SIGNALLING PATHWAYS INVOLVED IN TNFα-INDUCED CYTOPROTECTION: ROLE OF REACTIVE OXYGEN SPECIES

LYDIA LACERDA
(Student number: PRYLYD001)

Submitted for the Degree of Master of Science in Medicine

Faculty of Medicine, University of Cape Town
August, 2005

Supervisor: **Dr S. Lecour PharmD, PhD**
(Hatter Institute for Cardiology Research, University of Cape Town)
Co-supervisor: **Professor L. H. Opie PhD; DSc; DPhil**
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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 to be submitted for another degree in this or any other university.

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ACKNOWLEDGEMENTS

Heartfelt thanks to Sandrine Lecour, PhD. I could not have wished for a more inspired, knowledgeable and motivated supervisor who gave me encouragement and guidance at all times which has enabled me to complete this Masters degree.

My grateful thanks to my co-supervisor, Lionel H. Opie, PhD. D.Sc. DPhil for his thought-provoking advice, scientific direction, and many an interesting discussion.

In addition, I would like to thank my colleagues in the laboratory at the Hatter Institute for Cardiology Research, in particular Joy McCarthy for always being willing to share her knowledge and for giving sound advice. To all the others, a big thank you for always being there to bounce ideas around and keep me sane!

A special thank you to Sylvia Dennis, secretary "extraordinaire" who does so much to help but always stays in the background. Without you, I would be lost indeed!

To my parents: this is the culmination of a dream. Thank you for your moral support during your lifetimes. I am sure that you can see me now and are proud.

To my husband, Jorge: thank you for bearing with me whilst I did this. Your quiet support and confidence in me was invaluable, your computer advice was irreplaceable!!!

To my sons, Miguel and Philippe: thank you for your understanding and patience when supper wasn't ready on time! And for liking "fast food" so much! And for all advice and suggestions and help with computer problems!

Finally, I'd like to thank two people without whom life in the lab would have been much harder. Firstly, Victor Claassens, my "muscle man" who is always there to deal with the heavy stuff!! Thank you so much! And last, but not least, Patricia Van der Walt who keeps the glassware coming and the waterbaths full – thanks, Pat!

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ABBREVIATIONS

ADAM:	a disintegrin and metalloprotease
ADP:	Adenosine diphosphate
ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
CCCP:	Carbonyl cyanide 3-chlorophenylhydrazone
COX:	Cyclo-oxygenase
$CuSO_4$:	Copper sulphate
DAG:	Diacyl glycerol
DCFH-DA:	2'7'-dichlorofluorescin diacetate
$DiOC_6$:	3',3'-dihexyloxacarbocyanine iodide
DMEM:	Dulbecco's Modified Eagles Medium
DMSO:	Dimethyl sulfoxide
EDTA:	Ethylene diamine tetra-acetic acid
ETC:	Electron transport chain
FCS:	Foetal calf serum
Gi-protein:	Pertussis toxin sensitive inhibitory GTP-binding protein
GDP:	Guanosine diphosphate
HS:	Horse serum
IPC:	Ischemic preconditioning
JC-1:	3,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine
KCl:	Potassium chloride
KH_2PO_4:	Potassium dihydrogen phosphate
MAPK:	Mitogen activated protein kinase
mK_{ATP}:	Mitochondrial potassium ATP channel
MnSOD:	Manganese superoxide dismutase
MPG:	N-2-mercaptopropionyl-glycine

mPTP:	Mitochondrial permeability transition pore
NAD(P)H:	Nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase system
NAC:	N-acetyl-L-cysteine
Na_2CO_3:	Sodium bicarbonate
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NFκB:	Nuclear factor kappa B
NOE:	N-oleoylethanolamine
PBS:	Phosphate buffered saline
PC:	Preconditioning
PKC:	Protein kinase C
PLC:	Phospholipase C
PMT:	Photomultiplier tubes
PI:	Propidium iodide
RCI:	Respiratory Control Index
ROS:	Reactive oxygen species
2-SPBN:	N-tert-Butyl-a-(2-sulfophenyl) nitrone sodium
SI:	Simulated ischemia
SMase:	sphingomyelinase
SWOP:	Second window of protection
TNFα:	Tumour necrosis factor alpha
TNFβ:	Tumour necrosis factor beta
TNFR1:	TNFα receptor 1
TNFR2:	TNFα receptor 2
XO:	Xanthine oxidase

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ABSTRACT

Introduction: Tumour necrosis factor alpha (TNFα) is a pleiotropic cytokine which has both beneficial and deleterious effects. It has previously been shown in our laboratory that TNFα can mimic ischemic preconditioning (IPC). However, the signalling pathways involved in this protection remain incompletely understood. One potential protective pathway involves the generation of reactive oxygen species (ROS), which are known to be activated by TNFα.

Hypothesis: It was therefore hypothesized that TNFα-induced cytoprotection requires the generation of ROS. In addition, it was postulated that this ROS generation originates in the mitochondria.

Methods: C_2C_{12} myotubes, subjected to a simulated ischemic/reperfusion insult, were preconditioned with either a short period of hypoxia (IPC) or by a low dose of TNFα (0.5ng/ml). Cell viability was assessed using the propidium iodide technique and the trypan blue exclusion test. ROS scavengers were added to investigate the role of ROS in the protection conferred by TNFα. ROS generation was evaluated using 2'7'-dichlorofluorescin diacetate (DCFH-DA) and flow cytometry. The source of ROS production was investigated with a fluorescent imaging technique, by co-staining with Mitotracker Red CM-H_2XRos and DCFH-DA. In addition, the effect of TNFα on the bioenergetics of mitochondria was evaluated by investigation of respiratory parameters, proton leak, inner mitochondrial membrane potential and rate of ATP synthesis in isolated mouse heart mitochondria.

Results: Both TNFα and IPC stimuli improved cell viability in cells subjected to simulated ischemia/reperfusion but this protection was lost in the presence of ROS scavengers. Exposure of C_2C_{12} myotubes to TNFα was associated with an increase of ROS production that was abolished in the presence of ROS scavengers. The fluorescent imaging technique showed that the TNFα-induced ROS appeared to be localised mainly within the mitochondria.

Using isolated mouse heart mitochondria, it was demonstrated that exposure to TNFα (0.5ng/ml) increased the proton leak and reduced the respiratory control index (RCI). In addition, TNFα depolarised the inner mitochondrial membrane and decreased the rate of ATP synthesis. However, the presence of ROS scavengers abolished the effect of TNFα on RCI and proton leak.

In conclusion, these results strongly suggest that TNFα-induced cytoprotection requires the generation of ROS, most likely originating in the mitochondria. Furthermore, the data obtained from isolated mitochondria demonstrated for the first time that TNFα had an effect on the bioenergetics of the mitochondria, dependent on the intra-mitochondrial ROS, but independent of its binding to its specific cell surface receptors. Further investigations are required to demonstrate whether this direct effect of TNFα on the mitochondria may be involved in the production of ROS required for the cytoprotection.

A. INTRODUCTION

1. The Concept of Preconditioning

1.1 Introduction

The World Health Organisation has stated that by the year 2020, the major cause of death world wide will be acute coronary occlusion or "heart attack" (see review[1]). At present, in the United States, 1.5 million people per year develop myocardial infarction and 500 000 will die from this. For more than 25 years many attempts have been made to reduce myocardial infarct size. Various drug therapies such as ß-blockers, calcium channel blockers and anti-inflammatory compounds have been used in this pursuit. However, their effectiveness in limiting the infarct size as an end effector is small.[2] In order to prevent heart attacks it is of importance to understand how this event may be prevented or how to reduce the morbidity when it happens.

1.2 Definition

1.2.1 Ischemia/reperfusion

The word "ischemia" is derived from the Latin terms, *isch* meaning "to hold back" and *aemia* meaning "blood". Ischemia consequently means a lack of blood. In the heart, an abnormal narrowing of the coronary artery decreases the blood supply to the myocardium to cause ischemia, resulting in dysfunction and/or death of heart muscle cells (myocardial infarction).[3] Ischemic heart disease plays an important role in the increasing epidemic of heart related deaths. Feuerstein et al[4] reported that ischemic heart disease was responsible for a large part of cardiac failure.

Animal studies demonstrated that once the myocardium becomes severely ischemic, restoration of blood flow (reperfusion) is a prerequisite for myocardial salvage.[5] These studies also showed that reperfusion after an ischemic episode may be correlated with deleterious changes such as arrhythmias, enzyme release or severe intramyocardial haemorrhage.[5] These changes were termed "reperfusion injuries" as they generally occurred when blood flow was restored. However, the concerns about the clinical significance of these reperfusion injuries were put aside for many years by the results of clinical studies. It is only recently that it has been

demonstrated that reperfusion therapy may be more beneficial than the ischemic state.

1.2.2 Heart preconditioning

In 1986 Murry et al[5] discovered an intrinsic mechanism of profound protection which they described as ischemic preconditioning. They showed that four cycles of 5 minutes of ischemia with intermittent reperfusion could limit the infarct size by 75%. This phenomenon has been recognized as the strongest form of *in vivo* protection against myocardial ischemic injury. In their experiment, Murry et al[5] showed that the brief episodes of ischemia and reperfusion protected the heart (myocardium) against sub-lethal ischemia and this became known as *classical* preconditioning or the first window of protection. The preconditioned state is quite transient but very robust, lasting for up to 6 hours. A delayed form of protection reappears within 24 hours of the preconditioning stimulus and lasts for up to 72 hours. This is referred to as the *second window of protection (SWOP).*[6] Less robust than *classical* preconditioning, although more prolonged, *SWOP* occurs between 12 and 72 hours after a preconditioning stimulus. More recently, a study demonstrated that induction of foetal anaemia during pregnancy could enhance a long term cardioprotection.[7] This study introduced a possible new window of protection.

1.3 Different preconditioning stimuli

1.3.1 Ischemic preconditioning

Ischemic preconditioning (IPC) requires a brief period of ischemia followed by a short period of reperfusion. Li et al[8] showed that once a maximal response is achieved, further ischemic stimulation has no additional effect against reperfusion injures. Classically, to precondition a rat heart using the Langendorff perfusion model and considering the first window of protection, requires 2 cycles of 5 minutes coronary occlusion followed by 5 minutes of reperfusion.

1.3.2 Pharmacological preconditioning

In 1991 Liu et al[9] reported that stimulation of the adenosine A_1 receptor could mimic ischemic preconditioning. Over the last ten years, many

studies have shown that any agonist of Gi coupled receptors could act as a preconditioning trigger. These include adenosine, acetylcholine, bradykinin, the opioids, angiotensin II and endorphin.[10-15] A brief exposure to ethanol can also confer protection.[16] However, it should be noted that variable concentrations as well as different types of preconditioning triggers are required for different species.

In 2000, Horton et al[17] showed that a threshold must be reached in the heart with regard to the dose of stimulus used before the heart can be protected against further damage. Thus both the type of agent as well as the required concentration used to precondition, is critical.

1.3.3 Remote preconditioning

This form of preconditioning is the most poorly understood. In 1993 Przyklenk et al[18] reported in dogs that brief ischemia in one region of the heart could enhance cardioprotection in a remote region. It was hypothesised that during a preconditioning stimulus some compounds are released and could act as a preconditioning trigger in another part of the heart. Verdouw et al[19] confirmed this hypothesis by showing that occlusion of a mesenteric artery in the rat could initiate heart protection.

1.3.4 Other forms of preconditioning

Several other non-receptor-triggered forms of preconditioning have been described. In a study done in the isolated rat heart it was reported that a short period of elevated Ca^{2+} could mimic ischemic preconditioning.[20] In this same study, the protection could be abolished by verapamil, a calcium channel blocker. A transient period of heat stress (hyperthermia) can also confer cardioprotection,[21] but the mechanisms of this preconditioning effect still remain to be elucidated.

Other forms of preconditioning such as pacing, hypoxia and stretch of the myocardial fibres are most likely to induce their protection through release of receptor ligands due to negative energy balance.[22-24]

1.4 Preconditioning Pathways

1.4.1 Preconditioning and G-Protein Coupled Receptors

Thornton et al[25] postulated that once the heart has been stimulated by a trigger, such as adenosine, the ligands activated receptor is thought to couple to pertussis toxin sensitive inhibitory GTP-binding proteins (Gi-proteins) in order to transduce their signal. Schultz et al[26] showed similar findings. These Gi-proteins can move freely within the lipid bilayer of the cell.[27] GDP is bound to the Gi-proteins in their inactive state but when the Gi proteins are stimulated, the bound GDP is released to bind to the GTP thus activating this protein. The original signal is amplified by the movement of these Gi-proteins throughout the cell and many downstream molecules are subsequently activated. The role of these Gi-proteins in preconditioning is controversial. Evidence both for[28] and against[25, 29] a protective role exists in the rat model. Sumeray et al[30] performed experiments involving the blockade of the Gi-proteins in other animal models using pertussis toxin and they showed that the protection induced by ischemic preconditioning was attenuated.

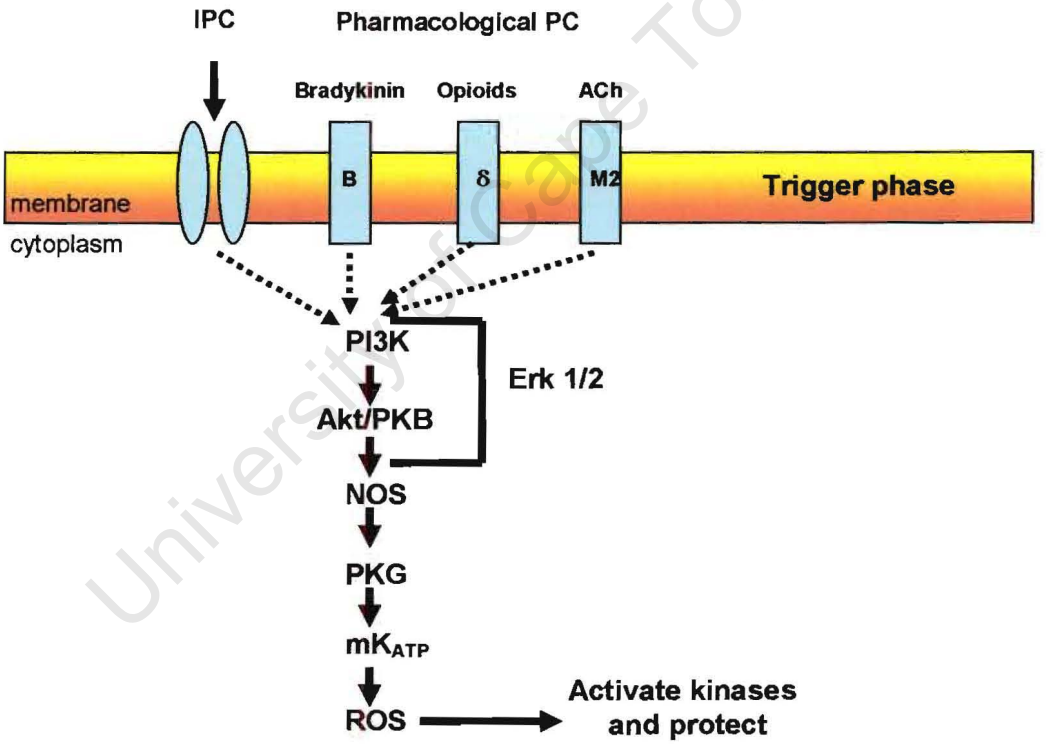
1.4.2 Triggers versus Mediators

In order to understand the different pathways involved in preconditioning it is convenient to think in terms of triggers, mediators, and end-effectors. Somewhere in the signal transduction pathways between the trigger signal and the end-effector is a memory element that is set by the preconditioning protocol and keeps the heart in a preconditioned state. Any step which takes place after the prolonged ischemic insult (index ischemia) can be classified as a mediator as it only exerts its activity after the index ischemia has begun. This can also include the end-effectors where the signal for protection presumably terminates. Triggers are therefore those interventions occurring before the index ischemia, (see review[1]). Many authors consider the opening of the mitochondrial K_{ATP} channel as the end-effector.[31-34] However, the existence of the channel is under speculation as, to date, it has not been cloned. Recent research by Hausenloy et al[131] demonstrated that if the mitochondrial permeability transition pore (mPTP) was kept closed at reperfusion, this afforded protection to the myocardium. Since effective inhibition of mPTP opening at reperfusion may provide protection against ischemia/reperfusion injury,

the mPTP has been postulated by this group as the end effector in preconditioning.

In ischemic preconditioning or with different pharmacological preconditioning mimetics, such as acetylcholine, bradykinin, opioids and ischemia a proposed trigger cascade involves the activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB). This activation results in a subsequent stimulation of nitric oxide synthase (NOS), protein kinase G (PKG), the mitochondrial potassium dependent ATP channel (mK_{ATP}) and reactive oxygen species (ROS). This cascade in turn, stimulates the activation of the protective kinases such as protein kinase C (PKC)[35] (Figure 1).

Some triggers can also act as mediators (eg: PI3K and Erk).



Adapted from Critz et al, Vasc Pharmacol, 2005, 42: 201-209

Figure 1-1: Proposed triggering cascade in classical preconditioning. *Ischemic preconditioning (IPC), bradykinin, opioids and acetylcholine act as preconditioning stimuli to activate the protective pathway in classical preconditioning.*
IPC = ischemic preconditioning; PI3K = Phosphatidylinositol 3-kinase; Akt/PKB = Protein kinase B; NOS = Nitric oxide synthase; PKG = Protein kinase G; mK_{ATP} = mitochondrial potassium adenosine triphosphate channel; ROS = reactive oxygen species

2. TNFα and cardiovascular preconditioning

2.1 Definition

Tumour necrosis factor alpha (TNFα) is a multifunctional cytokine that has been implicated in diverse physio and physiopathological events such as inflammation, cellular survival, growth, differentiation and apoptosis.[36, 37] It was first discovered and described in tumour masses and belongs to a family of signalling molecules that exist as type II membrane proteins characterized by the C-terminus being extra-cytoplasmic.[38] Two isoforms of TNF have been identified (TNFα and TNFβ) and they share similar activities. The more abundant of the two peptides is TNFα and it is thought to be the peptide that mediates cardiac homeostasis. It achieves its various effects by binding to one of its cognate receptors TNFα Receptor 1 (TNFR 1) or TNFα Receptor 2 (TNFR 2).[36] Both of these receptors are expressed in the myocardium.[39] Recently, a mitochondrial binding protein for TNFα (mTNFR) has been discovered.[40]

2.2 TNFα and ischemia/reperfusion

The production of cardiac TNFα has been well documented during acute cardiac ischemia with or without reperfusion.[36, 41] Controversy surrounds the role of TNFα as it has both beneficial and deleterious effects. Many authors have shown that there is a direct correlation between serum TNFα levels and the severity and progression of heart failure.[42-44] Kubota et al[45] demonstrated that mice which overexpress TNFα specifically in the heart develop myocarditis. However, in support of its beneficial role, Lecour et al[46] showed that TNFα can protect the heart in a dose and time-dependent manner. There is considerable support for TNFα in its protective role, depending on whether it is present for a short while in the myocardium or if it is present for a prolonged period.[47] Rabbits that had been given TNFα intravenously 24 hours prior to a sustained ischemic insult showed improved contractile function and a decrease in the secretion of lactate dehydrogenase.[48] The free radical scavenger, manganese superoxide dismutase (MnSOD) was also shown to be increased with the TNFα pre-treatment. This upregulation of MnSOD, possibly through transactivation of the nuclear transcription factor B (NF_kB), may be a link to the pathway through which TNFα may offer

protection from ischemia, as NF_kB is thought to upregulate a large number of cytoprotective genes.[49]

Kurrelmeyer et al[50] performed an elegant experiment in 2000 in which they illustrated that the loss of TNFα signalling resulted in a smaller capacity of the heart to resist an ischemic insult. The authors assessed infarct size 24 hours' after subjecting TNFR1 and/or TNFR2 receptor knock out mice to an acute coronary occlusion. It was found that the infarct size in the combined knock out mouse was much larger than the infarct size of the wild type or when the TNFR1 or TNFR2 receptors were knocked out individually. In addition, when the receptors were knocked out individually, the infarct size was similar to that of the wild type mouse. These results suggested that TNFα signalling does confer protection, probably via the activation of both receptors.

In contrast to these studies, Maekawa et al[51] demonstrated in TNFα knock out mice that once reperfusion was initiated with its known inflammatory reaction, a deficiency in TNFα signalling resulted in a smaller myocardial infarction. In wild type control mice that underwent the same ischemia/reperfusion protocol, anti-TNFα antibodies diminished the infarct size, thus confirming the putative deleterious effect of TNFα. In order to resolve these different outcomes one must surmise (1) that TNFα may promote an intrinsic cell survival programme in response to ischemia and (2) that this protective effect is attenuated by the inflammatory cascade induced by TNFα during reperfusion. Whether the effects of TNFα are deleterious or beneficial is probably dependent on the absolute levels within the distinct temporal periods of ischemia and reperfusion.

2.3 TNFα and ischemic preconditioning

TNFα is one of many inflammatory cytokines produced by the heart.[52, 53] In fact, the amount of TNFα per gram produced by the heart equals that produced either by the liver or the spleen.[52] Kapadia et al[52] demonstrated that a wide variety of cells, including smooth muscle, endothelial and macrophages produce TNFα and surprisingly, that TNFα is also produced by cardiomyocytes themselves. Thus myocardial function can be affected by local TNFα production and the development of techniques to limit production of TNFα by the heart may improve post-ischemic myocardial function.[36] In a

study done by Meldrum et al,[36] it was shown that ischemia/reperfusion increases cardiac TNFα levels after 40 minutes of reperfusion. In the same experiment, IPC or pharmacological preconditioning with adenosine decreased this TNFα production. Adenosine has also been reported to exert cardioprotective effects through other mechanisms.[54]

Smith et al[55] showed that endogenous biosynthesis of TNFα occurred during ischemic preconditioning and that in ischemic preconditioning TNFα is required to attain a tolerant phenotype against ischemia/reperfusion-induced infarction in the murine heart. From their data, it was concluded that TNFα is required to induce classical ischemic preconditioning and that it seems to confer this protection via the mitochondrial K_{ATP} channel (mK_{ATP}). In 2002 Lecour et al[46] showed that TNFα in a low concentration acts as a preconditioning mimetic agent in the isolated rat heart and that the cardioprotective effects seem to signal through the mK_{ATP} as well as via a novel sphingolipid signalling pathway.

2.4 TNFα and mechanisms of protection

TNFα is responsible for the activation of multiple downstream signalling cascades including p38-Mitogen-activated protein kinase (MAPK). The major MAPKs found in cardiac tissue include the extracellular signal-regulated kinases (ERK1/ERK2), the p46 and p54 c-Jun NH_2-terminal kinase/stress-activated protein kinases (JNK/SAPKs) and the α and β isoforms of p38.[132] p38 MAPK is known to act as a trigger in ischemic preconditioning.[133] Tanno et al[56] showed that a low concentration of TNFα could mimic ischemic preconditioning independently of p38MAPK activation. In contrast, higher non-protective doses of TNFα did activate p38MAPK. Recently, strong evidence has emerged to show that cardioprotection at reperfusion by TNFα is independent of activation of the prosurvival kinases, Akt (PKB) and ERK 1/2.[134] These data taken together suggest the possibility that multiple pathways may exist in relation to the trigger phase of preconditioning as has already been suggested by Yellon et al (see review [1]). The signalling pathways involved in IPC and TNFα preconditioning may therefore diverge.

Other pathways that TNFα is known to activate are NFκB[57] and PKC (pro survival),[35, 58] caspases (anti-survival)[59, 60] and the sphingolipids (either pro or anti survival).[46, 61] In addition to PKC, involvement of tyrosine kinases in

ischemic preconditioning, TNFα-induced preconditioning and preconditioning with adenosine has been suggested in earlier studies, using tyrosine kinase inhibitors.[62-64] It has also been demonstrated that a PKC-independent pathway, triggered by a tyrosine kinase mechanism could precondition. This study showed that TNFα was released in the heart after preconditioning by the activation of *a d*isintegrin *a*nd *m*etalloprotease (ADAM 17) and that infarct size in rabbit heart was reduced when ADAM 17 was activated.[65] These authors speculate that cardiomyocytes are the primary source of TNFα in response to ischemic preconditioning.

2.5 TNFα and the sphingolipid pathway

Sphingolipids are a miscellaneous family of phospholipids and glycolipids which are built upon a long chain sphingoid backbone, generally sphingosine. Over the last few years, many studies provided evidence implicating the sphingolipids as second messengers in multiple signalling pathways initiated on binding of TNFα to its receptor, TNFR1/p55.[66-68]

A schematic of sphingolipid biochemistry is shown in Figure 1-2.

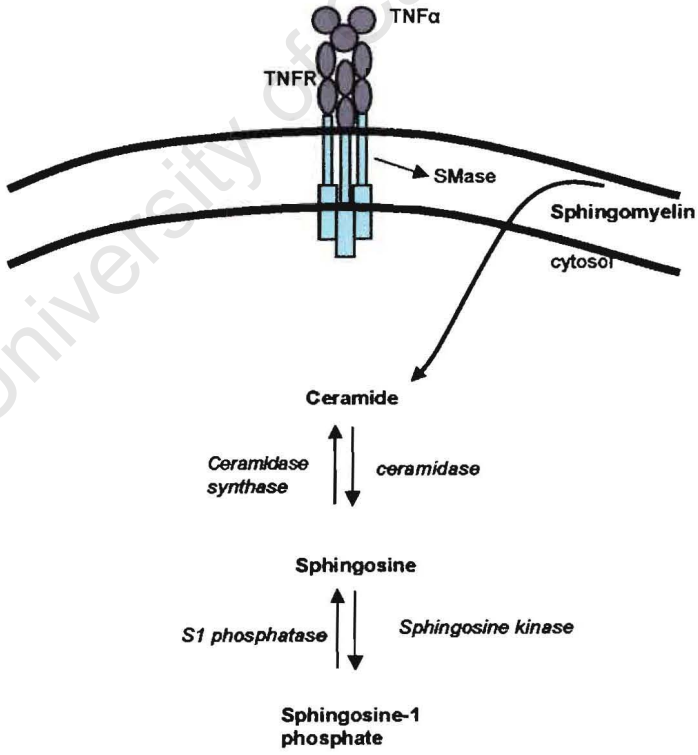


Figure 1-2: Schematic of sphingolipid biochemistry.

SMase = sphingomyelinase; TNFR = TNFα receptor; S1P Phosphatase = sphingosine-1-phosphate phosphatase.

Briefly, in the presence of the enzyme, ceramidase, the ceramide is activated to form sphingosine which can in turn be recycled back to ceramide in the presence of the enzyme, ceramide synthase, or forms sphingosine-1-phosphate in the presence of sphingosine kinase.

TNFα, with its broad spectrum of properties has been postulated to mediate sphingolipid signalling in order to contribute to the cell survival program activated by preconditioning. TNFα when binding to its receptors leads to activation of sphingomyelinase (SMase) to form ceramide from sphingomyelin. The sphingomyelinases are present in sub-cellular sites.

In 2002 Lecour et al[46] showed that the protection afforded with TNFα and ischemic preconditioning was lost in the presence of N-oleoylethanolamine (NOE), an inhibitor of ceramidase. In addition, a recent study showed that the activation of sphingosine kinase played an important role in IPC.[61] Interestingly, sphingolipids are involved in TNFα preconditioning, but are not known to play a role in the preconditioning that follows transient global ischemia, suggesting that the signalling pathways involved may be divergent.[46]

2.6 TNFα and the mitochondria

The mitochondria are very complex organelles found in the cytoplasm of all eukaryotic cells. An important function of the mitochondria is to convert energy found in nutrient molecules (substrates) and store it in the form of adenosine triphosphate (ATP).The cells utilise the ATP to provide energy for various enzymes to carry out a variety of cellular functions.

A mitochondrial binding protein for TNFα (mTNFR) has recently been discovered.[40, 69] This finding suggests the presence of a pathway capable of delivering TNFα directly from the cell surface to the mitochondria.[40] Therefore the effect of TNFα may be due in part to a direct effect on mitochondria. In 2003 Busquets et al[69] demonstrated a clear uncoupling of the mitochondria with the addition of a low concentration of TNFα to isolated rat liver mitochondria. This would indicate that intracellular effects can be exerted by TNFα. In addition, mitochondria derived reactive oxygen species have been implicated as pivotal signalling molecules in the cellular response to preconditioning triggers.[70, 71]

3. Reactive Oxygen Species

3.1 Definition

Any species capable of independent existence that contains one or more unpaired electrons is termed a "free radical".[72] Among the free radicals are the **R**eactive **O**xygen **S**pecies (ROS). The most commonly used definition of ROS is that they consist of free radicals where the unpaired electron or electrons are in the *oxygen* orbital. When the free electron is in the *nitrogen* orbital, such as nitric oxide, this is also termed a free radical but it belongs to a group known as the **R**eactive **N**itrogen **S**pecies (RNS).

Examples of ROS are the hydroxyl radical ($^{\bullet}OH$) and superoxide ($O_2^{\bullet}$). Hydrogen peroxide because of its rapid decomposition giving rise to $^{\bullet}OH$ and $O_2^{\bullet}$ is regarded as a reactive oxygen species, but it is not a true one.

Harmful chemicals may be formed when ROS react in order to gain or lose an electron. For many years the attention given to ROS was mainly focussed on their deleterious effects. ROS are strong oxidants and can damage other molecules and the cell structures of which they form a part. This damage can vary in magnitude and various intracellular defence mechanisms exist to neutralize or control the potentially destructive reactivity of ROS. Some defence mechanisms are simple molecules like vitamins E and C, while some are enzymes like superoxide dismutase (SOD), catalase and glutathione, (see review[67]). These compounds collectively act as ROS scavengers.

3.2 Main Sources of ROS

There are five main sources of ROS production within the cell:

(i) Reduced nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase system (NAD(P)H) is the main source of ROS in vascular cells. This is a membrane associated enzyme, composed of five subunits, catalyzing the one-electron reduction of oxygen, using NADH or NADPH as the electron donor.[73]

(ii) Xanthine oxidase (XO) is physiologically present in the heart where it catalyses hypoxanthine to xanthine and xanthine to uric acid, the two terminal steps of purine metabolism. Both these steps generate

superoxide. This excess ROS is central to the role of XO in heart failure.[74]

(iii) Cyclo-oxygenase (COX) exists in two major isoforms, COX-1 and COX-2. COX-1 is present in most cells and is responsible for constitutive prostaglandin formation. COX-2 is induced in response to stress, which includes ROS.[75, 76]

(iv) Nitric oxide (NO) is a regulatory gas that acts in many different ways in the cardiovascular system and is generated by the nitric oxide synthase (NOS) family of proteins. Three isoenzymes of NOS have been identified: the first is found in neuronal and epithelial cells, the second in cytokine-induced cells and the third in endothelial cells. The second isoform of NOS can be induced by lipopolysaccharide and cytokines in a multitude of different cells.[77, 78] In addition, ROS can be formed in cardiomyocytes by the uncoupling of NOS which releases an electron that may combine with the oxygen radical to generate peroxynitrite, a powerful oxidant.[143]

(v) Mitochondrial ROS: There are at least nine known sites that are capable of generating superoxide anion, a progenitor of ROS, (see review[79]). Complex 1 of the mitochondrial electron transport chain (ETC) has been accepted as a major site of mitochondrial ROS production,[80] and Complex III of the ETC has also been implicated as an important site in the generation of mitochondrial ROS.

There are many different ways of detecting the presence of ROS. The most reliable method is electron spin resonance with the spin trapping technique in which the presence of unpaired electrons trapped within a stable molecule can be detected. Unfortunately, this technique is very complex and requires the use of an expensive machine. A less specific but more affordable technique to different research laboratories consists of the use of fluorometric dyes such as 2'7'-dichlorofluorescin diacetate (DCFH-DA) which fluoresces when oxidized by ROS and emits a signal at a certain wavelength.[81-84]

3.3 ROS and ischemia/reperfusion

A fascinating example of ROS-mediated damage is ischemia/reperfusion injury. During ischemia, an organ not only suffers from lack of oxygen supply

but also from the ROS that are produced when oxygen is restored at reperfusion. Many studies have demonstrated that ischemia/reperfusion generates ROS, including hydroxyl radicals, superoxide anions and hydrogen peroxide.[85] [86] Studies have shown that these robust amounts of ROS which are present at reperfusion are thought to be deleterious.[87-89] In 1997 Vanden Hoek et al[90] performed a study on ventricular cardiomyocytes from chick embryos demonstrating that the generation of ROS induced during ischemia/reperfusion was decreased by the addition of two different ROS scavengers, MPG and phenanthroline, implicating ROS generation during ischemia prior to reperfusion.

Woo et al[91] have shown that ROS are important regulatory molecules implicated in the signalling cascade triggered by TNFα, although these events are not yet well characterised.

3.4 ROS and ischemic preconditioning

Evidence also exists that ROS may be involved in the protection afforded by IPC and pharmacological preconditioning. In a study done in chick ventricular cardiomyocytes, it was demonstrated that the increased protection afforded by acetylcholine-induced preconditioning was abolished by N-2-mercaptopropionyl-glycine (MPG), a ROS scavenger.[81] In 2001, Forbes et al[71] showed that preconditioning with diazoxide promoted the generation of ROS in cardiomyocytes and that N-acetyl-L-cysteine (NAC), a ROS scavenger, was effective in attenuating the improvement in post-ischemic function and energetics. These experiments suggest that the ROS can be involved in protection when generated as a trigger mechanism. Previous studies tested the theory that preconditioning might be mediated by an increase in antioxidant defences. However, Chen et al,[92] demonstrated that the addition of NAC during the preconditioning protocol abolished the protection. This suggested that ROS were protective.

ROS can also act as triggers of IPC. Cleveland et al[93] and Baines et al[94] demonstrated that the protection of IPC could be blocked by an infusion of dimethylthiourea, a ROS scavenger or by MPG, a diffusible ROS scavenger. The mechanism by which ROS precondition the myocardium has not yet been elucidated but it is known that they can activate G-proteins,[95] protein kinases[96] and ATP-dependent potassium channels.[97-99]

3.5 ROS and the mitochondria

After more than 30 years of study, several important mechanistic details of mitochondrial ROS production have been revealed but the bigger picture still remains to be elucidated. The bulk of oxidative pathways are harboured in the mitochondria. These pathways are packed with various redox carriers that theoretically can leak single electrons to oxygen, thereby converting it to the superoxide anion. Recent studies have implicated mitochondrial derived ROS as pivotal signalling molecules in the cellular response to preconditioning triggers.[70, 71]

Studies regarding the mechanistic aspects of mitochondrial ROS generation were carried out by Boveris and Chance in 1973.[100] They found that the mechanism of superoxide generation was located in Complex III of the electron transport chain (ETC). These findings were confirmed in 1980 by Grigolava et al.[101]

A second source of ROS generation in the ETC has been proposed at Complex I but the precise nature of the site and its significance to the production of mitochondrial ROS remain obscure.[102] Kushnareva et al[102] showed that cytochrome-c release causes ROS production in a site proximal to the rotenone inhibitory site in complex I of the ETC. One of the earliest studies demonstrated that superoxide can be produced in the presence of NADH by Complex I.[103] Although Complex I has been widely accepted as a major source of mitochondrial ROS production,[80, 104-106] there are still many issues that need to be addressed.

3.6 ROS and TNFα

Many studies have shown that TNFα produces ROS in various cell lines.[107,109] These studies used high concentrations of TNFα which led to the ROS production and subsequent cytotoxicity. In these studies the major source of TNFα-induced ROS production has been identified as the mitochondria. Garcia-Ruiz et al,[109] using high concentrations of TNFα, demonstrated that ceramide mediated the cytotoxicity of TNFα by increased generation of ROS in the mitochondria, principally at Complex III of the electron transport chain (ETC). Corda et al,[110] using a low concentration of TNFα demonstrated a rapid mitochondrial production of ROS at the ubisemiquinone site (between Complex II and III) in human endothelial cells.

They showed that this effect occurs via a ceramide-dependent signalling pathway initiated by SMase. Evidence exists that TNFα can bind to a receptor on the inner mitochondrial membrane[40] but whether it directly activates the production of ROS in the mitochondria remains uncertain.

B. OBJECTIVES

Objectives

Classic ischemic preconditioning (IPC) is described as brief episodes of ischemia/reperfusion which protect the myocardium from the damage induced by subsequent more prolonged ischemia. TNFα is a pleiotropic cytokine implicated in both beneficial (cell survival) and deleterious events including inflammation, growth and apoptosis.[36,37] Recently it has been shown that in a dose and time-dependent manner, TNFα can mimic ischemic preconditioning.[46] In addition, TNFα is known to produce intracellular reactive oxygen species (ROS) and the mitochondria have been identified as a major source of ROS production.[109,110]

Hypothesis

In the present study, it was proposed that activation of TNFα-induced cytoprotection is mediated by ROS generation. In addition, it was postulated that these ROS originate in the mitochondria.

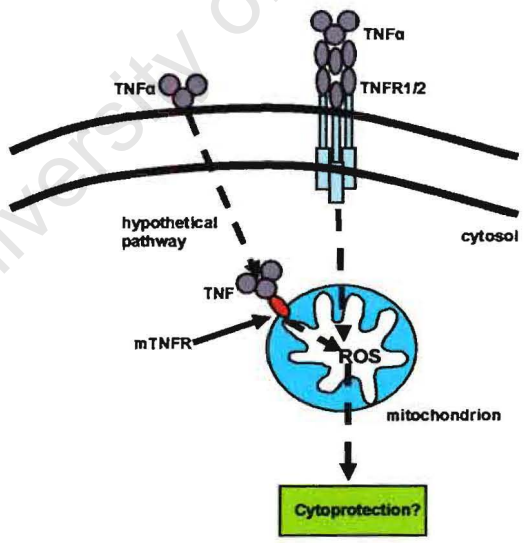


Figure 2-1: Schematic of proposed hypothesis.

TNFα = tumour necrosis factor alpha; TNFR1/2 = tumour necrosis factor receptor 1 or 2; mTNFR = mitochondrial tumour necrosis factor receptor; ROS = reactive oxygen species. *TNFα can either bind to its cell surface receptors inducing ROS production in mitochondria or it can act independently of this pathway, binding directly to the mTNFR of isolated mitochondria, inducing intra-mitochondrial ROS production.*

To evaluate this hypothesis, the following objectives were pursued:

1. Using C_2C_{12} myotubes subjected to simulated ischemia/reperfusion, the cytoprotective effect of TNFα was evaluated in the presence or absence of ROS scavengers. The production and the localisation of ROS within the mitochondria were measured using 2'7'-dichlorofluorescin diacetate dye and Mitotracker Red CM-H_2XRos.

2. Using isolated mouse heart mitochondria the effect of TNFα on the bioenergetics of the mitochondria in the presence or absence of ROS scavengers was assessed, using a Clarke type electrode.

C. MATERIALS

AND

METHODS

1. TNFα-induced cytoprotection is mediated via ROS

1.1 Cell culture

Skeletal muscle is able to undergo classical preconditioning using a mechanism which is virtually identical to that of the heart.[111] *A murine skeletal muscle cell line, C_2C_{12}, obtained from the European Collection of Cell Cultures (Centre for Applied Microbiology and Research, UK) was therefore chosen. These cells were sub cloned from mouse skeletal cells using hind limb muscle.*[112] *They are rapidly dividing myoblasts that can be differentiated into multinucleated myotubes, and are thus similar to cardiomyocytes. The tissue culture flasks do not require any prior coating in order for these cells to adhere.*

Cells were stored in cryovials in liquid nitrogen at -196°C and in order to grow the cells the cryovial was first thawed for 30-40 seconds in a 37°C water bath. The contents were carefully pipetted out into a 25 cm^2 tissue culture flask containing 5 ml of fresh culture medium consisting of Dulbecco's Modified Eagles Medium (DMEM); (Highveld Biological, RSA) supplemented with 10% Foetal Calf Serum (FCS); (Highveld Biological, RSA). Penicillin (1%); (Highveld Biological, RSA) and Streptomycin (1%); (Highveld Biological, RSA) were also added. The cells were placed in a 5% CO_2 incubator maintained at 37°C and 95% humidity until the flask was 90-95% confluent when they were trypsinised with a Trypsin/EDTA solution made up in phosphate buffered saline (PBS), containing 0.25% trypsin(w/v);(Highveld Biological, RSA) and 0.2% EDTA(w/v);(Sigma, Germany). A sufficient volume of Trypsin/EDTA to cover the cell layer was added to the cells for 3 minutes at 37°C after which it was neutralized with double the volume of DMEM containing 10% FCS. The cell suspension was centrifuged for 5 minutes at 1000 rpm. The cell pellet was resuspended in a sufficient volume of medium to split into either 1:3 or 1:4 ratios depending on the size of flask to be used. When a sufficient number of flasks had been seeded for the experiment, these were allowed to reach 80% confluency before differentiation which was achieved by

changing the medium to DMEM containing 1% Horse Serum (HS);(Sigma, Germany), penicillin (1%) and streptomycin (1%). The C_2C_{12} myoblast cells differentiate into myotubes over a period of 3-4 days and are only used 8-10 days post differentiation. During this period, the cells were fed every second day using fresh DMEM with 1% HS, penicillin (1%) and streptomycin (1%).

1.2 Preconditioning Protocol

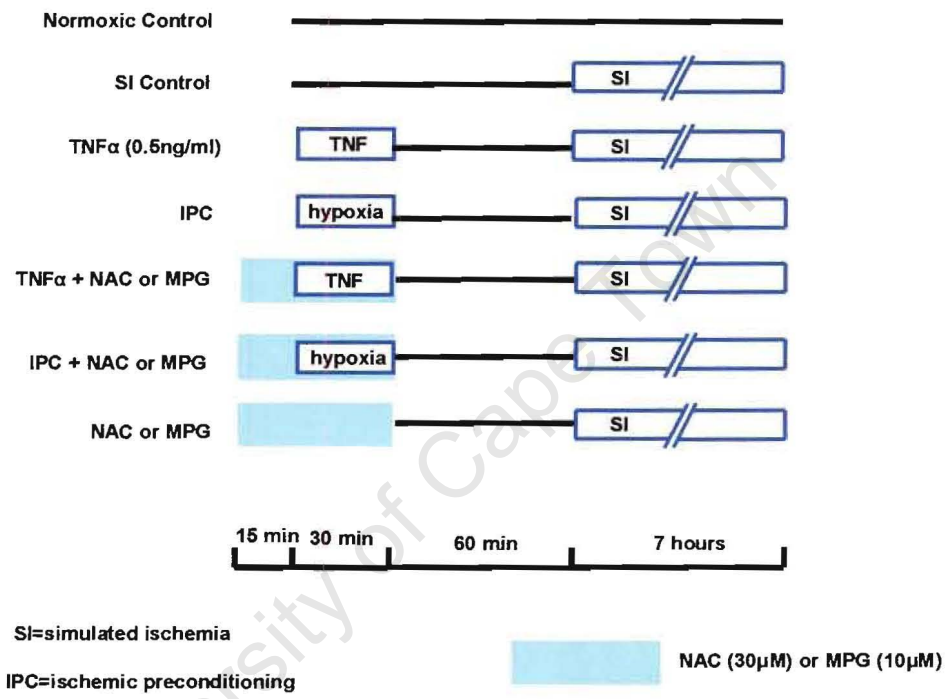


Figure 3-1: *Schematic representation of preconditioning protocols*

In physiological ischemia, the pH decreases, potassium levels increase and metabolic activity is compromised. Therefore in order to simulate the ischemia which occurs in the heart, a simulated ischemic buffer with a low pH, a high content of KCl, and 2-Deoxy-d-Glucose (2-DG) is used. The addition of 2-DG prevents glycolysis, thereby subjecting the cells to metabolic inhibition and the pH of the buffer is reduced from 7.2 to 6.4. The protocol to simulate *ischemia* was adapted from Esumi et al[113] and has been described previously for C_2C_{12} myotubes.[32] In brief, the

simulated ischemic buffer contained in mM: 137 NaCl, 12 KCl, 0.5$MgCl_2$, 0.9 $CaCl_2$, 20 Hepes, 20 2-Deoxy-d-Glucose (2-DG) adjusted to a pH of 6.4. A Sanyo Multigas Incubator with 5% CO_2, 94% N_2, and 1% O_2 provided a hypoxic environment. All experiments were performed in 25 cm^2 tissue culture flasks. The protocol consisted of the following groups:

A normoxic control group consisted of cells which were kept in their normal environment for the duration of the experiment but were subjected to the same number of washes with PBS as were the treated cells.

In a simulated ischemic (SI) control group, the cells were washed twice with PBS before addition of buffer and incubation for 7 hours in the hypoxic incubator with SI buffer, pH 6.4.

TNFα-treated cells (recombinant murine TNFα, PeproTech, USA, 0.5ng/ml in DMEM with 1% HS) were incubated for 30 minutes in the standard 5% CO_2 incubator. TNFα was washed out with two washes of PBS, fresh DMEM (1%HS) was added and the cells were reincubated for 60 minutes in the standard 5% CO_2 incubator. After a further 2 washes with PBS the cells were incubated with SI buffer for 7 hours in the hypoxic incubator.

In the IPC group the cells were incubated for 30 minutes in the hypoxic incubator in SI buffer, pH 6.2 without 2-DG, as we did not wish to inhibit glycolysis during IPC. This was followed by 2 washes with PBS and reincubation in DMEM (1% HS) for 60 minutes in the standard 5% CO_2 incubator. After a further 2 washes with PBS the cells were incubated with SI buffer for 7 hours in the hypoxic incubator.

To demonstrate whether reactive oxygen species (ROS) were involved in the protective effect of TNFα, two ROS scavengers, N-acetyl-L-cysteine (NAC 30μM);(Sigma, Germany) or N-2-mercaptopropionyl-glycine (MPG 10μM);(Sigma, Germany) were added to the cells 15 minutes prior to the addition of TNFα (0.5 ng/ml) and continued for the 30 minutes incubation with TNFα in the standard 5% CO_2 incubator or, in the case of IPC, 15 minutes in DMEM (1% HS) in the standard 5% CO_2 incubator followed by incubation with the SI buffer (no 2-DG, pH 6.2) for 30 minutes in the hypoxic incubator. Cells are then reincubated for 60 minutes in DMEM (1% HS) in the standard 5% CO_2 incubator followed by 2 washes with PBS before incubating in SI buffer for 7 hours in the hypoxic incubator.

The ROS scavenger control groups consisted of NAC or MPG added for 15 minutes in DMEM (1% HS). The cells are washed twice in PBS and incubated in DMEM (1% HS) with NAC or MPG for 30 minutes in the standard incubator. After two further washes with PBS, cells are reincubated in fresh DMEM (1% HS) for 60 minutes in the standard incubator, washed in PBS and incubated in SI buffer for 7 hours in the hypoxic incubator.

1.3 Cell viability analysis

1.3.1 Propidium Iodide

Cell viability was evaluated using a flow cytometer (FACS Calibur, Becton-Dickinson, USA) to measure the uptake of propidium iodide (PI);(Sigma, Germany) which is known to bind to DNA and become fluorescent.[82] However, if the cell membrane is intact PI will not be able to enter the cell and translocate to the nucleus. Consequently it is used in immunofluorescent staining protocols to identify dead cells. Flow cytometry detects the presence of individual cells that are held in a thin stream of fluid and passed through one or more laser beams causing the light to scatter and fluorescent dyes to emit light at various frequencies. Photomultiplier tubes (PMT) convert light to electrical signals and cell data is collected. Once the C_2C_{12} myotubes had undergone the experimental protocol outlined above, the flasks were trypsinised and cells resuspended in their normal growth medium. PI (1.5μM) was added and the cells reincubated for 5 minutes in the standard 5% CO_2 incubator before analysis on the flow cytometer. PI fluorescence was measured in a population of 1 x 10^4 cells which was then analysed by means of CellQuest software.

1.3.2 Trypan Blue

The Trypan Blue exclusion technique for cell viability was also used. In this method trypsinised cells are resuspended in PBS and mixed with an equal volume of trypan blue (4μM); (Sigma, Germany) which enters cells in which the membrane is not intact, dyeing them blue. Stained cells are loaded onto a Neubauer haemocytometer and evaluated by light microscopy. The ratio of blue cells, to grey cells or those in which cell

membranes are still intact is then calculated and expressed as a percentage.

1.4 Respiratory parameters

Consumption of oxygen during cellular respiration by the mitochondria is utilized for bioenergetic functions, the principal one being the conversion of ADP to ATP. ATP is used by enzymes to perform a wide range of cellular functions. In whole cells the respiration which occurs as a result of the utilisation of their endogenous substrates is termed State 2 respiration. In 2001 Minners et al[32] showed that IPC and pharmacological preconditioning increased cellular respiration prior to the index ischemia. It was decided for this study, to establish whether TNFα had a similar effect on State 2 respiration in whole C_2C_{12} myotubes, which would suggest that the cells were protected from ischemia by the TNFα stimulation. As MPG has been reported to have inconsistent effects on models of ischemia and reperfusion,[144] N-tert-Butyl-a-(2-sulfophenyl) nitrone sodium (2-SPBN), (Sigma, Germany) was chosen as a second ROS scavenger to confirm previous results with MPG. Two time points were examined:

a) *Immediately prior to the 7 hour simulated ischemic insult as shown in Figure 3-2.*

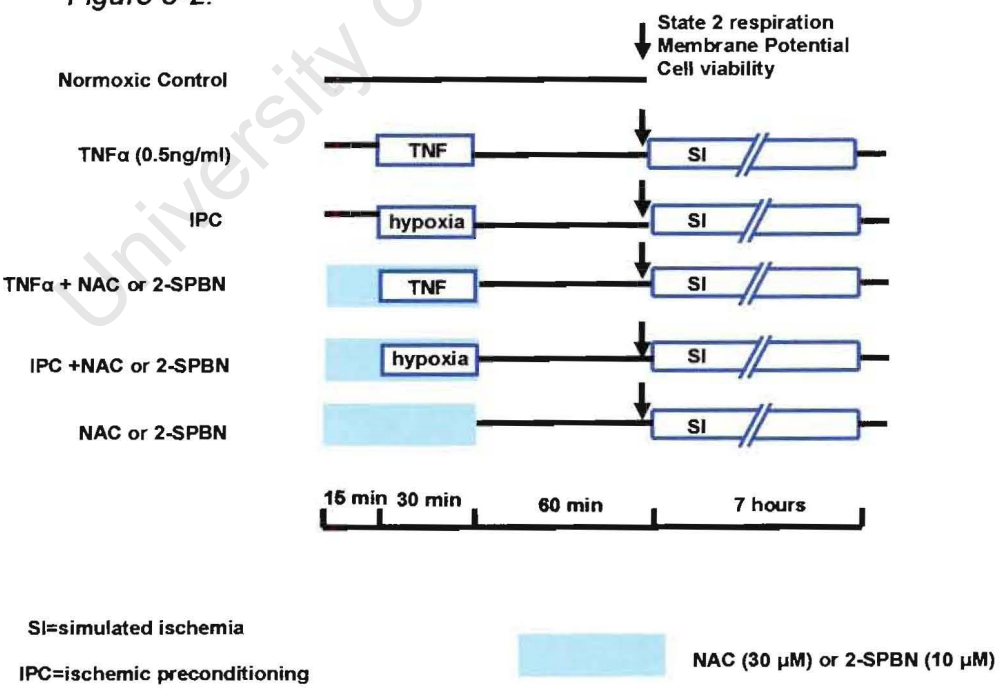


Figure 3-2: ***Schematic representation of State 2, Membrane Potential and cell viability protocols immediately prior to 7hr simulated ischemia.***

or

b) *Immediately after the full preconditioning protocol, with 7 hours of simulated ischemic insult as shown in Figure 3-3.*

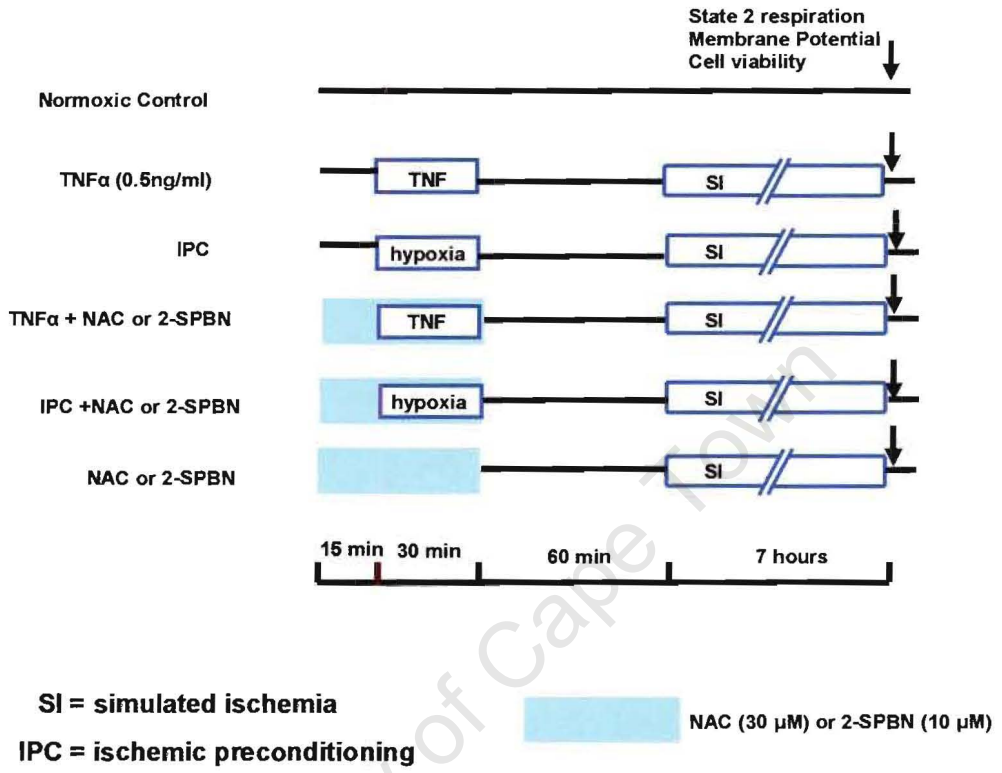


Figure 3-3: ***Schematic representation of State 2, Membrane Potential and cell viability protocols after 7hr simulated ischemia.***

Respiration studies were performed using an Oxytherm respirometer equipped with a Clark-type electrode and a Peltier temperature control unit (Oxytherm, Hansatech, Norfolk, UK). The respirometer was calibrated using PBS and a temperature of 37°C was maintained throughout. 1ml of PBS was then placed in the chamber and a baseline reading was taken to verify the calibration.

Each experiment had a normoxic control. In the second set of experiments, a simulated ischemic control was included. In both sets of experiments, cell viability was measured using PI. Using the percentage viability obtained, respiration values from viable cells only were calculated by means of a factor.

After trypsinisation, cells were resuspended in PBS at a concentration of 2 x 10^6cells/ml. 1x10^6 cells were added to the chamber and State 2 respiration was determined at the time points shown in Figure 3-2 and Figure 3-3.

1.5 Inner Mitochondrial Membrane Potential Analysis

Cells with a high energy requirement need to maintain their inner mitochondrial membrane potential for their performance. Relative changes in inner mitochondrial membrane potential have been widely measured by lipophilic fluorescent cations.[114, 115] A variety of these cations are currently available. To assess mitochondrial uncoupling, the inner mitochondrial membrane potential was evaluated using 3,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl-carbocyanine (JC-1). This dye exhibits potential-dependent accumulation in mitochondria which is indicated by a fluorescence emission shift from green (525nm) (FL1) to red (590nm) (FL2) after being excited at 488nm. A decrease in the red/green fluorescence intensity ratio therefore signifies mitochondrial depolarisation or a reduced membrane potential. To limit dye specific findings, use was made of a second cationic dye, Dihexyloxacarbocyanine iodide ($DiOC_6$), which was excited at 488nm and the resultant fluorescence analysed at 525nm (FL1).[116,117]Propidium iodide (FL3) was added to exclude dead cells from the analysis, by the selection of only viable cells on the flow cytometer and gating these cells, using CellQuest software. As PI is excited at 525nm and emitted at 550nm to 630nm, we were able to select the emission wavelength in order to compensate for the cross over in emission spectrums between FL1, FL2 and FL3, using the software provided.

Cells described in **1.4** above, were used to evaluate the inner mitochondrial membrane potential. JC-1 (Sigma, Germany) was dissolved in DMSO (0.25mM) and aliquots were stored at -20°C. $DiOC_6$ (Sigma, Germany) was dissolved in ethanol (1mM) and aliquots were stored at -20°C. Membrane potential was evaluated at the end point in each set of experiments as follows: After the different treatments (see Figure 3-2 and Figure 3-3) the cells were trypsinised, counted and resuspended in PBS. A 400µl aliquot was placed in a tube and 8µl JC-1 (final concentration 5µM) added to the tube. The tube was incubated at 37°C for 10 minutes after which 4µl PI (final concentration 1.5µM) was added and the tube reincubated for a further 5

minutes. To a second tube containing 400µl of the cell suspension, 4µl DiOC6 (final concentration 20nM) and 4µl PI (final concentration 1.5µM) were added and the tube incubated for 5 minutes at 37°C. To validate appropriate responses to the dyes, the uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP 0.1µM); (Sigma, Germany) or the hyperpolarising agent oligomycin (1.2µM); (Fluka, Germany) were incubated for 5 minutes with the cells. The cells were analysed on a fluorometric analysis cell sorter (FACSCalibur, Becton Dickinson, USA) and the mean fluorescence was calculated for each sample. All data were relative levels versus control. The remainder of the cell suspension was used for the respiration study as outlined in 1.4 above.

1.6 Detection and quantification of ROS production

A crucial signalling step in the cell-protective phenomenon of preconditioning is the production of reactive oxygen species.[70, 92] TNFα is known to mimic preconditioning and also to produce ROS. For this study it was decided to investigate whether TNFα-induced ROS were involved in the cytoprotective pathway. Various methods have been developed for the detection of different types of free radicals including superoxide, hydrogen peroxide and peroxinitrite.[118]

In this study the fluorometric probe, 2'7' dichlorofluorescin diacetate (DCFH-DA); (Molecular Probes, USA) was used. The non-fluorescent DCFH-DA rapidly penetrates the cell membrane and is deacetylated by intracellular esterases. Alkaline and oxidative hydrolysis also plays a role. The non-fluorescent compound dichlorofluorescin (DCFH) is formed and trapped in the cytosol where it is preferentially oxidised by hydrogen peroxide and superoxide to the fluorescent dichlorofluorescein (DCF). DCFH-DA was dissolved in DMSO to give a stock solution of 15mM and aliquots were stored at -20°C.

C_2C_{12} myotubes were trypsinised and resuspended in their normal growth medium after the preconditioning stimulus described in 1.2. DCFH-DA (5µM) was added and the myotubes reincubated at 37°C for 10 minutes. PI (1.5µM) was added and the cells reincubated for a further 5 minutes. The myotubes were divided into two equal aliquots. The first aliquot was used to give untreated baseline measurements for each time point, in

order to compensate for any background fluorescence generated by the growth medium, and the second aliquot was treated with TNFα (0.5ng/ml). DCF fluorescence was analysed every minute from zero to 3 minutes and thereafter at 5 minutes, 10 minutes, 15 minutes, 20 minutes 30 minutes, 60 minutes and 120 minutes using a FACS Calibur (Becton Dickinson, USA) flow cytometer. DCFH-DA is excited at 488nm and the fluorescein signal is analysed at 525nm (FL1). PI was added to exclude dead cells from the analysis (FL3). Less than 5% of C_2C_{12} myotubes were found to be PI positive. 1 x 10^4 PI negative cells were analysed in each sample. The fluorescence data was plotted on a histogram and the geometric mean calculated for each sample.[119] The same experiments were repeated with the addition of the ROS scavenger, MPG.

1.7 Source of ROS production after TNFα stimulation

To determine whether the ROS produced by the addition of TNFα to the C_2C_{12} myotubes were generated by the mitochondria, the fluorescent dye, Mitotracker Red CM-H_2XRos (M7513, Molecular Probes, USA) was used. This probe fluoresces only when it enters an actively respiring cell where it is oxidised to the fluorescent mitochondrion-selective probe and then sequestered in the mitochondria. This signal from this dye is excited at 488nm and emitted at around 600nm and can therefore be used in conjunction with DCFH-DA which is excited at 488nm and is emitted at 525nm.

The C_2C_{12} cells were grown on coverslips and differentiated as described previously. The myotubes were then stained simultaneously with DCFH-DA (5μM) and Mitotracker Red CM-H_2XRos (100nM) for 10 minutes at 37°C. The coverslip was then placed on a glass slide and the cells were visualised using a Nikon Eclipse E400 fluorescent microscope at 60X magnification and fitted with a Nikon DMX1200 digital camera. Once a suitable field had been selected and the image captured using Simple PCI software (Compix Inc, Imaging systems, Pennsylvania, USA), 0.5ng/ml TNFα was added directly to the cells being visualised and the cells were monitored for ROS production, with images being captured at 1 min, 3 min, 5 min and 10 min. These images were then superimposed over the image of the same field showing the mitochondria as stained with the DCFH-DA and mitotracker dye. Hydrogen peroxide was used as a positive control.

2. Effect of exogenous TNFα in Isolated Mouse Heart Mitochondria: role of ROS

As a result of the data obtained using the C_2C_{12} cell line and the discovery in recent years of a mitochondrial binding protein for TNFα, it was decided to investigate whether TNFα could have a direct effect on isolated heart mitochondria and whether this effect was mediated via activation of intra-mitochondrial ROS.

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 (NIH publication No. 85-23, revised 1996).

2.1 Isolation of Mitochondria

10-12 week old male Black 6 x 129 mice (25-30g) were anaesthetized with sodium pentabarbitone (50mg kg^{-1}intra-peritoneally) and heparinised to prevent the blood clotting (25 International Units, intra-peritoneally). A sufficient depth of anaesthesia was achieved once the pedal reflex had disappeared. The heart was then rapidly excised and kept in ice-cold isolation buffer (0.18M KCl, 0.01M EDTA, pH to 7.4 with 2M Tris).[120] The atria and connective tissue were trimmed off and the remaining ventricles minced on ice. The homogenate was rinsed until all visible blood had been eliminated (4 rinses with ~ 5X w/v ice-cold isolation buffer). After rinsing, the tissue was gently homogenized with a 5ml glass homogeniser (Tenbroek, Netherlands) and the homogenate centrifuged at 2930 rpm (Christ MK202, Germany) to eliminate the larger intracellular constituents. The supernatant was carefully transferred into a clean, cold tube and centrifuged at 4500 rpm to isolate the mitochondria.[120] The resulting pellet was resuspended gently in 50µl of ice-cold isolation buffer and was kept on ice for up to 3 hours. The protein content of the pellet was determined using the method of Lowry.[121]

2.2 Lowry Protein Determination

Duplicate 1:200 dilutions of mitochondrial suspension were made using sterile distilled water. 1ml of CTC reagent was added and the samples thoroughly mixed. After incubation for 10 minutes at room temperature, 0.5ml of Folin's Reagent (Merck, Germany) was added with continuous mixing as

the colour complex was formed immediately. The samples were incubated at room temperature for a further 30 minutes and then read at 750nm on a Varian 130 dual beam spectrophotometer.

CTC reagent consisted of the following:

Na_2CO_3 (20%w/v) and $CuSO_4.5H_2O$ (0.2%w/v) to which an equal volume of dipotassium tartrate (0.4%w/v) was added.

Folin-Ciocalteu's phenol reagent was diluted 1:6 with double distilled water before use.

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

2.3 Mitochondrial Respiration Studies

There are four states of mitochondrial respiration or utilisation of oxygen, which are defined as follows:

a) *State1* is the respiration resulting from mitochondrial use of endogenous substrates.

b) *State 2* is that which occurs in the absence of ADP.

c) *State 3* is the respiratory state in which phosphorylation of ADP to ATP occurs and is where a considerable amount of oxygen is utilised.

d) *State 4* is reached when all ADP has been converted to ATP.

Respiration studies were performed using an Oxytherm respirometer equipped with a Clark-type electrode and a Peltier temperature control unit (Oxytherm, Hansatech, Norfolk, UK). The respirometer was calibrated using the mitochondrial incubation buffer (25mM Tris, 250mM Sucrose, 8.5mM KH_2PO_4, pH to 7.4 with 2M Tris). All experiments were performed at 25°C.

The respiration protocols are shown in Figure 3-4.

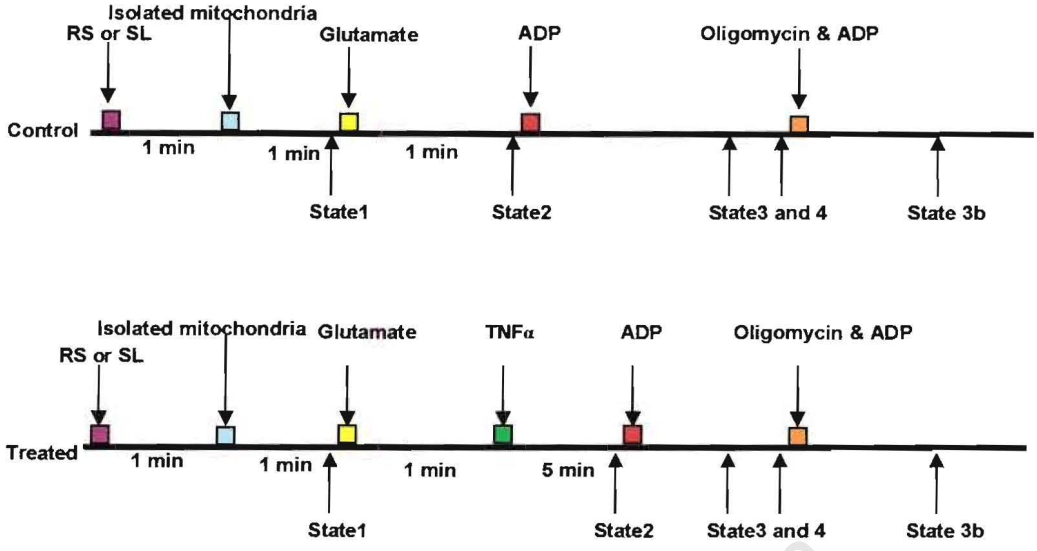


Figure 3-4: Schematic of respiration protocol. RS=ROSscavengers; SL=sphingolipid inhibitors.

Isolated mitochondria were added to mitochondrial incubation buffer in the Oxytherm chamber, left to stabilise for 1 min and then substrate (10mM) was added. After 1 min, State 3 respiration was initiated by the addition of ADP (350µM). Once State 4 was reached, oligomycin (1.2µM) was added to achieve State 3b which was used to calculate the proton leak. The treated group had TNFα (0.5ng/ml) added 1 min after the substrate.

A baseline reading of buffer alone was taken to verify the calibration of the Oxytherm respirometer. 25µl of the mitochondrial suspension (isolated as in 2.1) was then added to the 341µl of buffer and the plunger inserted. After 1 minute, a second reading was taken to determine State 1 respiration after which 20µl of glutamate (10mM) was added and State 2 respiration measured 1 minute later. State 2 respiration represents the utilization of the substrate in the absence of ADP. After stabilization, 14µl of ADP (350µM); (Roche, Germany) was added to trigger State 3 respiration. Conversion of ADP to ATP consumes a relatively large amount of oxygen during State 3.

The mitochondria progressed to State 4 respiration in which all the ADP had been utilized and a steady state of respiration again occurred.

The following groups were considered in the protocol outlined in Figure 3-4:

1) Untreated control mitochondria

2) 0.5ng/ml TNFα – added 1 min after stabilization with glutamate

3) TNFα + 30μM NAC

4) TNFα +10 μM 2-SPBN

5) TNFα + 5μM NOE

6) NAC

7) 2-SPBN

The ROS scavengers and sphingolipid inhibitors were added to the buffer 1 minute prior to the addition of isolated mitochondria.

The concentrations given are final concentrations within the chamber.

2.4 Proton Leak

The ATP synthase is specifically blocked by oligomycin. Therefore any oligomycin insensitive state 3 mitochondrial respiration which occurs will represent mitochondrial proton leak which is an index of the state of mitochondrial uncoupling.

To determine the rate of oligomycin induced mitochondrial proton leak, oligomycin (1.2μM) as well as an additional 14μl ADP (100mM stock) was added immediately after State 4 respiration (Figure 3-4). State 3b is the oligomycin insensitive respiration. The percentage of proton leak can be calculated as a result of the ratio between State 3b respiration and State 3 respiration.

2.5 Respiratory Control Index (RCI)

The respiratory control index (RCI) is a simple ratio of State 3 respiration/State 4 respiration and is an index of the viability and coupling of the mitochondrial population. An RCI equal to or greater than 3 is an index of a good mitochondrial isolation. Therefore mitochondria which had an RCI of less than 3 were discarded in our experiments.

2.6 Mitochondrial Inner Membrane Potential

The generation of mitochondrial ATP is dependent on the integrity of the inner mitochondrial membrane potential. This needs to be maintained in cells with high energy requirements such as the cardiomyocytes. However, mild uncoupling of the mitochondria is thought to be protective. To observe whether TNFα depolarises the isolated mitochondrial inner membrane, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbezimadazolyl-carbocyanine iodide) was used. JC-1 is one of the lipophilic fluorescent cations that have been widely used to measure relative changes in inner mitochondrial membrane potential.[114, 115]

JC-1 (2.5mM in DMSO) was diluted 1:100 in PBS, prewarmed to 37°C and 84µl of this was added to 341µl mitochondrial incubation buffer containing 25µl mitochondrial suspension, 20µl glutamate (10mM final concentration) and 14µl ADP (350µM final concentration). The final concentration of the JC-1 was thus 5µM. The suspension was kept at 37°C for 5 minutes and then analysed on a Becton-Dickson FACS (fluorescence activated cell sorter) Calibur flow cytometer. The JC-1 was excited at 488nm and the monomer signal (green) was emitted at 525nm (FL1). Simultaneously, the aggregate signal (red) was emitted at 590nm (FL2). As a control, unstained mitochondria were analysed. After obtaining the readings for membrane potential, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added at a final concentration of 0.1uM (20ul of a 1µM stock solution) in order to confirm that it was membrane potential and not mitochondrial volume or size being measured.

A second potentiometric dye, $DiOC_6$ (3',3'-dihexyloacarbocyanine iodide) was used to confirm the results obtained with JC-1. A 1mM stock solution was made up in ethanol, dispensed into Eppendorf tubes and stored at minus 20°C. An aliquot was diluted 1:1000 to yield a 1µM solution of which 40µl was added to the isolated mitochondria as for the JC-1. After 5 minutes at room temperature, the sample was analysed on the flow cytometer. $DiOC_6$ was excited at 488nm and the signal was emitted at 525nm (FL1). Unstained mitochondria were again used as a control and CCCP was used to abolish the membrane potential after staining (0.1µM final concentration).

2.7 Rate of mitochondrial ATP synthesis

The rate of ATP synthesis is the rate at which mitochondria phosphorylate ADP to ATP. By measuring this rate it can be seen which mitochondria are in a better bioenergetic state and more resilient to stress factors.

For this study a modified method of Budnikov was used.[122] In order to measure the amount of ADP that was phosphorylated to ATP over a timed period the following reaction was set up:

The control was prepared as follows:

898µl of synthesis buffer (final concentrations of: 150mM sucrose, 75mM KCl, 3µM rotenone, 5mM KH_2PO_4, 2.5mM $MgCl_2$) was warmed to 25°C. 1µl of mitochondrial suspension (prepared as in 2.1) was added. After 30 seconds of equilibration, 100µl succinate was added (final concentration 10mM) and allowed to equilibrate for 60 seconds.

TNFα (0.5ng/ml) was added to another identically prepared sample

2µl ADP (350µM) was added to the control and TNFα-treated mitochondria and the clock started immediately. A zero time sample of 5µl of each was taken and thereafter for every 10 seconds up to 60 seconds. The 5µl samples were added directly into 50µl cold 2.5% trichloroacetic acid to arrest the reaction and release the ATP. The solution was neutralized with 10µl of 1M Tris base, and frozen at -80°C for later assay.

The assay was carried out as follows: Duplicate 10µl samples from each assay time point were added into separate wells of a 96 well plate. A standard curve consisted of serial dilutions of sodium ATP (Roche, Germany) from 10^{-5}M to 10^{-9}M and 10µl aliquots were used in duplicate for the assay. 175µl of double distilled water was added to each well. Firefly luciferin/luciferase assay reagent, FFL (FL-AAM); (Sigma, Germany) was prepared by adding 5ml of double distilled water to one vial. The vial was allowed to stand for up to 30 minutes to dissolve properly. The reagent was added to a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, California, USA) and the 96 well plate inserted. 25µl of the FFL was added automatically by the luminometer to each well for the assay and the amount of relative light signal emitted was read, using the Veritas Software Rev 1.1.

Once the assay was completed, Graphpad Prism 3 was used to plot the log of relative light units (RLU) versus the concentration. The unknowns are then read off the standard curve by entering the log RLU's and allowing the programme to calculate the concentrations.

2.8 Role of ROS and the sphingolipid pathway

TNFα is known to stimulate the sphingolipid pathway and it has been shown previously that the cytoprotective effect of TNFα was abolished by NOE in the isolated perfused rat heart.[46] *In order to determine whether the sphingolipid pathway was mediated by TNFα-induced ROS in isolated mitochondria two inhibitors of this pathway were employed, imipramine, and N-oleoylethanolamine (NOE). Imipramine prevents TNFα activating endosomal acid sphingomyelinase (aSMase), thus inhibiting sphingomyelin from forming ceramide. NOE inhibits the enzyme ceramidase without which the ceramide cannot form sphingosine*

To determine whether NOE (Sigma, Germany) and imipramine (Sigma, Germany) could abrogate the effect of TNFα on the respiration, NOE or imipramine was added immediately prior to the mitochondria in the Oxytherm chamber and allowed to stabilise for 1 minute before adding TNFα (0.5ng/ml). After 5 minutes, State 2 respiration was measured. The same procedure as in 2.3 (see Figure 3-4) was then followed to obtain State 3 and State 4 respiration as well as the proton leak.

Chemicals: Unless otherwise mentioned all chemicals used were obtained from Merck, Germany.

3. Statistical Analysis

Results were expressed as mean values ± standard error of the mean (s.e.m) and were analysed by One Way Analysis of Variance (Anova) with Tukey-Kramer Multiple Comparisons test, using GraphPad InStat version 3.01 (GraphPad Software, San Diego, California, USA). Differences were considered statistically significant at values of $p<0.05$. When a comparision of two events is given, the Student's t test was used.

D. RESULTS

1. TNFα-induced cytoprotection is mediated by ROS

1.1 TNFα induces cytoprotection in C_2C_{12} myotubes

a) Cell viability

Cells were used 8-10 days post–differentiation, after reaching the mature myotube stage.

C_2C_{12} myotubes that were maintained in their normal environment for the full preconditioning protocol had a viability of 93±2% (PI technique) or 84±3% (Trypan blue exclusion test) respectively. Myotubes incubated for 7 hours in hypoxic buffer under hypoxic conditions (simulated ischemia, SI), showed a drastic reduction in viability to 34±1% (PI) or 24±6% (Trypan blue). Ischemic preconditioning protocol (IPC) improved myotube viability to 74±2% and 78±3% with PI and Trypan blue, respectively. Similarly TNFα used as a preconditioning mimetic improved the viability to 75±1% (PI) and 81±2% (Trypan blue). (*Figures 4-1 and 4-2*).

- Propidium Iodide Technique

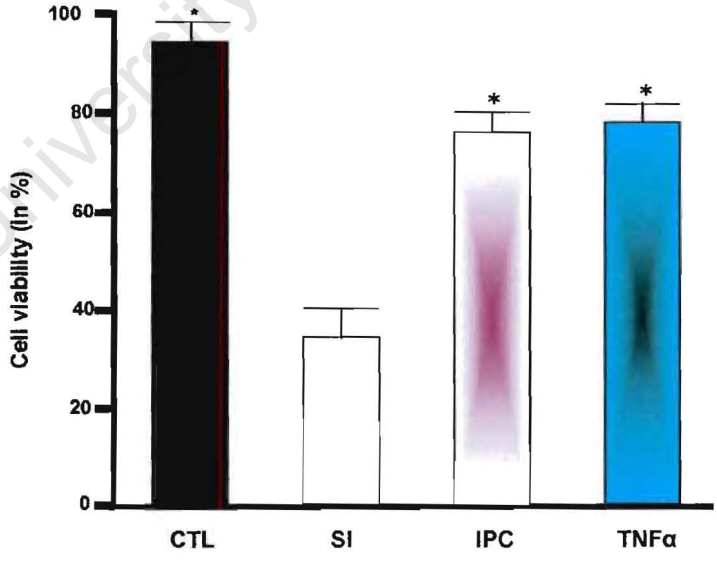


Figure 4-1: Cell Viability (using Propidium Iodide technique) in C_2C_{12} myotubes. *Cell viability (expressed as a percentage) was dramatically reduced in cells subjected to 7hr simulated ischemia (SI). Ischemic preconditioning (IPC) or TNFα (0.5ng/ml) restored the cell viability.*
**p<0.001 versus SI, n = 6.*

- Trypan Blue exclusion test

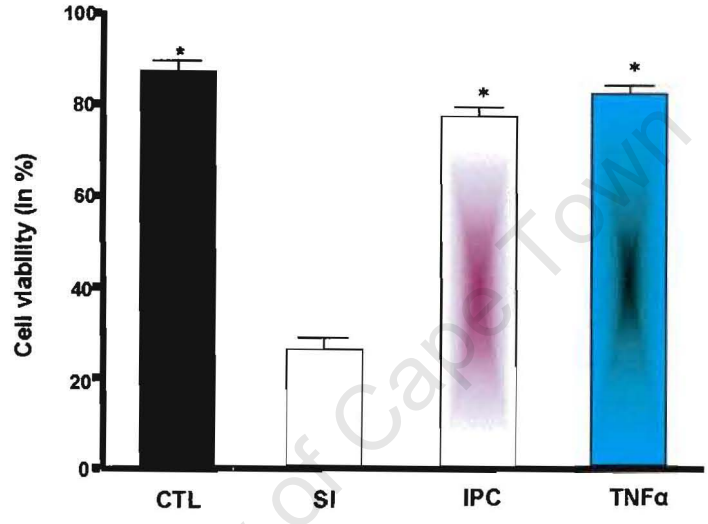


Figure 4-2: Cell Viability (using Trypan blue exclusion technique) in C_2C_{12} myotubes. *Cell viability (expressed as a percentage) was dramatically reduced in cells subjected to 7hr simulated ischemia (SI). Ischemic Preconditioning (IPC) or TNFα (0.5ng/ml) restored the cell viability.*
**p<0.001 versus SI, n = 6.*

b) *Respiratory parameters*

State 2 respiration was evaluated immediately prior to the 7 hour simulated ischemic period or immediately post 7 hour simulated ischemia.

- *Pre-simulated ischemia analysis*

Prior to simulated ischemia, State 2 respiration was markedly increased in the IPC and TNFα groups compared to the normoxic control (15.6±0.9 and 21.6±1.3 respectively versus control 8.2±0.5 $p<0.01$), (*Figure 4-3*).

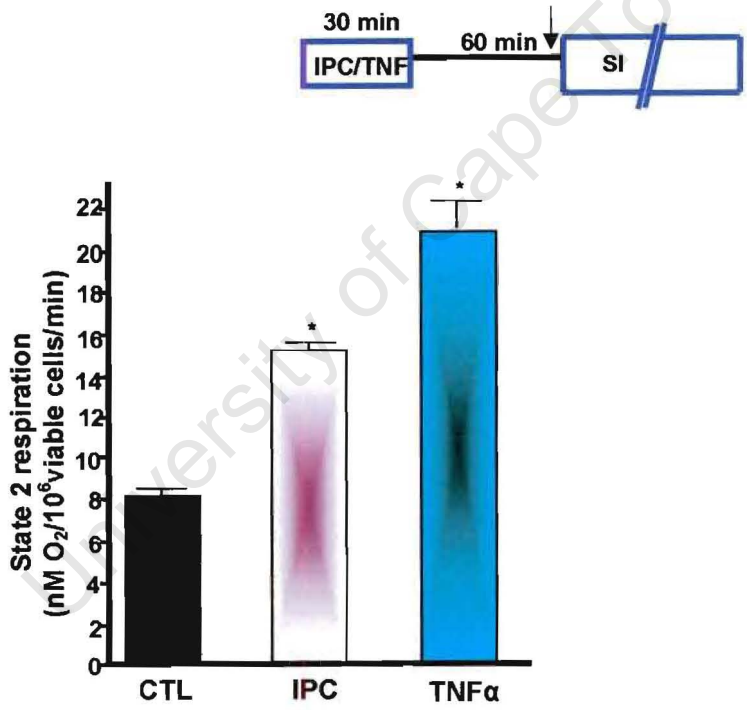


Figure 4-3: State 2 respiration in C_2C_{12} myotubes prior to the ischemic insult. *Both ischemic preconditioning (IPC) and TNFα (0.5ng/ml) increase State 2 respiration of C2C12 myotubes. State 2 respiration was expressed as nM of oxygen/million viable cells/min.*
**$p<0.01$ versus control, $n = 8$.*

- *Post-simulated ischemia analysis*

Post 7 hour simulated ischemia, State 2 respiration in the simulated ischemic control was increased compared to the normoxic control (25.8±0.9 versus 5.2±0.5 $p<0.05$). State 2 respiration values for the IPC group and the TNFα group were restored to normoxic values (5.4±0.4 and 6.6±1.4 respectively versus normoxic control 5.2±0.5). (*Figure 4-4*).

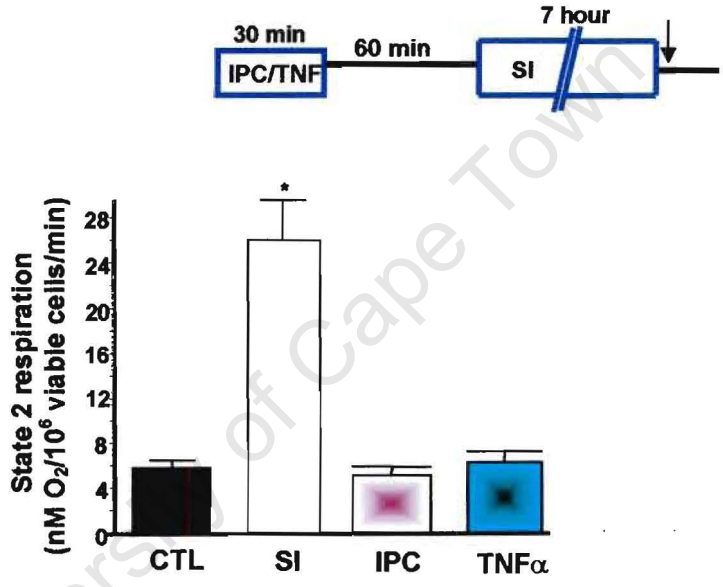


Figure 4-4: State 2 respiration in C2C12 myotubes immediately after the ischemic insult. *State 2 respiration of the simulated ischemic group (SI) is markedly increased versus control. Both ischemic preconditioning (IPC) and TNFα (0.5ng/ml) restore State 2 respiration to normoxic values. State 2 respiration was expressed as nM of oxygen/million viable cells/min.*

**$p<0.05$ versus control, $n \geq 8$.*

c) *Inner Mitochondrial Membrane Potential*

- *Pre-ischemic analysis*

The inner membrane potential was evaluated both immediately prior to or immediately post 7 hours simulated ischemic insult.

Both the IPC group and TNFα-treated myotubes that were examined prior to the SI demonstrated a modest depolarisation of the membrane potential compared to the normoxic control, 199±2 arbitrary units (A.U.) and 245±1 A.U. respectively, versus control 281±2 A.U., $p<0.05$. (*Figure 4-5)*

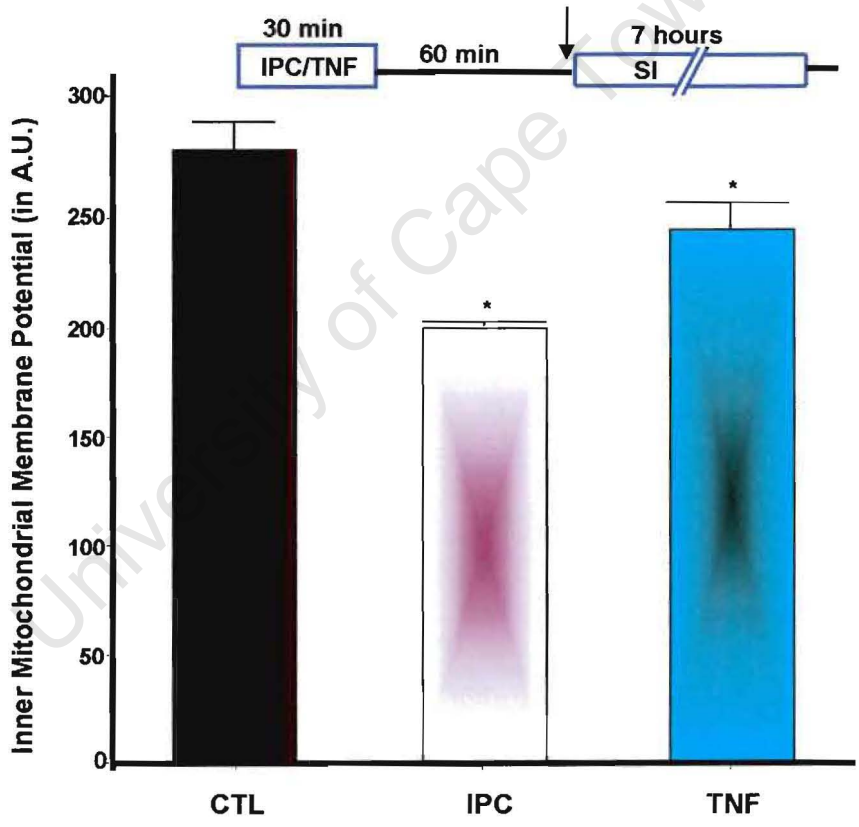


Figure 4-5: Membrane potential in C2C12 myotubes, using JC-1, prior to 7 hr SI. *The IPC group shows a pronounced depolarisation immediately prior to the 7hr simulated ischemic insult (SI). The TNFα-treated group (0.5ng/ml) demonstrated a modest depolarisation of the membrane. Membrane potential was measured in arbitrary units.*
**p<0.05 versus control, n = 8.*

- *Post- ischemic analysis*

After 7 hours of simulated ischemia, cells were depolarised versus the control group (174±7 A.U. and 232±6 A.U. respectively, $p<0.05$). Both IPC and TNFα restored the membrane potential to control values. (*Figure 4-6*)

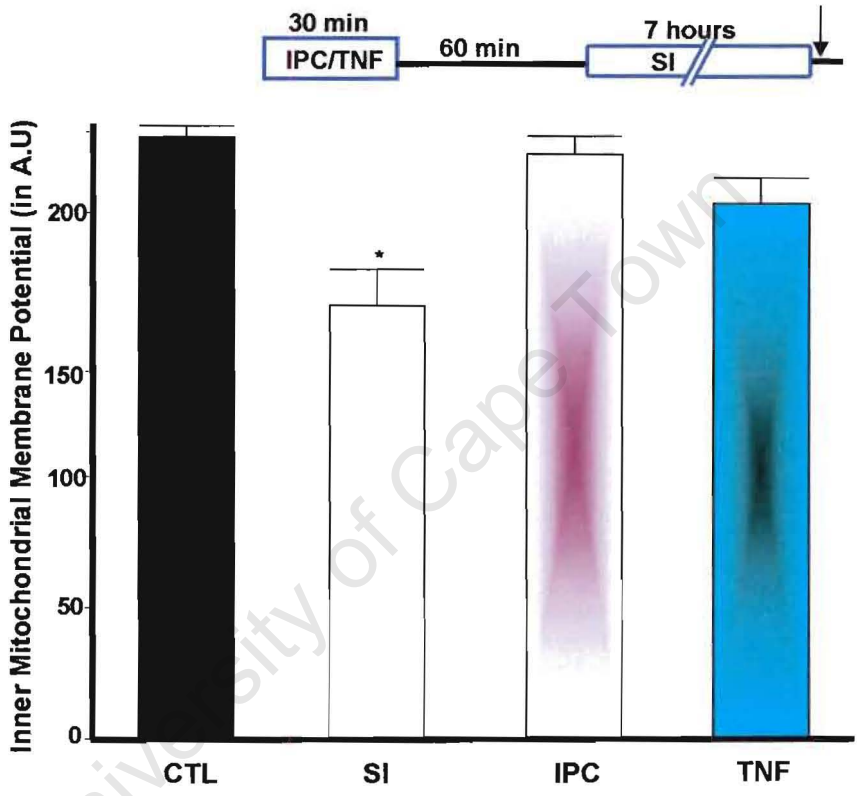


Figure 4-6: Membrane potential of C_2C_{12} myotubes, using JC-1 after 7hour simulated ischemia. *The C_2C_{12} myotubes which were subjected to 7hr simulated ischemia (SI) showed a robust depolarisation of their inner mitochondrial membrane. IPC and TNFα restored the membrane potential to normoxic values. Membrane potential was measured in arbitrary units (A.U.).*
**$p<0.05$ versus control, $n = 8$.*

1.2 TNFα-induced cytoprotection is abolished with ROS scavengers

a) Cell viability

Two ROS scavengers, NAC and MPG were added to the preconditioning protocol 15 minutes prior to IPC or addition of TNFα.

When used on their own, as preconditioning mimetics, both NAC and MPG had no protective effect. The viability for the normoxic, simulated ischemic, IPC and TNFα groups was similar to the values previously reported in Figure 4-1 and Figure 4-2. Addition of NAC to the preconditioning protocol reduced the viability from 85±1% to 55±1% for IPC and from 89±2% to 56±1% for TNFα, $p<0.001$. Addition of MPG reduced the viability to 27±7% for IPC and 15±1% for TNFα, $p<0.001$. (*Figure 4-7*)

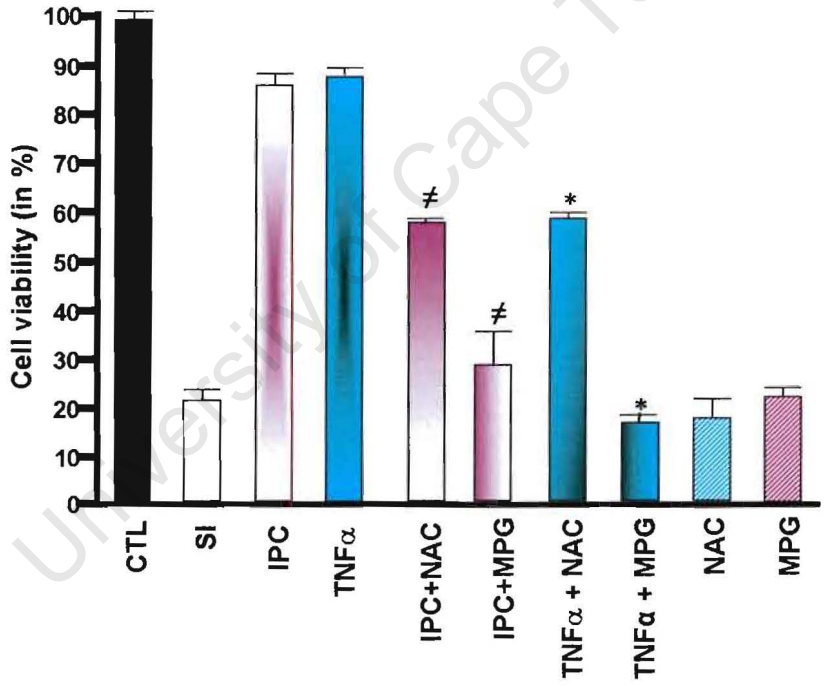


Figure 4-7: Effect of ROS scavengers on the cytoprotective effect of IPC and TNFα (Propidium Iodide technique). *Addition of N-acetyl-L-cysteine (NAC; 30μM) or N-2-mercaptopropionyl-glycine (MPG; 10μM) during the preconditioning stimulus reduced the viability of IPC and TNFα (0.5ng/ml) groups. Cell viability was expressed in percentage using Propidium Iodide technique.*
*≠p<0.001 versus IPC; *p<0.001 versus TNFα, n = 6.*

Similar results were obtained using Trypan blue technique. Addition of NAC reduced the viability from 87±2% to 55±1% for IPC and from 79±1% to 56±0.8% for TNFα, p<0.001. Addition of MPG reduced the viability to 41.5±0.4% for IPC and 18.1±0.6% for TNFα, p<0.001. (*Figure 4-8*)

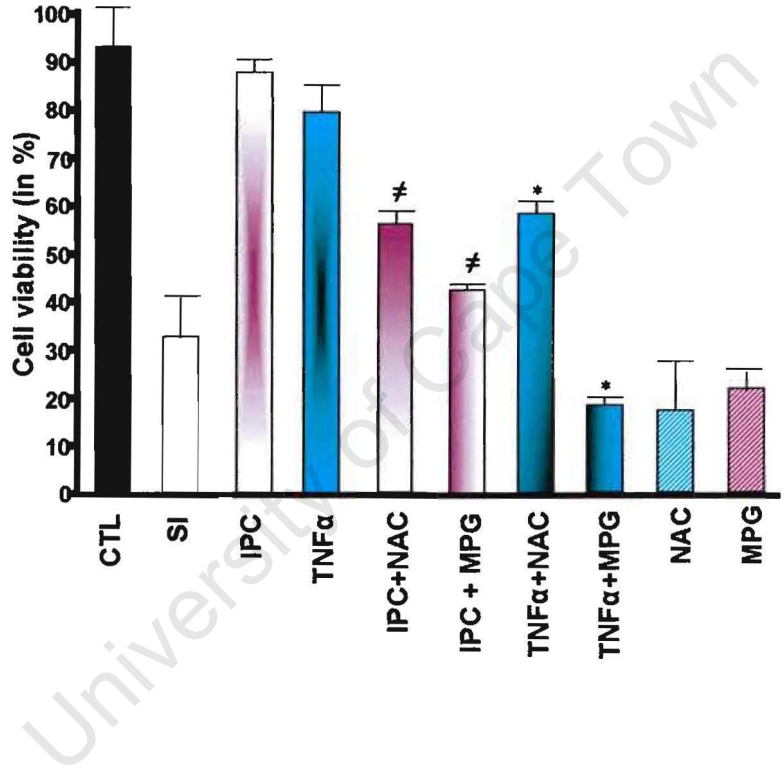


Figure 4-8: Effect of ROS scavengers on the cytoprotective effect of TNFα and IPC (Trypan Blue exclusion test). *Addition of N-acetyl-L-cysteine (NAC; 30μM) or 2-N-mercaptopropionyl-glycine (MPG; 10μM) during the preconditioning stimulus reduced the viability of IPC and TNFα (0.5ng/ml) groups. Cell viability was expressed in percentage using Trypan Blue exclusion technique.*
*≠p<0.001 versus IPC; *p<0.001 versus TNFα, n = 6.*

b) *Respiratory parameters*

- *Pre-ischemic analysis*

The presence of ROS scavengers abolished the increase in State 2 respiration previously observed with IPC or TNFα immediately prior to SI. Addition of NAC decreased the respiration from 15.5±0.6 to 5.7±0.3 for IPC and from 21.5±0.6 to 8.5±1 for TNFα, $p<0.01$. The second ROS scavenger, 2-SPBN decreased the respiration to 5.8±0.2 for IPC and 7.9±0.3 for TNFα, $p<0.01$. (*Figure 4-9*)

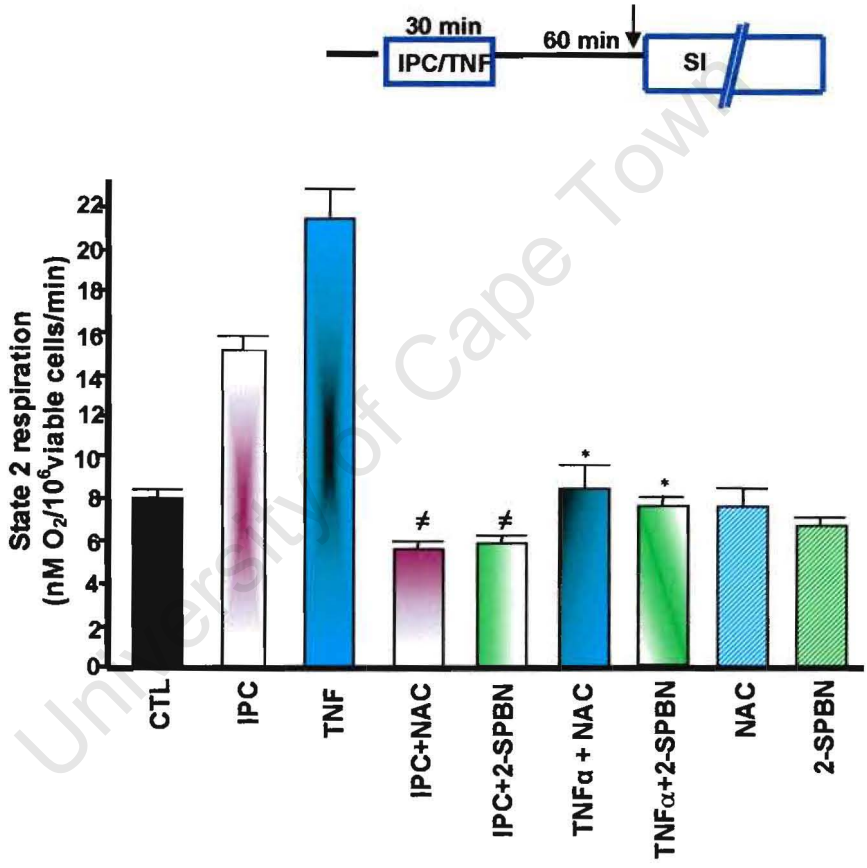


Figure 4-9: Effect of ROS scavengers on State 2 respiration in C_2C_{12} myotubes measured immediately before the ischemic insult. *The addition of NAC (30μM) or 2-SPBN (10μM) abolishes the increase in State 2 respiration induced by IPC or TNFα (0.5ng/ml). The ROS scavengers alone have no effect on State 2 respiration. State 2 respiration was expressed as nM of oxygen/million viable cells/min.*
*≠$p<0.01$ versus IPC; *$p<0.01$ versus TNFα, $n = 8$.*

- *Post-ischemic analysis*

Immediately after 7 hours SI, State 2 respiration in the simulated ischemic group was significantly raised to 25.8±0.95 versus the normoxic control of 4.8±0.5 $p<0.05$. State 2 respiration of both IPC and TNFα groups was restored to control group values. Addition of NAC reversed this effect from 4.5±0.6 to 9.1±0.9 for IPC, $p<0.05$ and 5.4±0.5 to 16.1±0.8, for TNFα, $p<0.05$. Addition of 2-SPBN reduced State 2 respiration to 12.2±0.8 for IPC and 17.3±0.9 for TNFα, $p<0.05$. (*Figure 4-10*)

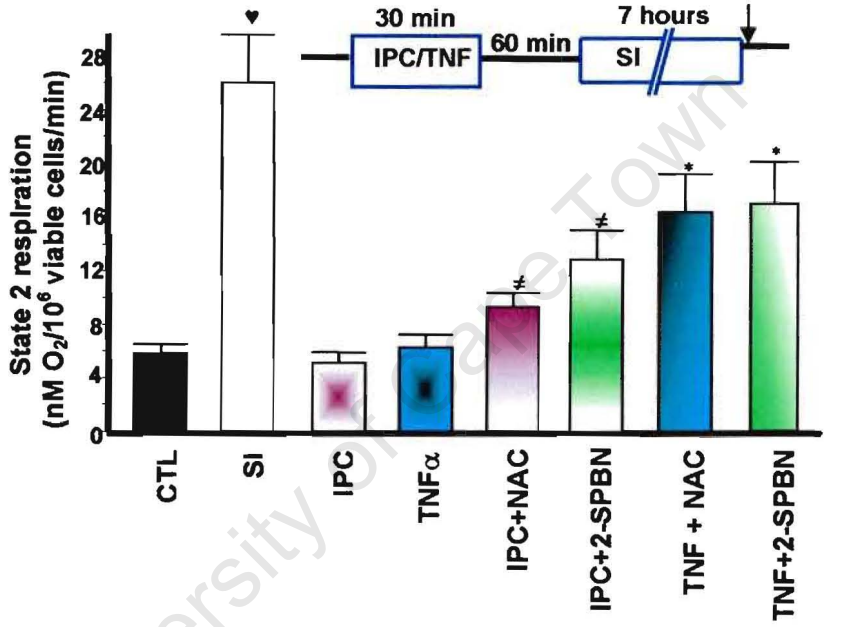


Figure 4-10: Effect of ROS scavengers on State 2 respiration in C_2C_{12} myotubes immediately after the ischemic insult. *State 2 respiration of the simulated ischemic group (SI) is markedly increased. Both ischemic preconditioning (IPC) and TNFα restore State 2 respiration to normoxic values. The addition of NAC (30μM) or 2-SPBN (10μM) significantly increases the respiration in the IPC and TNFα groups. NAC and 2-SPBN had no effect when administered on their own (not shown).*
State 2 respiration was expressed as nM of oxygen/million viable cells/min.

*≠$p<0.05$ versus IPC $n \geq 8$ *$p<0.05$ versus TNFα, ♥$p<0.001$ versus control, $n \geq 8$.*

1.3 Detection and quantification of ROS production

The detection and quantification of ROS was performed using the fluorometric probe 2'7'dichlorofluorescin diacetate (DCFH-DA) and a flow cytometer. An untreated control was run for each time point as endogenous ROS is present in cells. The results were normalised against the controls. A

short burst of ROS was observed lasting on average for 3 minutes after the addition of TNFα, a maximum increase of 45±1% being observed at 2 minutes. Addition of MPG decreased the amount of ROS generated in the untreated cells (15±1%). When MPG was added to the TNFα–treated cells, the ROS production was drastically decreased (60±1%) over the 3 minute period versus TNFα, $p<0.05$. (*Figure 4-11*)

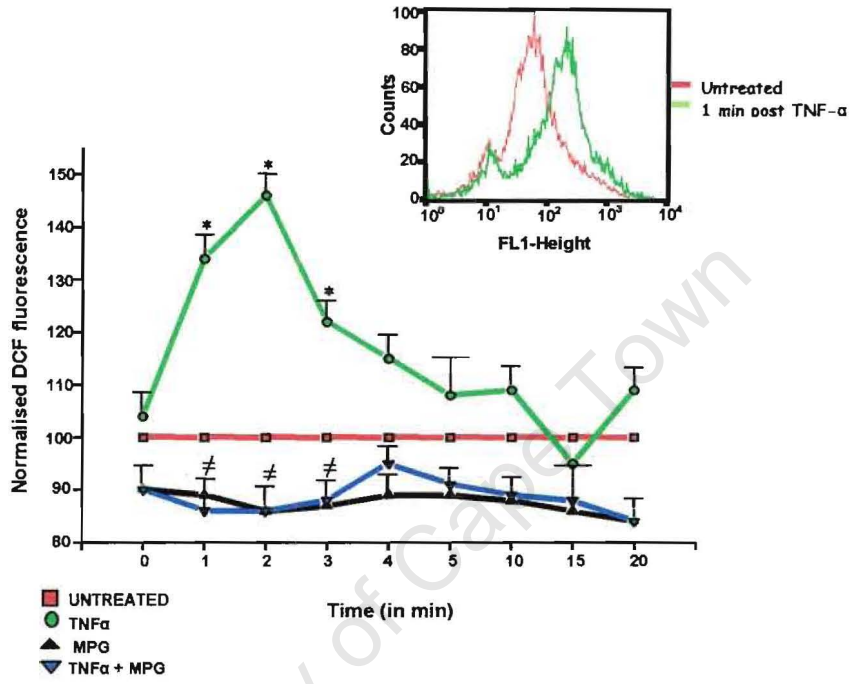


Figure 4-11: Effect of TNFα on DCF fluorescence in C_2C_{12} myotubes. *Cells were preincubated with DCF (5μM) for 15 min. Time 0 min corresponds to addition of TNFα (0.5ng/ml). There is a burst of ROS production 1 min after addition of TNFα which is lost by 4 min. Addition of MPG (10μM) abrogates the TNFα-induced ROS as well as endogenous ROS in untreated cells. Insert is a histogram showing the right shift in DCF signal one min following the addition of TNFα. DCF fluorescence was normalised to the untreated group.*
**p<0.01 versus untreated control, n = 9.*
≠p<0.05 versus TNFα, n = 6.

1.4 Source of ROS production after TNFα stimulation

A fluorescent imaging technique was used to determine the possible source of ROS production after TNFα stimulation. C_2C_{12} myotubes were co-stained with DCFH-DA (5μM) and Mitotracker Red CM-H_2XRos (100nM). TNFα (0.5ng/ml) is added and the increase in intensity of fluorescence within the mitochondria is indicative of TNFα-induced ROS production. (Figure 4-12)

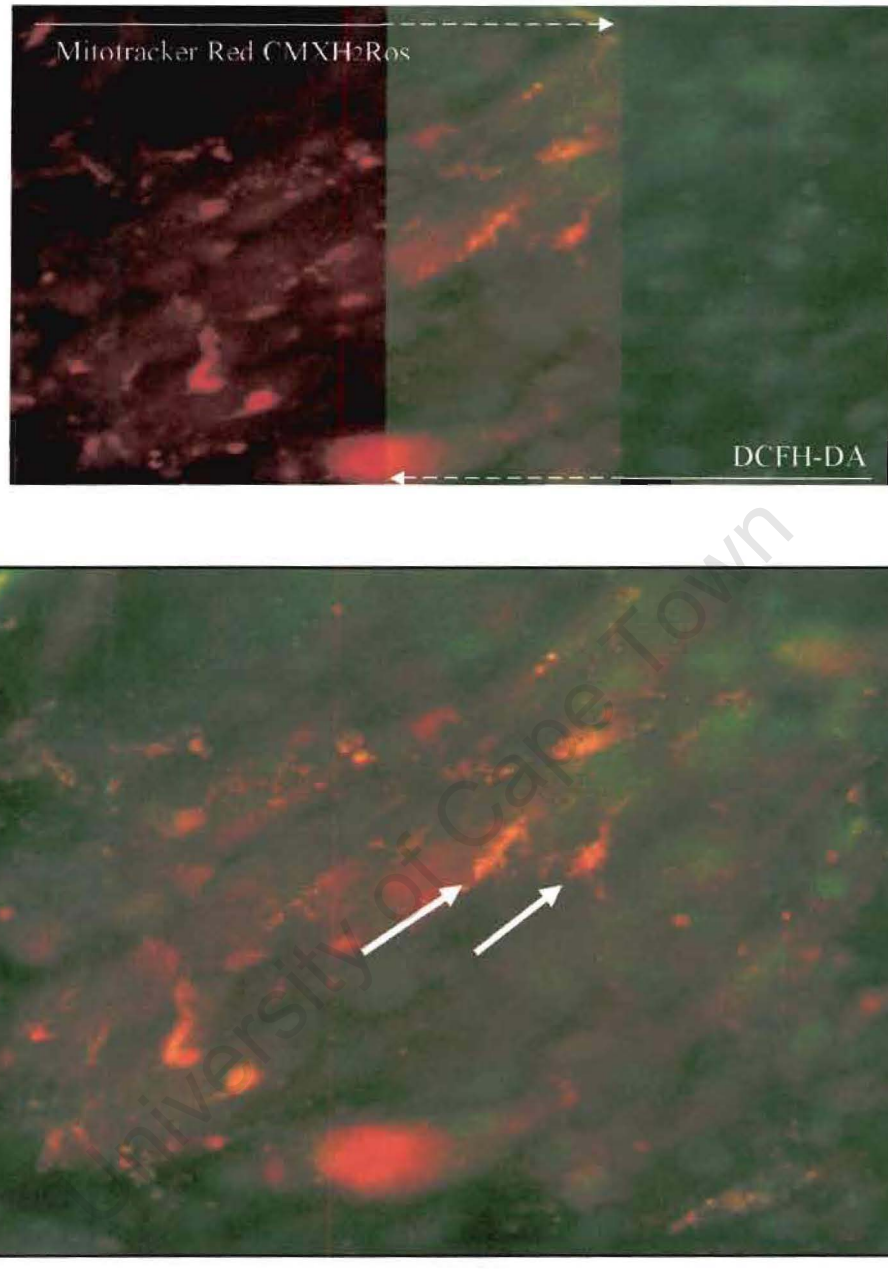


Figure 4-12: Mitochondria are a possible source of ROS production after TNFα stimulation of C_2C_{12} myotubes. *DCFH-DA (5μM) fluoresces in the presence of ROS (yellow) and Mitotracker-Red CM-H_2XRos (100nM) stains the mitochondria in red. When TNFα (0.5ng/ml) is added and the images merged, the resulting orange depicts the TNFα-induced ROS production, most of which appears to be in the mitochondria (arrows).*

2. Exogenous TNFα exerts a direct effect on isolated mouse heart mitochondria: role of ROS

2.1 Direct effect of TNFα on bioenergetic functions of isolated mouse heart mitochondria

a) *Mitochondrial respiration*

TNFα (0.5ng/ml) added directly to isolated mouse heart mitochondria decreases State 3 respiration significantly from 1.44±0.33 (control) to 0.84±0.15 (TNFα), $p<0.05$ (*Figure 4-13*).

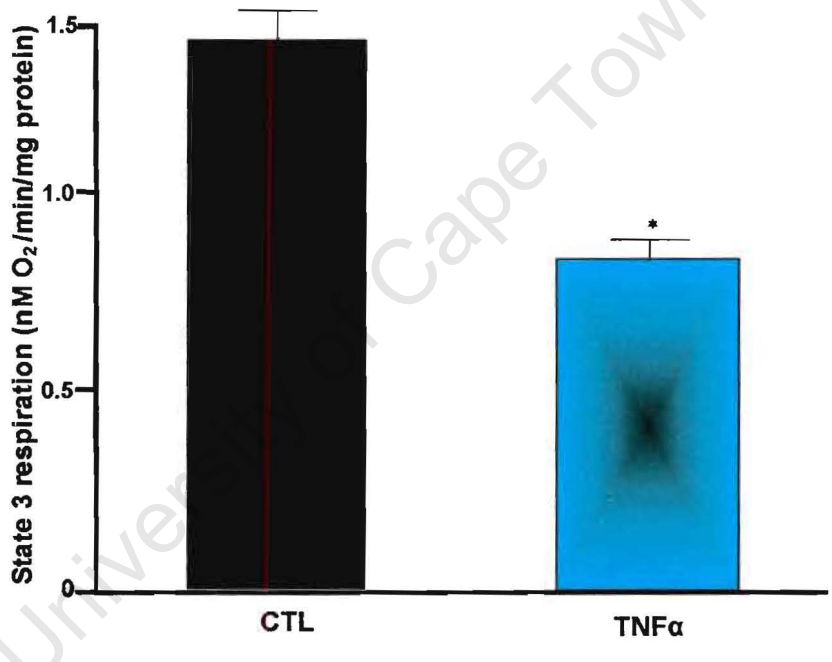


Figure 4-13: Effect of TNFα on State 3 respiration in isolated mouse heart mitochondria. *Addition of TNFα (0.5ng/ml) directly to isolated mouse heart mitochondria significantly decreases State 3 respiration. Respiration was expressed in nM of oxygen/minute/mg protein.*
**p<0.05 versus control, n = 9.*

The respiratory control index (RCI) was also significantly reduced from 7.6±1.1 (control) to 4.1±0.5 (TNFα) respectively, $p<0.05$. (*Figure 4-14*)

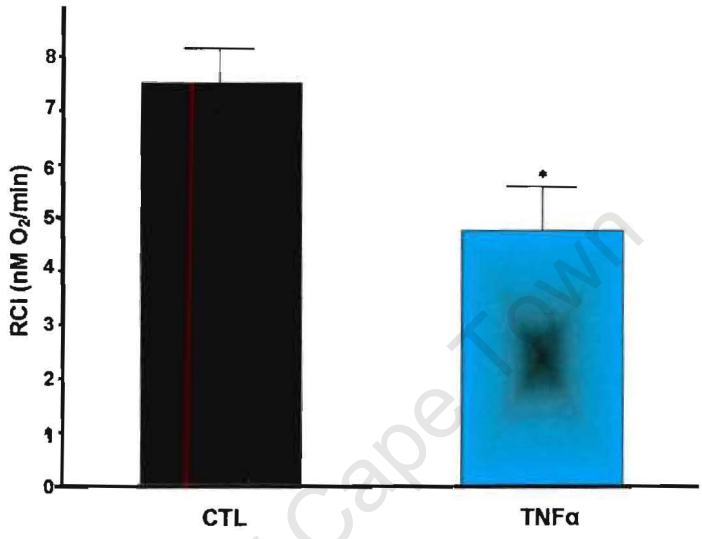


Figure 4-14: Effect of TNFα on the RCI of the isolated mouse heart mitochondria. *The RCI (State 3/State 4 respiration), showed a significant decrease when TNFα (0.5ng/ml) is added to the isolated mouse heart mitochondria. RCI was expressed as nM of oxygen/min. *p<0.01 versus control, n = 9.*

b) Mitochondrial proton leak

Addition of TNFα to isolated heart mitochondria increased the proton leak from 17±1% (control) to 25±2% (TNFα), p<0.01. (*Figure 4-15*)

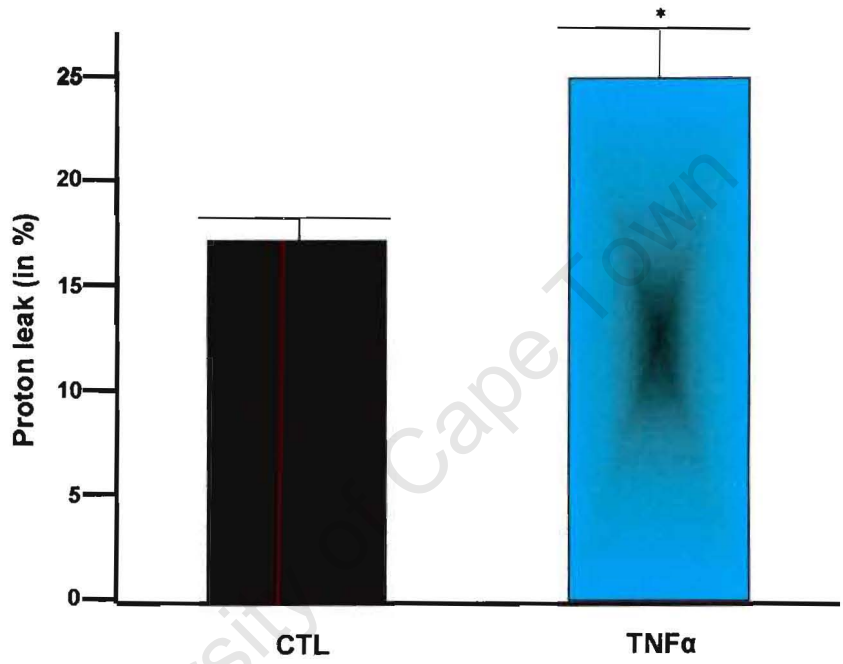


Figure 4-15: Effect of TNFα on Proton Leak in isolated mouse heart mitochondria. *Oligomycin (1µg/ml) was added to the isolated mouse heart mitochondria during State 3 respiration. The resulting oligomycin-insensitive respiration was used to calculate the proton leak. Mitochondria to which TNFα (0.5ng/ml) was added showed an increase in proton leak. Proton leak was expressed as a percentage.*
**p<0.01 versus control, n = 9.*

c) *Inner Mitochondrial Membrane Potential*

Isolated heart mitochondria treated with TNFα (0.5ng/ml) exhibited a modest degree of depolarisation, 79.5±3.1% (TNFα) compared with the control group, $p<0.05$. Results have been normalised against the control. (*Figure 4-16*)

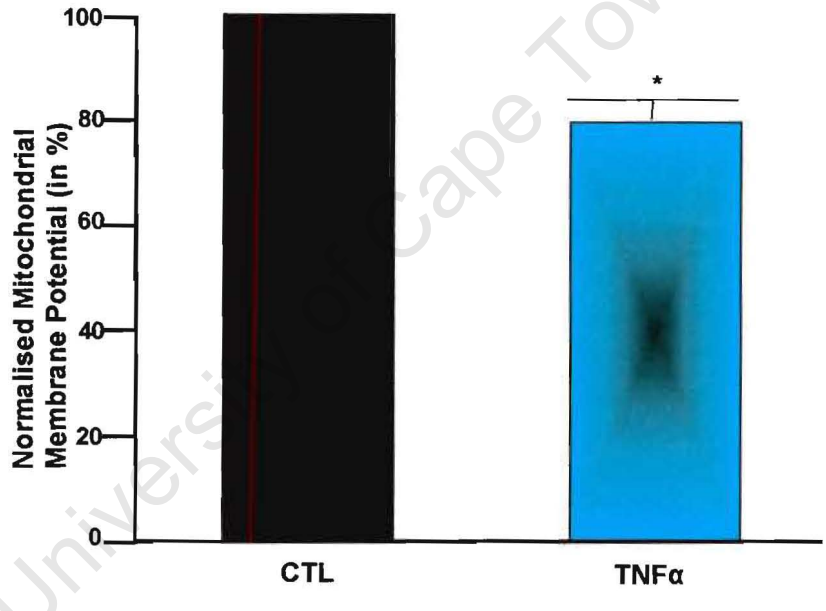


Figure 4-16: Effect of exogenous TNFα on the mitochondrial inner membrane potential. *The isolated mouse heart mitochondria were incubated with JC-1 (5μM) for 5 minutes at 37°C before analysis on the flow cytometer. The TNFα-treated mitochondria exhibited a modest degree of depolarisation as reflected by the aggregate (red) signal of JC-1. The results have been normalised and expressed as a percentage.*
**p<0.05 versus control, n = 10.*

d) *Mitochondrial ATP synthesis*

The rate of ATP synthesis was decreased from 0.53±0.06 (control) to 0.23±0.07 (TNFα) with addition of TNFα (0.5ng/ml) to isolated heart mitochondria, $p<0.05$ (*Figure 4-17*).

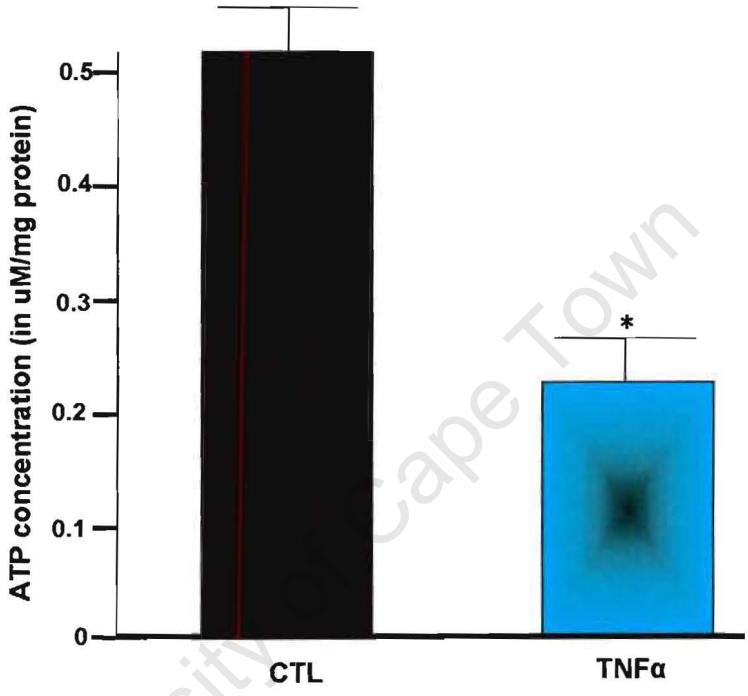


Figure 4-17: Effect of exogenous TNFα on ATP synthesis in isolated mouse heart mitochondria. *The addition of TNFα (0.5ng/ml) to isolated heart mitochondria decreased the rate of ATP synthesis. The concentration of ATP was expressed in μM/mg protein. *p<0.05 versus control, n=6.*

2.2 ROS scavengers abrogate the direct effect of TNFα in isolated mouse heart mitochondria

a) *Mitochondrial Respiration*

As described previously (Figure 3-14), treatment with TNFα decreases the RCI. RCI of control mitochondria was 4.4±0.2. Addition of ROS scavenger, NAC (30μM) to isolated heart mitochondria treated with TNFα (0.5ng/ml) abolished the effect of TNFα on the RCI from 1.8±0.2 for TNFα to 4.4±0.2 for TNFα+NAC, $p<0.05$. NAC alone had no effect. (*Figure 4-18*)

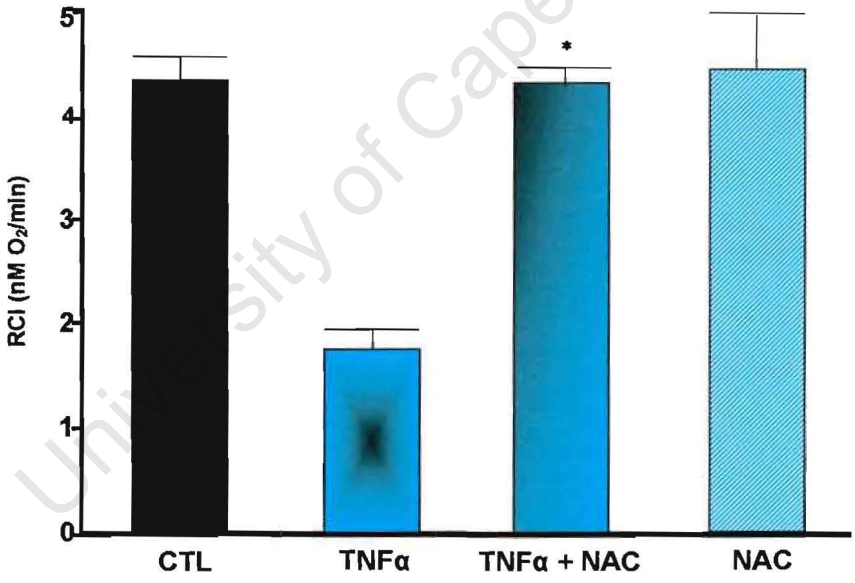


Figure 4-18: Effect of NAC on the RCI of TNFα-treated isolated mouse heart mitochondria. *The addition of TNFα (0.5ng/ml) in the presence of NAC (30μM) to the isolated mouse heart mitochondria abrogates the effect of TNFα on the respiratory control index (RCI). RCI was expressed as nM of oxygen/min.*
**p<0.01 versus TNFα, n = 6.*

Similarly, addition of a second ROS scavenger 2-SPBN, to isolated heart mitochondria treated with TNFα (0.5ng/ml) abrogates the effect of TNFα on the RCI (1.9±0.5 for TNFα and 3.9±0.6 for TNFα+2-SPBN, $p<0.05$). RCI of control mitochondria was 3.9±0.5. 2-SPBN alone had no effect. (*Figure 4-19*)

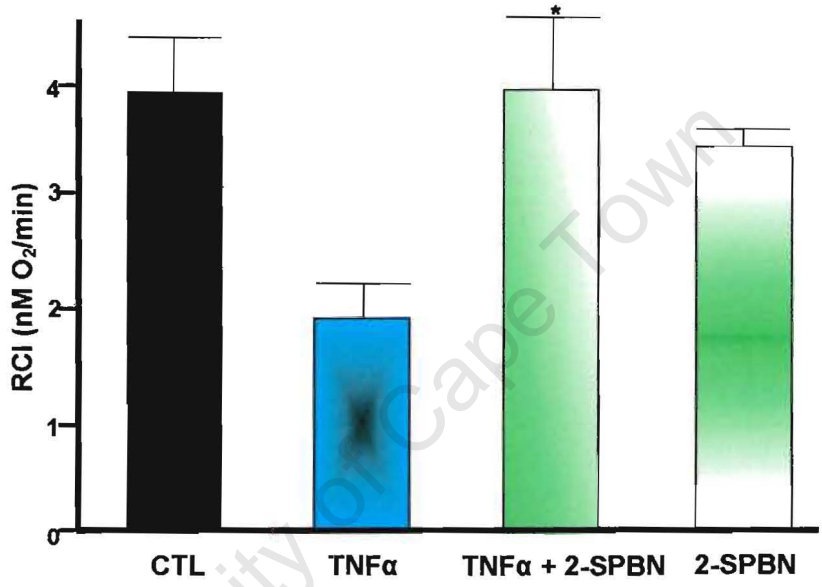


Figure 4-19: Effect of 2-SPBN on the respiratory control index of TNFα-treated mouse mitochondria. *The addition of 2-SPBN (10μM) in the presence of TNFα (0.5ng/ml) to the isolated mouse heart mitochondria abrogates the effect of TNFα on the respiratory control index (RCI). RCI was expressed as nM of oxygen/min.*
**p<0.05 versus TNFα, n = 6.*

b) *Mitochondrial Proton Leak*

ROS scavengers 2-SPBN or NAC were added to the buffer 1 minute prior to the addition of isolated heart mitochondria. TNFα (0.5ng/ml) was added after a further minute. ROS scavengers abolished the effect of TNFα (36.6±4.1% for TNFα and 16.2±0.4% for TNFα+2-SPBN, $p<0.01$). Control mitochondria had a proton leak of 15.7±0.6%. Similar results were obtained with NAC (not shown). 2-SPBN alone had no effect on isolated mitochondria (*Figure 4-20*).

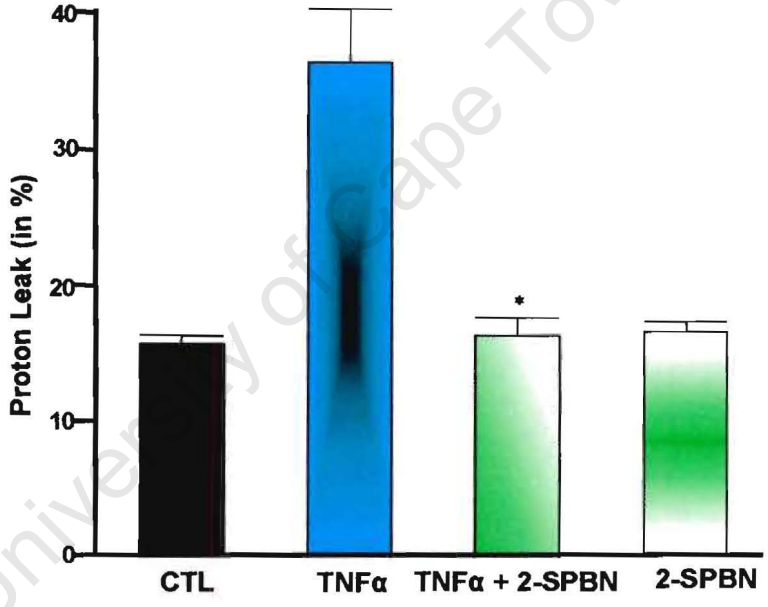


Figure 4-20: Effect of ROS scavengers on TNFα-induced increase in Proton Leak in isolated mouse heart mitochondria. *The isolated heart mitochondria were incubated with TNFα (0.5ng/ml) in the presence of 2-SPBN (10μM) or NAC (30μM). Oligomycin (1ug/ml) was added once State 3 respiration was achieved and the resulting oligomycin-insensitive respiration used to calculate the proton leak. The ROS scavengers abrogated the effect of TNFα on proton leak. Proton leak was expressed as a percentage.*
**p<0.001 versus TNFα, n = 6.*

2.3 Sphingolipid inhibitors abrogate the direct effect of TNFα on isolated mouse heart mitochondria

a) Mitochondrial Respiration

The mitochondria were treated with/without TNFα in the presence of imipramine, a sphingolipid antagonist. Imipramine alone had no effect on isolated mitochondria. Addition of imipramine to TNFα-treated mitochondria abolished the effect of TNFα on the RCI (1.4±0.1 for TNFα and 3.8±0.2 for TNFα+imipramine, $p<0.01$). Control mitochondria had an RCI of 4.2±0.3 (*Figure 4-21*).

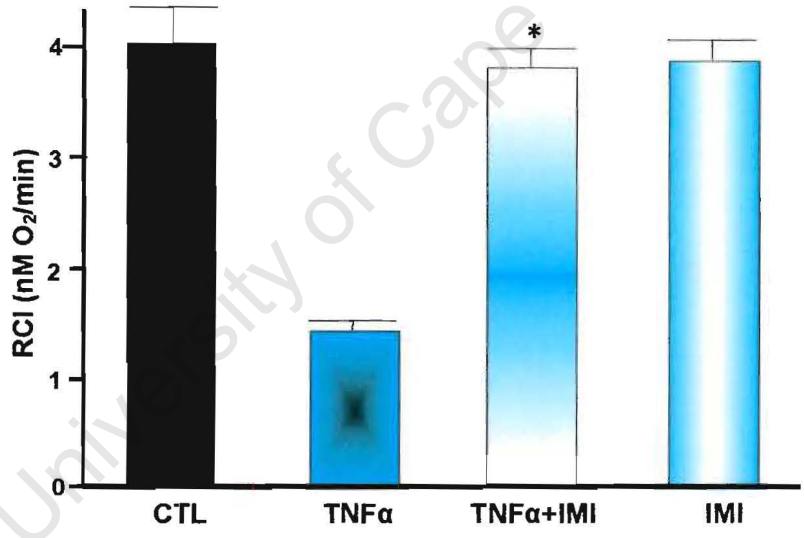


Figure 4-21: Effect of imipramine on the RCI of TNFα-treated isolated mouse heart mitochondria. *The addition of TNFα (0.5ng/ml) in the presence of imipramine (IMI) (10μM) to the isolated mouse heart mitochondria abrogates the effect of TNFα (0.5ng/ml) on the respiratory control index (RCI). RCI was expressed as nM of oxygen/min.*
**p<0.01 versus TNFα, n = 6.*

Similar results were obtained with a second sphingolipid inhibitor, NOE. NOE alone had no effect but the effect of TNFα on the RCI was abrogated (Control 4.1 ±1.7, TNFα 1.0±0.5, TNFα +NOE 3.9±1.7, p<0.01). (*Figure 4-22*)

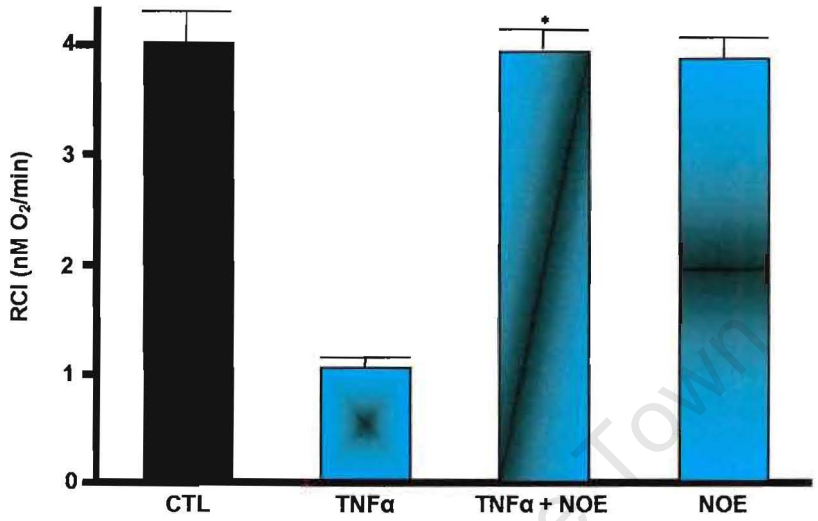


Figure 4-22: Effect of NOE on the RCI of TNFα-treated isolated mouse heart mitochondria. *The addition of N-oleoethanolamine (NOE) (5μM) in the presence of TNFα (0.5ng/ml) to the isolated mouse heart mitochondria abrogates the effect of TNFα on the respiratory control index (RCI). RCI was expressed as nM of oxygen/min.*
**p<0.05 versus TNFα, n = 6.*

b) *Mitochondrial Proton Leak*

Control mitochondria had a proton leak of 14.6±0.9%. Sphingolipid inhibitor, NOE or imipramine was added immediately prior to the addition of TNFα (0.5ng/ml) to isolated heart mitochondria. Addition of NOE had no effect on control mitochondria, but abolished the effect TNFα on the proton leak, reducing it from 50.4±5% for TNFα to 16.6±0.7% for TNFα+NOE, $p<0.01$. Similar results were obtained with imipramine (not shown). (*Figure 4-23*)

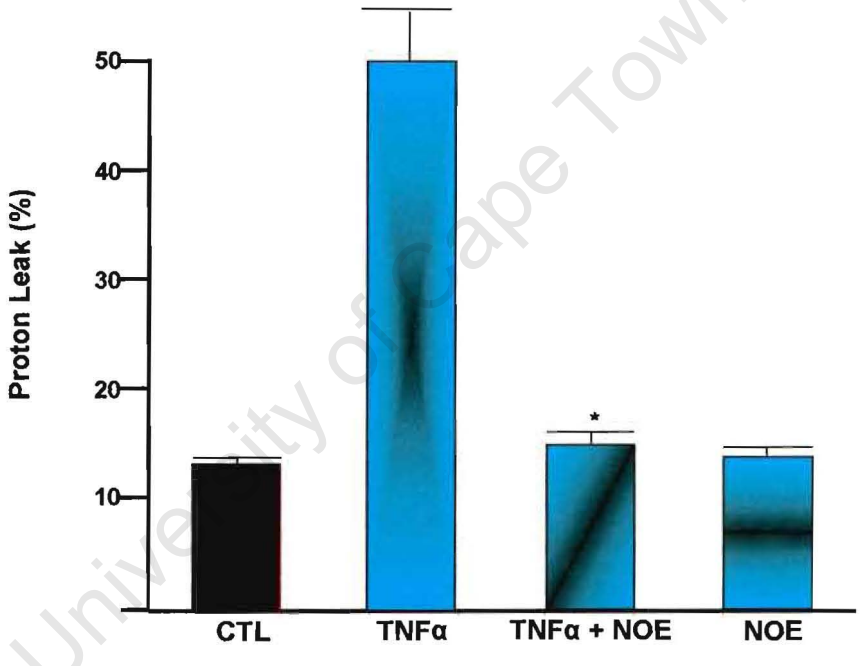


Figure 4-23: Effect of Sphingolipid inhibitors on TNFα-induced increase in Proton Leak in isolated mouse heart mitochondria. *The isolated heart mitochondria were incubated with TNFα (0.5ng/ml) in the presence of NOE (5μM) or imipramine (10μM). Oligomycin (1ug/ml) was added once State 3 respiration was achieved and the resulting oligomycin-insensitive respiration used to calculate the proton leak. The sphingolipid inhibitors abrogated the effect of TNFα on proton leak. Proton leak was expressed as a percentage.*

**p<0.001 versus TNFα, n = 6.*

E. DISCUSSION

The results of this study demonstrate in C_2C_{12} myotubes that the protection conferred by TNFα during a simulated ischemia/reperfusion protocol is mediated by the production of ROS which most likely originate in the mitochondria. The data obtained from isolated mouse heart mitochondria confirm the previous study in isolated rat liver mitochondria by Busquets et al[69] in which it was demonstrated that TNFα had a direct effect on the bioenergetics of the mitochondria independently of its binding to the TNFα cell surface receptor. In addition, the present study shows, for the first time to our knowledge, that this direct mitochondrial action of TNFα is mediated by activation of intra-mitochondrial ROS and stimulation of the sphingolipid pathway.

TNFα confers cytoprotection

Using an isolated rat heart model, Lecour et al[46] previously reported that TNFα can mimic IPC in a dose and time response manner and that 0.5ng/ml appears to be the best dose to confer cardioprotection. Similarly, Meiring[126] found that the same dose was required in C_2C_{12} myotubes to obtain the best cytoprotective effect following a simulated ischemic insult. Data obtained from the C_2C_{12} myotubes in the present study confirm that 0.5ng/ml of TNFα confers a dramatic cytoprotective effect improving the cell viability by 60%.

To evaluate cell viability in C_2C_{12} myotubes both propidium iodide (PI) and trypan blue exclusion techniques were used. The results obtained with PI analysis in the ischemic preconditioning protocol conformed to those described previously by Minners et al.[32] Data with the trypan blue technique did not differ from PI showing that both methods are validated to measure cell viability.

Previous evidence presented by Minners et al[32] in ischemic or pharmacologically preconditioned cells showed that the inner mitochondrial membrane of cells was depolarised and oxygen consumption was increased prior to the simulated ischemic insult. The data presented in this thesis conform to that of Minners et al[32] for IPC and in addition, show that membrane potential and cellular respiration were restored to normal levels immediately ***post*** simulated ischemia, in both IPC and TNFα groups.

TNFα-induced cytoprotection is mediated by ROS

The role of ROS in mediating preconditioning is becoming increasingly apparent.[70] It has been reported that low doses of ROS can mimic ischemic preconditioning[123] and that the ROS scavenger, MPG, can abrogate a single episode of preconditioning.[94] ROS may protect via activation of different kinases such as protein kinase C,[94] or by acting on the mitochondrial permeability transition pore.[124] Using an isolated rat heart model, Lecour et al[125] showed, that perfusion of TNFα (0.5ng/ml) was associated with an increase of ROS and that the cardioprotective effect of TNFα was blocked in the presence of MPG. This provides firm evidence for the production and a role of ROS in TNFα-induced cardioprotection. The source of ROS and their mechanism of action, however, remained to be delineated.

The data obtained from C_2C_{12} myotubes in the present study, confirmed the production and role of ROS in TNFα-induced cytoprotection as addition of ROS scavengers abolished this cytoprotective effect. In addition, after TNFα stimulation of the cells, an increase in ROS production was seen. This provided firm evidence that it was the reactive oxygen species generated by TNFα which conferred the protection seen in the simulated ischemia/reperfusion protocol.

TNFα generates ROS within the mitochondria

Mitochondria have been identified as a major source of ROS production.[127] Vanden Hoek et al [70] used chick cardiomyocytes to show that ROS originate from the mitochondria during a hypoxic insult. This experiment demonstrated that ROS were generated during the hypoxic preconditioning rather than during the reoxygenation period. Using the same model of chick cardiomyocytes, Vanden Hoek et al[70] showed that mitochondrial ROS can activate signalling cascades involved in IPC. Goossens et al[107] provided firm evidence on the induction of ROS formation in mitochondria upon TNFα stimulation in L929 cells. In a study by Corda et al [110] it was demonstrated that TNFα induced a rapid increase in mitochondrial ROS production in human endothelial cells.

By co-staining with Mitotracker Red CM-H_2XRos and DCFH-DA and using a fluorescent imaging technique, we have shown that the ROS generated by the protective dose of TNFα in C_2C_{12} myotubes most likely originated within the mitochondria.

Although all previous papers have shown a deleterious effect of ROS produced in the mitochondria following a TNFα stimulation,[107,109,130] we have demonstrated that ROS has a protective effect when TNFα was used at a low concentration.

TNFα has a direct effect on the bioenergetic functions of the heart mitochondria

A mitochondrial binding protein as well as an intracellular trafficking pathway for TNFα has recently been discovered.[40,69] Busquets et al[69] showed that the addition of a low concentration of TNFα to isolated mouse liver mitochondria decreased the respiratory control index (RCI) of the mitochondria suggesting that TNFα acted directly on the mitochondria.

Isolated mouse heart mitochondria were used in the present study as a model to determine whether TNFα could exert a direct effect on heart mitochondria, independently of its binding to the TNFα cell-surface receptors.

One limitation of this study is the question of mitochondrial purity. Various techniques such as LDH release into the cytosol[69] or Percoll gradient centrifugation[109] have been used to determine mitochondrial purity. However, these were done in liver mitochondria where a reasonable amount of mitochondria were obtained. In contrast, minute quantities of mitochondria are obtained from a mouse heart. Percoll centrifugation is therefore impossible. Likewise the amount of LDH release into the cytosol would give erroneous results firstly, because of the scarcity of mitochondria and secondly, as a result of relatively low centrifugation speeds, the cytosolic fraction could be contaminated by the mitochondrial fraction. High speed centrifugation is also detrimental for bioenergetic studies as although "pure" mitochondria are obtained, they are no longer actively respiring and the bioenergetics are therefore greatly compromised, with the RCI dropping to below 1 (RCI ≥ 3 is accepted as a viable preparation). A technique using two

different centrifugal speeds was therefore chosen. The first centrifugation at a low speed (2930 rpm) is sufficient to pellet the larger intracellular constituents without damaging the mitochondria. The supernatant is carefully collected and the mitochondria are pelleted by centrifuging at 4500 rpm. The cytosolic fractions, having a much lower mass than the mitochondria, are not pelleted but remain in this second supernatant and are discarded, leaving the mitochondrial pellet. A relatively "pure" mitochondrial preparation without compromising the bioenergetics of the mitochondria is obtained with this technique.

Data obtained from the present study, confirm the decrease in State 3 respiration and RCI, as shown in the liver mitochondria by Busquets et al.[69] In addition, it was shown for the first time in isolated heart mitochondria, that membrane potential and rate of ATP synthesis were decreased when the mitochondria were stimulated with a low dose of TNFα. These mitochondria exhibited what appeared to be a less efficient energetic state and may have been "preconditioned" to withstand a more severe insult. However, when ROS scavengers, NAC and 2-SPBN were added prior to the TNFα stimulus these effects were abolished. This confirmed that TNFα had a direct effect on the bioenergetics of isolated heart mitochondria and provided strong evidence that this effect was mediated via mitochondrial ROS. Interestingly, sphingolipid inhibitors, imipramine and NOE also abrogated the effect of TNFα on isolated heart mitochondria, suggesting the involvement of the sphingolipid pathway. It is known that there is a mitochondrial pool of ceramide[128].TNFα has been shown to increase the intra-mitochondrial levels of ceramide through the activation of at least two pathways: one is mediated by sphingomyelinase and the other is dependent on ceramide synthesis.[129] Because ceramide is known to generate ROS,[109,110] it is appropriate to stipulate that TNFα activates ROS generation via the ceramide pathway in the model of isolated heart mitochondria used in this study.

Conclusion

TNFα is a naturally occurring cytokine produced by a wide variety of cell types. Depending on concentration and time conditions, TNFα can have both deleterious and beneficial effects. Many authors have shown the deleterious

effects of TNFα, such as the elevated circulating levels of TNFα in severe chronic heart failure and lethal myocarditis in mice with cardiac-specific overexpression of TNFα.[42-45] In contrast, Lecour et al[46] have shown that low doses of TNFα can be beneficial in isolated rat heart and other research has shown similar benefits.[50] Similarly, the beneficial or deleterious effects of reactive oxygen species appear to be concentration and time-dependent, with many authors showing that large amounts of ROS are present during ischemic/reperfusion injury.[87-89] Conversely, the presence of small amounts of ROS have beneficial effects.[71,81]

The data obtained from C_2C_{12} myotubes show that ROS generated by TNFα in a low dose is cytoprotective during ischemia/reperfusion. This study also demonstrated that TNFα-induced ROS originated mostly within the mitochondria of the myotubes. Data obtained from the isolated heart mitochondria confirm that exogenous TNFα has a direct effect on mitochondria. In addition, it has been shown for the first time that the direct effect of TNFα on the mitochondrial bioenergetics is abolished by ROS scavengers and sphingolipid inhibitors, providing a link between intra-mitochondrial ROS generation and the activation of sphingolipids. However, the link between cytoprotection in ischemia/reperfusion and the direct mitochondrial action of TNFα has yet to be established.

The changes seen in mitochondrial bioenergetics after direct stimulation with TNFα reflect mitochondrial function immediately prior to the index ischemia/reperfusion insult. The results obtained from isolated heart mitochondria demonstrate for the first time, that the TNFα-treated mitochondria exhibit features consistent with respiratory uncoupling, as shown by the depolarisation of the inner mitochondrial membrane potential and the decrease in rate of ATP synthesis. Thus, these mitochondria appear to have a less efficient bioenergetic state than those that have not been "preconditioned" with TNFα, suggesting that uncoupling of oxidation from phosphorylation by preconditioning would make the mitochondria more resilient to a lethal insult. Together, the data from the cells and the isolated heart mitochondria strongly suggest that treatment with a low concentration of TNFα improves the tolerance of mitochondria to subsequent lethal ischemia. We speculate that this could be mediated by the production of

intra-mitochondrial ROS and subsequent activation of intra-mitochondrial sphingolipids.

Future direction

The exact effect of uncoupling oxidative phosphorylation in preconditioning has not yet been established. The generation of ROS by the mitochondria as signalling intermediates in the preconditioning program has been postulated by Vanden Hoek et al,[70] Forbes et al,[71] and Yao et al.[135,136] In contrast, Carmody et al[137] and Halmosi et al[138] showed that a more robust generation of ROS by mitochondria could have detrimental effects on cellular survival and integrity. Previous data have shown that there may be a potential role for enhanced mitochondrial uncoupling as a regulatory process which may limit excessive ROS generation in the context of ischemia.[140-142] The data presented in this study demonstrate that the intra-mitochondrial ROS generated by the direct action of a low dose of TNFα on the mitochondria stimulates the ceramide pathway. It is possible to speculate that this may be a "pro-survival" pathway which can confer cytoprotection during ischemia/reperfusion. However, the data on the isolated heart mitochondria were obtained under normoxic conditions. Future research plans include the establishment of a mitochondrial model of "ischemia/reperfusion" to explore whether the stimulation of ROS production and activation of sphingolipids in the mitochondria by TNFα, independently of its binding to TNFα cell surface receptors, can have a protective effect during ischemia/reperfusion. In addition, the establishment of this model will pave the way for the investigation of other preconditioning triggers and their signalling pathways during ischemia/reperfusion.

In conclusion, a new approach to the study of the effects of TNFα on the heart may be initiated from the present work. New strategies and targets may be identified to enable the treatment of cardiac disease in the future by understanding the mechanisms involved in the direct signalling pathway within the mitochondria.

F. PUBLICATIONS

Publications and Abstracts

Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DM, Sack MN. Ischemic and pharmacological preconditioning in Girardi cells and C_2C_{12} myotubes induce mitochondrial uncoupling. Circ. Res. 2001; 89: 787-92

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Lacerda L, Yellon DM, Smith RM. Role for Reactive Oxygen Species in TNFα-induced myocyte protection. J. Mol Cell Biol. 2004; 37: 246 abstract no. B114

Lacerda L, McCarthy J, Opie LH, Lecour S. TNFα uncouples respiration in isolated heart mitochondria. J.Mol Cell Biol. 2005; 38: 1040 abstract no. 116

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