment of Malignant Tumors by Repeated Innoculations of Erysipelas: With a report of Ten Original Cases. Am J Med *10*, 487-511.

Costa, A. D., Garlid, K. D., West, I. C., Lincoln, T. M., Downey, J. M., Cohen, M. V., and Critz, S. D. (2005). Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria. Circ Res *97*, 329-336.

Crestanello, J. A., Lingle, D. M., Kamelgard J, *et al*. (1996). Ischemic preconditioning decreases oxidative stress during reperfusion: a chemiluminescence study. J Surg Res *65*, 53-58.

Critz, S. D., Cohen, M. V., and Downey, J. M. (2005). Mechanisms of acetylcholine- and bradykinin-induced preconditioning. Vascul Pharmacol *42*, 201-209.

Crompton, M., Costi, A., and Hayat, L. (1987). Evidence for the presence of a reversible Ca2+-dependent pore activated by oxidative stress in heart mitochondria. Biochem J *245*, 915-918.

Crompton, M., Ellinger, H., and Costi, A. (1988). Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J *255*, 357-360.

Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000). Serine/threonine protein kinases and apoptosis. Exp Cell Res *256*, 34-41.

Csonka, C., Szilvassy, Z., Fulop, F., Pali, T., Blasig, I. E., Tosaki, A., Schulz, R., and Ferdinandy, P. (1999). Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. Circulation *100*, 2260-2266.

Curb, J. D., Abbott, R. D., Rodriguez, B. L., Masaki, K., Chen, R., Sharp, D. S., and Tall, A. R. (2004). A prospective study of HDL-C and cholesteryl ester transfer protein gene mutations and the risk of coronary heart disease in the elderly. J Lipid Res *45*, 948-953.

D'Andrea, M. R., Derian, C. K., Leturcq, D., Bake, S. M., Brunmark ,A., Ling, P., Darrow, A. L, Santulli, R. J, Brass, L. F., and Andrade-Gordon, P. (1998). Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. J Histochem Cytochem *46*, 157-164.

da Silva, R., Gramp, T., Pasch, T., Schaub, M.C., and Zaugg, M. (2004). Differential activation of mitogen-activated protein kinases in ischemic and anesthetic preconditioning. Anesthesiology *100*, 59-69.

Damiano, B. P., D'Andrea, M. R., de Garavilla, L., Cheung, W. M., and Andrade-Gordon, P. (1999). Increased expression of protease activated receptor-2 (PAR-2) in balloon-injured rat carotid artery. Thromb Haemost *81*, 808-814.

Dana, A., Skarli, M., Papakrivopoulou, J., and Yellon, D. M. (2000). Adenosine A(1) receptor induced delayed preconditioning in rabbits: induction of p38 mitogen-activated protein kinase activation and Hsp27 phosphorylation via a tyrosine kinase- and protein kinase C-dependent mechanism. Circ Res *86*, 989-997.

Daniel, C., Schaub, K., Amann, K., Lawler, J., and Hugo, C. (2007). Thrombospondin-1 is an endogenous activator of TGF-beta in experimental diabetic nephropathy in vivo.Diabetes *56*, 2982-2989.

Darling, C. E., Jiang, R., Maynard, M., Whittaker, P., Vinten-Johansen, J., and Przyklenk, K. (2005). Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. Am J Physiol Heart Circ Physiol *289*, H1618-1626.

De Taeye, B. M., Novitskaya, T., McGuinness, O. P., Gleaves, L., Medda, M., Covington, J. W., and Vaughan, D. E. (2007). Macrophage TNF-alpha contributes to insulin resistance and hepatic steatosis in diet-induced obesity. Am J Physiol Endocrinol Metab *293*, E713-725.

Deswal, A., Petersen, N. J., Feldman, A. M., Young, J. B., White, B. G., and Mann, D. L. (2001). Cytokines and cytokine receptors in advanced heart failure: an analysis of the cytokine database from the Vesnarinone trial (VEST). Circulation *103*, 2055-2059.

Deuchar, G. A., Opie, L. H., and Lecour, S. (2007). TNFalpha is required to confer protection in an in vivo model of classical ischaemic preconditioning. Life Sci *80*, 1686-1691.

Di Filippo, C., Marfella, R., Cuzzocrea, S., Piegari, E., Petronella, P., Giugliano, D., Rossi, F., and D'Amico, M. (2005). Hyperglycemia in streptozotocin-induced diabetic rat increases infarct size associated with low levels of myocardial HO-1 during ischemia/reperfusion. Diabetes *54*, 803-810.

Di Lisa, F., and Bernardi, P. (2006). Mitochondria and ischemia-reperfusion injury of the heart: fixing a hole. Cardiovasc Res *70*, 191-199.

Di Lisa, F., Canton, M., Menabo, R., Kaludercic, N., and Bernardi, P. (2007). Mitochondria and cardioprotection. Heart Fail Rev *12*, 249-260.

Di Lisa, F., Menabo, R., Canton, M., Barile, M., and Bernardi, P. (2001). Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. J Biol Chem *276*, 2571-2575.

Diamond, M. J., Gujral, J., and Kapoor, D. (2005). Percutaneous intervention of left main coronary artery disease: a fresh look at a class III recommendation. South Med J *98*, 1153-1154.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature *399*, 601-605.

Dinh, W., Futh, R., Nickl, W., Krahn, T., Ellinghaus, P., Scheffold, T., Bansemir, L., Bufe, A., Barroso, M. C., and Lankisch, M. (2009). Elevated plasma levels of TNF-alpha and interleukin-6 in patients with diastolic dysfunction and glucose metabolism disorders. Cardiovasc Diabetol *8*, 58.

Dixon, R., Davidson SM, Wynne AM, Yellon DM, and Smith, C. C. (2009). The cardioprotective actions of leptin are lost in the Zucker obese (fa/fa) rat. J Cardiovasc Pharmacol *53*, 311-317.

Donahue, R. P., Abbott, R. D., Bloom, E., Reed, D. M., and Yano, K. (1987). Central obesity and coronary heart disease in men. Lancet *1*, 821-824.

Donato, M., D'Annunzio, V., Berg, G., Gonzalez, G., Schreier, L., Morales, C., Wikinski, R. L., and Gelpi, R. J. (2007). Ischemic postconditioning reduces infarct size by activation of A1 receptors and K+(ATP) channels in both normal and hypercholesterolemic rabbits. J Cardiovasc Pharmacol *49*, 287-292.

Dong, J. W., Vallejo, J. G., Tzeng, H. P., Thomas, J. A., and Mann, D. L. (2010). Innate immunity mediates myocardial preconditioning through Toll-like receptor 2 and TIRAP-dependent signaling pathways. Am J Physiol Heart Circ Physiol *298*, H1079-1087.

Donzelli, S., Switzer, C. H., Thomas, D. D., Ridnour, L. A., Espey, M. G., Isenberg, J. S., Tocchetti, C. G., King, S. B., Lazzarino, G., Miranda, K. M., *et al.* (2006). The activation of metabolites of nitric oxide synthase by metals is both redox and oxygen dependent: a new feature of nitrogen oxide signaling. Antioxid Redox Signal *8*, 1363-1371.

Dorge, H., Schulz, R., Belosjorow, S., Post, H., van de Sand, A., Konietzka, I., Frede, S., Hartung, T., Vinten-Johansen, J., Youker, K. A., *et al.* (2002). Coronary microembolization: the role of TNF-alpha in contractile dysfunction. J Mol Cell Cardiol *34*, 51-62.

Downey, J. M., Davis, A. M., and Cohen, M. V. (2007). Signaling pathways in ischemic preconditioning. Heart Fail Rev *12*, 181-188.

du Toit, E. F., Smith, W., Muller, C., Strijdom, H., Stouthammer, B., Woodiwiss, A. J., Norton, G. R., and Lochner, A. (2008). Myocardial susceptibility to ischemic-reperfusion injury in a prediabetic model of dietary-induced obesity. Am J Physiol Heart Circ Physiol *294*, H2336-2343.

Duchen, M. R., McGuinness, O., Brown, L. A., and Crompton, M. (1993). On the involvement of a cyclosporin A sensitive mitochondrial pore in myocardial reperfusion injury. Cardiovasc Res *27*, 1790-1794.

Ducimetiere, P., Richard, J., and Cambien, F. (1986). The pattern of subcutaneous fat distribution in middle-aged men and the risk of coronary heart disease: the Paris Prospective Study. Int J Obes *10*, 229-240.

Ebrahim, Z., Yellon, D. M., and Baxter, G. F. (2007). Attenuated cardioprotective response to bradykinin, but not classical ischaemic preconditioning, in DOCA-salt hypertensive left ventricular hypertrophy. Pharmacol Res *55*, 42-48.

Edmunds, N., Woodward B. (1998). Effects of tumour necrosis factor-alpha on the coronary circulation of the rat isolated perfused heart: a potential role for thromboxane A2 and sphingosine.Br J Pharmacol *124*, 493-498.

Emilsson, K., Waldestedt, C., Sun M K, Nystedt, S., Owman C, and Sundelin, J. (1997). Vascular effects of protease-activated receptor-2 agonist peptide. J Vasc Res *34*, 267-272.

Engler, R. (1987). Consequences of activation and adenosine-mediated inhibition of granulocytes during myocardial ischemia. Fed Proc *46*, 2407-2412.

Fantinelli, J. C., and Mosca, S. M. (2007). Comparative effects of ischemic pre and postconditioning on ischemia-reperfusion injury in spontaneously hypertensive rats (SHR). Mol Cell Biochem *296*, 45-51.

Fargeas, M. J., Fioramonti, J., and Bueno, L. (1993). Central action of interleukin 1 beta on intestinal motility in rats: mediation by two mechanisms. Gastroenterology *104*, 377-383.

Feng, J., Fischer, G., Lucchinetti, E., Zhu, M., Bestmann, L., Jegger, D., Arras, M., Pasch, T., Perriard, J. C., Schaub, M. C., and Zaugg, M. (2006). Infarct-remodeled myocardium is receptive to protection by isoflurane postconditioning: role of protein kinase B/Akt signaling. Anesthesiology *104*, 1004-1014.

Ferdinandy, P., Schulz, R, Baxter G F (2007). Interaction of Cardiovascular Risk Factors with Myocardial Ischemia/Reperfusion Injury, Preconditioning, and Postconditioning. Pharmacol Rev *59*, 418-458.

Ferrari, R., and Hearse, D. J. (1997). Reperfusion Injury: Does It Exist and Does It Have Clinical Relevance? J Thromb Thrombolysis *4*, 25-34.

Flaherty, M. P., Guo, Y., Tiwari, S., Rezazadeh, A., Hunt, G., Sanganalmath, S. K., Tang, X. L., Bolli, R., and Dawn, B. (2008). The role of TNF-alpha receptors p55 and p75 in acute myocardial ischemia/reperfusion injury and late preconditioning. J Mol Cell Cardiol *45*, 735-741.

Forrat, R., Sebbag, L., Wiernsperger, N., Guidollet, J., Renaud, S., de Lorgeril, M. (1993). Acute myocardial infarction in dogs with experimental diabetes. Cardiovasc Res *27*, 1908-1912.

Frangogiannis, N. G., Lindsey, M. L., Michael, L. H., Youker, K. A., Bressler, R. B., Mendoza, L. H., Spengler, R. N., Smith, C. W., and Entman, M. L. (1998). Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation *98*, 699-710.

Frassdorf, J., Weber, N. C., Obal, D., Toma, O., Mullenheim, J., Kojda, G., Preckel, B., and Schlack, W. (2005). Morphine induces late cardioprotection in rat hearts in vivo: the involvement of opioid receptors and nuclear transcription factor kappaB. Anesth Analg *101*, 934-941, table of contents.

Frias, M. A., James, R. W., Gerber-Wicht, C., and Lang, U. (2009). Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. Cardiovasc Res *82*, 313-323.

Frühbeck G. (2006) Intracellular signalling pathways activated by leptin. Biochem J *393*: 7-20.

Fryer, R. M., Pratt, P. F., Hsu, A. K., and Gross, G. J. (2001). Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. J Pharmacol Exp Ther *296*, 642-649.

Fuglesteg, B. N., Suleman, N., Tiron, C., Kanhema, T., Lacerda, L., Andreasen, T. V., Sack, M. N., Jonassen, A. K., Mjos, O. D., Opie, L. H., and Lecour, S. (2008). Signal transducer and activator of transcription 3 is involved in the cardioprotective signalling pathway activated by insulin therapy at reperfusion. Basic Res Cardiol *103*, 444-453.

Gao, Q., Xia, Q., Cao, C. M., Zhang, S. Z., and Bruce, I. C. (2004). Role of the mitochondrial permeability transition pore in TNF-alpha-induced recovery of ventricular contraction and reduction of infarct size in isolated rat hearts subjected to ischemia/reperfusion. Conf Proc IEEE Eng Med Biol Soc *5*, 3622-3624.

Gao, Q., Zhang, S. Z, Cao, C. M., Bruce, I. C., and Xia, Q. (2005). The mitochondrial permeability transition pore and the Ca2+ ac.tivated K+ channel contribute to the cardioprotection conferred by tumor necrosis factor alpha. Cytokine *32*, 199-205.

Garfinkel, L. (1985). Overweight and cancer. Ann Intern Med *103*, 1034-1036.

Garlid, K. D., Paucek, P., Yarov-Yarovoy, V., and others (1997). Cardioprotective effect of diaoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. Circ Res *81*, 1072-1082.

Ginks, W. R., Sybers, H. D., Maroko, P. R., Covell, J. W., Sobel, B. E., and Ross, J., Jr. (1972). Coronary artery reperfusion. II. Reduction of myocardial infarct size at 1 week after the coronary occlusion. J Clin Invest *51*, 2717-2723.

Giroir, P. B., Horton, J.W., White, J., McIntyre, K.L., Lin, C.Q. (1994). Inhibition of tumor necrosis factor prevents myocardial depression during burn shock. Am J Physiol Heart Circ Physiol *267*, H118-H124.

Gomez, L., Paillard, M., Thibault, H., Derumeaux, G., and Ovize, M. (2008). Inhibition of GSK3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. Circulation *117*, 2761-2768.

Gomez, L., Li B, Mewton, N., Sanchez, I., Piot, C., Elbaz, M., and Ovize M. (2009). Inhibition of mitochondrial permeability transition pore opening: translation to patients. Cardiovasc Res *83*, 226-233.

Gonon, A. T., Gourine, A. V., and Pernow, J. (2000). Cardioprotection from ischemia and reperfusion injury by an endothelin A-receptor antagonist in relation to nitric oxide production. J Cardiovasc Pharmacol *36*, 405-412.

Goodman, M. D., Koch, S. E., Fuller-Bicer, G A., *et al.* (2008). Regulating RISK: a role for JAK-STAT signalling in postconditioning? Am J Physiol Heart Circ Physiol *295*, H1649-H1656.

Gordon, D. J., Probstfield, J. L., Garrison, R. J., Neaton, J. D., Castelli, W. P., Knoke, J. D., Jacobs, D. R, Jr., Bangdiwala, S., and Tyroler, H. A. (1989). High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation *79*, 8-15.

Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B., and Dawber, T. R. (1977). High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med *62*, 707-714.

Goto, M., Liu, Y., Yang, X. M., Ardell, J. L., Cohen, M. V., and Downey, J. M. (1995). Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. Circ Res *77*, 611-621.

Gray, M. O., Karliner, J. S., and Mochly-Rosen, D. (1997). A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J Biol Chem *272*, 30945-30951.

Gray, M. O., Zhou, H. Z., Schafhalter-Zoppoth, I., Zhu, P., Mochley-Rosen, D., *et al*. (2004). Preservation of base-line hemodynamic function and loss of inducible cardioprotection in adult mice lacking protein kinase C epsilon. J Biol Chem *279*, 3596-3604.

Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995). The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell *83*, 793-802.

Griffiths, E. J., and Halestrap, A. P. (1993). Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. J Mol Cell Cardiol *25*, 1461-1469.

Grisham, M. B., Jourd'Heuil, D., and Wink, D. A. (1999). Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. Am J Physiol *276*, G315-321.

Gross, E. R., and Gross, G. J. (2006). Ligand triggers of classical preconditioning and postconditioning. Cardiovasc Res *70*, 212-221.

Gross, E. R., Hsu, A. K., and Gross, G. J. (2004). Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. Circ Res *94*, 960-966.

Gross, E. R., Hsu, A. K., and Gross, G. J. (2006). The JAK/STAT pathway is essential for opioid-induced cardioprotection: JAK2 as a mediator of STAT3, Akt, and GSK-3 beta. Am J Physiol Heart Circ Physiol *291*, H827-834.

Gross, E. R., Hsu, A. K., and Gross, G. J. (2007). Diabetes abolishes morphine-induced cardioprotection via multiple pathways upstream of glycogen synthase kinase-3beta. Diabetes *56*, 127-136.

Gross, G. J. (2003). Role of opioids in acute and delayed preconditioning. J Mol Cell Cardiol *35*, 709-718.

Grunfeld, C., Zhao, C., Fuller, J., *et al*. (1996). Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters. J Clin Invest *97*: 2152-2157.

Gu, Q., Yang, X. P., Bonde, P., DiPaula, A., Fox-Talbot, K., and Becker, L. C. (2006). Inhibition of TNF-alpha reduces myocardial injury and proinflammatory pathways following ischemia-reperfusion in the dog. J Cardiovasc Pharmacol *48*, 320-328.

Gurevitch, J., Frolkis, I., Yuhas, Y., Lifschitz-Mercer, B., Berger, E., Paz, Y., Matsa, M., Kramer, A., and Mohr, R. (1997). Anti-tumor necrosis factor-alpha improves myocardial recovery after ischemia and reperfusion. J Am Coll Cardiol *30*, 1554-1561.

Hadour, G., Ferrera, R., Sebbag, L., Forrat, R., Delaye, J., and de Lorgeril, M. (1998). Improved myocardial tolerance to ischaemia in the diabetic rabbit. J Mol Cell Cardiol *30*, 1869-1875.

Hale, S. L., and Kloner, R. A. (2010). Cardioprotection with adenosine-regulating agent, GP531: effects on no-reflow, infarct size, and blood flow following ischemia/ reperfusion in the rabbit. J Cardiovasc Pharmacol Ther *15*, 60-67.

Halestrap, A. P., and Brenner, C. (2003). The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. Curr Med Chem *10*, 1507-1525.

Halestrap, A. P., Clarke, S. J., and Javadov, S. A. (2004). Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res *61*, 372-385.

Halestrap, A. P., Kerr, P. M., Javadov, S., and Woodfield, K. Y. (1998). Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochim Biophys Acta *1366*, 79-94.

Halkos, M. E., Kerendi, F., Corvera, J. S., Wang, N. P., Kin, H., Payne, C. S., Sun, H. Y., Guyton, R. A., Vinten-Johansen, J., and Zhao, Z. Q. (2004). Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. Ann Thorac Surg *78*, 961-969; discussion 969.

Hamann, A., Benecke, H., Le Marchand-Brustel, Y., Susulic, V. S., Lowell, B. B., and Flier, J. S. (1995). Characterization of insulin resistance and NIDDM in transgenic mice with reduced brown fat. Diabetes *44*, 1266-1273.

Hamid, S. A., and Baxter, G. F. (2005). Adrenomedullin limits reperfusion injury in experimental myocardial infarction. Basic Res Cardiol *100*, 387-396.

Hamid, S. A., Totzeck, M., Rasssaf, T., and Baxter, G. F. (2007). Adrenomedullin augments nitrite production and protects against ischaemia-reperfusion injury in mouse heart. J Mol Cell Cardiol *42 (suppl)*, S195.

Hanley P. J, *et al.* (2005). K(ATP) channels and preconditioning: a re-examination of the role of mitochondrial K(ATP) channels and an overview of alternative mechanisms. J Mol Cell Cardiol *39*, 17-50.

Hansson, G. K. (2005). Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med *352*, 1685-1695.

Hattori, R., Maulik, N., Otani, H., Zhu, L., Cordis, G., Engelman, R. M., Siddiqui, M. A., and Das, D. K. (2001). Role of STAT3 in ischemic preconditioning. J Mol Cell Cardiol *33*, 1929-1936.

Hausenloy, D., Wynne, A., Duchen, M., and Yellon, D. (2004a). Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. Circulation *109*, 1714-1717.

Hausenloy, D. J. (2009). Signalling pathways in ischaemic postconditioning. Thromb Haemost *101*, 626-634.

Hausenloy, D. J., Duchen, M. R., and Yellon, D. M. (2002). Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. Cardiovasc Res *60*, 617-625.

Hausenloy, D. J., Mocanu, M. M., and Yellon, D. M. (2004b). Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. Cardiovasc Res *63*, 305-312.

Hausenloy, D. J., Mwamure, P. K., Venugopal, V., Harris, J., Barnard, M., Grundy, E., Ashley, E., Vichare, S., Di Salvo, C., Kolvekar, S., *et al.* (2007). Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. Lancet *370*, 575-579.

Hausenloy, D. J., Tsang, A., and Yellon, D. M. (2005). The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. Trends Cardiovasc Med *15*, 69-75.

Hausenloy, D. J., and Yellon, D. M. (2004). New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res *61*, 448-460.

Hausenloy, D. J., and Yellon, D. M. (2006). Survival kinases in ischemic preconditioning and postconditioning. Cardiovasc Res *70*, 240-253.

Hausenloy, D. J., Tsang, A., and Yellon, D.M. (2006b). Postconditioning does not protect the diabetic heart. J.Mol.Cell Cardiol *40*, 958.

Hausenloy, D. J., and Yellon, D. M. (2007a). Preconditioning and postconditioning: united at reperfusion. Pharmacol Ther *116*, 173-191.

Hausenloy, D. J., and Yellon, D. M. (2007b). Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. Heart Fail Rev *12*, 217-234.

Headrick, J. P., Willems, L., Ashton, K. J., Holmgren, K., Peart, J., and Matherne, G. P. (2003). Ischaemic tolerance in aged mouse myocardium: the role of adenosine and effects of A1 adenosine receptor overexpression. J Physiol *549*, 823-833.

Herskowitz, A., Choi, S., Ansari, A. A., and Wesselingh, S. (1995). Cytokine mRNA expression in postischemic/reperfused myocardium. Am J Pathol *146*, 419-428.

Heusch, G., Boengler, K., and Schulz, R. (2008). Cardioprotection: nitric oxide, protein kinases, and mitochondria. Circulation *118*, 1915-1919.

Heusch, G., Buchert, A., Feldhaus, S., and Schulz, R. (2006). No loss of cardioprotection by postconditioning in connexin 43-deficient mice. Basic Res Cardiol, *101*, 354-356.

Heusch, G., Boengler, K., and Schulz, R. (2010). Inhibition of mitochondrial permeability transition pore opening: the holy grail of cardioprotection. Basic Res Cardiol, *105*, 151-154.

Higuchi, Y., McTiernan, C. F., Frye, C. B., McGowan, B. S., Chan, T. O., and Feldman, A. M. (2004). Tumor necrosis factor receptors 1 and 2 differentially regulate survival, cardiac dysfunction, and remodeling in transgenic mice with tumor necrosis factor-alpha-induced cardiomyopathy. Circulation *109*, 1892-1897.

Hofmann, C., Lorenz, K., Braithwaite, S. S., Colca, J. R., Palazuk, B. J., Hotamisligil, G. S., and Spiegelman, B. M. (1994). Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. Endocrinology *134*, 264-270.

Hoole, S. P., Heck, P. M., Sharples, L., Khan, S. N., Duehmke, R., Densem, C. G., Clarke, S. C., Shapiro, L. M., Schofield, P. M., O'Sullivan, M., and Dutka, D. P. (2009). Cardiac Remote Ischemic Preconditioning in Coronary Stenting (CRISP Stent) Study: a prospective, randomized control trial. Circulation *119*, 820-827.

Hopper, R. A., Forrest, C. R., Xu, H., Zhong, A., He, W., Rutka, J., Neligan, P., and Pang, C. Y. (2000). Role and mechanism of PKC in ischemic preconditioning of pig skeletal muscle against infarction. Am J Physiol Regul Integr Comp Physiol *279*, R666-676.

Hossain, P. (2007). Obesity and Diabetes in the Developing World - A Growing Challenge. N Engl J Med *356*, 213-215.

Hotamisligil, G. (1993). Adipose expression of tumor necrosis factor alpha: direct role in obesity-linked insulin resistance. Science *259*, 87-91.

Hotamisligil, G. S. (2003). Inflammatory pathways and insulin action. Int J Obes Relat Metab Disord *27 Suppl 3*, S53-55.

Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L., and Spiegelman, B. M. (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest *95*, 2409-2415.

Hotamisligil, G. S., Budavari, A., Murray, D., and Spiegelman, B. M. (1994a). Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha. J Clin Invest *94*, 1543-1549.

Hotamisligil, G. S., Johnson, R. S., Distel, R. J., Ellis, R., Papaioannou, V. E., and Spiegelman, B. M. (1996). Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. Science *274*, 1377-1379.

Hotamisligil, G. S., Murray D L, Choy L N, and Spiegelman, B. M. (1994b). Tumour necrosis factor alpha inhibits signalling from the insulin receptor. Proc Natl Acad Sci USA *91*, 4854-4858.

Hsu, H., Xiong, J., and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495-504.

Hu, K., Duan, D., Li, G. R., *et al.* (1996). Protein kinase C activates ATP-sensitive K+ current in human and rabbit ventricular myocytes. Circulation Research *78*, 492-498.

Huang, J. S., Guh, J. Y., Chen, H. C., Hung, W. C., Lai, Y. H., and Chuang, L. Y. (2001). Role of receptor for advanced glycation end-product (RAGE) and the JAK/STAT-signaling pathway in AGE-induced collagen production in NRK-49F cells. J Cell Biochem *81*, 102-113.

Ichikawa, Y., Miura, T., Nakano, A., Miki, T., Nakamura, Y., Tsuchihashi, K., and Shimamoto, K. (2004). The role of ADAM protease in the tyrosine kinase-mediated trigger mechanism of ischemic preconditioning. Cardiovasc Res *62*, 167-175.

Ikezoe, T., Saito, T., Bandobashi, K., Yang, Y., Koeffler, H. P., and Taguchi, H. (2004). HIV-1 protease inhibitor induces growth arrest and apoptosis of human multiple myeloma cells via inactivation of signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2. Mol Cancer Ther *3*, 473-479.

Iliodromitis, E. K., Zoga, A., Vrettou, A., Andreadou, I., Paraskevaidis, I. A., Kaklamanis, L., and Kremastinos, D. T. (2006). The effectiveness of postconditioning and preconditioning on infarct size in hypercholesterolemic and normal anesthetized rabbits. Atherosclerosis *188*, 356-362.

Irwin, M. W., Mak, S., Mann, D. L., Qu, R., Penninger, J. M., Yan, A., Dawood, F., Wen, W. H., Shou, Z., and Liu, P. (1999). Tissue expression and immunolocalization of tumor necrosis factor-α in postinfarction dysfunctional myocardium. Circulation *99*, 1492-1498.

Ishihara, M., Inoue, I., Kawagoe, T., Shimatani, Y., Kurisu, S., Nishioka, K., Kouno, Y., Umemura, T., Nakamura, S., and Sato, H. (2001). Diabetes mellitus prevents ischemic preconditioning in patients with a first acute anterior wall myocardial infarction. J Am Coll Cardiol *38*, 1007-1011.

Javadov, S. A., Clarke, S., Das, M., Griffiths, E. J., Lim, K. H., and Halestrap, A. P. (2003). Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. J Physiol *549*, 513-524.

Jennings, R. B., Reimer, K A (1991). The cell biology of acute myocardial ischemia. Ann Rev Med *42*, 225-246.

Jiang, R., Zatta, A., Kin, H., Wang, N., Reeves, J. G., Mykytenko, J., Deneve, J., Zhao, Z. Q., Guyton, R. A., and Vinten-Johansen, J. (2007). PAR-2 activation at the time of reperfusion salvages myocardium via an ERK1/2 pathway in in vivo rat hearts. Am J Physiol Heart Circ Physiol *293*, H2845-2852.

Jiang, X., Shi, E., Nakajima, Y., and Sato, S. (2006). COX-2 mediates morphine-induced delayed cardioprotection via an iNOS-dependent mechanism. Life Sci *78*, 2543-2549.

Jiang, Y., Woronicz, J. D., Liu, W., and Goeddel, D. V. (1999). Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. Science *283*, 543-546.

Jin, G., Hayashi T, Kawagoe J, Takizawa T, Nagata T, Nagano I, Syoji M, and K., A. (2005). Deficiency of PAR-2 gene increases acute focal ischemic brain injury.J Cereb Blood Flow Metab *25*, 302-313.

Jin, Z. Q., Goetzl, E. J., and Karliner, J. S. (2004). Sphingosine kinase activation mediates ischemic preconditioning in murine heart. Circulation *110*, 1980-1989.

Jin, Z. Q., Karliner, J. S., and Vessey, D. A. (2008). Ischaemic postconditioning protects isolated mouse hearts against ischaemia/reperfusion injury via sphingosine kinase isoform-1 activation. Cardiovasc Res *79*, 134-140.

Jinkun, X., Tian, W., Zhang, L., and others (2009). Morphine prevents the mitochondrial permeability transition pore opening through NO/cGMP/PKG/Zn2+/GSK-3 β signal pathway in cardiomyocytes. Am J Physiol Heart Circ Physiol *298*, H601-H608.

Jones, S. J., Ledgerwood, E. C., Prins, J. B., Galbraith, J., Johnson, D. R., Pober, J. S., and Bradley, J. R. (1999). TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. J Immunol *162*, 1042-1048.

Jonassen, A.K., Aasum, E., Riemersma, R.A., Mjos, O.D., Larsen, T. (2000). Glucose-insulin-potassium reduces infarct size when administered during reperfusion. Cardiovasc Drugs Ther *14*, 616-623.

Jonassen, A.K., Sack, M., Mjos, O.D., Yellon, D.M. (2001). Myocardial protection by insulin in reperfusion injury requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ Res *89*, 1191-1198.

Jooste, P. L., Steenkamp, H. J., Benade, A. J., and Rossouw, J. E. (1988). Prevalence of overweight and obesity and its relation to coronary heart disease in the CORIS study. S Afr Med J *74*, 101-104.

Juhaszova, M., Zorov, D. B., Kim, S. H., Pepe, S., Fu, Q., Fishbein, K. W., Ziman, B. D., Wang, S., Ytrehus, K., Antos, C. L., *et al.* (2004). Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest *113*, 1535-1549.

Juhaszova, M., Zorov, D. B., Yaniv, Y., Nuss, H. B., Wang, S., and Sollott, S. J. (2009). Role of glycogen synthase kinase-3beta in cardioprotection. Circ Res *104*, 1240-1252.

Kanety, H., Hemi, R., Papa, M. Z., and Karasik, A. (1996). Sphingomyelinase and ceramide suppress insulin-induced tyrosine phosphorylation of the insulin receptor substrate-1. J Biol Chem *271*, 9895-9897.

Kannel, W. B., and McGee, D. L. (1979). Diabetes and cardiovascular risk factors: the Framingham study. Circulation *59*, 8-13.

Kaplan, N. M., Victor, R. G. (2010). Clinical Hypertension. 10th edition, publishers: Wolters Kluwer/Lippincott Williams and Wilkins.

Katakam, P. V., Jordan, J. E., Snipes, J. A., Tulbert, C. D., Miller, A. W., and Busija, D. W. (2007). Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. Am J Physiol Regul Integr Comp Physiol *292*, R920-926.

Kelly, R. (2008). Sphingosine-1-phosphate induced cardioprotection is mediated by STAT-3. J Mol Cell Cardiol *44*, 740.

Kern, P. A., Ranganathan, S., Li, C., Wood, L., and Ranganathan, G. (2001). Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab *280*, E745-751.

Kern, P. A., Saghizadeh, M., Ong, J. M., Bosch, R. J., Deem, R., and Simsolo, R. B. (1995). The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest *95*, 2111-2119.

Kern, P. A., (1997). Potential role of TNFα and lipoprotein lipase as candidate genes for obesity. J Nutr *127*, 1917S-1922S.

Kersten, J. R., Montgomery, M. W., Ghassemi, T., Gross, E. R., Toller, W. G., Pagel, P. S., and Warltier, D. C. (2001). Diabetes and hyperglycemia impair activation of mitochondrial K(ATP) channels. Am J Physiol Heart Circ Physiol *280*, H1744-1750.

Kersten, J. R., Toller, W. G., Gross, E. R., Pagel, P. S., and Warltier, D. C. (2000). Diabetes abolishes ischemic preconditioning: role of glucose, insulin, and osmolality. Am J Physiol Heart Circ Physiol *278*, H1218-1224.

Kevin, L. G., Camara, A. K., Riess, M. L., Novalija, E., and Stowe, D. F. (2003). Ischemic preconditioning alters real-time measure of O2 radicals in intact hearts with ischemia and reperfusion. Am J Physiol Heart Circ Physiol *284*, H566-574.

Kim, J. Y., Park, H. K., Yoon, J. S., Kim, S. J., Kim, E. S., Ahn, K. S., Kim, D. S., Yoon, S. S., Kim, B. K., and Lee, Y. Y. (2008). Advanced glycation end product (AGE)-induced proliferation of HEL cells via receptor for AGE-related signal pathways. Int J Oncol *33*, 493-501.

Kimura, H., Shintani-Ishida K, Nakajima M, Liu S, Matsumoto K, and Yoshida, K. (2006). Ischemic preconditioning or p38 MAP kinase inhibition attenuates myocardial TNF alpha production and mitochondria damage in brief myocardial ischemia.Life Sci *78*, 1901-1910.

Kin, H. (2008). Inhibition of myocardial apoptosis by postconditioning is associated with attenuation of oxidative stress-mediated nuclear factor- kappaB translocation and TNF alpha release. Shock *6*, 761-768.

Kin, H., Zatta, A. J., Lofye, M. T., Amerson, B. S., Halkos, M. E., Kerendi, F., Zhao, Z. Q., Guyton, R. A., Headrick, J. P., and Vinten-Johansen, J. (2005a). Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res *67*, 124-133.

Kin, H., Zatta, A. J., Lofye, M. T., and others (2005b). Activation of opioid receptors mediates the infarct size reduction by Postconditioning. J Mol Cell Cardiol *38*, 827-832.

Kin, H., Zhao, Z. Q., Sun, H. Y., Wang, N. P., Corvera, J. S., Halkos, M. E., Kerendi, F., Guyton, R. A., and Vinten-Johansen, J. (2004). Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res *62*, 74-85.

Kis, A., Baxter, G. F., and Yellon, D. M. (2006). Limitation of myocardial reperfusion injury by AMP579, an adenosine A1/A2A receptor agonist: role of A2A receptor and Erk1/2. Cardiovasc Drugs Ther *17*, 415-425.

Kloner, R. A. (1993). Does reperfusion injury exist in humans? J Am Coll Cardiol *21*, 537-545.

Kloner, R. A., Forman, M. B., Gibbons, R. J., Ross, A. M., Alexander, R. W., and Stone, G. W. (2006). Impact of time to therapy and reperfusion modality on the efficacy of adenosine in acute myocardial infarction: the AMISTAD-2 trial. Eur Heart J *27*, 2400-2405.

Kloner, R. A., Shook, T., Przyklenk, K., Davis, V. G., Junio, L., Matthews, R. V., Burstein, S., Gibson, M., Poole, W. K., Cannon, C. P., and et al. (1995). Previous angina alters in-hospital outcome in TIMI 4. A clinical correlate to preconditioning? Circulation *91*, 37-45.

Koistinen, H. A., Bastard, J. P., Dusserre, E., Ebeling, P., Zegari, N., Andreelli, F., Jardel, C., Donner, M., Meyer, L., Moulin, P., *et al.* (2000). Subcutaneous adipose tissue expression of tumour necrosis factor-alpha is not associated with whole body insulin resistance in obese nondiabetic or in type-2 diabetic subjects. Eur J Clin Invest *30*, 302-310.

Kolesnick, R. N., and Kronke, M. (1998). Regulation of ceramide production and apoptosis. Annu Rev Physiol *60*, 643-665.

Kristiansen, S. B., Lofgren, B., Stottrup, N. B., Khatir, D., Nielsen-Kudsk, J. E., Nielsen, T. T., Botker, H. E., and Flyvbjerg, A. (2004). Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. Diabetologia *47*, 1716-1721.

Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. Physiol Rev *87*, 99-163.

Kroeze, W. K., Sheffler, D. J., and Roth, B. L. (2003). G-protein-coupled receptors at a glance. J Cell Sci ***116*, 4867-4869.**

Krogh-Madsen, R., Plomgaard, P., Moller, K., Mittendorfer, B., and Pedersen, B. K. (2006). Influence of TNF-alpha and IL-6 infusions on insulin sensitivity and expression of IL-18 in humans. Am J Physiol Endocrinol Metab *291*, E108-114.

Krumins, S. A., Faden, A. I., and Feuerstein, G. (1985). Opiate binding in rat hearts: modulation of binding after hemorrhagic shock. Biochem Biophys Res Commun *127*, 120-128.

Kubota, T., McTiernan, C. F., Frye, C. S., Demetris, A. J., and Feldman, A. M. (1997). Cardiac-specific overexpression of tumor necrosis factor-alpha causes lethal myocarditis in transgenic mice. J Card Fail *3*, 117-124.

Kupatt, C., Habazettl, H., Goedecke, A., Wolf, D. A., Zahler, S., Boekstegers, P., Kelly, R. A., and Becker, B. F. (1999). Tumor necrosis factor-alpha contributes to ischemia- and reperfusion-induced endothelial activation in isolated hearts. Circ Res *84*, 392-400.

Kurrelmeyer, K. M., Michael, L. H., Baumgarten, G., Taffet, G. E., Peschon, J. J., Sivasubramanian, N., Entman, M. L., and Mann, D. L. (2000). Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. Proc Natl Acad Sci U S A *97*, 5456-5461.

Kuzuya, T., Hoshida, S., Yamashita, N., Fuji, H., Oe, H., Hori, M., Kamada, T., and Tada, M. (1993). Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res *72*, 1293-1299.

Labruto, F., Yang, J., Vaage, J., and Valen, G. (2005). Role of tumor necrosis factor alpha and its receptor I in preconditioning by hyperoxia. Basic Res Cardiol *100*, 198-207.

Lacerda, L., Smith, R. M., Opie, L., and Lecour, S. (2006). TNFalpha-induced cytoprotection requires the production of free radicals within mitochondria in C2C12 myotubes. Life Sci *79*, 2194-2201.

Lacerda, L., Somers, S., Opie, L. H., and Lecour, S. (2009). Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. Cardiovasc Res *84*, 201-208.

Lacerda, L., McCarthy, J., Mungly, S.F.K., Lynn, E.G., Sack, M.N., Opie, L.H., Lecour, S. (2010). Basic Res Cardiol DOI 10.1007/s00395-010-1113-4.

Lantz, M., Malik, S., Slevin, M. L., and Olsson, I. (1990). Infusion of tumor necrosis factor (TNF) causes an increase in circulating TNF-binding protein in humans. Cytokine *2*, 402-406.

Lapidus, L., Bengtsson, C., Larsson, B., Pennert, K., Rybo, E., and Sjostrom, L. (1984). Distribution of adipose tissue and risk of cardiovascular disease and death: a 12 year follow up of participants in the population study of women in Gothenburg, Sweden. Br Med J (Clin Res Ed) *289*, 1257-1261.

Larsson, B., Bjorntorp, P., and Tibblin, G. (1981). The health consequences of moderate obesity. Int J Obes *5*, 97-116.

Larsson, B., Svardsudd, K., Welin, L., Wilhelmsen, L., Bjorntorp, P., and Tibblin, G. (1984). Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913. Br Med J (Clin Res Ed) *288*, 1401-1404.

Laskey, W. K. (2005). Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: a pilot study. Catheter Cardiovasc Interv *65*, 361-367.

Latini, R., Bianchi, M., Correale, E., Dinarello, C.A., Fantuzzi, G., Fresco, C., Maggioini, A.P., Mengozzi, M., Romano, S., Shapiro, L. (1994). Cytokines in acute myocardial infarction: selective increase in circulating tumor necrosis factor, its soluble receptor, and interleukin 1 receptor antagonist. J Cardiovasc Pharmacol *23*, 1-6.

Lawson, C. S., and Downey, J. M. (1993). Preconditioning: state of the art myocardial protection. Cardiovasc Res *27*, 542-550.

Lecour, S. (2009a). Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol *47*, 32-40.

Lecour, S. (2009b). Multiple protective pathways against reperfusion injury: a SAFE path without Aktion? J Mol Cell Cardiol *46*, 607-609.

Lecour, S., Rochette, L., and Opie, L. (2005a). Free radicals trigger TNF alpha-induced cardioprotection. Cardiovasc Res *65*, 239-243.

Lecour, S., Smith, R. M., Woodward, B., Opie, L. H., Rochette, L., and Sack, M. N. (2002). Identification of a novel role for sphingolipid signaling in TNF alpha and ischemic preconditioning mediated cardioprotection. J Mol Cell Cardiol *34*, 509-518.

Lecour, S., Suleman, N., Deuchar, G. A., Somers, S., Lacerda, L., Huisamen, B., and Opie, L. H. (2005b). Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (Akt and extracellular signal-regulated kinase). Circulation *112*, 3911-3918.

Ledgerwood, E. C., Pober, J. S., and Bradley, J. R. (1999). Recent advances in the molecular basis of TNF signal transduction. Lab Invest *79*, 1041-1050.

Leesar, M. A., Stoddard, M. F., Manchikalapudi, S., and Bolli, R. (1999). Bradykinin-induced preconditioning in patients undergoing coronary angioplasty. J Am Coll Cardiol *34*, 639-650.

Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., and Herman, B. (1997). The mitochondrial permeability transition in toxic, hypoxic and reperfusion injury. Mol Cell Biochem *174*, 159-165.

Levine, B. (1990). Elevated circulating levels of tunor necrosis factor in severe chronic heart failure. N Engl J Med *323*, 236-241.

Lew, E. A. (1985). Mortality and weight: insured lives and the American Cancer Society studies. Ann Intern Med *103*, 1024-1029.

Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H., Chen, E. Y., and Goeddel, D. V. (1991). Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci U S A *88*, 2830-2834.

Libby, P. (2002). Inflammation in atherosclerosis. Nature *420*, 868-874.

Lim, S. Y., Davidson, S. M., Hausenloy, D. J., and Yellon, D. M. (2007). Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. Cardiovasc Res *75*, 530-535.

Liu, G. S., C, R. S., Olsson, R. A., Mullane,K., Walsh, R. S., and Downey, J. M. (1994). Evidence that the A3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. Cardiovasc Res *28*, 1057-1061.

Liu, G. S., Thornton, J., Van Winkle, D. M., Stanley, A. W., Olsson, R. A., and Downey, J. M. (1991). Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. Circulation *84*, 350-356.

Liu, H., Huang, Q., Shi, B., Eksarko, P., Temkin, V., and Pope, R. M. (2006). Regulation of Mcl-1 expression in rheumatoid arthritis synovial macrophages. Arthritis Rheum *54*, 3174-3181.

Liu, R. Y., Fan, C., Mitchell, S., Chen, Q., Wu, J., and Zuckerman, K. S. (1998a). The role of type I and type II tumor necrosis factor (TNF) receptors in the ability of TNF-alpha to transduce a proliferative signal in the human megakaryoblastic leukemic cell line Mo7e. Cancer Res *58*, 2217-2223.

Liu, X., Das, Anindita., Merino,Vanessa F., Bader, Michael and Kukreja, R. C. (2008). Loss of Myocardial Ischemic Postconditioning in Adenosine A1 and Bradykinin B2 Receptors Gene Knockout Mice. Circulation *118*, 532-537.

Liu, X., Wei, J., Peng, D. H., Layne, M. D., and Yet, S. F. (2005). Absence of heme oxygenase-1 exacerbates myocardial ischemia/reperfusion injury in diabetic mice. Diabetes *54*, 778-784.

Liu, Y., and Downey, J. M. (1993). Preconditioning against infarction in the rat heart does not involve a petussis toxin sensitive G protein. Cardiovasc Res *27*, 608-611.

Liu, Y., Sato T, O'Rourke B, *et al.* (1998b). Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? Circulation *97*, 2463-2469.

Liu, Y., Thornton, J. D., Cohen, M. V., Downey, J. M., and Schaffer, S. W. (1993). Streptozotocin-induced non-insulin-dependent diabetes protects the heart from infarction. Circulation *88*, 1273-1278.

Lochner, A., Marais, E., Genade, S., and Moolman, J. A. (2000). Nitric oxide: a trigger for classic preconditioning? Am J Physiol Heart Circ Physiol *279*, H2752-2765.

Loukogeorgakis, S. P., van den Berg, M. J., Sofat, R., Nitsch, D., Charakida, M., Haiyee, B., de Groot, E., MacAllister, R. J., Kuijpers, T. W., and Deanfield, J. E. (2010). Role of NADPH oxidase in endothelial ischemia/reperfusion injury in humans. Circulation *121*, 2310-2316.

Lubert, S. (1988). Biochemistry (W.H. Freeman and Company).

Ma, G., Al-Shabrawey, M., Johnson, J. A., Datar, R., Tawfik, H. E., Guo, D., Caldwell, R. B., and Caldwell, R. W. (2006). Protection against myocardial ischemia/reperfusion injury by short-term diabetes: enhancement of VEGF formation, capillary density, and activation of cell survival signaling. Naunyn Schmiedebergs Arch Pharmacol *373*, 415-427.

MacDonald, I. (1998). Energy expenditure in humans: The influence of activity, diet and the sympathetic nervous system. In: Kopelman PG; Stock MJ, eds. Clinical Obesity, Oxford: Blackwell Science.

MacEwan, D. J. (2002). TNF receptor subtype signalling: differences and cellular consequences. Cell Signal *14*, 477-492.

Maekawa, N., Wada, H., Kanda, T., Niwa, T., Yamada, Y., Saito, K., Fujiwara, H., Sekikawa, K., and Seishima, M. (2002). Improved myocardial ischemia/reperfusion injury in mice lacking tumor necrosis factor-alpha. J Am Coll Cardiol *39*, 1229-1235.

Maekawa, N., Wada, H., Saito, K., Fujii, H., Niwa, K., Fujigaki, S., Kirii, H., Takatsu, H., Sekikawa, K., and Seishima, M. (2000). Myocardial ischemia/reperfusion injury in mice lacking tumor necrosis factor-α. Circulation *102*, II-77 (Abstract).

Magazine, H. I., King, J. M., and Srivastava, K. D. (1996). Protease activated receptors modulate aortic vascular tone. Int J Cardiol *53 Suppl*, S75-80.

Mahaffey, K. W., Puma, J. A., Barbagelata, N. A., DiCarli, M. F., Leesar, M. A., Browne, K. F., Eisenberg, P. R., Bolli, R., Casas, A. C., Molina-Viamonte, V., *et al.* (1999). Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. J Am Coll Cardiol *34*, 1711-1720.

Mann, D. L. (2002). Inflammatory mediators and the failing heart: past, present, and the foreseeable future. Circ Res *91*, 988-998.

Mann, D. L. (2003). Stress-activated cytokines and the heart: from adaptation to maladaptation. Annu Rev Physiol *65*, 81-101.

Manson, J. E., Willett W C, Stampfer M J, Colditz GA, Hunter DJ, Hankinson, S. E., Hennekens, C. H., and Speizer, F. E. (1995). Body weight and mortality among women. N Engl J Med *333*, 677-685.

Mantzoros, CS., Moschos, S., Avraamopoulos, I., Kaklamani, V., Liolios, A., Doulgerakis, D.E., Griveas, I., Katsilambros, N and Flier J.S. Leptin Concentrations in Relation to Body Mass Index and the Tumor Necrosis Factor-α System in Humans. J Clin Endocrinol and Metab *82:* 3408-3413.

Marber, M. S., Latchman, D. S., Walker, J. M., and Yellon, D. M. (1993). Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. Circulation *88*, 1264-1272.

Marceau, F., Hess, J. F., and Bachvarov, D. R. (1998). The B1 receptors for kinins. Pharmacol Rev *50*, 357-386.

Marfella, R., Di Filippo, C., Esposito, K., Nappo, F., Piegari, E., Cuzzocrea, S., Berrino, L., Rossi, F., Giugliano, D., and D'Amico, M. (2004). Absence of inducible nitric oxide synthase reduces myocardial damage during ischemia reperfusion in streptozotocin-induced hyperglycemic mice. Diabetes *53*, 454-462.

Margalet, V.S., Romero, C.M., Alvarez, J.S., Goberna, R., Najib, S., Yanes, C.G. (2003). Role of leptin as an immunomodulator of blood mononuclear cells: mechanism of action. Clin Exp Immunol *133*: 11-19.

Marinissen, M. J., and Gutkind, J. S. (2001). G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci *22*, 368-376.

Marino, M., Dunn, A., Grail, D., Inglese, M., Noguchi, Y., Richards, E., Jungbluth, A., Wada, H., Moore, M., Williamson, B., *et al.* (1997). Characterization of tumor necrosis factor-deficient mice.Proc Natl Acad Sci USA 1997 Jul 22;94(15):8093-8098.

Maroko, P. R., Kjekshus, J. K., Sobel, B. E., Watanabe, T., Covell, J. W., Ross, J., Jr., and Braunwald, E. (1971). Factors influencing infarct size following experimental coronary artery occlusions. Circulation *43*, 67-82.

Maroko, P. R., Libby, P., Ginks, W. R., Bloor, C. M., Shell, W. E., Sobel, B. E., and Ross, J., Jr. (1972). Coronary artery reperfusion. I. Early effects on local myocardial function and the extent of myocardial necrosis. J Clin Invest *51*, 2710-2716.

Marques, J. M., Belza, I., Holtmann, B., Pennica, D., Prieto, J., and Bustos, M. (2007). Cardiotrophin-1 is an essential factor in the natural defense of the liver against apoptosis. Hepatology *45*, 639-648.

Marsche, G., *et al.* (2007). Hypochlorite-modified albumin colocalizes with RAGE in the artery wall and promotes MCP-1 expression via RAGE-ERK1/2 MAP-kinase pathway. The FASEB Journal *21*, 1145-1152.

Marzilli, M., Orsini, E., Marraccini, P., and Testa, R. (2000). Beneficial effects of intracoronary adenosine as an adjunct to primary angioplasty in acute myocardial infarction. Circulation *101*, 2154-2159.

Mason, R. B., Pluta, R. M., Walbridge, S., Wink, D. A., Oldfield, E. H., and Boock, R. J. (2000). Production of reactive oxygen species after reperfusion in vitro and in vivo: protective effect of nitric oxide. J Neurosurg *93*, 99-107.

Massie, B. M., and Shah, N. B. (1997). Evolving trends in the epidemiologic factors of heart failure: rationale for preventive strategies and comprehensive disease management. Am Heart J *133*, 703-712.

Matsui, T., Li, L., del Monte, F., Fukui, Y., Franke, T. F., Hajjar, R. J., and Rosenzweig, A. (1999). Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. Circulation *100*, 2373-2379.

Matsumori, A., Yamada, T., Suzuki, H., Matoba, Y., and Sasayama, S. (1994). Increased circulating cytokines in patients with myocarditis and cardiomyopathy. Br Heart J *72*, 561-566.

Maulik, N., Engelman, D. T., Watanabe, M., Engelman, R. M., Maulik, G., Cordis, G. A., and Das, D. K. (1995). Nitric oxide signaling in ischemic heart. Cardiovasc Res *30*, 593-601.

Maulik, N., Watanabe, M., Zu, Y. L., Huang, C. K., Cordis, G. A., Schley, J. A., *et al.*(1996). Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. FEBS Lett *396*, 233-237.

Maulik, N., Yoshida, T., Zu, Y. L., Sato, M., Banerjee, A., and Das, D. K. (1998). Ischemic preconditioning triggers tyrosine kinase signaling: a potential role for MAPKAP kinase 2. Am J Physiol *275*, H1857-1864.

Maury, C. P., and Teppo, A. M. (1989). Circulating tumour necrosis factor-alpha (cachectin) in myocardial infarction. J Intern Med *225*, 333-336.

Meldrum, D., Cain, B.S., Cleveland, J.C. Jr., Meng, X., Ayala, A., Banerjee, A., and Harken, A.. (1997). Adenosine decreases post-ischaemic cardiac TNF-alpha production: anti-inflammatory implications for preconditioning and transplantation. Immunology *92*, 472-477.

Meldrum, D. R. (1998). Tumor necrosis factor in the heart. Am J Physiol *274*, R577-R595.

Meldrum, D. R., Cleveland, J. C., Jr., Cain, B. S., Meng, X., and Harken, A. H. (1998a). Increased myocardial tumor necrosis factor-alpha in a crystalloid-perfused model of cardiac ischemia-reperfusion injury. Ann Thorac Surg *65*, 439-443.

Meldrum, D. R., Meng, X., Dinarello, C. A., Ayala, A., Cain, B. S., Shames, B. D., Ao, L., Banerjee, A., and Harken, A. H. (1998b). Human myocardial tissue TNFalpha expression following acute global ischemia in vivo. J Mol Cell Cardiol *30*, 1683-1689.

Menash, G. A. (2008). Ischaemic heart disease in Africa. Heart *94*, 836-843.

Miura, K., Kano, S., Nakai, T., Satoh, K., Hoshi, K., and Ichihara, K. (1997). Inhibitory effects of glibenclamide and pertussis toxin on the attenuation of ischemia-induced myocardial acidosis following ischemic preconditioning in dogs. Jpn Circ J *61*, 709-714.

Mizukami, Y., and Yoshida, K. (1997). Mitogen-activated protein kinase translocates to the nucleus during ischaemia and is activated during reperfusion. Biochem J *323 (Pt 3)*, 785-790.

Mocanu, M. M., Bell, R. M., and Yellon, D. M. (2002). PI3 kinase and not p42/p44 appears to be implicated in the protection conferred by ischemic preconditioning. J Mol Cell Cardiol *34*, 661-668.

Monden, Y., Kubota, T., Inoue, T., Tsutsumi, T., Kawano, S., Ide, T., Tsutsui, H., and Sunagawa, K. (2007). Tumor necrosis factor-alpha is toxic via receptor 1 and protective via receptor 2 in a murine model of myocardial infarction. Am J Physiol Heart Circ Physiol *293*, H743-753.

Montecucco, F., Lenglet, S., Braunersreuther, V., Burger, F., Pelli, G., Bertolotto, M., Mach, F., and Steffens, S. (2009). CB(2) cannabinoid receptor activation is cardioprotective in a mouse model of ischemia/reperfusion. J Mol Cell Cardiol *46*, 612-620.

Moolman, J. A., Genade, S., Tromp, E., Opie, L.H., Lochner, A. (1997). Ischaemic preconditioning does not protect hypertrophied myocardium against ischaemia. S Afr Med J *87*, C151-C156.

Morello, S., Vellecco, V., Roviezzo, F., Maffia, P., Cuzzocrea, S., Cirino, G., *et al.* (2005). A protective role for proteinase activated receptor 2 in airways of lipopolysaccharide-treated rats. Biochem Pharmacol *19*, 223-230.

Moro, C., Jouan, M. G., Rakotovao, A., Toufektsian, M. C., Ormezzano, O., Nagy, N., Tosaki, A., de Leiris, J., and Boucher, F. (2007). Delayed expression of cytokines after reperfused myocardial infarction: possible trigger for cardiac dysfunction and ventricular remodeling. Am J Physiol Heart Circ Physiol *293*, H3014-3019.

Moss, M. L., Jin, S. L., Milla, M. E., Bickett, D. M., Burkhart, W., Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Hassler, D., *et al.* (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature *385*, 733-736.

Mozaffari, M., Schaffer, SW. (2008). Effect of pressure overload on cardioprotection of mitochondrial KATP channels and GSK-3beta: interaction with the MPT pore. Am J Hypertens *21*, 570-575.

Mubagwa, K., and Flameng, W. (2001). Adenosine, adenosine receptors and myocardial protection: an updated overview. Cardiovasc Res *52*, 25-39.

Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation *74*, 1124-1136.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., *et al.* (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell *85*, 817-827.

Nakamura, T., Terajima, T., Ogata, T., Ueno, K., Hashimoto, N., Ono, K., and Yano, S. (2006). Establishment and Pathophysiological Characterization of Type 2 Diabetic Mouse Model Produced by Streptozotocin and Nicotinamide. Biol Pharm Bull *29*, 1167-1114.

Nakano, A., Baines, C. P., Kim, S. O., Pelech, S. L., Downey, J. M., Cohen, M. V., and Critz, S. D. (2000). Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: evidence for involvement of p38 MAPK. Circ Res *86*, 144-151.

Nakano, M., Knowlton, A. A., Dibbs, Z., and Mann, D. L. (1998). Tumor necrosis factor-alpha confers resistance to hypoxic injury in the adult mammalian cardiac myocyte. Circulation *97*, 1392-1400.

Napoli, C., Cicala, C., Wallace, J. L., de Nigris, F., Santagada, V., Caliendo, G., Franconi, F., Ignarro, L. J., and Cirino, G. (2000). Protease-activated receptor-2 modulates myocardial ischemia-reperfusion injury in the rat heart. Proc Natl Acad Sci U S A *97*, 3678-3683.

Napoli, C. De Nigris, F., Cicala C, Wallace, JL, Caliendo G, Condorelli M, Santagada V, Cirino G (2002). Protease-activated receptor-2 activation improves efficiency of experimental ischemic preconditioning. Am J Physiol Heart Circ Physiol *282*, H2004-H2010.

Nawata, T., Takahashi, N., Ooie, T., Kaneda, K., Saikawa, T., and Sakata, T. (2002). Cardioprotection by streptozotocin-induced diabetes and insulin against ischemia/reperfusion injury in rats. J Cardiovasc Pharmacol *40*, 491-500.

Negoro, S., Kunisada, K., Fujio, Y., Funamoto, M., Darville, M. I., Eizirik, D. L., Osugi, T., Izumi, M., Oshima, Y., Nakaoka, Y., *et al.* (2001). Activation of signal transducer and activator of transcription 3 protects cardiomyocytes from hypoxia/reoxygenation-induced oxidative stress through the upregulation of manganese superoxide dismutase. Circulation *104*, 979-981.

Nelson, S. K., Wong, G. H., and McCord, J. M. (1995). Leukemia inhibitory factor and tumor necrosis factor induce manganese superoxide dismutase and protect rabbit hearts from reperfusion injury. J Mol Cell Cardiol *27*, 223-229.

Nestruck, A. C., and Davignon, J. (1986). Risks for hyperlipidemia. Cardiol Clin *4*, 47-56.

Neves, S. R., Ram, P. T., and Iyengar, R. (2002). G protein pathways. Science *296*, 1636-1639.

Nieto-Vazquez, I., Fernandez-Veledo, S., Kramer, D. K., Vila-Bedmar, R., Garcia-Guerra, L., and Lorenzo, M. (2008). Insulin resistance associated to obesity: the link TNF-alpha. Arch Physiol Biochem *114*, 183-194.

Nishihara, M., Miura T, Miki T, Sakamoto J, Tanno M, Kobayashi H, Ikeda Y, Ohori K, Takahashi A, and Shimamoto, K. (2006). Erythropoietin affords additional cardioprotection to preconditioned hearts by enhanced phosphorylation of glycogen synthase kinase-3 beta. Am J Physiol Heart Circ Physiol *291*, H748-755.

Nishinakamura, H., Minoda, Y., Saeki, K., Koga, K., Takaesu, G., Onodera, M., Yoshimura, A., and Kobayashi, T. (2007). An RNA-binding protein alphaCP-1 is involved in the STAT3-mediated suppression of NF-kappaB transcriptional activity. Int Immunol *19*, 609-619.

Nishino, Y., Webb, I. G., Davidson, S. M., Ahmed, A. I., Clark, J. E., Jacquet, S., Shah, A. M., Miura, T., Yellon, D. M., Avkiran, M., and Marber, M. S. (2008). Glycogen synthase kinase-3 inactivation is not required for ischemic preconditioning or postconditioning in the mouse. Circ Res *103*, 307-314.

Norton, E. D., Jackson, E. K., Turner, M. B., Virmani, R., and Forman, M. B. (1992). The effects of intravenous infusions of selective adenosine A1-receptor and A2-receptor agonists on myocardial reperfusion injury. Am Heart J *123*, 332-338.

Numaguchi, K., Shimokawa, H., Nakaike, R., Egashira, K., and Takeshita, A. (1996). PKC inhibitors prevent endothelial dysfunction after myocardial ischemia-reperfusion in rats. Am J Physiol *270*, H1634-1639.

Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994). Molecular cloning of a potential proteinase activated receptor. Proc Natl Acad Sci U S A *91*, 9208-9212.

Nystedt, S., Larsson, A.K., Aberg, H., and Sundelin, J. (1995). The mouse proteinase-activated receptor-2 cDNA and gene. Molecular cloning and functional expression.J Biol Chem *270*, 5950-5955.

Ockaili, R., Emani, V. R., Okubo, S., Brown, M., Krottapalli, K., and Kukreja, R. C. (1999). Opening of mitochondrial KATP channel induces early and delayed cardioprotective effect: role of nitric oxide. Am J Physiol *277*, H2425-2434.

Ofei, F., Hurel, S., Newkirk, J., Sopwith, M., and Taylor, R. (1996). Effects of an engineered human anti-TNFα antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. Diabetes *45*, 881-885.

Okubo, S., Tanabe, Y., Takeda, K., Kitayama, M., Kanemitsu, S., Kukreja, R. C., and Takekoshi, N. (2004). Ischemic preconditioning and morphine attenuate myocardial apoptosis and infarction after ischemia-reperfusion in rabbits: role of delta-opioid receptor. Am J Physiol Heart Circ Physiol *287*, H1786-1791.

Olafsson, B., Forman, M. B., Puett, D. W., Pou, A., Cates, C. U., Friesinger, G. C., and Virmani, R. (1987). Reduction of reperfusion injury in the canine preparation by intracoronary adenosine: importance of the endothelium and the no-reflow phenomenon. Circulation *76*, 1135-1145.

Oldenburg, O., Qin, Q., Sharma A.R., Cohen, MV., Downey, J.M., and Benoit, J. (2002). Acetylcholine leads to free radical production d,ependent on K(ATP) channels, G(i) proteins, phosphatidylinositol 3-kinase and tyrosine kinase.Cardiovasc Res *55*, 544-552.

Oldenburg, O., Qin, Q., Krieg, T., Yang, X. M., Philipp, S., Critz, S. D., Cohen, M. V., and Downey, J. M. (2004). Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. Am J Physiol Heart Circ Physiol *286*, H468-476.

Oliff, A., Defeo-Jones, D., Boyer, M., Martinez, D., Kiefer, D., Vuocolo, G., Wolfe, A., and Socher, S. H. (1987). Tumors secreting human TNF/cachectin induce cachexia in mice. Cell *50*, 555-563.

Omura, T., Yoshiyama, M., Shimada, T., Shimizu, N., Kim, S., Iwao, H., Takeuchi, K., and Yoshikawa, J. (1999). Activation of mitogen-activated protein kinases in in vivo ischemia/reperfused myocardium in rats. J Mol Cell Cardiol *31*, 1269-1279.

Opie, L. (2006). The Metabolic Syndrome - Does it exist?, Vol II (Cape Town, Stanford Writers, University of Cape Town press).

Opie, L. H. (1989). Reperfusion injury and its pharmacologic modification. Circulation *80*, 1049-1062.

Orchard, T. J., Costacou, T., Kretowski, A., and Nesto, R. W. (2006). Type 1 diabetes and coronary artery disease. Diabetes Care *29*, 2528-2538.

Osario, J. Diabetes and cardiovascular risk: insights from clinical trials. Nature Reviews *7, 239*

Ovize, M., Obadia, J.F., Janier, M., Andre-Fouet X (1997). Lethal Myocardial Reperfusion Injury: A Right Target for the Clinician? J Thromb and Thrombolysis *4*, 149-152.

Ozog, M., Bernier, S.M., Bates, D.C., Chatterjee, B., Lo, C.W., and Naus, C. (2004). The complex of ciliary neurotrophic factor-ciliary neurotrophic factor receptor alpha upregulates connexin43 and intracellular coupling in astrocytes via the Janus tyrosine kinase/signal transducer and activator of transcription pathway. Mol Biol Cell *15*, 4761-4774.

Pain, T., Yang, X. M., Critz, S. D., Yue, Y., Nakano, A., Liu, G. S., Heusch, G., Cohen, M. V., and Downey, J. M. (2000). Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circ Res *87*, 460-466.

Pan, H. L., Chen, S. R., Scicli, G. M., and Carretero, O. A. (2000). Cardiac interstitial bradykinin release during ischemia is enhanced by ischemic preconditioning. Am J Physiol Heart Circ Physiol *279*, H116-121.

Park, S. S., Zhao, H., Jang, Y., Mueller, R. A., and Xu, Z. (2006a). N6-(3iodobenzyl)-adenosine-5'-N-methylcarboxamide confers cardioprotection at reperfusion by inhibiting mitochondrial permeability transition pore opening via glycogen synthase kinase 3 beta. J Pharmacol Exp Ther *318*, 124-131.

Park, S. S., Zhao, H., Mueller, R. A., and Xu, Z. (2006b). Bradykinin prevents reperfusion injury by targeting mitochondrial permeability transition pore through glycogen synthase kinase 3beta. J Mol Cell Cardiol *40*, 708-716.

Paulson, D. J. (1997). The diabetic heart is more sensitive to ischemic injury. Cardiovasc Res *34*, 104-112.

Peart, J. N., Gross, E. R., Reichelt, M. E., Hsu, A., Headrick, J. P., and Gross, G. J. (2008). Activation of kappa-opioid receptors at reperfusion affords cardioprotection in both rat and mouse hearts. Basic Res Cardiol *103*, 454-463.

Peiretti, F., Deprez-Beauclair, P., Bonardo, B., Aubert, H., Juhan-Vague, I., Nalbone, G. (2003). Identification of SAP97 as an intracellular binding partner of TACE. J Cell Sci *116*, 1949-1957.

Penna, C., Cappello, S., Mancardi, D., *et al.*(2006a). Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Research in Cardiology *101*, 168-179.

Penna, C., Mancardi, D., Rastaldo, R., Losano, G., and Pagliaro, P. (2007). Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. Cardiovasc Res *75*, 168-177.

Penna, C., Mancardi, D., Tullio, F., and Pagliaro, P. (2008). Postconditioning and intermittent bradykinin induced cardioprotection require cyclooxygenase activation and prostacyclin release during reperfusion. Basic Res Cardiol *103*, 368-377.

Penna, C., Mancardi, D., Tullio, F., and Pagliaro, P. (2009). Intermittent adenosine at the beginning of reperfusion does not trigger cardioprotection. J Surg Res *153*, 231-238.

Penna, C., Rastaldo, R., Mancardi, D., *et al*. (2006b). Post-conditioning induced cardioprotection requires signaling througha redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Research in Cardiology *101*, 180-189.

Pepe, S., Xiao, R. P., Hohl, C., Altschuld, R., and Lakatta, E. G. (1997). 'Cross talk' between opioid peptide and adrenergic receptor signaling in isolated rat heart. Circulation *95*, 2122-2129.

Peraldi, P., and Spiegelman, B. M. (1997). Studies of the mechanism of inhibition of insulin signaling by tumor necrosis factor-alpha. J Endocrinol *155*, 219-220.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., *et al.* (1998). An essential role for ectodomain shedding in mammalian development. Science *282*, 1281-1284.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem *83*, 346-356.

Pi-Sunyer, F. X. (1990). Obesity and diabetes in blacks. Diabetes Care *13*, 1144-1149.

Pi-Sunyer, F. X. (1991). Health implications of obesity. Am J Clin Nutr *53*, 1595S-1603S.

Ping, P., Takano, H., Zhang, J., Tan, X. L., Qiu, Y., Li, R. C., *et al.* (1999a). Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. Circulation Research *84*, 587-604.

Ping, P., Zhang ,J., Cao, X., Li, R. C., Kong, D., Tang, X. L., *et al.* (1999b). PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. Am J Physiol *276*, H1468-1481.

Ping, P., Zhang, J., Huang, S., Cao, X., Tang, X. L., Li, R. C., *et al.* (1999c). PKC-dependent activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits. Am J Physiol *277*, H1771-1785.

Ping, P., Zhang, J., Qiu, Y., Tang, X. L,, Manchikalapudi, S., Cao, X., *et al.* (1997). Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circulation Research *81*, 404-414.

Ping, P., Zhang, J., Zheng, Y. T., Li, R. C., Dawn, B., Tang, X. L, *et al.* (1999d). Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits. Circulation Research *85*, 542-550.

Pinhas-Hamiel, O., and Zeitler, P. (2000). "Who is the wise man?--The one who foresees consequences:". Childhood obesity, new associated comorbidity and prevention. Prev Med *31*, 702-705.

Piot, C., Croisille, P., Staat, P., Thibault, H., Rioufol, G., Mewton, N., Elbelghiti, R., Cung, T. T., Bonnefoy, E., Angoulvant, D., *et al.* (2008). Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med *359*, 473-481.

Podar, T., Solntsev, A., Karvonen, M., Padaiga, Z., Brigis, G., Urbonaite, B., Viik-Kajander, M., Reunanen, A., and Tuomilehto, J. (2001). Increasing incidence of childhood-onset type 1 diabetes in 3 Baltic countries and Finland. Diabetologia *44*, B17-20.

Poirier, P., Despres, J. P. (2003). Waist circumference, visceral obesity, and cardiovascular risk. J Cardiopulm Rehabil *23*, 161-169.

Poirier, P., Giles, T. D., Bray, G. A., Hong, Y., Stern, J. S., Pi-Sunyer, F. X., and Eckel, R. H. (2006). Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Circulation *113*, 898-918.

Prasad, M., and Jones, R. (1992). Enhanced membrane protein kinase C activity in myocardial ischaemia. Basic Res Cardiol *87*, 19-26.

Prisant, L. M. (2005). Hypertensive heart disease. J Clin Hypertens (Greenwich) *7*, 231-238.

Przyklenk, K. (1997). Lethal Myocardial "Reperfusion Injury": The Opinions of Good Men. J Thromb Thrombolysis *4*, 5-6.

Przyklenk, K., Bauer, B., Ovize, M., Kloner, R. A., and Whittaker, P. (1993). Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation *87*, 893-899.

Przyklenk, K., Maynard, M., Darling, C. E., and Whittaker, P. (2008). Aging mouse hearts are refractory to infarct size reduction with post-conditioning. J Am Coll Cardiol *51*, 1393-1398.

Przyklenk, K., Maynard, M., Greiner, D.L., and Whittaker, P. (2010). Cardioprotection with Postconditioning: Loss of efficacy in Murine Models of Type-2 and Type-1 Diabetes. Antiox Redox Signal(in print).

Quinlan, C. L., Costa, A. D., Costa, C. L., Pierre, S. V., Dos Santos, P., and Garlid, K. D. (2008). Conditioning the heart induces formation of signalosomes that interact with mitochondria to open mitoKATP channels. Am J Physiol Heart Circ Physiol *295*, H953-H961.

Quintana, M., Hjemdahl, P., Sollevi, A., Kahan, T., Edner, M., Rehnqvist, N., Swahn, E., Kjerr, A. C., and Nasman, P. (2003). Left ventricular function and cardiovascular events following adjuvant therapy with adenosine in acute myocardial infarction treated with thrombolysis, results of the ATTenuation by Adenosine of Cardiac Complications (ATTACC) study. Eur J Clin Pharmacol *59*, 1-9.

Radeff-Huang, J., Seasholtz, T. M., Matteo, R. G., and Brown, J. H. (2004). G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. J Cell Biochem *92*, 949-966.

Rafiee, P., Shi, Y., Su, J., Pritchard, K.A. Jr., Tweddell, J.S., and Baker, J. (2005). Erythropoietin protects the infant heart against ischemia-reperfusion injury by triggering multiple signaling pathways. Basic Res Cardiol *100*, 187-197.

Rakhit, R. D., Edwards, R. J., Mockridge, J. W., Baydoun, A. R., Wyatt, A. W., Mann, G. E., and Marber, M. S. (2000). Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes. Am J Physiol Heart Circ Physiol *278*, H1211-1217.

Ramani, K., Lust, W. D., Whittingham, T. S., and Lesnefsky, E. J. (1996). ATP catabolism and adenosine generation during ischemia in the aging heart. Mech Ageing Dev *89*, 113-124.

Raphael, J., Gozal, Y., Navot, N., Zuo, Z. (2010). Hyperglycemia inhibits anesthetic-induced postconditioning in the rabbit heart via modulation of phosphotidylinositol-3-kinase/Akt and endothelial nitric oxide synthase signaling. J Cardiovasc Pharmacol 55 (4), 348-357.

Rauchhaus, M., Doehner, W., Francis, D. P., Davos, C., Kemp, M., Liebenthal, C., Niebauer, J., Hooper, J., Volk, H. D., Coats, A. J., and Anker, S. D. (2000). Plasma cytokine parameters and mortality in patients with chronic heart failure. Circulation *102*, 3060-3067.

Ravingerova, T., Neckar, J., and Kolar, F. (2003). Ischemic tolerance of rat hearts in acute and chronic phases of experimental diabetes. Mol Cell Biochem *249*, 167-174.

Ravingerova, T., Stetka, R., Volkovova, K., Paneza, D., Dzurba, A., Ziegelhoffer,A., Styk, J. (2000). Acute diabetes modulates response to ischemia in isolated rat heart. Mol Cell Biochem *210*,143-151.

Reddy, P., Slack, J. L., Davis, R., Cerretti, D. P., Kozlosky, C. J., Blanton, R. A., Shows, D., Peschon, J. J., and Black, R. A. (2000). Functional analysis of the domain structure of tumor necrosis factor-alpha converting enzyme. J Biol Chem *275*, 14608-14614.

Reichelt, M. E., Willems, L., Molina, J. G., Sun, C. X., Noble, J. C., Ashton, K. J., Schnermann, J., Blackburn, M. R., and Headrick, J. P. (2005). Genetic deletion of the A1 adenosine receptor limits myocardial ischemic tolerance. Circ Res *96*, 363-367.

Reil, J. C., Gilles, S., Zahler, S., Brandl, A., Drexler, H., Hultner, L., Matrisian, L. M., Welsch, U., and Becker, B. F. (2007). Insights from knock-out models concerning postischemic release of TNFalpha from isolated mouse hearts. J Mol Cell Cardiol *42*, 133-141.

Rentoukas, I., Giannopoulos, G., Kaoukis, A., Kossyvakis, C., Raisakis, K., Driva, M., Panagopoulou, V., Tsarouchas, K., Vavetsi, S., Pyrgakis, V., and Deftereos, S. (2010). Cardioprotective role of remote ischemic periconditioning in primary percutaneous coronary intervention: enhancement by opioid action. JACC Cardiovasc Interv *3*, 49-55.

Riksen, N. P., Smits, P., Rongen, G.A. (2004). Ischaemic preconditioning: from molecular characterisation to clinical application - part I. J Med *62*, 353-362.

Romano, M. A., Seymour, E. M., Berry, J. A., McNish, R. A., and Bolling, S. F. (2004). Relative contribution of endogenous opioids to myocardial ischemic tolerance. J Surg Res *118*, 32-37.

Rosenvinge, A., Krogh-Masden, R., Baslund, B., and Pedersen, B. K. (2007). Insulin resistance in patients with rheumatoid arthritis: effect of anti-TNF alpha therapy. Scand J Rheumatol *36*, 91-96.

Ross, A. M., Gibbons, R. J., Stone, G. W., Kloner, R. A., and Alexander, R. W. (2005). A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). J Am Coll Cardiol *45*, 1775-1780.

Rothwell, N. J. (1993). Cytokines and thermogenesis. Int J Obes Relat Metab Disord *17 Suppl 3*, S98-101; discussion 115.

Roytblat, L., Rachinsky, M., Fisher, A., Greemberg, L., Shapira, Y., Douvdevani, A., and Gelman, S. (2000). Raised interleukin-6 levels in obese patients. Obes Res *8*, 673-675.

Rylatt, D. B., Aitken, A., Bilham, T., Condon, G. D., Embi, N., and Cohen, P. (1980). Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated by glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase. Eur J Biochem *107*, 529-537.

Rytter, L., Troelsen, S., and Beck-Nielsen, H. (1985). Prevalence and mortality of acute myocardial infarction in patients with diabetes. Diabetes Care *8*, 230-234.

Sabri, A., Muske, G., Zhang, H., Pak, E., Darrow, A., Andrade-Gordon, P., and Steinberg, S. F. (2000). Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. Circ Res *86*, 1054-1061.

Sack, M. (2002). Tumor necrosis factor-alpha in cardiovascular biology and the potential role for anti-tumor necrosis factor-alpha therapy in heart disease. Pharmacol Ther *94*, 123-135.

Sack, M. N., Disch, D. L., Rockman, H. A., and Kelly, D. P. (1997). A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. Proc Natl Acad Sci U S A *94*, 6438-6443.

Sack, M. N., Smith, R. M., and Opie, L. H. (2000). Tumor necrosis factor in myocardial hypertrophy and ischaemia--an anti-apoptotic perspective. Cardiovasc Res *45*, 688-695.

Sadat, U. (2009). Signaling pathways of cardioprotective ischemic preconditioning. Int J Surg *7*, 490-498.

Safranow, K., Dziedziejko, V., Rzeuski, R., Czyzycka, E., Wojtarowicz, A., Binczak-Kuleta, A., Jakubowska, K., Olszewska, M., Ciechanowicz, A., Kornacewicz-Jach, Z., *et al.* (2009). Plasma concentrations of TNF-alpha and its soluble receptors sTNFR1 and sTNFR2 in patients with coronary artery disease. Tissue Antigens *74*, 386-392.

Saghizadeh, M., Ong, J. M., Garvey, W. T., Henry, R. R., and Kern, P. A. (1996). The expression of TNF alpha by human muscle. Relationship to insulin resistance. J Clin Invest *97*, 1111-1116.

Sanada, K., Okano, T., and Fukada, Y. (2002). Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. J Biol Chem *277*, 267-271.

Sarraf, P., Frederich, R. C., Turner, E.M., Ma, G., Jaskowiak, N.T., Rivet, D.J., Flier, J.S., Lowell, B.B., Fraker, D.L., Alexander, H.R. Multiple Cytokines and Acute Inflammation Raise Mouse Leptin Levels: Potential Role in Inflammatory Anorexia. J Exp Med *185*: 171-175

Sato T, O'Rourke B, *et al.* (1998). Modulation of mitochondrial ATP-dependent K+ channels by protein kinase C. Circulation Research *83*, 110-114.

Schrader, J., Haddy, F. J., and Gerlach, E. (1977). Release of adenosine, inosine and hypoxanthine from the isolated guinea pig heart during hypoxia, flow-autoregulation and reactive hyperemia. Pflugers Arch *369*, 1-6.

Schreyer, S. A., Chua, S. C. Jr., and LeBoeuf, R. C. (1998). Obesity and diabetes in TNF-alpha receptor-deficient mice. J Clin Invest *102*, 402-411.

Schultz, J., Hsu, A.K., and Gross, G. (1997). Ischemic preconditioning and morphine-induced cardioprotection involve the delta (delta)-opioid receptor in the intact rat heart. J Mol Cell Cardiol *29*, 2187-2195.

Schultz, J. E., Hsu, A. K., and Gross, G. J. (1996). Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. Circ Res *78*, 1100-1104.

Schulz, R. (2008). TNFalpha in myocardial ischemia/reperfusion: damage vs. protection. J Mol Cell Cardiol *45*, 712-714.

Schulz, R., Boengler, K., Totzeck, A., Luo, Y., Garcia-Dorado, D., and Heusch, G. (2007). Connexin 43 in ischemic pre- and postconditioning. Heart Fail Rev *12*, 261-266.

Schulz, R., and Heusch, G. (2009). Tumor necrosis factor-alpha and its receptors 1 and 2: Yin and Yang in myocardial infarction? Circulation *119*, 1355-1357.

Schulz, R., Post, H., Vahlhaus, C., and Heusch, G. (1998). Ischemic preconditioning in pigs: a graded phenomenon: its relation to adenosine and bradykinin. Circulation *98*, 1022-1029.

Schwartz, L. M., and Lagranha, C. J. (2006). Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischemia-reperfusion injury in pigs. Am J Physiol Heart Circ Physiol *290*, H1011-1018.

Shang, L., Ananthakrishnan, R., Li, Q., Quadri, N., Abdillahi, M., Zhu, Z., Qu, W., Rosario, R., Toure, F., Yan, S. F., *et al.* RAGE modulates hypoxia/reoxygenation injury in adult murine cardiomyocytes via JNK and GSK-3beta signaling pathways. PLoS One *5*, e10092.

Sharma, H. S., and Das, D. K. (1997). Role of cytokines in myocardial ischemia and reperfusion. Mediators Inflamm *6*, 175-183.

Sharrett, A. R., Ballantyne, C. M., Coady, S. A., Heiss, G., Sorlie, P. D., Catellier, D., and Patsch, W. (2001). Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. Circulation *104*, 1108-1113.

Shi, E., Jiang, X., Bai, H., Gu, T., Chang, Y., and Wang, J. (2003). Cardioprotective effects of morphine on rat heart suffering from ischemia and reperfusion. Chin Med J (Engl) *116*, 1059-1062.

Sivaraman, V., Mudalagiri, N. R., Di Salvo, C., Kolvekar, S., Hayward, M., Yap, J., Keogh, B., Hausenloy, D. J., and Yellon, D. M. (2007). Postconditioning protects human atrial muscle through the activation of the RISK pathway. Basic Res Cardiol *102*, 453-459.

Skyschally, A., van Caster, P., Boengler, K., Gres, P., Musiolik, J., Schilawa, D., Schulz, R., and Heusch, G. (2009). Ischemic postconditioning in pigs: no causal role for RISK activation. Circ Res *104*, 15-18.

Smith, C. C., Mocanu, M. M., Davidson, S. M., Wynne, A. M., Simpkin, J. C., and Yellon, D. M. (2006). Leptin, the obesity-associated hormone, exhibits direct cardioprotective effects. Br J Pharmacol *149*, 5-13.

Smith, R. M., McCarthy, J., and Sack, M. N. (2001). TNF alpha is required for hypoxia-mediated right ventricular hypertrophy. Mol Cell Biochem *219*, 139-143.

Smith, R. M., Suleman, N., Lacerda, L., Opie, L. H., Akira, S., Chien, K. R., and Sack, M. N. (2004). Genetic depletion of cardiac myocyte STAT-3 abolishes classical preconditioning. Cardiovasc Res *63*, 611-616.

Smith, R. M., Suleman, N., McCarthy, J., and Sack, M. N. (2002). Classic ischemic but not pharmacologic preconditioning is abrogated following genetic ablation of the TNFalpha gene. Cardiovasc Res *55*, 553-560.

Smith, S. C., and Allen, P. M. (1992). Neutralization of endogenous tumor necrosis factor ameliorates the severity of myosin-induced myocarditis. Circ Res *70*, 856-863.

Smith C.C.T., Mocanu, M.M., Davidson, S.M., Wynne, A.M., Simpkin, J.C., Yellon, D.M.(2006). Leptin, the obesity-associated hormone, exhibits direct cardioprotective effects. Br J Pharmacol *149*: 5-13.

Smith C.C.T., Dixon, R.A., Wynne, A.M., Theodorou, L., Ong, S-G., Subrayan, S., Davidson, S., Hausenloy, D.J., Yellon, D.M. (2010). Leptin-induced cardioprotection involves JAK/STAT signaling that may be linked to the mitochondrial permeability transition pore. Am J Physiol Heart Circ Physiol (article in press). doi:10.1152/ajpheart.00092.2010.

Somers, S., Lacerda, L., Opie, L., and Lecour, S. (2005). NFκ B triggers TNF induced cardioprotection in C2C12. J Mol Cell Cardiol *38*, 1040-1041.

Speechly-Dick, M. E., Baxter, G. F., and Yellon, D. M. (1994). Ischaemic preconditioning protects hypertrophied myocardium. Cardiovasc Res *28*, 1025-1029.

Staat, P., Rioufol, G., Piot, C., Cottin, Y., Cung, T. T., L'Huillier, I., Aupetit, J. F., Bonnefoy, E., Finet, G., Andre-Fouet, X., and Ovize, M. (2005). Postconditioning the human heart. Circulation *112*, 2143-2148.

Stevens, R. J., Coleman, R. L., Adler, A. I., Stratton, I. M., Matthews, D. R., and Holman, R. R. (2004). Risk factors for myocardial infarction case fatality and stroke case fatality in type 2 diabetes: UKPDS 66. Diabetes Care *27*, 201-207.

Steyn, K., Fourie, J., Rossouw, J. E., Langenhoven, M. L., Joubert, G., and Chalton, D. O. (1990). Anthropometric profile of the coloured population of the Cape Peninsula. S Afr Med J *78*, 68-72.

Suleman, N., Opie L, and Lecour, S. (2006). Ischaemic postconditioning confers cardioprotection via phosphorylation of STAT-3. J Mol Cell Cardiol *40*, 977 Abstract No. 155.

Suleman, N., Somers, S., Smith, R., Opie, L. H., and Lecour, S. C. (2008). Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning. Cardiovasc Res *79*, 127-133.

Sumeray, M. S., Rees, D. D., and Yellon, D. M. (2000). Infarct size and nitric oxide synthase in murine myocardium. J Mol Cell Cardiol *32*, 35-42.

Sun, H. Y., Wang, N. P., Halkos, M., Kerendi, F., Kin, H., Guyton, R. A., Vinten-Johansen, J., and Zhao, Z. Q. (2006). Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signaling pathways. Apoptosis *11*, 1583-1593.

Takeo, S., Tanonaka, K., Miyake, K., and Imago, M. (1988). Adenine nucleotide metabolites are beneficial for recovery of cardiac contractile force after hypoxia. J Mol Cell Cardiol *20*, 187-199.

Tanno, M., Gorog, D. A., Bellahcene, M., Cao, X., Quinlan, R. A., and Marber, M. S. (2003). Tumor necrosis factor-induced protection of the murine heart is independent of p38-MAPK activation. J Mol Cell Cardiol *35*, 1523-1527.

Tartaglia, L. A., Ayres, T. M., Wong, G. H., and Goeddel, D. V. (1993). A novel domain within the 55 kd TNF receptor signals cell death. Cell *74*, 845-853.

Tatsumi, T., Matoba, S., Kobara, M., Keira, N., Kawahara, A., Tsuruyuma, K., *et al*. (1998). Energy metabolism after ischemic preconditioning in streptozotocininduced diabetic rat hearts. J Am Coll Cardiol *31*, 707-715.

Thirunavukkarasu, M., Varma, S., Penumathasa, K.S., Juhasz, B., Zhan, L., Otani, H., Bagchi, D., Das, D.K., Maulik, N. (2007). Resveratrol alleviates cardiac dysfunction in streptozotocin-induced diabetes: role of nitric oxide, thioredoxin, and heme oxygenase. Free Radic Biol Med *43*, 720-729.

Thornton, J. D., Liu, G. S., and Downey, J. M. (1993). Pretreatment with pertussis toxin blocks the protective effects of preconditioning: evidence for a G-protein mechanism. J Mol Cell Cardiol *25*, 311-320.

Tio, R. A., Tobe, T. J., Bel, K. J., de Langen, C. D., van Gilst, W. H., and Wesseling, H. (1991). Beneficial effects of bradykinin on porcine ischemic myocardium. Basic Res Cardiol *86*, 107-116.

Tobe, T. J., de Langen, C. D., Tio, R. A., Bel, K. J., Mook, P. H., and Wesseling, H. (1991). In vivo effect of bradykinin during ischemia and reperfusion: improved electrical stability two weeks after myocardial infarction in the pig. J Cardiovasc Pharmacol *17*, 600-607.

Tomai, F., Crea, F., Chiariello, L., and Gioffre, P. A. (1999). Ischemic preconditioning in humans: models, mediators, and clinical relevance. Circulation *100*, 559-563.

Tong, H., Chen, W., Steenbergen, C., Murphy, E. (2000). Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. Circulation Research *87*, 309-315.

Tong, H., Imahashi, K., Steenbergen, C., and Murphy, E. (2002). Phosphorylation of glycogen synthase kinase-3beta during preconditioning through a phosphatidylinositol-3-kinase--dependent pathway is cardioprotective. Circ Res *90*, 377-379.

Torbenson, M., Yang, S. Q., Liu, H. Z., Huang, J., Gage, W., and Diehl, A. M. (2002). STAT-3 overexpression and p21 up-regulation accompany impaired regeneration of fatty livers. Am J Pathol *161*, 155-161.

Torre-Amione, G., Kapadia, S., Lee, J., Bies, R. D., Lebovitz, R., and Mann, D. L. (1995). Expression and functional significance of tumur necrosis factor receptors in human myocardium. Circulation *92*, 1487-1493.

Torti, F. M., Dieckmann, B., Beutler, B., Cerami, A., and Ringold, G. M. (1985). A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. Science *229*, 867-869.

Tosaki, A., Engelman, D. T., Engelman, R. M., and Das, D. K. (1996). The evolution of diabetic response to ischemia/reperfusion and preconditioning in isolated working rat hearts. Cardiovasc Res *31*, 526-536.

Tracey, K. J., Cerami, A. (1992). Pleiotrophic effects of TNF in infection and neoplasia: beneficial, inflammatory, catabolic,or injurious (New York Dekker).

Tsang, A., Hausenloy, D. J., Mocanu, M. M., Carr, R. D., and Yellon, D. M. (2005). Preconditioning the diabetic heart: the importance of Akt phosphorylation. Diabetes *54*, 2360-2364.

Tsang, A., Hausenloy, D. J., Mocanu, M. M., and Yellon, D. M. (2004). Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res *95*, 230-232.

Tschope, C., Heringer-Walther, S., Koch, M., Spillmann, F., Wendorf, M., and Hauke, D., *et al*. (2000). Myocardial bradykinin B2 receptor expression at different time points after induction of myocardial infarction. J Hyperten *18*.

Tschope, C., Koch, M., Spillmann, F, Heringer-Walther, S., Mochmann, H. C., and Strauss, H., *et al.* (1999). Upregulation of the cardiac bradykini.n B2 receptors after myocardial infarction. Immunopharmacology *44*, 111-117.

Turner, R. C., Millns, H., Neil, H. A., Stratton, I. M., Manley, S. E., Matthews, D. R., and Holman, R. R. (1998). Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). Bmj *316*, 823-828.

Uysal, K. T., Wiesbrock, S. M., and Hotamisligil, G. S. (1998). Functional analysis of tumor necrosis factor (TNF) receptors in TNF-alpha-mediated insulin resistance in genetic obesity. Endocrinology *139*, 4832-4838.

Uysal, K. T., Wiesbrock, S. M., Marino, M. W., and Hotamisligil, G. S. (1997). Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature *389*, 610-614.

Valeur, H. S., and Valen, G. (2009). Innate immunity and myocardial adaptation to ischemia. Basic Res Cardiol *104*, 22-32.

Vassilli, P. (1992). The pathophysiology of tumor necrosis factors. Annu Rev Immunol *10*, 411-452.

Ventura, C., Bastagli, L., Bernardi, P., Caldarera, C. M., and Guarnieri, C. (1989). Opioid receptors in rat cardiac sarcolemma: effect of phenylephrine and isoproterenol. Biochim Biophys Acta *987*, 69-74.

Venugopal, V., Hausenloy, D. J., Ludman, A., Di Salvo, C., Kolvekar, S., Yap, J., Lawrence, D., Bognolo, J., and Yellon, D. M. (2009). Remote ischaemic preconditioning reduces myocardial injury in

patients undergoing cardiac surgery with cold-blood cardioplegia: a randomised controlled trial. Heart *95*, 1567-1571.

Vessey, D. A., Li, L., Kelley, M., and Karliner, J. S. (2008). Combined sphingosine, S1P and ischemic postconditioning rescue the heart after protracted ischemia. Biochem Biophys Res Commun *375*, 425-429.

Vince, J. E., Pantaki, D., Feltham, R., Mace, P. D., Cordier, S. M., Schmukle, A. C., Davidson, A. J., Callus, B. A., Wong, W. W., Gentle, I. E., *et al.* (2009). TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-{kappa}b and to prevent tnf-induced apoptosis. J Biol Chem *284*, 35906-35915.

Vinten-Johansen, J., Zhao, Z. Q., Jiang, R., and Zatta, A. J. (2005). Myocardial protection in reperfusion with postconditioning. Expert Rev Cardiovasc Ther *3*, 1035-1045.

Vogel, W.M., Apstein, C.S. (1988). Effects of alloxan-induced diabetes on ischemia-reperfusion injury in rabbit hearts. Circ Res *62*, 975-982.

Walker, A. R. (1998). Africa health/ill-health: is it all gloom and doom? Natl Med J India *11*, 241-242.

Wang, G., Wu, S., Pei, J.M., Yu, XC., Wong, T.M. (2001). Kappa- but not delta-opioid receptors mediate effects of ischemic preconditioning on both infarct and arrhythmia in rats.Am J Physiol Heart Circ Physiol *280*, H384-391.

Wang, J., Gao, Q., Shen, J., Ye, T. M., and Xia, Q. (2007). Kappa-opioid receptor mediates the cardioprotective effect of ischaemic postconditioning. Zhejiang Da Xue Xue Bao Yi Xue Ban *36*, 41-47.

Wang, P., Tate, J.M., and Lloyd, S. G. (2008). Low carbohydrate diet decreases myocardial insulin signaling and increases susceptibility to myocardial ischemia. Life Sci *83*, 836-844.

Wang, P., and Zweier, J. L. (1996). Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. J Biol Chem *271*, 29223-29230.

Wang, Y., *et al*. (1999). Role of protein kinase C in mitochondrial KATP channel-mediated protection against Ca2+ overload injury in rat myocardium. Circulation Research *84*, 1156-1165.

Wautier, M. P., Chappey, O., Corda, S., Stern, D. M., Schmidt, A. M., and Wautier, J. L. (2001). Activation of NADPH oxidase by AGE links oxidant stress to altere gene expression via RAGE. Am J Physiol Endocrinol Metab *280*, E685-E694.

Wegrzyn, J., Potla, R., Chwae, Y. J., Sepuri, N. B., Zhang, Q., Koeck, T., Derecka, M., Szczepanek, K., Szelag, M., Gornicka, A., *et al.* (2009). Function of mitochondrial Stat3 in cellular respiration. Science *323*, 793-797.

Wei, L., Sun, D., Yin, Z., Yuan, Y., Hwang,A., Zhang, Y., Si, R., Zhang, R., Guo, W., Cao, F., and Wang, H. (2010). A PKC-beta inhibitor protects against cardiac microvascular ischemia reperfusion injury in diabetic rats. Apoptosis *15*, 488-498.

Weihrauch, D., Krolikowski, J. G., Bienengraeber, M., Kersten, J. R., Warltier, D. C., and Pagel, P. S. (2005). Morphine enhances isoflurane-induced postconditioning against myocardial infarction: the role of phosphatidylinositol-3-kinase and opioid receptors in rabbits. Anesth Analg *101*, 942-949, table of contents.

Weinbrenner, C., Liu, G. S., Cohen, M. V., and Downey, J. M. (1997). Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. J Mol Cell Cardiol *29*, 2383-2391.

Weselcouch, E. O., Baird, A. J., Sleph, P., and Grover, G. J. (1995). Inhibition of nitric oxide synthesis does not affect ischemic preconditioning in isolated perfused rat hearts. Am J Physiol *268*, H242-249.

WHO (1996). From World Health Report 1996 (World Health Organisation, Geneva). J Commun Dis *28*, 215-219.

WHO (2000). Obesity: Preventing and managing the global epidemic. World Health Organ Tech Rep Ser 2000 No894.

WHO (2005). Obesity Task Force.

WHO (2009). Fact Sheet No. 317.

Wink, D. A., and Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radic Biol Med *25*, 434-456.

Wittert, G., Hope, P., and Pyle, D. (1996). Tissue distribution of opioid receptor gene expression in the rat. Biochem Biophys Res Commun *218*, 877-881.

Xi, L., and Kukreja, R. C. (2000). Pivotal role of nitric oxide in delayed pharmacological preconditioning against myocardial infarction. Toxicology *155*, 37-44.

Xiao, C. Y., Chen, M., Zsengeller, Z., and Szabo, C. (2004). Poly(ADP-ribose) polymerase contributes to the development of myocardial infarction in diabetic rats and regulates the nuclear translocation of apoptosis-inducing factor. J Pharmacol Exp Ther *310*, 498-504.

Xu, J., Carretero, O. A., Y, S., Shesely E G, Rhaleb N E, and Liu, Y. H. (2005). Role of B1 kinin receptor in the regulation of cardiac function and remodeling after myocardial infarction. Hypertension *45*, 747-753.

Xu, Z., Downey, J. M., and Cohen, M. V. (2001). Amp 579 reduces contracture and limits infarction in rabbit heart by activating adenosine A2 receptors. J Cardiovasc Pharmacol *38*, 474-481.

Xuan, Y. T., Guo, Y., Han, H., Zhu, Y., and Bolli, R. (2001). An essential role of the JAK-STAT pathway in ischemic preconditioning. Proc Natl Acad Sci U S A *98*, 9050-9055.

Xuan, Y.T., Guo, Y., Zhu, Y., Wang, O.L., Rokosh, G., Messing, R.O., Bolli, R.(2005) Role of the Protein Kinase C-ε-Raf-1-MEK-1/2-p44/42 MAPK Signaling Cascade in the Activation of Signal Transducers and Activators of Transcription 1 and 3 and Induction of Cyclooxygenase-2 After Ischemic Preconditioning. Circulation, *112*, 1971-1978.

Xuan, Y.T., Guo, Y., Zhu, Y., Wang, O.L., Rokosh, G., Bolli, R. (2007). Endothelial Nitric Oxide Synthase Plays an Obligatory Role in the Late Phase of Ischemic Preconditioning by Activating the Protein Kinase Cε-p44/42 Mitogen-Activated Protein Kinase-pSer-Signal Transducers and Activators of Transcription1/3 Pathway. Circulation *116*, 535-544.

Yang, X. C., Liu, Y., Wang, L. F., Cui, L., Wang, T., Ge, Y. G., Wang, H. S., Li, W. M., Xu, L., Ni, Z. H., *et al.* (2007). Reduction in myocardial infarct size by postconditioning in patients after percutaneous coronary intervention. J Invasive Cardiol *19*, 424-430.

Yang, X. D., Tisch, R., Singer, S. M., Cao, Z. A., Liblau, R. S., Schreiber, R. D., and McDevitt, H. O. (1994). Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. J Exp Med *180*, 995-1004.

Yang, X. M., Krieg, T., Cui, L., Downey, J. M., and Cohen, M. V. (2004a). NECA and bradykinin at reperfusion reduce infarction in rabbit hearts by signaling through PI3K, ERK, and NO. J Mol Cell Cardiol *36*, 411-421.

Yang, X. M., Philipp, S., Downey, J. M., and Cohen, M. V. (2005). Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol *100*, 57-63.

Yang, X. M., Proctor, J. B., Cui, L., Krieg, T., Downey, J. M., and Cohen, M. V. (2004b). Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol *44*, 1103-1110.

Yang, X. P., Liu, Y. H., Scicli, G. M., Webb, C. R., and Carretero, O. A. (1997). Role of kinins in the cardioprotective effect of preconditioning: study of myocardial ischemia/reperfusion injury in B2 kinin receptor knockout mice and kininogen-deficient rats. Hypertension *30*, 735-740.

Yasmin, W., Strynadka, K. D., and Schulz, R. (1997). Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. Cardiovasc Res *33*, 422-432.

Yellon, D. M., Alkhulaifi, A. M., and Pugsley, W. B. (1993). Preconditioning the human myocardium. Lancet *342*, 276-277.

Yellon, D. M., and Downey, J. M. (2003). Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev *83*, 1113-1151.

Yellon, D. M., and Hausenloy, D. J. (2007). Myocardial reperfusion injury. N Engl J Med *357*, 1121-1135.

Yellon, D.M., and Jennings, R.B. (1992). Myocardial protection. The pathophysiology of reperfusion and reperfusion injury (New York, Raven).

Yokoyama, T., Nakano, M., Bednarczyk, J.L,, McIntyre, W., Entman, M., Mann, D.L. (1997). Tumor necrosis factor $-\alpha$ provokes a hypertrophic growth response in adult cardiomyocytes. Circulation *95*, 1247-1252.

Yokoyama, T., Vaca, L., Rossen, R. D., Durante, W., Hazarika, P., and Mann, D. L. (1993). Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. J Clin Invest *92*, 2303-2312.

Yoshida K., Kawamura, S., Mizukam, Y, *et al.*(1997). Implication of protein kinase C-alpha, delta and epsilon isoforms is ischemic precond.itioning in perfused rat hearts. J Biochem (Tokyo) *122*, 506-511.

Yudkin, J. S., Yajnik, C. S., Mohamed-Ali, V., and Bulmer, K. (1999). High levels of circulating proinflammatory cytokines and leptin in urban, but not rural, Indians. A potential explanation for increased risk of diabetes and coronary heart disease. Diabetes Care *22*, 363-364.

Yue, Y., Qin, Q., Cohen, M. V., Downey, J. M., and Critz, S. D. (2002). The relative order of mK(ATP) channels, free radicals and p38 MAPK in preconditioning's protective pathway in rat heart. Cardiovasc Res *55*, 681-689.

Zairis, M. N., Lyras, A. G., Makrygiannis, S. S., Psarogianni, P. K., Adamopoulou, E. N., Handanis, S. M., Papantonakos, A., Argyrakis, S. K., Prekates, A. A., and Foussas, S. G. (2004). Type 2 diabetes and intravenous thrombolysis outcome in the setting of ST elevation myocardial infarction. Diabetes Care *27*, 967-971.

Zatta, A. J., Kin, H., Yoshishige, D., *et al.* (2008). Evidence that cardioprotection by postconditioning involves preservation of myocardial opioid content and selective opioid receptor activation. Am J Physiol Heart Circ Physiol *294*, H1444-H1451.

Zhang, W. M., Jin, W. Q., and Wong, T. M. (1996). Multiplicity of kappa opioid receptor binding in the rat cardiac sarcolemma. J Mol Cell Cardiol *28*, 1547-1554.

Zhang, X., Zhang, J., Wei, H., and Tian, Z. (2007). STAT3-decoy oligodeoxynucleotide inhibits the growth of human lung cancer via down-regulating its target genes. Oncol Rep *17*, 1377-1382.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature *372*, 425-432.

Zhao, Z. Q., Corvera, J. S., Halkos, M. E., Kerendi, F., Wang, N. P., Guyton, R. A., and Vinten-Johansen, J. (2003). Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol *285*, H579-588.

Zhu, M., Feng, J., Lucchinetti, E., Fischer, G., Xu, L., Pedrazzini, T., Schaub, M. C., and Zaugg, M. (2006). Ischemic postconditioning protects remodeled myocardium via the PI3K-PKB/Akt reperfusion injury salvage kinase pathway. Cardiovasc Res *72*, 152-162.

Zuanetti, G., Latini, R., Maggioni, A. P., Santoro, L., and Franzosi, M. G. (1993). Influence of diabetes on mortality in acute myocardial infarction: data from the GISSI-2 study. J Am Coll Cardiol *22*, 1788-1794.