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# **The Role of Sublethal Stress on Mitochondria and the Development of Cardiac Preconditioning**

Submitted by Jan O. Minners, MD  
for the degree of Doctor of Philosophy (Medicine)

University of Cape Town Medical School  
January, 2003

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## Acknowledgements

It is with great pleasure that I acknowledge the support by a number of individuals without whom this thesis would not have been possible. Firstly, I would like to thank my supervisor Michael Sack for his incessant support and ability to balance intellectual guidance with academic freedom. His approach to science, his work ethic and his interpersonal skills represent the goalpost for which I will strive in my future career. To Lionel Opie, who initially accepted my application for a post at the University of Cape Town, I will always be thankful for laying the foundation of this work. It is a great honor to work with one of the great scientists in cardiovascular medicine. Derek Yellon is thanked for many stimulating discussions and his support during my stay at the Hatter Institute in London. I would like to thank my fellow researchers in London and Cape Town, in particular Lydia Lacerda for her tireless efforts in keeping the ball rolling. Financial support is acknowledged from the Wellcome Trust, the Aaron Beare Foundation, South Africa, and the South African Medical Research Council. Finally I would like to thank my family, in particular my wife Doerthe, for the encouragement and emotional backing throughout the years.

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## Publications arising from this work

### Papers

Minners J, van den Bos EJ, Yellon DY, Schwalb H, Opie LH, Sack MN. Dinitrophenol, Cyclosporine A, and Trimetazidine modulate preconditioning in the isolated rat heart – support for a mitochondrial role in cardioprotection. *Cardiovasc. Research* 2000; 47:68-73.

Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DY, Sack MN. Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res.* 2001; 89:787-92.

Minners J, McLeod CJ, Sack MN. Mitochondrial plasticity in classical ischaemic preconditioning - moving beyond the mitochondrial  $K_{ATP}$  channel. *Cardiovascular Research* resubmitted (Dec 2002) with minor changes.

Minners J, Lacerda L, McLeod CJ, Sack MN. Diazoxide induces free radical production via inhibition of succinate supported respiration in C2C12 myotubes. *Circulation Research* submitted (Dec 2002).

## Abstracts

Minners J, Opie LH. Dinitrophenol preconditions the isolated rat heart: Proposed role for mitochondrial inner membrane potential. *Circulation* 1999; 100: abstract supplement I-343.

Minners J, Yellon DM, Jonassen AK, Opie LH. Uncoupling of mitochondrial oxidative phosphorylation preconditions the isolated rat heart. *European Heart Journal* 1999; 20: abstract supplement 316.

Van den Bos EJ, Minners J, Opie LH, Sack MN. Trimetazidine abolishes ischaemic and drug-induced preconditioning: Does mitochondrial protection antagonize preconditioning-like cardioprotection? *Circulation* 2000; 102: abstract supplement II-269.

Minners J, Meiring JJ, van den Bos EJ, Opie LH, Sack MN. Inner mitochondrial membrane modulation in preconditioned myotubes: A putative role for mitochondrial homeostasis in enhanced tolerance to ischemia. *Circulation* 2000; 102: abstract supplement II-287.

Van den Bos EJ, Minners J, Opie LH, Sack MN. Trimetazidine (TMZ) abolishes drug-induced and attenuates ischaemic preconditioning - further support for a mitochondrial role in preconditioning. *European Heart Journal* 2000; 21: abstract supplement 671.

Van den Bos EJ, Minners J, Opie LH, Sack MN. Cyclosporine A-mediated preconditioning-like cardioprotection is abolished by trimetazidine - proposed role of mitochondrial homeostasis in preconditioning. *European Heart Journal* 2000; 21: abstract supplement 670.

Minners J, Lacerda L, Yellon DY Sack MN. Diazoxide modulates mitochondrial electron transfer chain activity during the preconditioned state in C2C12 myotubes. *Circulation* 2002; 106: abstract supplement II-231.

University of Cape Town

## Abstract

Cardiac preconditioning describes a phenomenon of cardioprotection whereby a preceding stimulus renders the heart more resistant to subsequent ischaemia/reperfusion induced cell death. Several triggers of preconditioning have been identified including adenosine, bradykinin and opioids. Paradoxically, triggers that would be considered noxious to the heart such as ischaemia itself – when applied for a short duration – also confer preconditioning-like cardioprotection. The intracellular signaling pathways activated by preconditioning triggers appear to converge on the mitochondrion, possibly via activation of a mitochondrial ATP sensitive potassium (mitoK<sub>ATP</sub>) channel. Potassium channel openers are known to confer protection in a variety of models and decrease mitochondrial inner membrane potential in isolated mitochondria as positively charged potassium ions move into the mitochondrial matrix. These findings led to the underlying hypothesis of this PhD that opening of the mitoK<sub>ATP</sub> channel results in a certain degree of mitochondrial stress as evidenced by changes in mitochondrial homeostasis, which in turn render the cell more resistant to a subsequent lethal injury. To begin to explore this hypothesis 2,4- dinitrophenol, a prototypical uncoupling agent known to decrease inner mitochondrial membrane potential, was given as a preconditioning mimetic. The protection seen in an isolated heart model following 2,4 dinitrophenol administration was comparable to that observed with ischaemic preconditioning.

To further pursue the underlying concept that mitochondrial stress is eventually protective, parameters of mitochondrial function were investigated in a cell culture model. Administration of the prototypical mitoK<sub>ATP</sub> channel opener diazoxide resulted in an acute increase in in-situ mitochondrial volume and a dose-dependent inhibition of succinate-supported respiration with a resultant increase in reactive oxygen species (ROS) generation. Interestingly, these results suggest a broader role for metabolic inhibition as an early component of cytoprotective signaling. To assess whether the perturbations of mitochondrial homeostasis are sustained throughout the preconditioned phase inner mitochondrial membrane potential, cellular oxygen consumption and ATP levels were investigated just prior to index ischaemia. Cells exposed to a variety of preconditioning stimuli (adenosine, diazoxide, and ischaemic preconditioning) showed decreased inner mitochondrial membrane potential, increased oxygen consumption and decreased ATP levels, a constellation resembling the uncoupled – and less energy efficient – state, which was elicited using DNP in the initial experiments involving isolated rat hearts.

In summary, the data on the cell protective phenomenon of preconditioning described in this dissertation suggest a complex regulatory program at the mitochondrial level in response to a preconditioning trigger. Firstly, I show that directly “stressing” the mitochondrion with an uncoupling agent is protective. Secondly, the mitochondrial response to the prototypical preconditioning mimetic diazoxide is characterized by substrate specific respiratory inhibition and subsequent free radical production. Thirdly, the preconditioned state (after washout of the trigger) is one of an uncoupled – and therefore less energy

efficient - mitochondrion. These perturbations in mitochondrial function probably activate as yet unidentified mechanisms to improve energetics during the subsequent ischaemia/reperfusion insult. Moreover, since diazoxide has direct effects on mitochondrial function the role of the mitoK<sub>ATP</sub> channel in cellular protection needs to be reassessed. Further characterization of the highly dynamic regulatory events involved is required to fully understand innate cellular protection and finally identify the elusive "end effector(s)" of preconditioning.

University of Cape Town

# Introduction

## History / Definition

Cardiac preconditioning describes a cell survival program whereby a trigger renders the heart partially resistant to subsequent ischaemia/reperfusion induced cell death. Murry et al in 1986 reported on a study in dogs subjected to 40min of coronary artery occlusion (Murry, Jennings et al. 1986). When the 40min occlusion was preceded by four cycles of 5min coronary artery occlusion interspersed with episodes of 5min reperfusion, the resultant infarct size was reduced from 29% of the area at risk to 7%. The authors termed this apparent cardio-protective phenomenon ischaemic preconditioning. Subsequently, ischaemic preconditioning has been found in all species investigated, including man (Walker, Walker et al. 1995). Despite major advances in the understanding of preconditioning since the publication of this seminal paper by Murry et al, a unifying hypothesis on the mechanisms of preconditioning is still lacking. The present dissertation tries to add to the understanding of this complex phenomenon by focusing on the role of the mitochondrion, the power-house of the cell, in preconditioning.

## Signaling

The cellular signaling cascade involved in preconditioning is conceptually divided into triggers, mediators, and end-effectors (Yellon, Baxter et al. 1998). This has greatly helped in our understanding of the complex signaling networks activated in preconditioning. In the following paragraphs the literature relating to signaling in preconditioning will be briefly reviewed (figure 1). The focus will be on the mitochondrial ATP-sensitive potassium (mitoK<sub>ATP</sub>) channel, since the information available on this later channel formed the basis for the experiments presented in this dissertation. For a more detailed review of the subject the reader is referred to references (Yellon, Baxter et al. 1998; Schulz, Cohen et al. 2001).

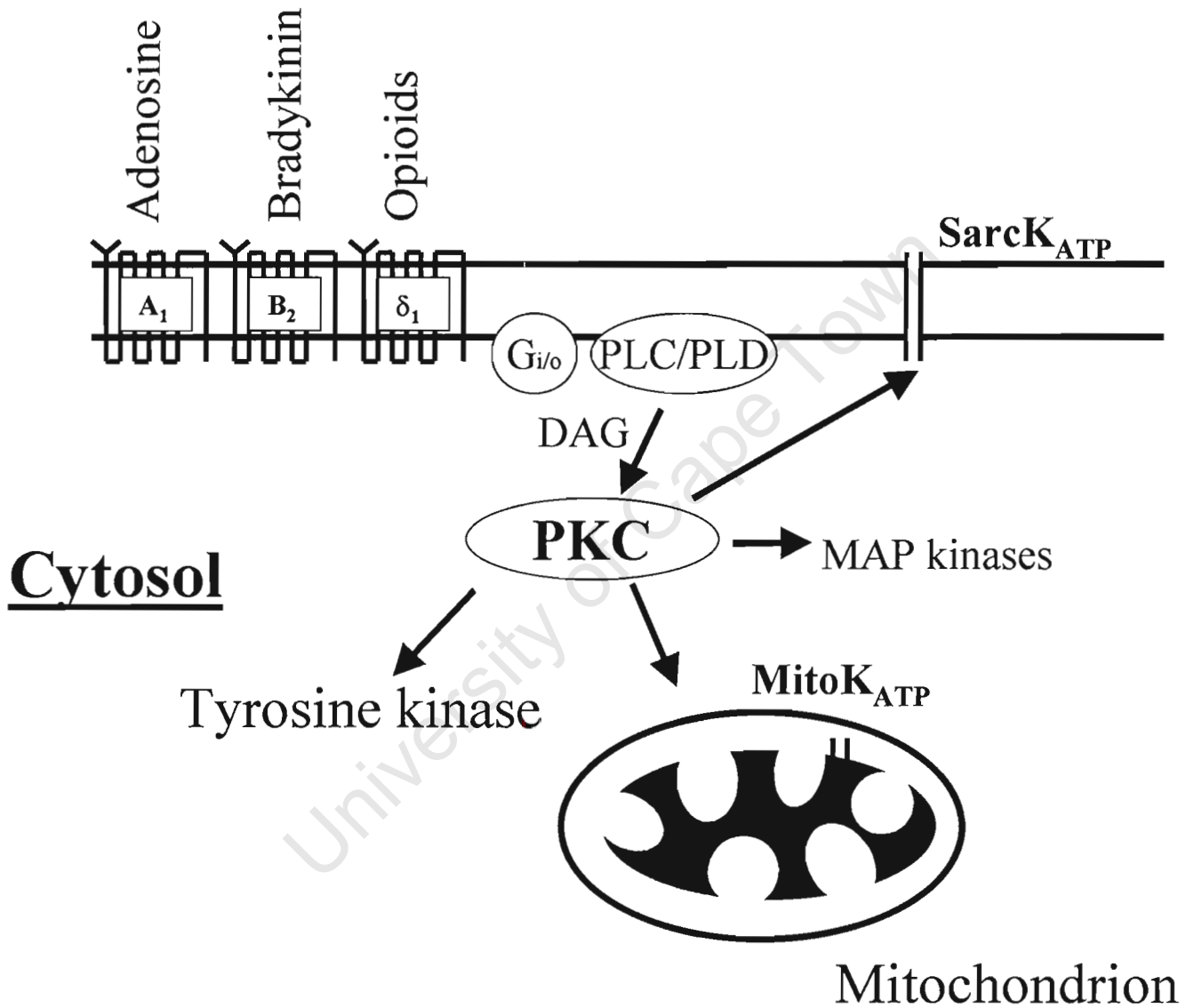
Triggers of preconditioning include a variety of paracrine and neuroendocrine receptor-mediated substances such as adenosine (Mullane and Bullough 1995), acetylcholine (Yao and Gross 1993), catecholamines (Banerjee, Locke-Winter et al. 1993), angiotensin II (Liu, Tsuchida et al. 1995), endothelin (Wang, Gallagher et al. 1996), bradykinin (Goto, Liu et al. 1995), and opioids (Schultz, Hsu et al. 1996). To which extent these substances are individually capable of inducing preconditioning is species, and model, dependent. For instance adenosine appears to play no role in preconditioning in the isolated rat heart (Li and Kloner 1993) but is important in the dog (Auchampach and Gross 1993). Receptor-independent triggers include free radicals (Tritto, D'Andrea et al. 1997; Vanden Hoek, Becker et al. 1998) and nitric oxide, both from an endogenous (Lochner, Marais et al. 2000) and an exogenous (Nakano, Liu et

al. 2000) source. Conceptually interesting is the observation that potentially noxious stimuli like ischaemia itself or calcium overload have been shown to trigger preconditioning (Murry, Jennings et al. 1986; Ashraf, Suleiman et al. 1994).

Mediators of preconditioning include G-proteins, phospholipase C, protein kinase C (PKC), tyrosine kinases (TK), and mitogen activated-kinases (MAPK) (Yellon, Baxter et al. 1998). G-proteins for instance couple bradykinin and adenosine to phospholipase C, the resultant diacylglycerol in turn activating PKC (Downey and Cohen 1995). A growing number of isoforms of PKC are described of which PKC $\epsilon$  appears to be the most commonly involved in preconditioning. However, again marked species differences exist with isoforms  $\alpha$  and  $\delta$  involved in canine (Kitakaze, Funaya et al. 1997) and rodent (Yoshida, Kawamura et al. 1997) models, respectively.

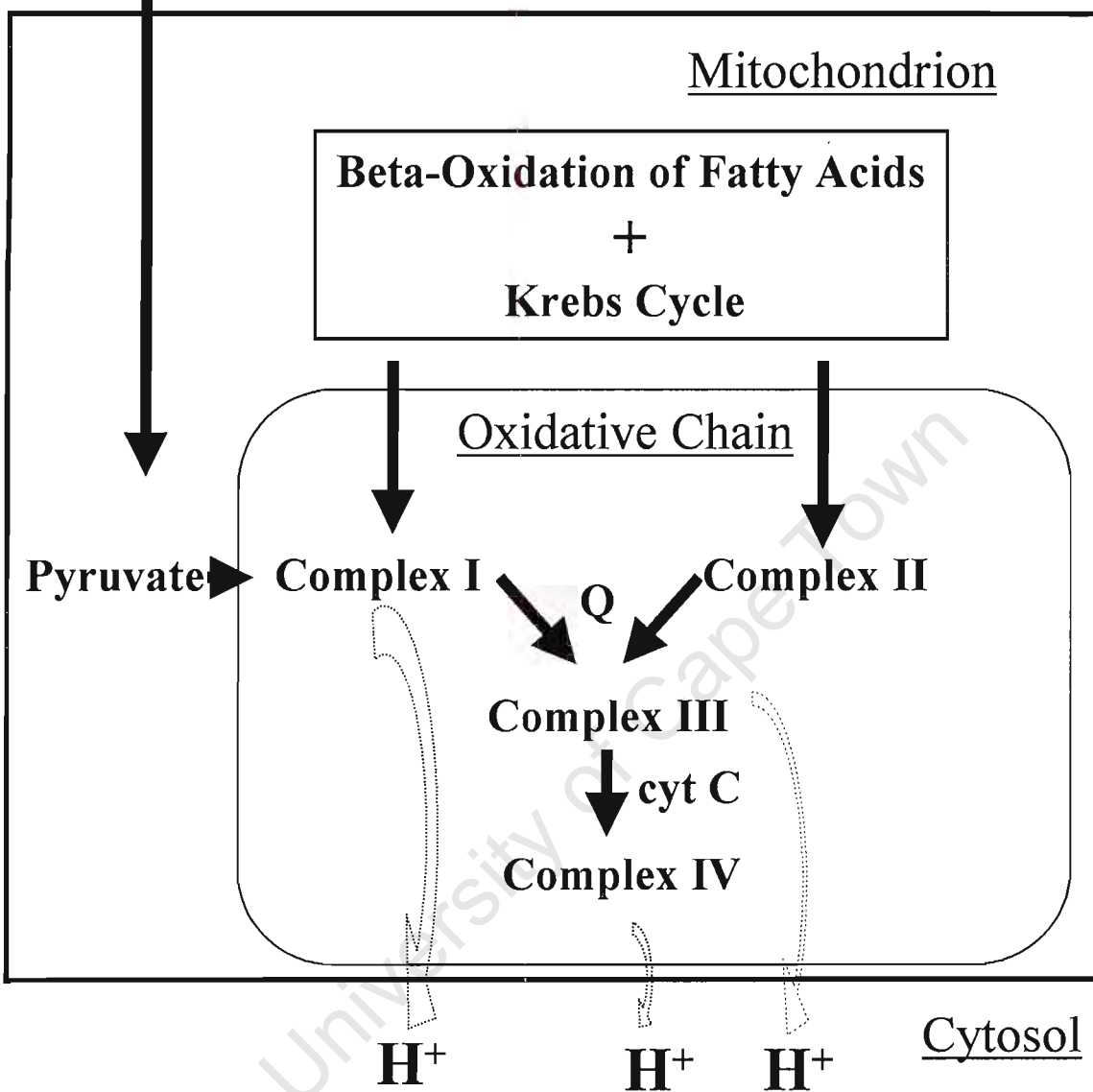
Several studies have shown that the intracellular signaling pathways converge on the mitoK<sub>ATP</sub> channel (Liu, Gao et al. 1996; Garlid, Paucek et al. 1997; Liu, Sato et al. 1998). Consequently, the mitoK<sub>ATP</sub> channel has been proposed to represent the end-effector of preconditioning (Gross and Fryer 1999). However, this proposition has been repeatedly challenged (Holmuhamedov, Jovanovic et al. 1998; Hanley, Mickel et al. 2002) and after more than a decade of intense research the end-effector of preconditioning is still a matter of debate. In the following paragraphs the current knowledge regarding K<sub>ATP</sub> channels will briefly be summarized.

# Extracellular Space



**Figure 1** Diagrammatic representation of current concepts regarding signal transduction following a preconditioning stimulus.

# Carbohydrates



**Figure 2** Major sources of redox equivalents and their relation to the respiratory chain. Extramitochondrial redox equivalents from glycolysis also feed into complex I and II (not shown), complex I = NADH:Ubiquinone oxidoreductase, complex II = succinate:ubiquinone oxidoreductase, complex III = ubiquinone:cytochrome c oxidoreductase, complex IV = cytochrome oxidase, Q = ubiquinone, cyt = cytochrome

Redox Pair	Standard reduction potential (E <sub>0</sub> ' Volt)
O <sub>2</sub> /H <sub>2</sub> O	+0.82
Cytochrome c; Fe <sup>3+</sup> /Fe <sup>2+</sup>	+0.22
Ubiquinone; ox/red	+0.11
NAD <sup>+</sup> /NADH	-0.32
H <sup>+</sup> /H <sub>2</sub>	-0.42
Succinate/alpha-ketoglutarate	-0.67

**Table 1** A selection of biologically important standard reduction potentials. Conventionally, standard reduction potentials are measured at pH 0.0 with the potential of the H<sub>2</sub> electrode set at zero. For biological systems it is more relevant to show standard reduction potential at pH 7.0, as in this table.

A highly selective pore, the ATP synthase ( $F_1F_0$  ATPase), is the physiologic site coupling the reentry of protons into the mitochondrion with the phosphorylation of ADP to ATP (figure 3). The high turnover of ADP/ATP calls for an efficient transport of nucleotides across the inner mitochondrial membrane which is ensured by the highly selective adenine nucleotide transporter (ANT).

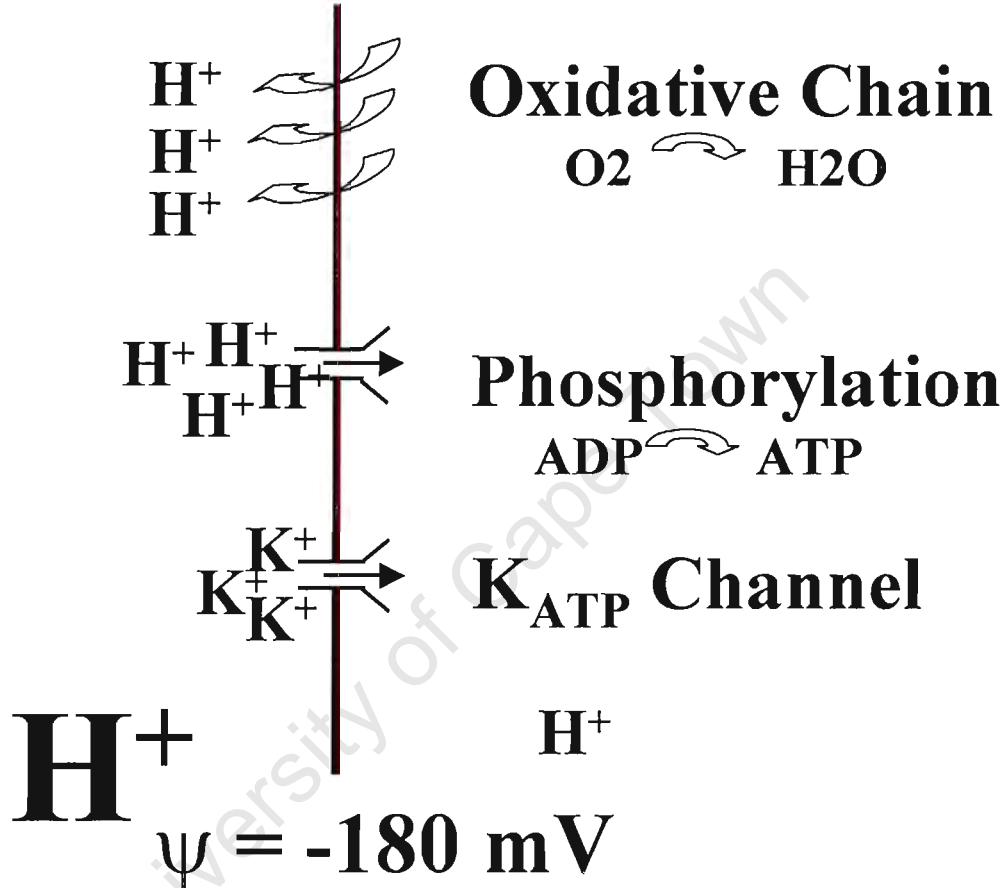
In addition to the structures described so far, the mitochondrial inner membrane contains multiple other channels, exchangers and transporters including IMAC, phosphate transporter, dicarboxylate transporter, tricarboxylate transporter, alpha-ketoglutarate transporter, glutamate/malate transporter, pyruvate, ornithine and carnitine transporter, calcium uniporter, potassium/proton antiporter, and the permeability transition pore.

Finally, relevant to preconditioning, the  $\text{mitoK}_{\text{ATP}}$  channel is located on the inner mitochondrial membrane (figure 3) as described by Inoue (Inoue, Nagase et al. 1991).

## Intermembrane

## Matrix

space Inner Mitochondrial Membrane



**Figure 3** Generation of the inner mitochondrial membrane potential as proposed by the chemo-osmotic hypothesis (Mitchell). The oxidative chain pumps protons ( $\text{H}^+$ ) from the matrix to the intermembrane space. A membrane potential ( $\Delta \psi$ ) and a pH gradient ( $\Delta \text{pH}$ , not shown) are established. Following the concentration gradient through the ATP synthase ( $\text{F}_1\text{F}_0$  ATPase)  $\text{H}^+$  provide the energy to phosphorylate ADP to ATP. The mitochondrial  $\text{K}_{\text{ATP}}$  channel is shown including the assumed net influx of potassium ions upon channel activation.

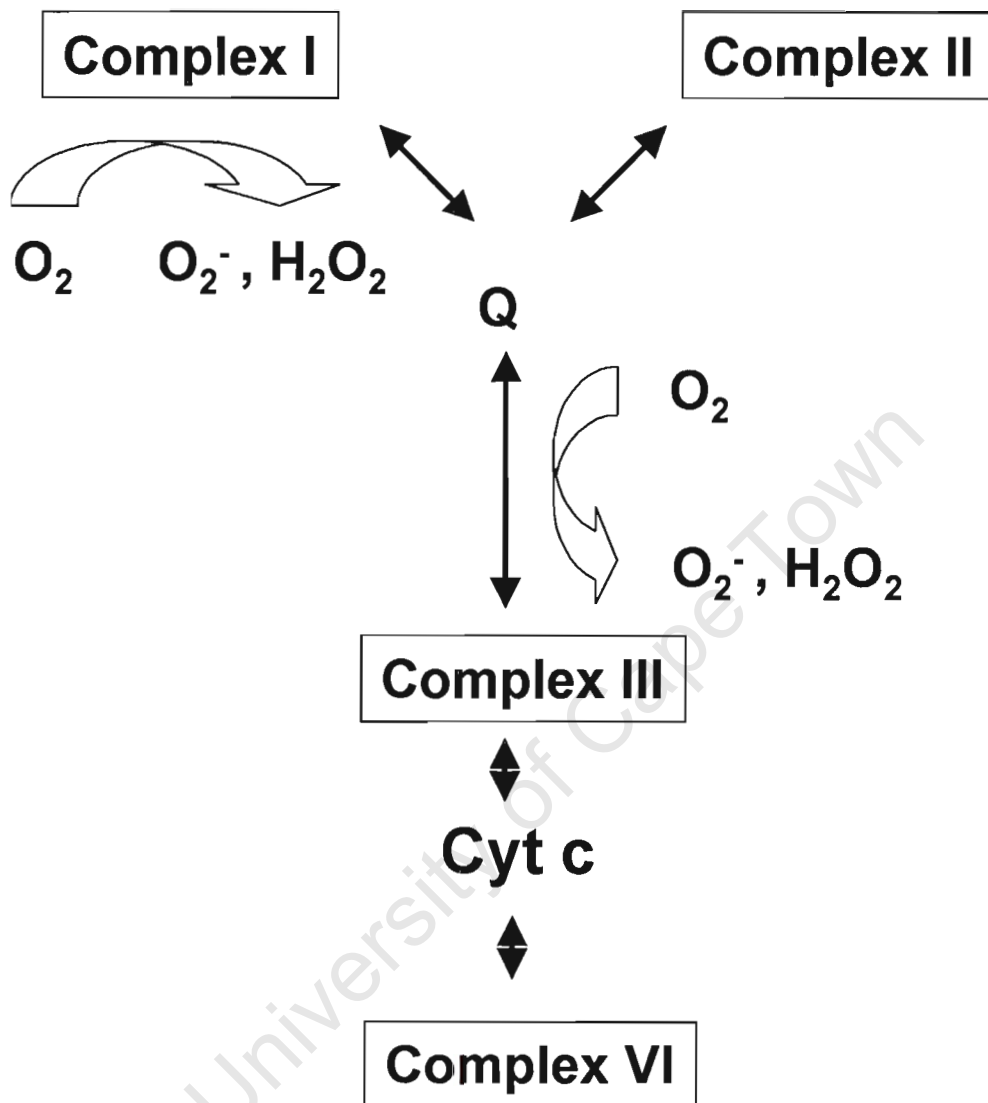
## Consequences of mitochondrial $K_{ATP}$ channel activation

Activation of the mito $K_{ATP}$  channel - be it via ischaemic preconditioning or via a pharmacological potassium channel opener - has been proposed to represent the possible end-effector of preconditioning (Gross and Fryer 1999). However, the question remains as to how activation of this particular channel might result in improved cell survival and decreased infarct size. Hypothetically, activation of the mito $K_{ATP}$  channel could have a variety of effects on mitochondrial function. Since, as mentioned, the positive charge on the outside greatly exceeds the charge on the matrix side of the inner mitochondrial membrane activation of the mito $K_{ATP}$  channel should result in a net flux of positively charged potassium ions from the intermembrane space into the matrix of the mitochondrion. This, in turn, would decrease inner mitochondrial membrane potential as, indeed, suggested by Szewczyk's work (Szewczyk, Mikolajek et al. 1993). Holmuhamedov also showed in isolated mitochondria from rat heart that the  $K_{ATP}$  channel openers pinacidil, cromakalim and levcromakalim in "protective" concentrations decrease inner mitochondrial membrane potential by between 10 and 25% (Holmuhamedov, Jovanovic et al. 1998).

The cycling of potassium via the  $K^+/H^+$  exchanger and the  $K^+$  uniporter is believed to be crucial in maintaining mitochondrial potassium homeostasis and thereby mitochondrial volume (Garlid 1996). Volume changes, in turn, have been shown to be intricately involved in mitochondrial metabolism (Halestrap 1989). Mito $K_{ATP}$  channel activation via modulation of potassium homeostasis could therefore play a pivotal role in the regulation of metabolism on the

mitochondrial level thereby providing an attractive hypothesis as to how opening the mitoK<sub>ATP</sub> channel could result in improved cell survival after an ischaemia/reperfusion insult. Indeed, K<sub>ATP</sub> channel openers pinacidil, cromakalim and diazoxide have been shown to increase volume of isolated mitochondria (Holmuhamedov, Jovanovic et al. 1998; Kowaltowski, Seetharaman et al. 2001). Whether this increase in mitochondrial volume is related to the preconditioning phenomenon is currently unclear.

Reactive oxygen species have been identified as important signaling molecules in cellular metabolism (Kamata and Hirata 1999; Irani 2000). Interestingly, antioxidants abolish preconditioning when present during the preconditioning stimulus (Chen, Gabel et al. 1995; Tritto, D'Andrea et al. 1997, Baines, 1997; Pain, Yang et al. 2000; Forbes, Steenbergen et al. 2001). Although baseline production of free radicals by mitochondria is low they can become the main source of ROS production within a cell under certain circumstances (Ambrosio, Zweier et al. 1993). Indeed, the source of ROS during the trigger phase of preconditioning has been traced to the mitochondrion, however the exact site of ROS production (figure 4) is a matter of debate (Vanden Hoek, Becker et al. 1998).



**Figure 4** Two sites of ROS production within the respiratory chain are indicated by arrows, the first within complex I, most likely linked to the FMN group, and the second at the transition from ubiquinone to complex III. complex I = NADH:Ubiquinone oxidoreductase, complex II = succinate:ubiquinone oxidoreductase, complex III = ubiquinone:cytochrome c oxidoreductase, complex IV = cytochrome oxidase, Q = ubiquinone, cyt = cytochrome

Opening the mitoK<sub>ATP</sub> channel might modulate ROS production during the trigger phase of preconditioning since diazoxide treatment acutely increases ROS in cardiomyocytes (Forbes, Steenbergen et al. 2001). Evidence to the complexity of ROS mediated signaling is the fact that potassium channels in turn exhibit increased activity after exposure to free radicals (Zhang, Chen et al. 2001) thereby indicating a potentially important link between mitoK<sub>ATP</sub> channel activation and free radical production.

Taken together, the data demonstrate a distinct mitochondrial response to mitochondrial K<sub>ATP</sub> channel activation (Gross and Fryer 1999) which might account for the cell protective properties of K<sub>ATP</sub> channel openers and – by inference – ischaemic preconditioning.

## Hypothesis

Mitochondria are intimately linked to the cycle of events leading to preconditioning. However, little is known about the possible mechanisms involved. Based on the findings presented in the introduction three questions were generated to explore the putative role of the mitochondrion in the cell protective phenomenon of preconditioning.

- If depolarisation of the inner mitochondrial membrane as triggered by  $\text{mitoK}_{\text{ATP}}$  channel activation is important in preconditioning, do other agents that decrease inner mitochondrial membrane potential also protect?
- Does direct stress on the mitochondrion activate the cardiac preconditioning program and constitute one of the downstream cellular events linking different triggers of preconditioning?
- Is short-term perturbation of mitochondrial homeostasis rather than mitochondrial protection necessary to evoke the preconditioned state?

Investigating these three aspects of preconditioning resulted in the underlying hypothesis of this thesis that

**Sublethal mitochondrial stress is critical for the development of the preconditioned phenotype.**

I initially investigated whether 2,4-dinitrophenol (DNP), an agent known to result in a profound decrease of inner mitochondrial membrane potential via uncoupling of oxidation from phosphorylation (Laiho and Trump 1975), could protect the heart against ischaemia-induced cell death. This simple hypothesis followed by the initial experiments demonstrating a protective effect of 2,4-dinitrophenol is the foundation of this dissertation, proving that sublethal mitochondrial stress is an integral component of the preconditioning program.

## **Project Aims**

The objectives of this work were to

- **Determine the effects of direct modulation of mitochondrial homeostasis on ischaemia/reperfusion injury in the heart.**
- **Investigate early mitochondrial signaling events ultimately resulting in preconditioning.**
- **Delineate and characterize the mitochondrial phenotype associated with the preconditioned state.**

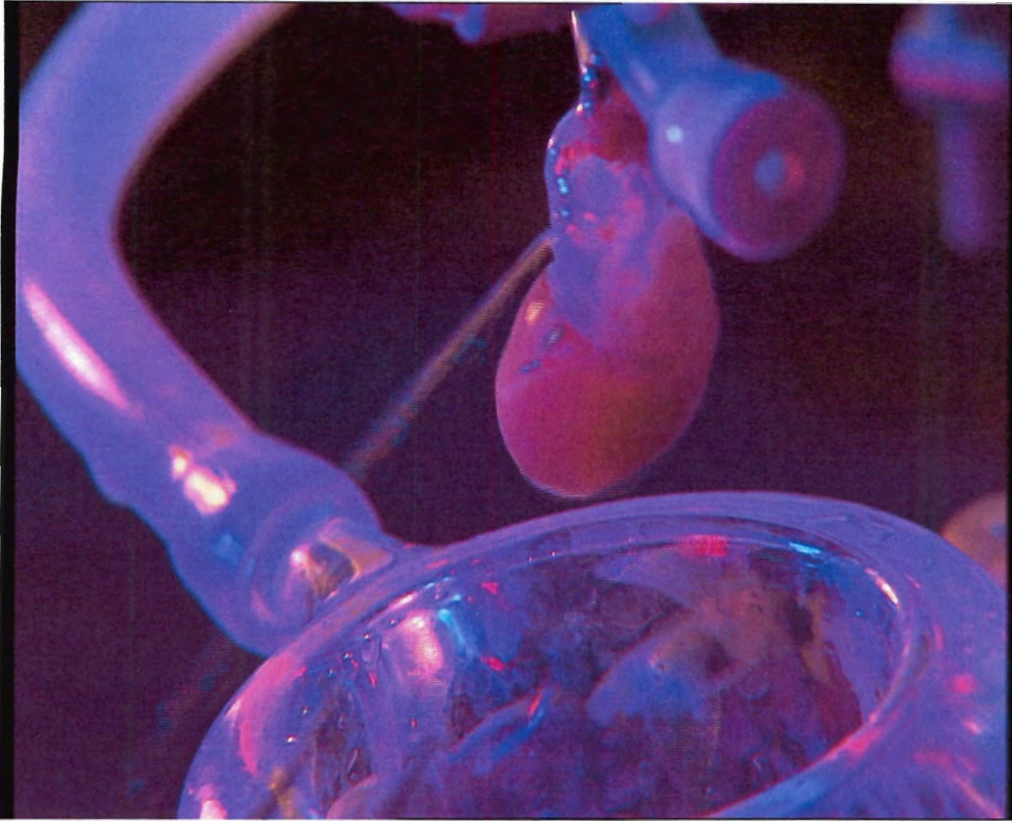
## **Materials and Methods**

### **Ethics**

All animal studies performed were approved by the Animal Research Review Committee of the University of Cape Town, and followed the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

### **The Langendorff perfused isolated rat heart model**

In order to begin evaluating the putative beneficial influence of mitochondrial stress on cell survival the initial studies were performed in the Langendorff-perfused isolated rat heart model. The experimental setup was modified from that described by Bugge and Ytrehus (Bugge and Ytrehus 1995). Male Long-Evans rats (250-300g) were anaesthetized with 70 mg.kg<sup>-1</sup> sodium pentobarbitone (intraperitoneally) and heparinized (200 IU i.v.). The heart was rapidly excised, immersed in ice cold modified Krebs-Henseleit buffer solution (NaCl 118, KCl 4.7, CaCl<sub>2</sub> 1.8, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.2 and glucose 11.0, units = mmol.l<sup>-1</sup>). Within one minute the heart was mounted onto a Langendorff apparatus and perfused retrogradely via the aorta with Krebs-Henseleit buffer (pH 7.4) at constant pressure (100 cm H<sub>2</sub>O, figure 5). The perfusate was oxygenated with 95% O<sub>2</sub>-5% CO<sub>2</sub> and maintained at 37 degrees Celsius throughout the experiment.

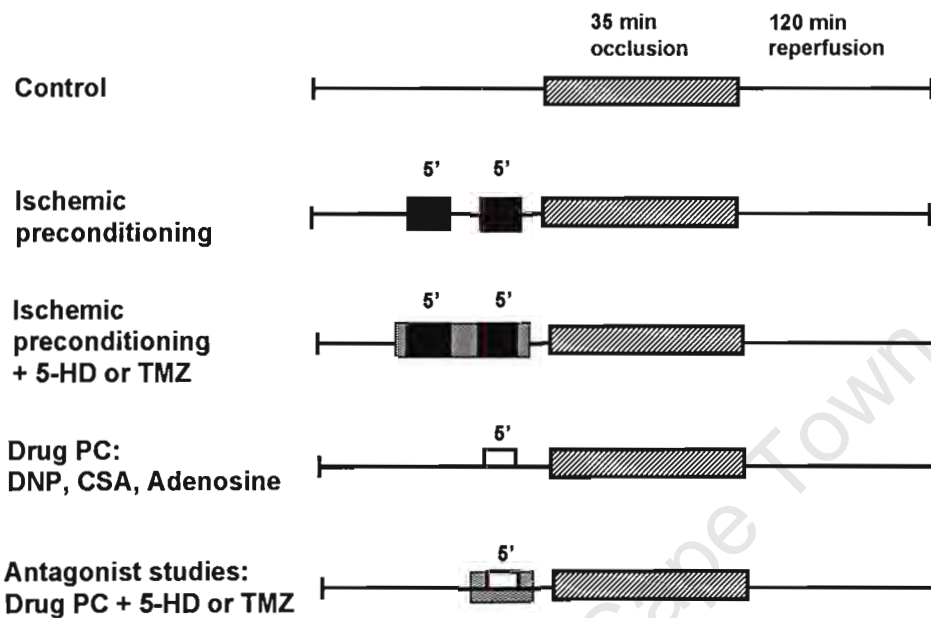


**Figure 5** The Langendorff perfused isolated rat heart model. The heart is mounted via the aorta and perfused retrogradely. A small incision at the level of the pulmonary artery allows for the drainage of the perfusate which can be quite vigorous as indicated by the visible jet of fluid.

A water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle via the left atrium. Left ventricular end-diastolic pressure was set to 4 mmHg as baseline. Myocardial temperature was measured by a thermoprobe inserted into a small incision in the pulmonary artery. A 6/0 silk suture was placed around the left coronary artery, close to its origin and the hearts were then allowed to stabilize for 15 minutes. The ends of the suture were threaded through the end of a Gilson pipette tip to produce a snare and locked with a second pipette tip. Regional ischaemia was induced by carefully tightening the silk suture around the coronary artery and clamping the pipette tip against the epicardial surface. 35 min of regional ischaemia were followed by 120 minutes of reperfusion. Heart rate (HR) and left ventricular developed pressure (LVDP = difference between LV systolic and diastolic pressures) were continuously displayed on a Lectromed recorder. Coronary flow was measured every 10 min throughout the experiment.

### **Isolated Rat Heart Perfusion Protocol**

The perfusion protocol is shown in figure 6. All hearts were allowed an equilibration period of at least 15 minutes and were consequently subjected to 35 minutes of regional ischaemia followed by 120 minutes of reperfusion. No further interventions were performed in the controls. IPC was elicited by two cycles of 5 minutes of global ischaemia interspersed with 5 minutes reperfusion prior to regional ischaemia.



**Figure 6** Graph summarizing the perfusion protocols for the different treatment groups. DNP denotes 2,4-dinitrophenol, CSA cyclosporin A, TMZ trimetazidine, 5-HD 5-hydroxydecanoate. DNP, CSA, and adenosine were given for 5 min with a 5 min washout period prior to the 35 min index ischemia. In the antagonist studies TMZ or 5-HD was given for 9 min covering the DNP, CSA adenosine perfusion and the ischemic preconditioning protocol. □ drug perfusion, ■ antagonist studies, ■ global ischemia, ▨ regional ischemia.

2,4-dinitrophenol (DNP, 50  $\mu\text{M}$ ), cyclosporine A (CSA, 0.2  $\mu\text{M}$ ), and adenosine (100  $\mu\text{M}$ ) were given for 5 min followed by 5 min of reperfusion before the regional ischaemia. Trimetazidine (TMZ, 1  $\mu\text{M}$ ) was given for 9 min, covering the period of drug treatment (DNP/TMZ, CSA/TMZ, Adeno/TMZ) and the IPC protocol (IPC/TMZ) as was 5-hydroxydecanoic acid (5-HD, 100  $\mu\text{M}$ , DNP/5-HD, CSA/5-HD and IPC/5-HD).

### **Measurement of risk zone and infarct size**

At the end of the experiment, the silk suture around the coronary artery was securely tied and a 5 mg/ml suspension of zinc-cadmium sulfide fluorescent microspheres (in 0.9% w/v saline) was slowly infused through the aorta to delineate the myocardial risk zone under ultraviolet light. The heart was then frozen overnight before being cut into 2 mm thick slices (4-5 slices per heart), defrosted, and stained by incubation for 15 minutes in 1% w/v triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4). Slices were then fixed in 10% v/v formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue. The area at risk and the area of infarcted tissue in the risk zone were determined using computerized planimetry (Summa Sketch III; Summa Graphics). The volume of infarcted tissue (I) and the risk zone (R) was then calculated by multiplying each area with the slice thickness and summing the products. The infarct size was expressed as the percentage of the risk zone infarcted (I/R ratio).

## Drugs

For isolated rat heart experiments DNP, CSA, adenosine and TTC were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DNP was dissolved in 0.9% saline, CSA in 99% ethanol and adenosine in 0.9% saline. DNP, CSA and adenosine were stored as a 1 mmol l<sup>-1</sup> stock solution. 5-HD was bought from RBI (Natick, MA, USA) and dissolved in Krebs-Henseleit solution on the day of the experiment. TMZ was obtained from Servier Pharmaceuticals, France, and dissolved in Krebs-Henseleit solution on the day of the experiment. For the experiments performed in cell culture diazoxide, DioC6, rotenone, glutamate, malate, and succinate were bought from Sigma. JC-1 was from Molecular Probes.

## Cell culture and preconditioning protocols in cells

To be able to more directly investigate parameters of mitochondrial function experiments were subsequently performed in cell culture. A human-derived atrial cell line (Girardi cells) and a mouse derived skeletal muscle cell line (C2C12 myotubes) were employed. Girardi cells were obtained from the European Collection of Cell Cultures. Cells were maintained in a 5% CO<sub>2</sub> atmosphere in MEM (Sigma, St. Louis, USA) supplemented with L-glut, NEAA, pen/strep and 10% FBS. When confluent adherent cells were used in the following preconditioning protocol. The normoxic control buffer was adapted from Esumi et al (Esumi, Nishida et al. 1991) and contained in mmol/L: 137 NaCl, 3.58 KCl, 0.49 MgCl<sub>2</sub>, 0.9 CaCl<sub>2</sub>, 4 HEPES and 11 glucose with a pH of

7.4. The simulated ischaemic buffer contained in mM: 118 NaCl, 3.58 KCl, 0.49 MgCl<sub>2</sub>, 0.9 CaCl<sub>2</sub>, 4 HEPES, 20 2-DG and 12 KCl adjusted to a pH of 6.2. The simulated ischaemic buffer was pre-gassed with 5% CO<sub>2</sub> and 95% argon for 15 minutes prior to use. A Sanyo multigas incubator with 5% CO<sub>2</sub>, 94% N<sub>2</sub>, and 1% O<sub>2</sub> provided the hypoxic environment. All experiments were performed in 6 well plates. Each experiment included a normoxic control, an ischaemic control and the various preconditioning protocols. In brief, the preconditioning trigger was either achieved by feeding the cells the simulated ischaemic buffer and placing them in the hypoxic chamber or keeping cells in a normoxic buffer supplemented with adenosine (0.1mM) or diazoxide (0.3mM) in a standard 5% CO<sub>2</sub> incubator for 30 minutes. 5-HD (0.1mM) was added when indicated. After one hour of reoxygenation/drug-free incubation cells were either trypsinized and transferred to the flowcytometer unit or underwent 6 hours of simulated ischaemia with subsequent cell viability measurements. A minimum of five 6-well plate experiments for each intervention was performed and results were normalized to the values obtained with the non-preconditioned controls.

C2C12 myoblasts were obtained from the American Type Culture Collection (Rockville, MD). The rapidly dividing myoblasts were maintained in a 5% CO<sub>2</sub> atmosphere in DMEM supplemented with 20% FBS until approximately 50 - 70% confluent (day 1-3). Differentiation to myotubes was initiated in 6 well plates by changing to a 2% HS supplemented DMEM media and by maintaining the cells in an 8% CO<sub>2</sub> environment. To promote full differentiation into multinucleated, thickened and elongated myotubes the serum in the media was progressively reduced to 0% for at least 24 hours prior to experimentation.

Mature myotubes at 100% confluence were used for experiments between days 7 to 10. The preconditioning protocol was similar as for Girardi cells (trigger 60min, index ischaemic insult 8 h).

### **Measurement of cell viability**

Cell viability was evaluated by LDH release into the medium which was measured spectrophotometrically (Roche, Switzerland) and via the measurement of propidium iodide uptake on flow cytometry (Boise, Gonzalez-Garcia et al. 1993). Propidium iodide fluorescence was measured in a population of  $1 \times 10^4$  cells and was expressed as percent change over ischaemic controls.

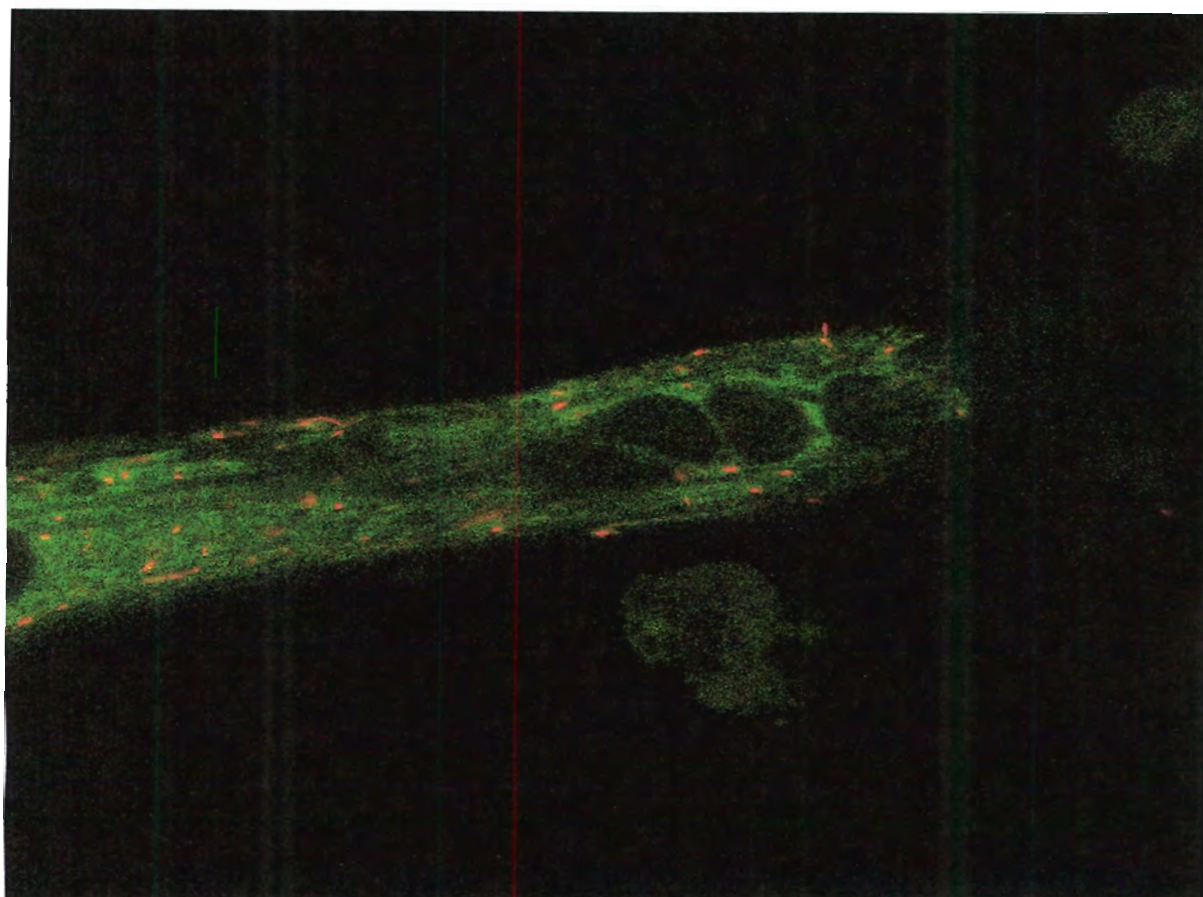
### **Inner mitochondrial membrane potential analysis**

Maintaining inner mitochondrial membrane potential is fundamental for the performance and survival of cells, particularly those with a high energy requirement. Lipophilic fluorescent cations have been widely used to monitor (Plasek and Sigler 1996; Mathur, Hong et al. 2000) relative changes in inner mitochondrial membrane potential. A growing variety of these cations is currently available. Two different dyes were used, namely DiOC<sub>6</sub>(3) (3'3'-dihexyloxacarbocyanine iodide) and JC-1(5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine) to limit dye specific findings to a minimum. Both dyes are carbocyanine derivatives and have previously been

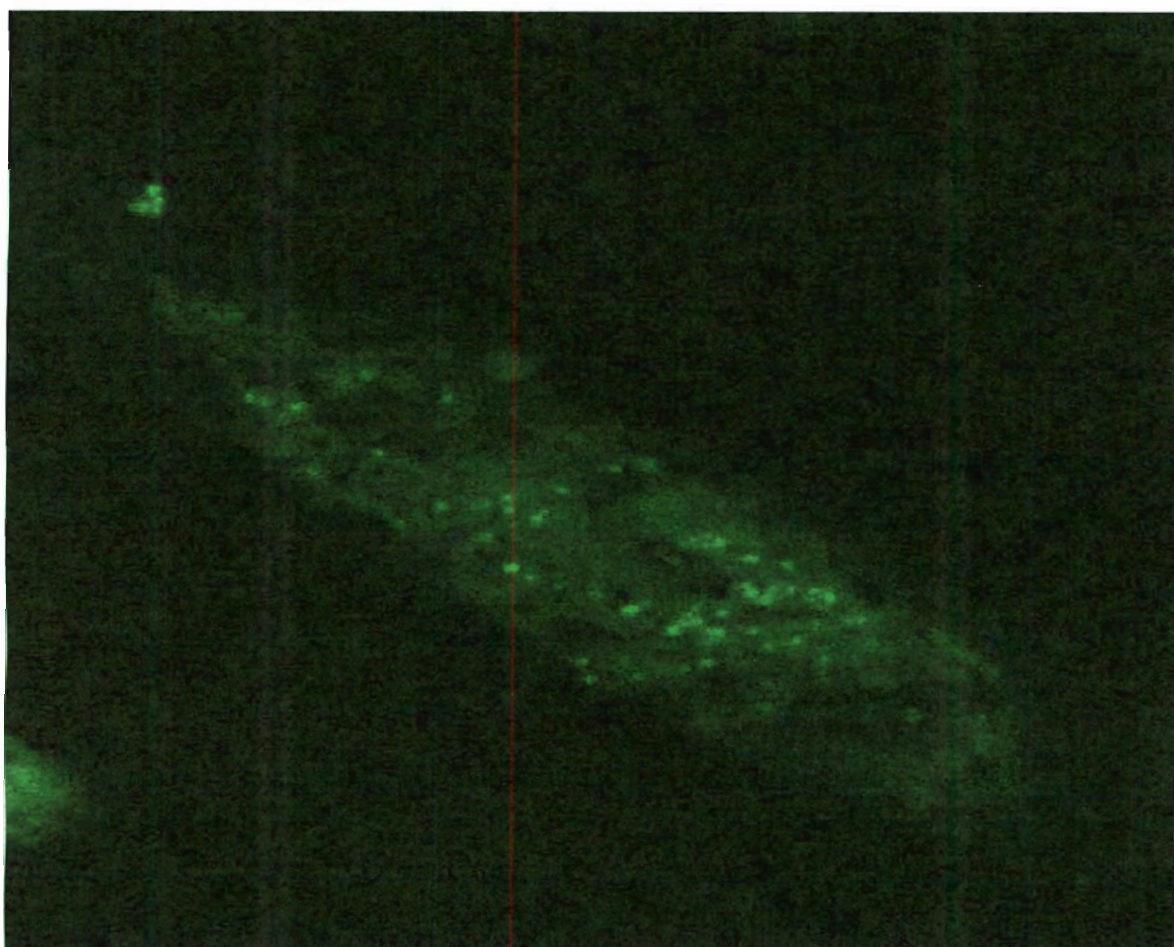
used in flow cytometric analysis (Cossarizza, Baccarani-Contri et al. 1993; Zamzami, Marchetti et al. 1995).

DiOC<sub>6</sub> was dissolved in ethanol and used at a final concentration of 10nM. Cells were stained for 30 min at 37° C, trypsinized and transferred on ice to the flow cytometry unit for analysis. JC-1 was dissolved in DMSO (5mg/ml) and aliquots of 40µL were stored at -20° C. Cells were stained with a final concentration of 10µg/ml of the dye for 30 min at 37° C to obtain good mitochondrial loading of the dye. Confocal microscopy was performed to confirm selective mitochondrial staining both in C2C12 myotubes and Girardi cells (Figures 7 and 8).

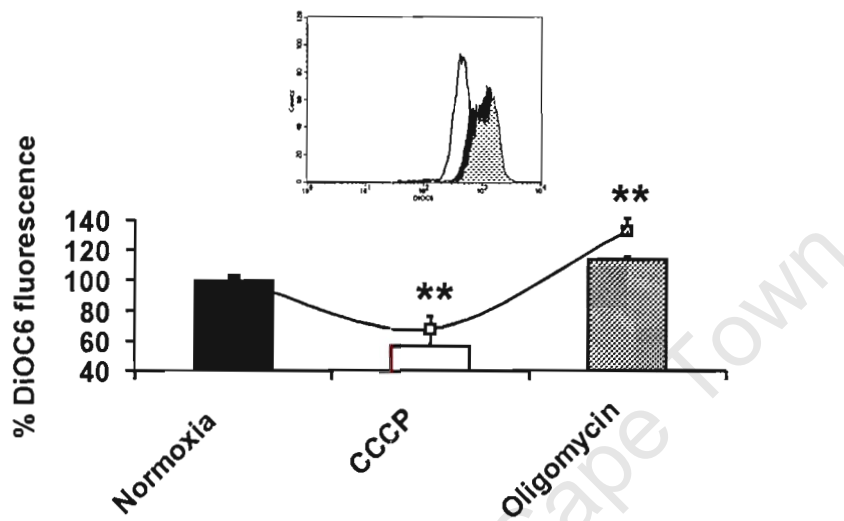
After trypsinization cells were resuspended in medium at a density of approximately 10<sup>6</sup>/ml and transferred on ice to the flow cytometer unit. To validate appropriate responses to both the uncoupling agent CCCP (0.1 mM) or the hyperpolarizing agent oligomycin (1.25 µg/ml) cells were incubated for 10 min with these agents (Figure 9). DiOC<sub>6</sub> was excited at 488nm and resultant fluorescence analyzed at 525 nm (FL1) on a FACSCalibur (Becton Dickinson) flow cytometer. JC-1 was excited at 488nm and the monomer signal (green) was analyzed at 525nm (FL1). Simultaneously, the aggregate signal (red) was analyzed at 590nm (FL2). One minute prior to mitochondrial membrane potential measurement propidium iodide (PI, 10µg/ml) was added to exclude dead cells (FL3) from the analysis. In Girardi cells the percentage of PI positive cells was <1%, in C2C12 myotubes <5%. 1 x 10<sup>4</sup> PI-negative cells were analyzed in each sample. DiOC<sub>6</sub> fluorescence data was plotted on a histogram (JC-1 data on a dot plot) and mean fluorescence was calculated for each sample. All other data are relative levels versus control.



**Figure 7** Confocal image of a mature C2C12 myotube stained with JC-1. Mitochondria with high inner membrane potential are stained red (aggregate signal), mitochondria with low membrane potential stain green (monomer signal). Multiple nuclei are discernible as black, oval structures.



**Figure 8** Confocal image of a mature C2C12 myotube stained with DiOC6. Mitochondria appear as green punctate structures.



**Figure 9** Tracking inner mitochondrial membrane potential in Girardi (bar graph) and C2C12 myotubes (line graph) using DiOC<sub>6</sub> (3'3'-dihexyloxycarbocyanine iodide). The uncoupling agent CCCP reduces and the ATPase inhibitor oligomycin increases DiOC<sub>6</sub> fluorescence in both cell types (\*\*p<0.01). The inset shows a flowcytometric overlay image of a Girardi cell experiment reflecting 10000 untreated (black), CCCP-treated (white) and oligomycin-treated (grey) cells.

## **Cellular ATP concentration**

Cellular ATP concentration was measured using a luciferase assay. Protein determination was performed on a 5 $\mu$ l sample of trypsinized, neutralised cells using the Lowry method. In brief, an adjusted volume of cell suspension to obtain 200  $\mu$ g of protein (100  $\mu$ g in C2C12 cells) was injected into three times the volume of boiling water. After 10 min of boiling, cells were placed on ice and allowed to cool for 10 min, then pulse spun in an Eppendorf centrifuge to pellet the cellular debris. 10  $\mu$ l (5 $\mu$ l in C2C12 cells) of supernatant was mixed with an equal amount of fresh firefly extract and assayed following the manufacturer's instructions supplied with the kit. To calculate the final concentration of ATP, expressed as  $\mu$ M/mg protein, an appropriate standard curve using the ATP standard supplied was generated ( $1 \times 10^{-5}$ M to  $1 \times 10^{-8}$ M ATP).

## **Oxygen consumption**

After trypsinization approximately  $3 \times 10^6$  cells were resuspended in 160 $\mu$ l of PBS. 5 $\mu$ l of cell suspension were used for protein assay and 5 $\mu$ l for a cell count. For measurements of oxygen consumption a respirometer equipped with a temperature control unit (Oxytherm, Hansatech, UK) was used. 800  $\mu$ l of PBS were placed into the oxygen electrode chamber and temperature was set to 37  $^{\circ}$ C. Baseline O<sub>2</sub> concentration at this temperature under constant stirring was found to be 0.210  $\mu$ mol/ml. 100 $\mu$ l of cell suspension was added and the decrease in oxygen concentration over time recorded. Rate of oxygen

consumption was calculated using software provided by the manufacturer and results were expressed as nmol oxygen/min/ $10^6$  cells.

Oxygen consumption in permeabilized cells (see below) was performed as in whole cells except that an “intracellular” buffer was used containing 0.25M Sucrose, 2mM  $\text{KH}_2\text{PO}_4$ , 5mM  $\text{MgCl}_2$ , 1mM EDTA, 1mM ADP, 20mM MOPS, 0.1% BSA, 10mM 2-DG. pH 7.4 (Pham, Robinson et al. 2000).

### **Cell permeabilization**

Cells were permeabilized to allow for the selective feeding of substrates into complex I and II of the oxidative chain. To establish which concentration of digitonin to use, preliminary experiments investigating PI uptake on flow cytometry were performed following a method described by Pham (Pham, Robinson et al. 2000). C2C12 cells showed 100% permeabilization with a concentration of  $70\mu\text{g}$  per million cells. This concentration of digitonin was therefore subsequently used in all experiments. Girardi cells were found to be very sensitive to digitonin permeabilization and showed no relevant oxygen consumption after digitonin treatment, regardless whether glutamate/malate or succinate was used as substrate. Therefore, experiments involving permeabilized cells were performed in C2C12 myotubes only.

## **Cell size measurements**

Cell coulter measurements were performed to investigate possible changes in cell volume by the different preconditioning protocols. Data obtained with an optical method (forward scatter data from flow cytometry, see below) had to be validated against a method of whole cell volume measurement not relying on optical input. To this end a Coulter<sup>R</sup> multisizer (Coulter Electronics Limited, England) was used which derives volume measurements from changes in impedance. In brief, the multisizer monitors the electrical current between two electrodes immersed in the conductive layer of liquid on either side of a small aperture. As each particle (cell) passes through the aperture, impedance between the electrodes is changed and an electrical pulse is generated. The magnitude of this pulse is proportional to the particle volume.  $10^4$  cells were investigated in each group and the mean volume was calculated by the software package (Accucomp<sup>R</sup>) provided by the manufacturer.

## **In-situ mitochondrial volume measurements**

Classically, in isolated mitochondria volume is investigated spectrophotometrically measuring the decrease in absorbance at 520nm. This region is chosen since it is devoid of interference with cytochromes or protein absorbances. To extend volume measurements to the in situ mitochondrion flowcytometric analysis using the forward scatter signal has been recommended (Vander Heiden, Chandel et al. 1997; Macouillard-Poulletier de, Belaud-

Rotureau et al. 1998). Incident light from an argon laser (FACSCalibur<sup>R</sup> flow cytometer, Becton Dickinson, San Jose, USA) is scattered by the cell and detected at low angles (0-10 degrees, figure 10). The larger an object (cell) the higher the forward scatter value.

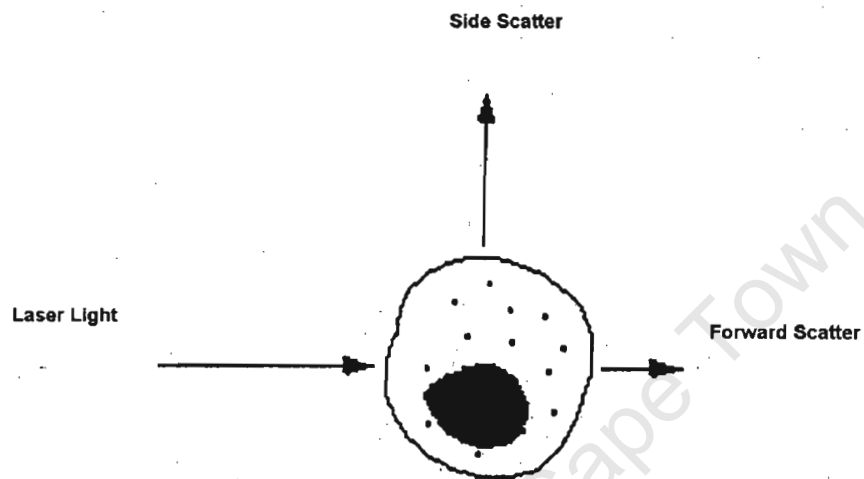
To be able to attribute the changes in forward scatter signal to changes in mitochondrial volume rather than cell volume it was essential to establish whether interventions resulted in a change in whole cell volume using an impedance-based technique (see above). Cell size was shown to stay constant, therefore the observed changes in the cellular refractive index (forward scatter) could be related to changes in mitochondrial volume and not to changes in whole cell volume (Quinlan, Thomas et al. 1983). Forward scatter data were obtained after linear amplification and plotted as a histogram and mean forward scatter value was calculated for  $1 \times 10^4$  cells in each sample.

## **ROS measurements**

Production of reactive oxygen species (ROS) is a pivotal signaling step in the cell-protective phenomenon of preconditioning (Chen, Gabel et al. 1995; Vanden Hoek, Becker et al. 1998). Multiple methods for the detection of different types of free radicals including superoxide and hydrogen peroxide as well as peroxynitrite have been developed and are critically assessed in reference (Tarpey and Fridovich 2001). In the present study the probe dichlorofluorescein diacetate (DCFH-DA) was used. The non-fluorescent DCFH-DA rapidly penetrates the cell membrane and is deacetylated by intracellular

esterases. Alkaline and oxidative hydrolysis also play a role. The resultant non-fluorescent compound dichlorofluorescein (DCFH) is trapped in the cytosol and preferentially oxidized by hydrogen peroxide and superoxide to fluorescent dichlorofluorescein (DCF).

After trypsinization cells were resuspended in serum-free medium and stained with 5 $\mu$ M DCFH-DA for 15 min at 37°C. Baseline measurements of DCF fluorescence were taken on a FACSCalibur (Becton Dickinson) flow cytometer. Subsequently, cells were incubated with DMSO as controls, diazoxide (100 $\mu$ M) and rotenone (10 $\mu$ M) as indicated. Sequential measurements were made at 5, 15 and 30 min. DCF was excited at 488nm and the signal was analyzed at 525nm (FL1). Propidium iodide (PI, 10 $\mu$ g/ml) was added to exclude dead cells (FL3) from the analysis. Less than 5% of C2C12 myotubes were found to be PI positive. 1 x 10<sup>4</sup> PI-negative cells were analysed in each sample. DCF fluorescence data was plotted on a histogram and mean fluorescence was calculated for each sample.



**Figure 10** Schematic depicting the refractory properties of a cell resulting in forward scatter and sideward scatter.

## Results

### I. Direct impairment of mitochondrial function preconditions

#### **Uncoupling oxidation from phosphorylation with 2,4 dinitrophenol confers preconditioning-like cardioprotection in the isolated rat heart**

To evaluate whether direct modulation of mitochondrial homeostasis may activate the cardiac preconditioning program I investigated whether the administration of 2,4-dinitrophenol (DNP), known to decrease inner mitochondrial membrane potential and considered the prototypical uncoupling agent (Laiho and Trump 1975) could protect against ischaemia-induced cell death. In a Langendorff-perfused isolated rat heart model DNP was given for 5 min (50 $\mu$ M) with a 5 minutes washout period. An ischaemic preconditioning group (two cycles of 5 minutes global ischaemia interspersed with 5 minutes reperfusion) served as positive and a group without preconditioning as negative control. Haemodynamic data of these studies comprising heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) are summarized in table 2. IPC reduced LVDP prior to the index ischaemia (by 26%,  $p < 0.05$ ). A similar reduction in LVDP was seen following the administration of DNP (52% reduction,  $p < 0.01$ ). An augmented CF, regularly found after an IPC protocol, was not evident in the DNP group.

**Table 2 Hemodynamic data for isolated rat heart experiments**

Group	Baseline	Preocclusion	Occlusion 34 min	Reperfusion 30 min	Reperfusion 120 min
<b>Heart Rate (/min)</b>					
Control	297 +/- 8	297 +/- 20	250 +/- 4	270 +/- 24	263 +/- 25
IPC	333 +/- 6	350 +/- 9 **	297 +/- 10 *	287 +/- 13	267 +/- 8
IPC/5-HD	310 +/- 11	330 +/- 11	283 +/- 6	290 +/- 9	250 +/- 9
IPC/TMZ	297 +/- 8	307 +/- 14	277 +/- 19	273 +/- 8	280 +/- 7
DNP	288 +/- 6	215 +/- 27 **	263 +/- 17	285 +/- 13	240 +/- 15
DNP/5-HD	301 +/- 9				
DNP/TMZ	280 +/- 10	257 +/- 25	260 +/- 17	263 +/- 13	243 +/- 10
CSA	297 +/- 6	293 +/- 13	270 +/- 41	250 +/- 15	263 +/- 13
CSA/5-HD	320 +/- 12	310 +/- 9	293 +/- 15	292 +/- 12	284 +/- 6
CSA/TMZ	333 +/- 3	320 +/- 16	287 +/- 15	307 +/- 12	300 +/- 21
Adenosine	323 +/- 10	280 +/- 15	260 +/- 23	260 +/- 20	256 +/- 20
Adenosine/TMZ	280 +/- 19	283 +/- 20	243 +/- 43	300 +/- 13	233 +/- 24
<b>Developed Pressure (mmHg)</b>					
Control	107 +/- 2	106 +/- 4	53 +/- 3	75 +/- 3	56 +/- 9
IPC	111 +/- 3	83 +/- 9 **	49 +/- 4	68 +/- 3	51 +/- 3
IPC/5-HD	117 +/- 4	84 +/- 5 **	48 +/- 5	65 +/- 3	52 +/- 3
IPC/TMZ	118 +/- 7	106 +/- 10	50 +/- 3	65 +/- 2	55 +/- 2
DNP	105 +/- 3	103 +/- 5 #	76 +/- 6 ***	76 +/- 5	49 +/- 6
DNP/5-HD	112 +/- 5				
DNP/TMZ	113 +/- 7	80 +/- 5 ***	64 +/- 8	75 +/- 6	45 +/- 7
CSA	104 +/- 3	91 +/- 6 *	58 +/- 7	69 +/- 5	47 +/- 5
CSA/5-HD	116 +/- 4	108 +/- 5	51 +/- 2	77 +/- 5	55 +/- 3
CSA/TMZ	103 +/- 1	86 +/- 4 **	51 +/- 3	86 +/- 2	65 +/- 2
Adenosine	111 +/- 5	109 +/- 7	67 +/- 9	69 +/- 3	61 +/- 4
Adenosine/TMZ	115 +/- 7	102 +/- 6	64 +/- 9	75 +/- 5	65 +/- 7
<b>Coronary Flow (ml/min)</b>					
Control	11 +/- 0.3	11 +/- 0.8	7 +/- 0.9	11 +/- 1.3	9 +/- 0.5
IPC	13 +/- 0.2	17 +/- 1.0 ***	7 +/- 0.7	11 +/- 0.9	7 +/- 0.9
IPC/5-HD	13 +/- 0.6	16 +/- 1.0 ***	7 +/- 0.4	9 +/- 0.9	7 +/- 0.4
IPC/TMZ	12 +/- 1.0	17 +/- 1.0 ***	7 +/- 1.0	9 +/- 0.3	8 +/- 0.2
DNP	12 +/- 0.5	13 +/- 1.4	9 +/- 1.6	11 +/- 1.6	8 +/- 1.4
DNP/5-HD	12 +/- 0.8				
DNP/TMZ	12 +/- 0.6	12 +/- 0.7	7 +/- 0.3	10 +/- 0.9	8 +/- 0.7
CSA	13 +/- 0.5	11 +/- 1.0	6 +/- 0.2	9 +/- 0.5	6 +/- 0.9
CSA/5-HD	13 +/- 1.0	13 +/- 1.1	6 +/- 0.3	12 +/- 1.0	8 +/- 0.8
CSA/TMZ	14 +/- 0.5	13 +/- 1.0	6 +/- 0.4	12 +/- 1.2	8 +/- 0.7
Adenosine	12 +/- 0.9	14 +/- 1.5	6 +/- 0.6	10 +/- 0.6	8 +/- 0.3
Adenosine/TMZ	12 +/- 1.4	13 +/- 2.3	6 +/- 0.9	10 +/- 1.0	7 +/- 0.4

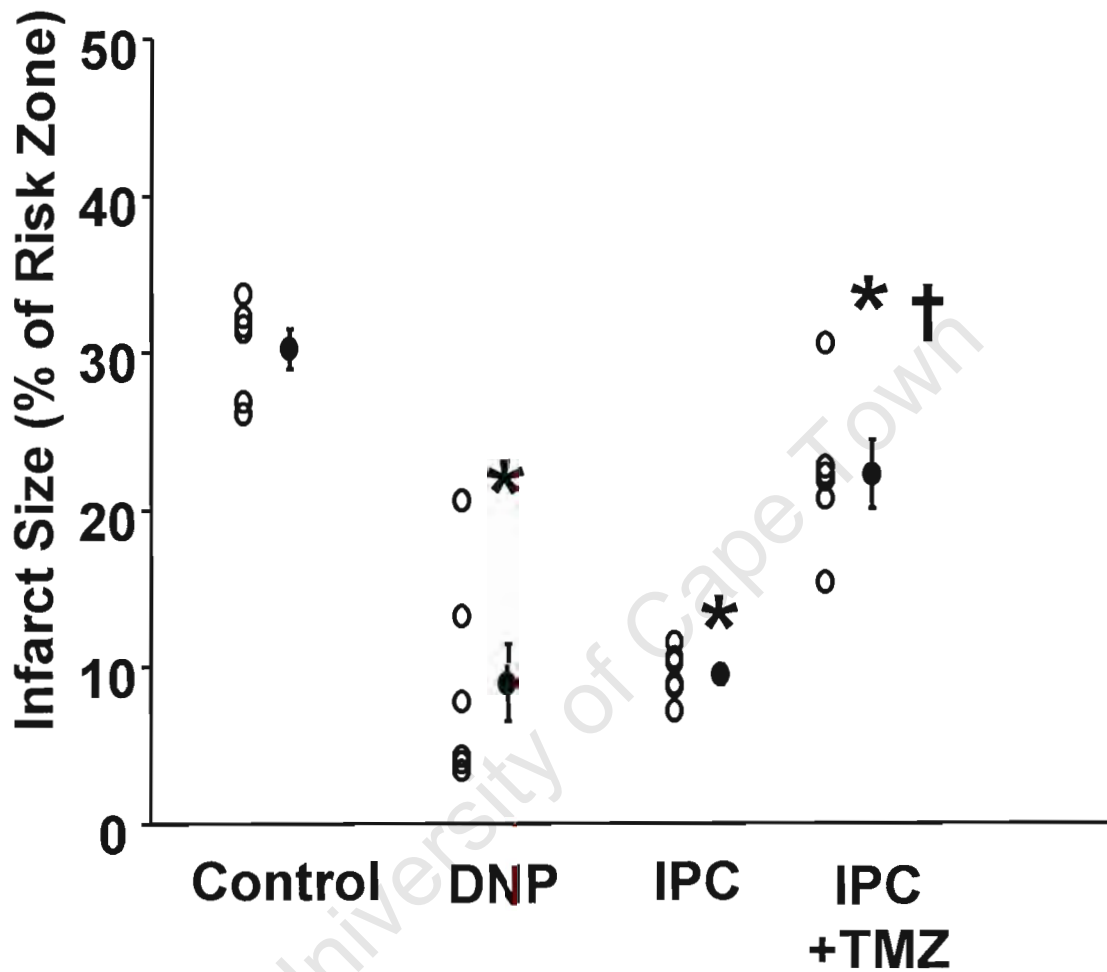
Values are given as mean +/- SEM, n=6 in each group. Preocclusion is defined as the time point prior to the 35 min index ischemia and occlusion is defined as the time point 34 min into the index ischemia.

IPC denotes ischemic preconditioning, DNP 2,4-dinitrophenol, CSA cyclosporin A, TMZ trimetazidine, 5-HD 5-hydroxydecanoate, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001 vs. control, # note, that DNP reduced LVDP acutely to 55 +/- 3 mmHg. This effect was reversed within 5 min of drug-free perfusion.

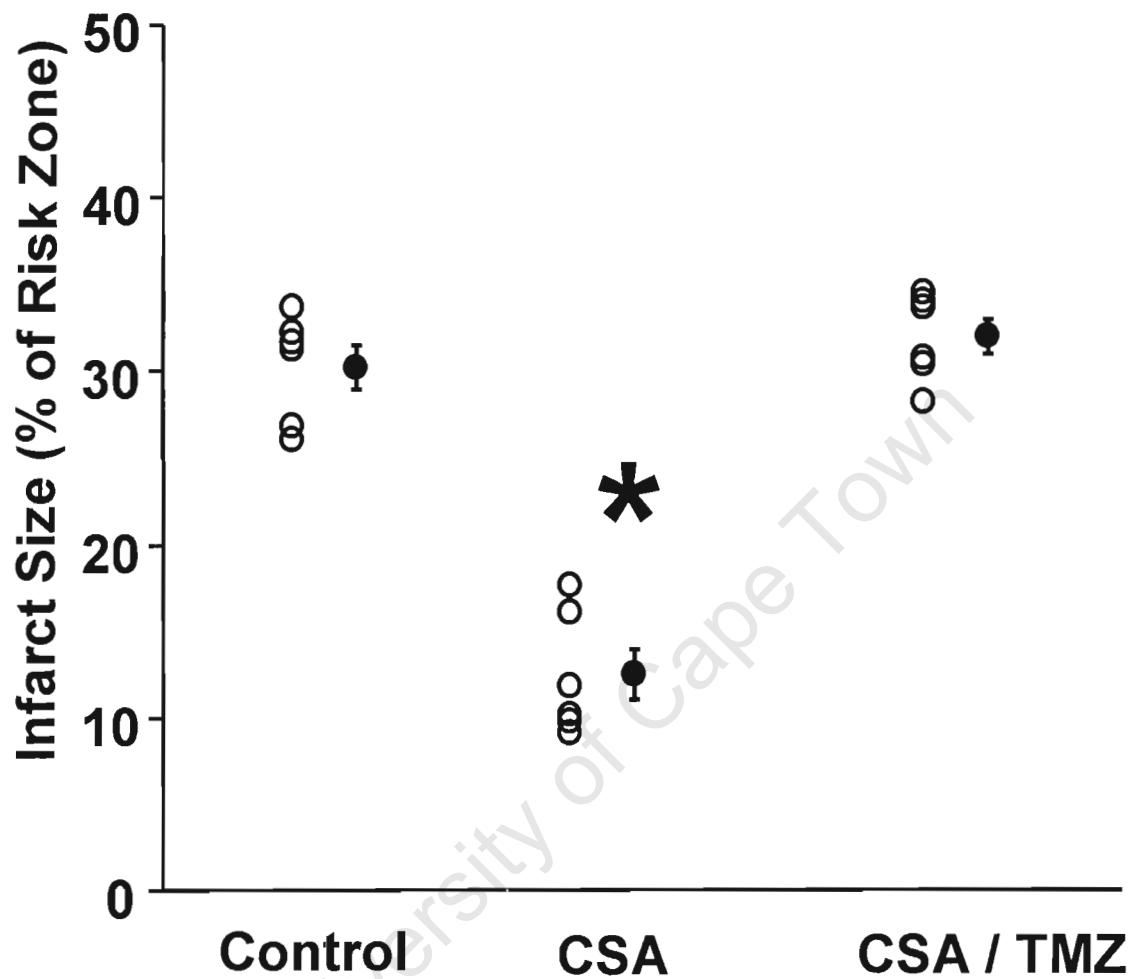
The primary endpoint in this study was infarct size expressed as percent of the area at risk (I/R ratio). Infarct size in control hearts was  $30.2 \pm 1.3\%$  of the risk zone (figure 11). DNP was as effective as ischaemic preconditioning in reducing infarct size (I/R ratio: DNP:  $9.0 \pm 2.4\%$ , IPC:  $9.5 \pm 0.6\%$ ,  $p < 0.001$  vs. controls), thereby showing that uncoupling mitochondrial oxidation from phosphorylation could act as a preconditioning trigger.

### **Cyclosporine A pretreatment protects ischaemic myocardium**

To further support the concept of mitochondrial involvement in preconditioning I evaluated whether cyclosporine A (CSA), which as one of its actions is a potent inhibitor of mitochondrial respiration between cytochromes b and c1 (Aupetit, Ghazi et al. 1988) and has been shown to dose-dependently inhibit cardiac metabolism (Niemann, Saeed et al. 2002), could reduce infarct size. Conversely, trimetazidine (TMZ), an anti-ischaemic drug widely used in France and Japan in patients with angina pectoris and known to inhibit CSA-induced impairment of mitochondrial function (Salducci, Chauvet-Monges et al. 1996), was used in an attempt to block CSA-mediated preconditioning. As shown previously (Griffiths and Halestrap 1993; Weinbrenner, Liu et al. 1998) CSA was protective, hearts showed an infarct size of  $12.5 \pm 1.4\%$  (controls  $30.2 \pm 1.3\%$ ,  $p < 0.001$ ). TMZ had no effect on infarct size when given on its own ( $33.1 \pm 1.6\%$ ) but completely blocked the CSA-mediated reduction in infarct size ( $31.9 \pm 1.0\%$ ,  $p = \text{NS}$  vs. controls, figure 12) supporting the notion that a mitochondrial mechanism might be responsible for the protection seen with CSA.



**Figure 11** Infarct size following a 35 min occlusion of the left anterior descending artery expressed as a percentage of the risk zone (I/R ratio). DNP and ischaemic preconditioning protect against infarction (\*  $p < 0.001$  vs. controls). This effect is attenuated by trimetazidine (TMZ, \*  $p < 0.001$  vs. IPC and †  $p < 0.007$  vs. controls).  $n = 6$  in each group.



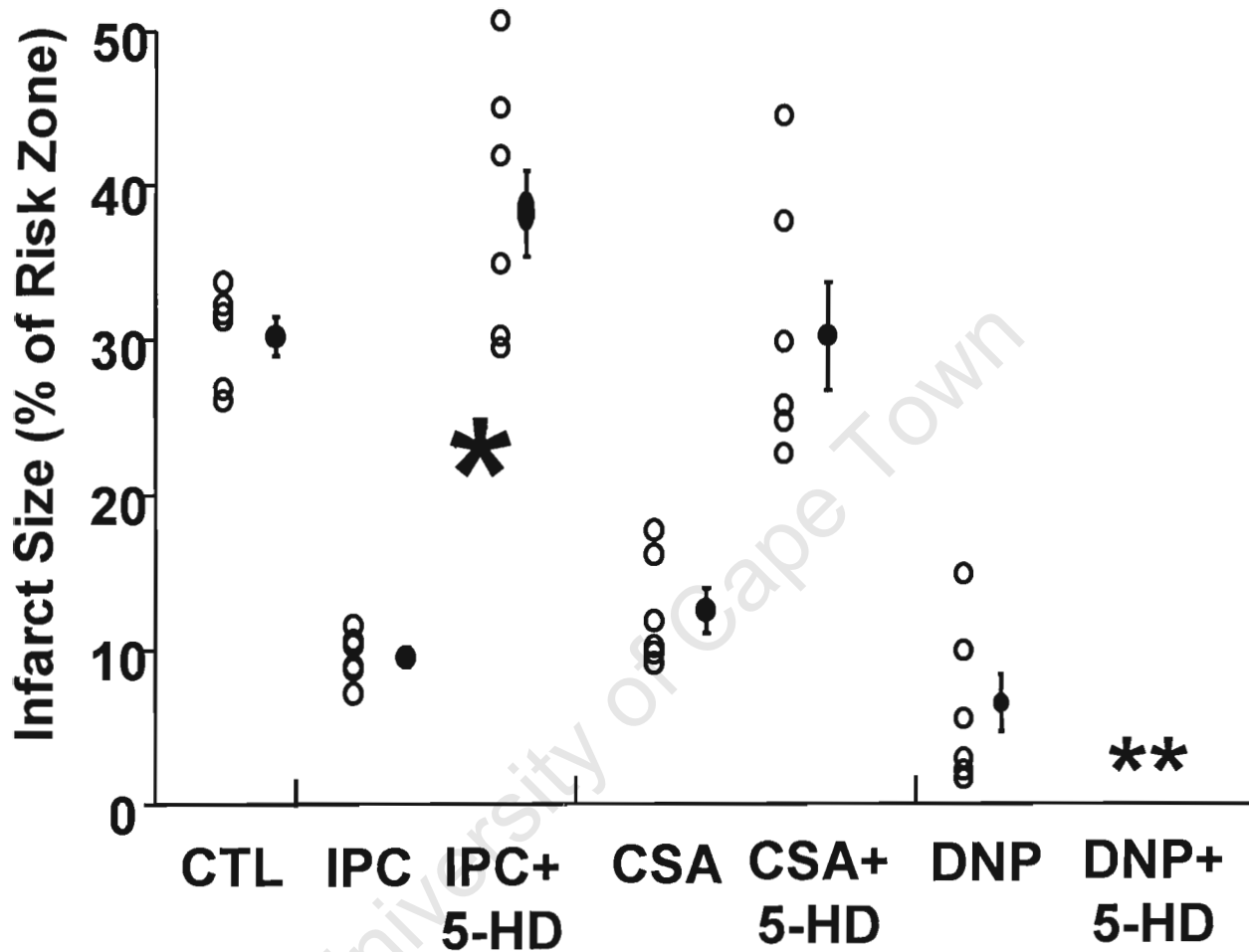
**Figure 12** Infarct size following a 35 min occlusion of the left anterior descending artery expressed as a percentage of the risk zone (I/R ratio). Cyclosporine A reduced infarct size which was reversed by the coadministration of trimetazidine, n = 6 in each group.

### **Trimetazidine partially blocks ischaemic preconditioning**

If mitochondrial stress exerts beneficial effects in preconditioning mitochondrial protection might actually be detrimental in this setting. To test this hypothesis the anti-ischaemic drug TMZ, known to protect the ischaemic mitochondrion, was investigated during an IPC protocol. In support of the underlying hypothesis the presence of TMZ limited the infarct reducing effects of IPC by about 50% (I/R ratio: IPC/TMZ  $22.2 \pm 2.0\%$ ,  $p < 0.001$  vs. IPC, figure 11).

### **The mitoK<sub>ATP</sub> channel blocker 5-hydroxydecanoate abolishes preconditioning**

5-hydroxydecanoate (5-HD) is a selective blocker of the mitoK<sub>ATP</sub> channel (Auchampach, Grover et al. 1992) with a 1000x higher affinity for the mitoK<sub>ATP</sub> over the sarcolemmal K<sub>ATP</sub> channel (Garlid, Paucek et al. 1997) and abolishes ischaemic preconditioning in a variety of models. The effects of 5-HD on ischaemic preconditioning, CSA and DNP-induced protection were investigated in the isolated rat heart model. The mitoK<sub>ATP</sub> blocker abrogated the protection seen with IPC and CSA (I/R ratio: IPC/5-HD  $33 \pm 1.3\%$ , CSA/5-HD  $32 \pm 4.1\%$ ). Unfortunately the effect of 5-HD on DNP mediated PC could not be evaluated in this model since the coadministration of DNP and 5-HD resulted in contracture in all hearts tested (figure 13). Nevertheless, the data on 5-HD obtained in the isolated rat heart model support the concept of mitochondrial involvement in preconditioning.



**Figure 13** Infarct size following a 35 min occlusion of the left anterior descending artery expressed as a percentage of the risk zone (I/R ratio). 5-HD abolishes the protection seen with ischaemic preconditioning, cyclosporine. \*\*The combination of 5-HD and DNP resulted in contracture in all hearts tested, n = 6 in each group.

## II. Early mitochondrial signaling

### **Preconditioning improves post-ischaemic cell viability in adherent transformed cell lines**

The main finding of the experiments described so far is that transient mitochondrial uncoupling using 2,4-dinitrophenol (DNP) in the isolated rat heart confers protection against myocardial infarction to a similar degree as ischaemic preconditioning (Minners, van den Bos et al. 2000). In order to assess parameters of mitochondrial function more directly, a whole cell model of preconditioning was employed investigating two distinct cell lines. Mitochondrial parameters were investigated in a human derived atrial cell line (Girardi cells) and in a skeletal mouse cell line (C2C12 myotubes).

To confirm that Girardi cells and C2C12 myotubes could be preconditioned cells were subjected to 8 hours of simulated ischaemia (pH 6.2, 1% oxygen, (Esumi, Nishida et al. 1991)). Preconditioning was achieved by either 30min of simulated ischaemia, adenosine (100 $\mu$ M) or diazoxide (100 $\mu$ M) treatment followed by a 30 min washout. Cell viability was assessed by measuring LDH release spectrophotometrically and propidium iodide uptake on flow cytometry. The results are summarized in table 3. Preconditioning in Girardi cells reduced LDH release to 65 $\pm$ 9% (simulated ischaemia), 68 $\pm$ 4% (adenosine), and 70 $\pm$ 5% (diazoxide) of non-preconditioned control cells ( $p < 0.01$  vs. controls). Using propidium iodide exclusion as an index of cell viability the corresponding values were 76 $\pm$ 6% (simulated ischaemia), 72 $\pm$ 4% (adenosine) and 74 $\pm$ 3% (diazoxide,  $p < 0.01$  vs. controls).

Co-administration of 5-HD during preconditioning abolished the protection against a subsequent simulated ischaemic insult. The LDH release following ischaemic preconditioning in the presence of 5-HD was  $83\pm 12\%$ , adenosine+5-HD was  $92\pm 4\%$  and following diazoxide+5-HD  $102\pm 5\%$  ( $p=n.s.$  vs. controls). In parallel, propidium iodide uptake was increased to  $107\pm 9\%$  in cells undergoing ischaemic preconditioning+5-HD, to  $105\pm 3\%$  in the adenosine+5-HD and to  $98\pm 5\%$  in the diazoxide+5-HD group ( $p=n.s.$  vs. controls). Similar cytoprotective effects of these preconditioning triggers were obtained using C2C12 myotubes (table 3).

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**Table 3 Cell survival in preconditioned vs. control cells**

<b>Girardi cells</b>	<b>LDH % vs. control</b>	<b>PI % vs. control</b>
Control	100 +/- 0	100 +/- 0
IPC	65 +/- 9	76 +/- 6 *
IPC/5-HD	83 +/- 12	107 +/- 9
Adenosine	68 +/- 4	72 +/- 4 *
Adenosine/5-HD	92 +/- 4	105 +/- 3
Diazoxide	70 +/- 5	74 +/- 3 *
Diazoxide/5-HD	102 +/- 5	98 +/- 5
<b>C2C12 myotubes</b>		
Control	100 +/- 0	100 +/- 0
IPC	58 +/- 5	48 +/- 3 *
IPC/5-HD	90 +/- 8	78 +/- 12
Adenosine	70 +/- 4	72 +/- 5 *
Adenosine/5-HD	81 +/- 6	84 +/- 4
Diazoxide	59 +/- 9	52 +/- 4 *
Diazoxide/5-HD	98 +/- 11	89 +/- 6

Values are given as mean +/- SEM, n>6 in each group. IPC = ischaemic preconditioning with simulated ischaemia, 5-HD = 5-hydroxydecanoate, all p<0.01 vs. non-preconditioned controls

## **Pharmacologic preconditioning with diazoxide acutely increases mitochondrial volume**

Modulation of mitochondrial volume has been described as a fundamental mechanism of respiratory control (Halestrap 1989). Since the experiments presented so far suggest the involvement and modulation of respiratory control in preconditioning, mitochondrial volume changes in the cellular model of preconditioning were assessed. Measuring the volume of in situ mitochondria is feasible using flowcytometric forward scatter (FS) data from whole cell measurements. Relative changes in mitochondrial volume (see material and methods (Quinlan, Thomas et al. 1983; Vander Heiden, Chandel et al. 1997; Macouillard-Poulletier de, Belaud-Rotureau et al. 1998) can be tracked as long as whole cell volume is unchanged. To circumvent methodological problems with the evaluation of mitochondrial parameters during the trigger ischaemia the following experiments were performed during pharmacological preconditioning with diazoxide.

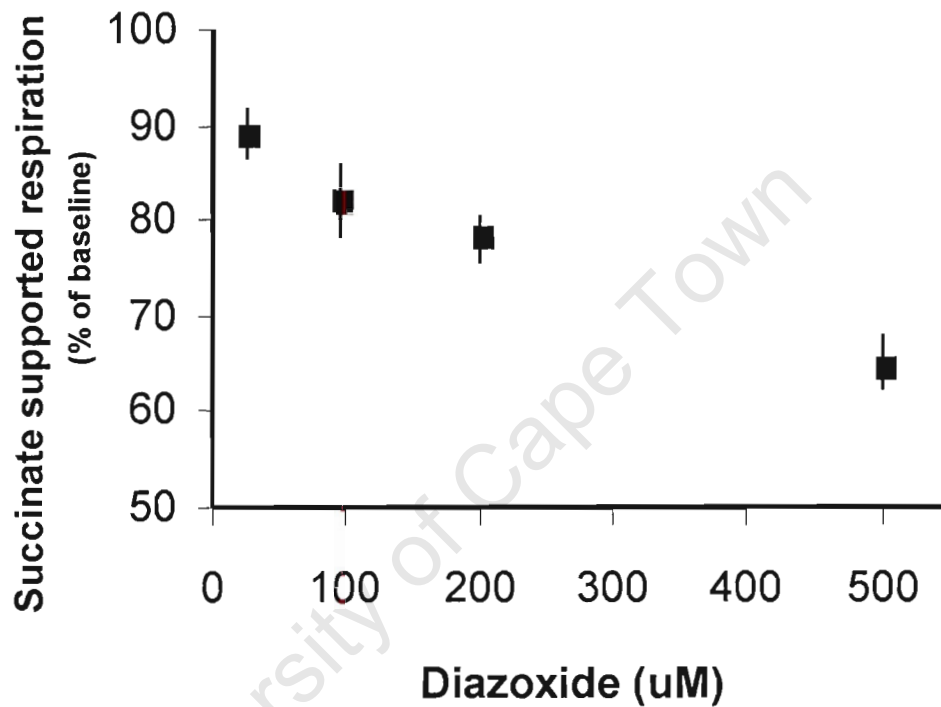
Firstly whole cell volume was investigated in the present model of preconditioning using a cell coulter. No differences in cell size were found (data not shown). Subsequently, forward scatter (FS) data obtained during the preconditioning experiments were analysed. These experiments were performed using the preconditioning mimetic diazoxide. CCCP (100 $\mu$ M) was used as a positive control which previously has been shown to reproducibly increase mitochondrial volume as assessed by forward scatter on flow cytometry (Macouillard-Poulletier de, Belaud-Rotureau et al. 1998). In the present study 5 min of incubation with diazoxide increased mitochondrial

volume to  $112 \pm 2\%$  ( $p < 0.001$  vs. DMSO treated controls). After 30 min the mitochondrial volume had returned to baseline ( $98 \pm 3\%$ ,  $p = \text{n.s.}$  vs. controls) highlighting the dynamic nature of changes in mitochondrial volume during the early phase of preconditioning.

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## **Diazoxide decreases succinate-supported respiration**

The prototypical potassium channel opener diazoxide may exert its cytoprotective effect via activation of the mitoKATP channel. However, mitoKATP channel independent effects of diazoxide on mitochondrial function have been described including the inhibition of mitochondrial respiration at the level of succinate dehydrogenase (complex II) (Schafer, Wegener et al. 1969; Ovide-Bordeaux, Ventura-Clapier et al. 2000; Hanley, Mickel et al. 2002). Our initial data in permeabilized C2C12 myotubes confirmed the previous observations in skinned cardiac muscle strips (Ovide-Bordeaux, Ventura-Clapier et al. 2000) in that a dose-dependent attenuation in succinate-supported mitochondrial respiration following the administration of diazoxide was found (figure 14). Expressed as the ratio respiratory rate before / respiratory rate after the addition of diazoxide the highest concentration of diazoxide used (500 $\mu$ M) diminished oxygen consumption to a value of  $0.65\pm 0.7$  or by approximately 35%. Moreover, the attenuation of oxygen consumption was sustained by the concentrations of diazoxide routinely used as a preconditioning-mimetic (30 $\mu$ M diazoxide  $0.89\pm 0.1$ , 100 $\mu$ M diazoxide  $0.82\pm 0.2$ , figure 14). This effect was electron transfer chain complex specific in that diazoxide had no effect on the rate of oxygen consumption in the presence of the complex I substrates glutamate and malate (100 $\mu$ M diazoxide  $1.03\pm 0.2$ , 500 $\mu$ M diazoxide  $1.04\pm 0.4$ ).

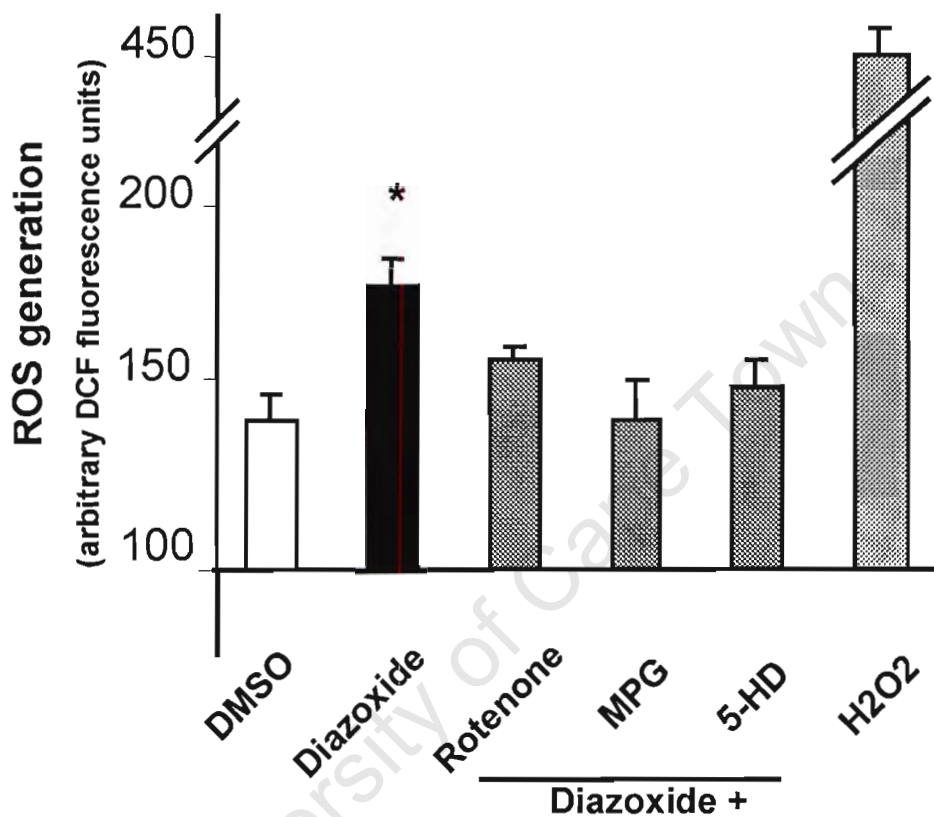


**Figure 14** Dose-dependent inhibition of succinate supported respiration by diazoxide. Oxygen consumption was measured with a Clarke-type electrode in digitonin permeabilized C2C12 cells respiring on succinate (10mM). After 5 min diazoxide was added and oxygen consumption evaluated for an additional 5 min. The data are expressed as the percentage change pre vs. post diazoxide addition. Diazoxide had no effect on glutamate/malate supported respiration

## Diazoxide increases ROS production in C2C12 myotubes

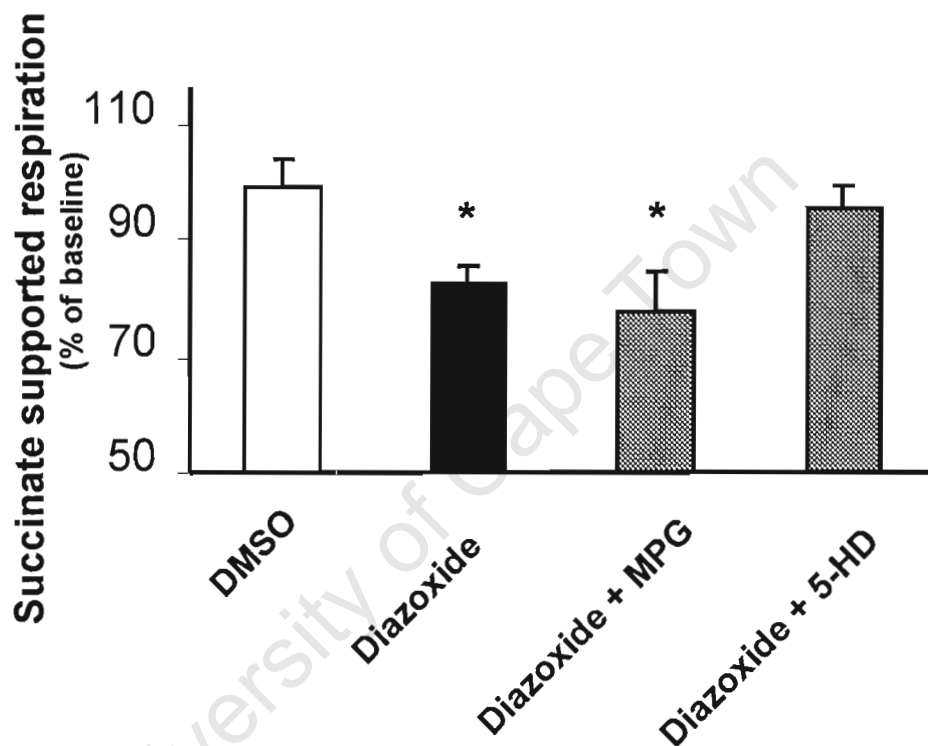
Inhibitors of the respiratory chain classically increase electron transfer chain ROS production (Ambrosio, Zweier et al. 1993). I therefore investigated whether the respiratory chain inhibition induced by diazoxide was sufficient to augment reactive oxygen species production. Incubation of the C2C12 myotubes with diazoxide (100 $\mu$ M) for 5 minutes significantly increased ROS production to 176 $\pm$ 4 arbitrary fluorescence units (a.u.) as measured by DCF signal on flow cytometry compared to DMSO treated control cells (140 $\pm$ 6 a.u.,  $p$ <0.01, figure 15). The fluorometric readings at 15 minutes incubation with diazoxide showed that this effect was sustained (DMSO controls: 163 $\pm$ 6 a.u. vs. diazoxide 205 $\pm$ 5 a.u., basal control = 100,  $p$ <0.01).

The sites of mitochondrial ROS production within the respiratory chain are still under investigation, although, most investigators agree that the major sites are within complexes I and III of the electron transfer chain (see introduction, figure 4). Interestingly, recent data suggest that the major physiologically relevant mitochondrial site of ROS generation may be within complex I (Kushnareva, Murphy et al. 2002; Liu, Fiskum et al. 2002). It was therefore explored whether a reverse flow of electrons through complex I might be responsible for the increase in ROS observed in response to diazoxide administration.



**Figure 15** Flowcytometric analysis of reactive oxygen species generation as indicated by DCF fluorescence in C2C12 myotubes. After baseline measurements cells were incubated with diazoxide (100uM) for 5 min. The complex I inhibitor rotenone (10uM), the antioxidant MPG (100uM) and the medium chain fatty acid 5-HD (500uM) abolished the diazoxide-induced increase in ROS generation (\* $p < 0.01$ , diazoxide vs. DMSO controls).

According to the work of Liu and colleagues (Liu and Gutterman 2002) I reasoned that if diazoxide-mediated ROS production was attenuated by an inhibitor of complex I, then the source of ROS would be via reverse electron flow through complex I. Hence diazoxide was administered in the presence of the complex I inhibitor rotenone (10 $\mu$ M). As illustrated in figure 15 rotenone abolished ROS formation in diazoxide treated cells (diazoxide/rotenone: 155 $\pm$ 10 a.u., p=n.s. vs. DMSO), implicating reverse flow of electrons through complex I was responsible for the increase in ROS observed. Rotenone had no effect on DMSO treated cells (DMSO/rotenone: 143 $\pm$ 7 a.u., p=n.s vs. DMSO controls). To establish whether respiratory inhibition precedes ROS formation the effects of the antioxidant MPG (100 $\mu$ M) were investigated. MPG abolished the diazoxide mediated increase in DCF signal (139 $\pm$ 6 a.u., figure 15) but had no effect on the inhibition of succinate-supported respiration (figure 16).



**Figure 16** 5-HD releases the diazoxide-induced respiratory inhibition. Succinate supported respiration was inhibited by 100uM diazoxide (\* $p < 0.01$  vs. DMSO controls), coadministration of MPG had no effect ( $p < 0.01$  vs DMSO controls). In contrast 5-HD abolished the decrease in respiration caused by diazoxide ( $p = n.s.$  vs. DMSO controls) but had no effect on respiration when given on its own (data not shown).

## **5-HD releases diazoxide-induced respiratory inhibition**

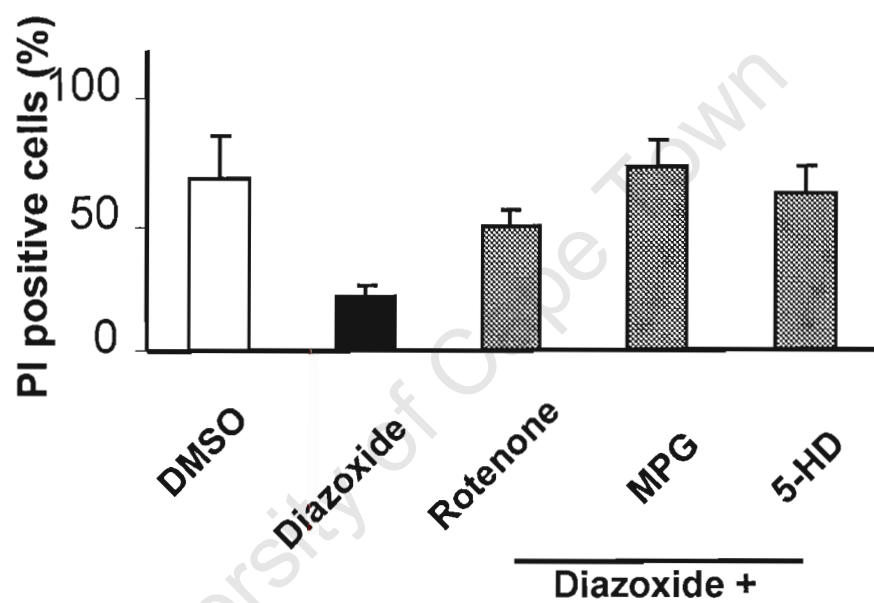
The medium chain fatty acid 5-hydroxydecanoate abolishes diazoxide-mediated protection hypothetically via inhibition of the mitoKATP channel (Auchampach, Grover et al. 1992; Liu, Sato et al. 1999). However, Hanley et al (Hanley, Mickel et al. 2002) recently presented data showing that 5-HD serves as a substrate for acyl-CoA synthase. These investigators hypothesized that 5-HD acyl-CoA could circumvent diazoxide-induced metabolic inhibition by feeding electrons into the respiratory chain at the level of ubiquinone. Here, data are presented to support this hypothesis in that the co-administration of 5-HD reverses the decrease in diazoxide-mediated inhibition of succinate-driven respiration (diazoxide:  $82\pm 4\%$  vs. diazoxide/5-HD:  $95\pm 5\%$ ,  $p < 0.001$ , figure 16).

## **Rotenone, MPG and 5-HD abolish diazoxide induced cellular protection**

In extension to the results on the cytoprotective effects of diazoxide presented in table 3 and in order to establish whether the above findings correlate with the protective properties of diazoxide, C2C12 myotubes cells were subjected to 7 hours of simulated ischaemia, as described above (Minners, Lacerda et al. 2001). Pretreatment with diazoxide (30min,  $100\mu\text{M}$ , 60min wash out) decreased propidium iodide (PI) positive cells from  $69\pm 16\%$  to  $22\pm 5\%$  ( $p < 0.001$ , figure 17). The cellular protection was abolished by MPG and 5-HD (diazoxide/MPG:  $72\pm 11\%$ , diazoxide/5-HD:  $62\pm 10\%$ ,  $p = \text{n.s.}$  vs. DMSO controls). Importantly,

rotenone also attenuated the protection afforded by diazoxide (diazoxide/rotenone  $49\pm 8\%$ ,  $p < 0.001$  vs. diazoxide,  $p < 0.01$  vs. DMSO controls), indicating that the described reverse electron flow through complex I is operational in diazoxide-mediated cellular protection. A general toxic effect of rotenone is unlikely as the coadministration of DMSO and rotenone was undistinguishable from DMSO controls (DMSO/rotenone:  $76\pm 13\%$ , DMSO controls:  $69\pm 16$ ,  $p = n.s.$ ).

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**Figure 17** Diazoxide-induced cellular protection is abolished by MPG and 5-HD, and attenuated by rotenone as indicated by propidium iodide (PI) exclusion (\*\* $p < 0.001$ , \* $p < 0.01$ ).

### **III. Characterizing the preconditioned state**

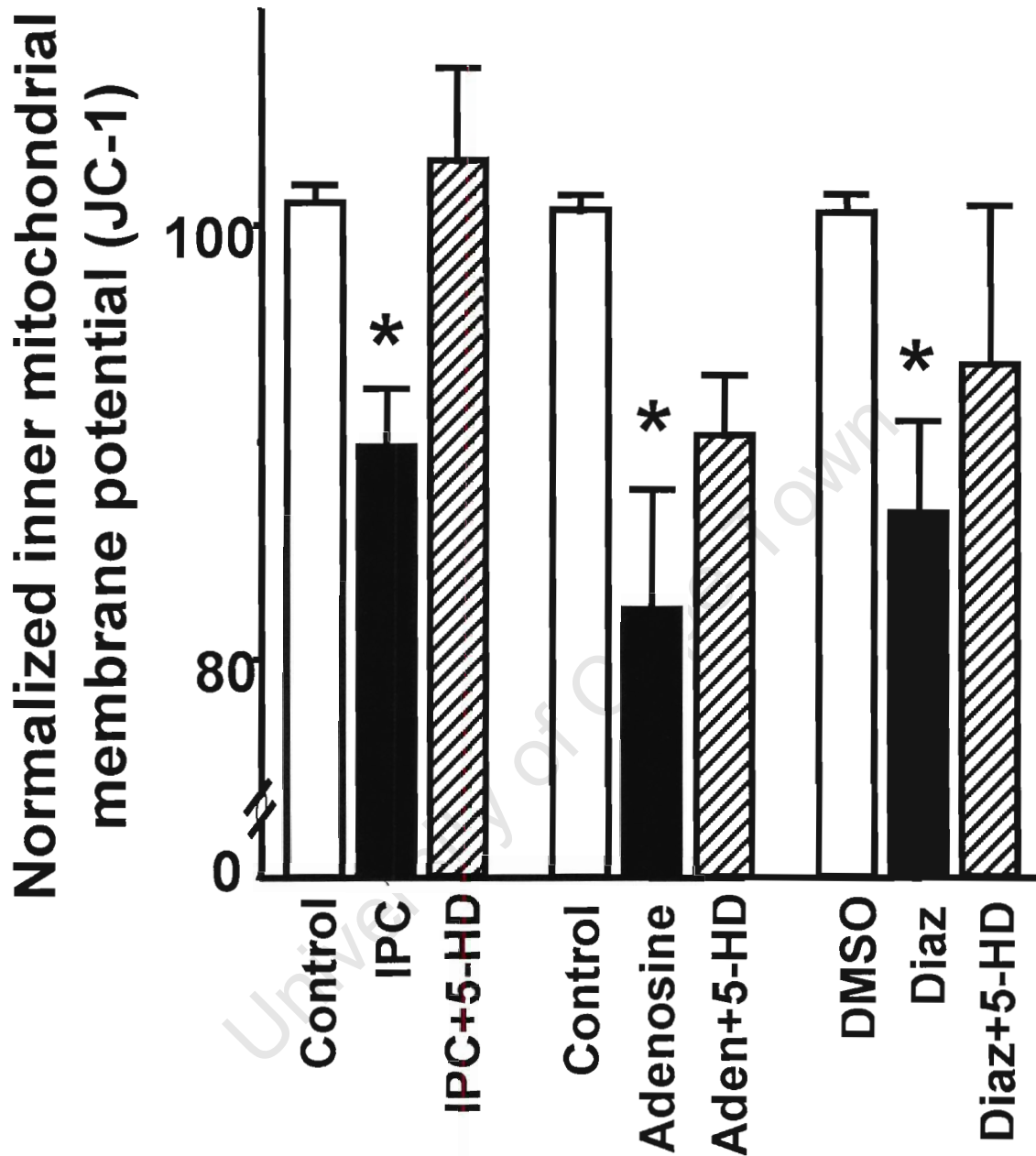
#### **Preconditioning depolarizes mitochondrial inner membrane potential**

The initial finding of my dissertation demonstrated that transient mitochondrial uncoupling using 2,4-dinitrophenol (DNP) in the isolated rat heart confers protection against myocardial infarction to a similar degree as ischaemic preconditioning (Minners, van den Bos et al. 2000). Mitochondrial uncoupling describes a dissociation of mitochondrial respiration from ATP synthesis and is characterized by a dissipation of the  $H^+$  gradient across the inner mitochondrial membrane and a subsequent increase in oxygen consumption that is not associated with an increase in ATP production (Bashan, Burdett et al. 1993). To evaluate whether modulation of mitochondrial parameters as seen by uncoupling is a sustained and universal component of the preconditioning program the preconditioned state was assessed following a multitude of preconditioning triggers. The cellular models described above involving a human derived atrial cell line (Girardi cells) and a skeletal mouse cell line (C2C12 myotubes) were employed to investigate inner mitochondrial membrane potential, oxygen consumption and whole cell ATP levels following simulated ischaemic, adenosine and diazoxide preconditioning.

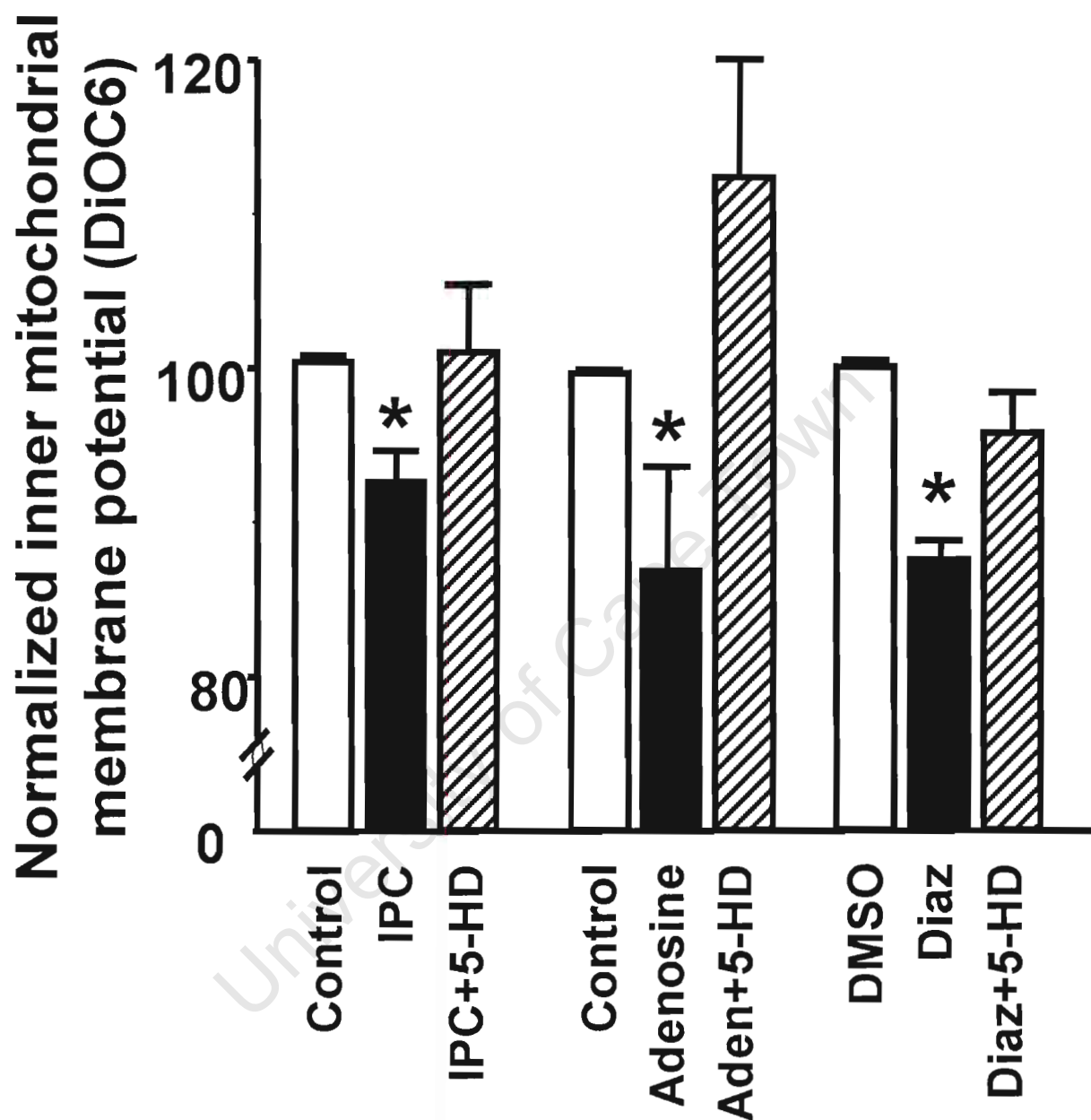
Experiments were performed during the preconditioned state, which is best represented by the time point just prior to the prolonged 7-8 hour index ischaemia. During this particular period preconditioned cells were found to exhibit a modestly depolarized inner mitochondrial membrane. Preconditioning with simulated ischaemia decreased mitochondrial membrane potential as

reflected by the aggregate (red) signal of JC-1 to  $90\pm 3\%$  in Girardi cells (figure 14). The corresponding value for adenosine was  $82\pm 7\%$  and for diazoxide  $87\pm 4\%$  ( $p < 0.05$  vs. control 100%). The co-administration of 5-HD abolished the inner mitochondrial membrane depolarization that had been induced by preconditioning - simulated ischaemia + 5-HD  $103\pm 5\%$ , adenosine + 5-HD  $90\pm 3\%$ , and diazoxide + 5-HD  $94\pm 5\%$  ( $p = \text{ns}$  vs. controls, figure 14).

A second potentiometric dye DiOC6 showed similar results (simulated ischaemia  $93\pm 5$ , adenosine  $87\pm 7\%$ , diazoxide  $88\pm 1\%$ ,  $p < 0.05$ , figure 15). Again, this depolarized phenotype was abolished by the co-administration of 5-HD (figure 15). The mitochondrial data using C2C12 cells is summarized in table 4, for this and all subsequent measurements.



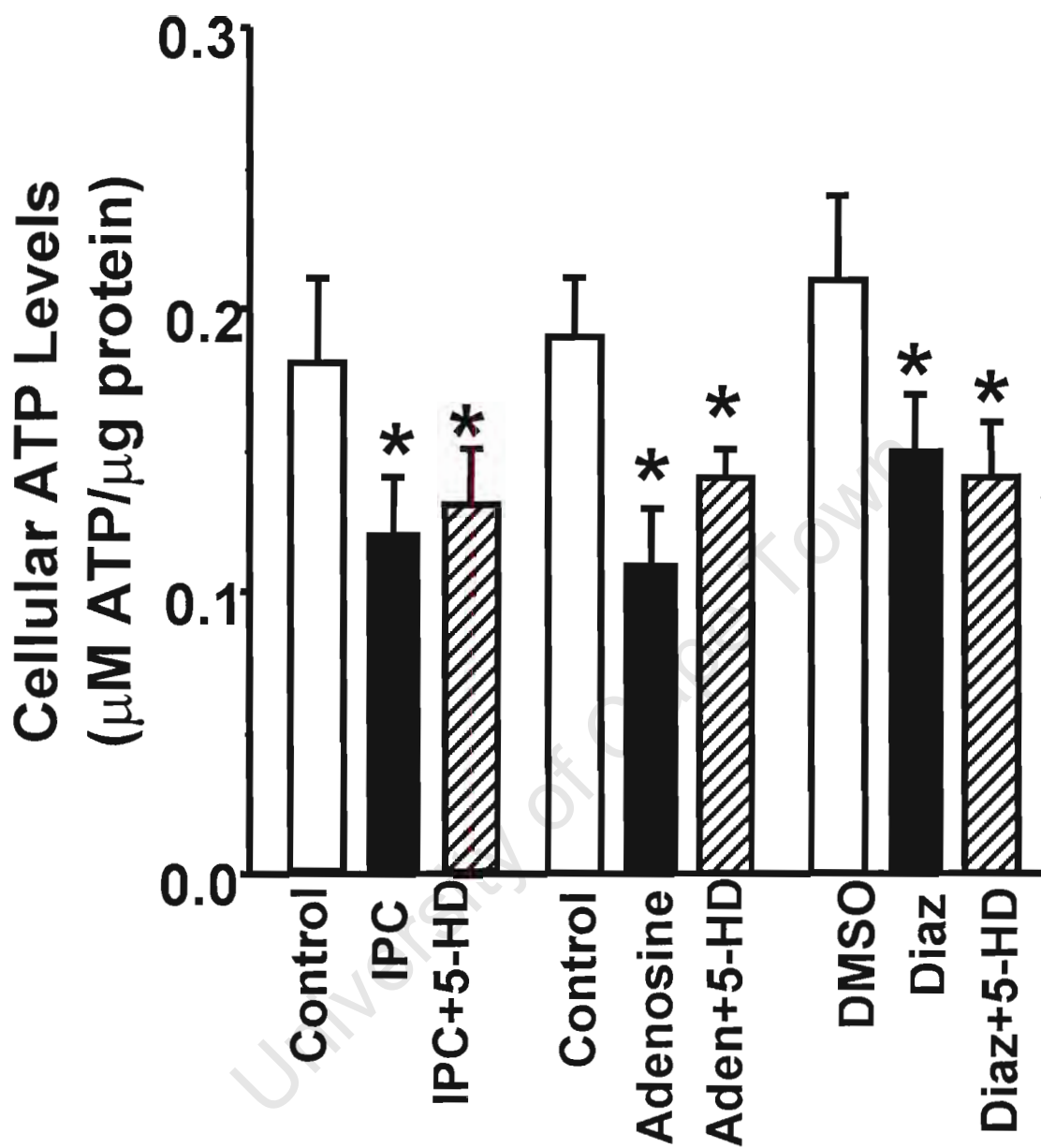
**Figure 18** Flowcytometric analysis of preconditioning-induced changes in inner mitochondrial membrane potential in Girardi cells. Staining with the potentiometric dye JC-1 revealed a modest mitochondrial depolarization in preconditioned cells prior to index ischemia. The mitochondrial  $K_{ATP}$  channel blocker 5-HD reversed the changes in mitochondrial membrane potential.  $n \geq 6$ , \*  $p < 0.05$  vs. non-preconditioned control cells.



**Figure 19** Flowcytometric analysis of preconditioning-induced changes in inner mitochondrial membrane potential in Girardi cells. Similarly to the findings with JC-1 staining with the potentiometric dye DiOC6 revealed a modest mitochondrial depolarization in preconditioned cells prior to index ischemia. The mitochondrial  $K_{ATP}$  channel blocker 5-HD reversed the changes in mitochondrial membrane potential.  $n \geq 6$ , \*  $p < 0.05$  vs. non-preconditioned control cells.

## Preconditioning decreases cellular ATP concentrations

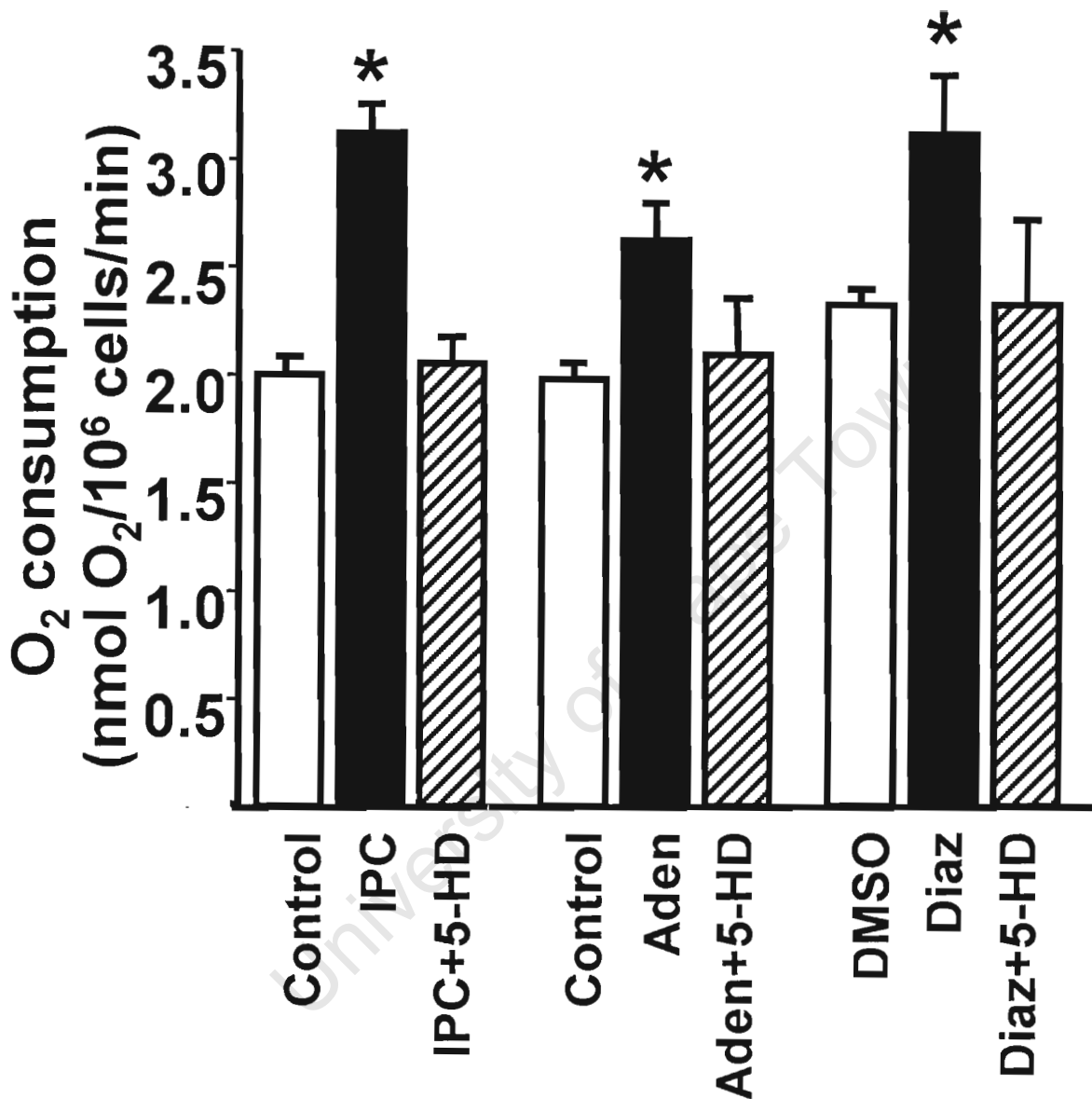
Similar to the results published by Murry and coworkers in 1986 (Murry, Jennings et al. 1986) preconditioned cells exhibit lower ATP levels than controls when investigated just prior to index ischaemia (control  $0.21 \pm 0.03$  nM/ $\mu$ g protein, simulated preconditioning ischaemia  $0.12 \pm 0.02$ , adenosine  $0.15 \pm 0.02$ , diazoxide  $0.11 \pm 0.02$ ,  $p < 0.05$ ,  $n = 6$ , figure 16). Interestingly, 5-HD the known antagonist of preconditioning induced cytoprotection did not modulate cellular ATP concentrations when co-administered with the preconditioning triggers (simulated ischaemia+5-HD  $0.13 \pm 0.02$  nM/ $\mu$ g protein, adenosine+5-HD  $0.14 \pm 0.02$ , diazoxide+5-HD  $0.14 \pm 0.01$ ,  $p < 0.05$  vs. control cells, figure 16).



**Figure 20** Preconditioning-induced changes in cellular ATP levels in Girardi cells. Using recombinant firefly luciferase bioluminescence cellular ATP levels are shown to be decreased in preconditioning with either simulated ischemia, adenosine or diazoxide. 5-HD had no significant effect on preconditioning induced attenuation of ATP levels.  $n \geq 10$ ,  $*p < 0.05$  vs. non-preconditioned cells.

## Oxygen consumption is increased in preconditioned cells

The classical definition of uncoupling oxidation from phosphorylation by increasing the proton leak via the inner mitochondrial membrane includes decreased membrane potential, decreased ATP production and resultant increase in oxygen consumption in an attempt to re-establish the proton gradient (Laiho and Trump 1975). In the previous two chapters the findings relating to membrane potential and ATP levels were described. The final parameter of mitochondrial function to be investigated in this study was, therefore, oxygen consumption, using a Clarke-type electrode. Control (non-preconditioned) Girardi cells consumed oxygen at a rate of  $2.3 \pm 0.1$  nM oxygen/min/ $1 \times 10^6$  cells. Preconditioning increased oxygen consumption (simulated ischaemia  $3.12 \pm 0.1$ , adenosine  $3.11 \pm 0.3$ , diazoxide  $2.6 \pm 0.2$ ,  $p < 0.05$ , figure 17). The increase in cellular oxygen consumption seen with PC was abolished by the co-administration of 5-HD (simulated ischaemia+5-HD  $2.05 \pm 0.1$  nM oxygen/min/ $1 \times 10^6$  cells, adenosine+5-HD  $2.3 \pm 0.4$ , diazoxide+5-HD  $2.1 \pm 0.3$ ,  $p = \text{n.s.}$  vs. controls, figure 17).



**Figure 21** Oxygen consumption in preconditioned Girardi cells. Oxygen consumption measurement using a Clark type electrode revealed that preconditioned cells exhibit increased oxygen consumption by approximately 25-30% compared to control cells. Co-administration of the mitochondrial  $K_{ATP}$  channel blocker 5-HD abolished the change in oxygen consumption.  $n \geq 5$ , \*  $p < 0.05$  vs. control cells.

**Table 4** Summarized data reflecting mitochondrial parameters during the preconditioned state in C2C12 myotubes.

**Inner mitochondrial membrane potential**

		JC-1 aggregate			
		n	mean (%)	SEM	p value
CONTROL		13	102.15	1.47	
ADENOSINE		7	89.57	3.06	<0.05
ADENO+5-HD		6	109.33	4.29	
DMSO		8	103.06	1.12	
DIAZOXIDE		6	91.4	1.75	<0.05
DIAZ+5-HD		6	98.0	2.88	
CONTROL		10	101.5	0.73	
IPC		7	83.9	4.78	<0.05
IPC+5-HD		7	100.3	4.24	
		DiOC6			
		n	mean (%)	SEM	p value
CONTROL		14	98.3	0.62	
ADENOSINE		8	92.5	2.38	<0.05
ADENO+5-HD		6	99.5	3.25	
DMSO		8	101.7	0.58	
DIAZOXIDE		6	87.6	1.84	<0.01
DIAZ+5-HD		6	104.7	3.01	
CONTROL		14	98.3	0.62	<0.05
IPC		5	95.0	1.05	
IPC+5-HD		5	102.6	2.32	
<b>Oxygen consumption (ng O<sub>2</sub>/min/10<sup>-6</sup> cells)</b>					
CONTROL		6	4.14	0.13	
ADENOSINE		6	5.49	0.27	<0.05
ADENO+5-HD		6	4.65	0.11	
DMSO		5	4.90	0.18	
DIAZOXIDE		5	5.73	0.15	<0.05
DIAZ+5-HD		5	4.76	0.22	
CONTROL		5	4.45	0.15	
IPC		5	6.05	0.26	<0.05
IPC+5-HD		5	4.13	0.27	
<b>ATP levels (micromol ATP/microgram protein)</b>					
CONTROL		5	0.28	0.01	
ADENOSINE		5	0.19	0.02	<0.05
ADENO+5-HD		5	0.14	0.01	<0.05
DMSO		6	0.31	0.02	
DIAZOXIDE		5	0.23	0.01	<0.05
DIAZ+5-HD		5	0.21	0.01	<0.05
CONTROL		6	0.31	0.01	
IPC		6	0.21	0.02	<0.05
IPC+5-HD		6	0.24	0.01	<0.05

## Discussion

The major findings of this thesis include

1. Modulation of mitochondrial homeostasis and in particular the application of sublethal mitochondrial stress induces preconditioning-like cardioprotection.
2. Pharmacological preconditioning directly modulates mitochondrial respiratory function and modulates mitochondrial ROS generation.
3. The preconditioned state is characterized by an uncoupled mitochondrial phenotype.

## **Mitochondrial stress induces preconditioning**

The first set of data provided in this thesis demonstrates that short-term administration of the uncoupling agent DNP has an ischaemic preconditioning-like protective effect against a subsequent ischaemia/reperfusion injury to the heart. Similarly, CSA, a potent inhibitor of the oxidative chain, preconditions the isolated rat heart. The anti-ischaemic drug trimetazidine (TMZ), thought to work in part via a mitochondrial 'protective' mechanism, reversed CSA-induced cardioprotection. Moreover, TMZ significantly reduced protection afforded by 'classical' ischaemic preconditioning. Taken together, the data presented strongly support the concept that mitochondrial stress may induce preconditioning-like cardioprotection.

### **DNP**

The protection afforded by a sublethal dose of the classical uncoupling agent DNP in the isolated rat heart was recently confirmed in a cellular model using isolated cardiac myocytes (Rodrigo, Lawrence et al. 2002). Rodrigo and coworkers showed that pretreatment with 50 $\mu$ M DNP for 7 min protected cells against injury caused by metabolic inhibition and reperfusion. Pretreated myocytes exhibited decreased calcium uptake, inner mitochondrial membrane potential and NADH levels. The latter two findings are consistent with the uncoupling properties of the compound, whereas the first was interpreted as indicating a possible mechanism by which DNP might exert its protective effect. In addition to modulating calcium handling treatment with DNP has been shown

to result in an approximate doubling of glucose transport in skeletal myotubes (Khayat, Tsakiridis et al. 1998). Interestingly, calcium also seems to play an important role in this PI3-kinase independent upregulation of glucose transport, since the addition of chelating agents resulted in an 80% reduction in glucose uptake. Alekseev and coworkers (Alekseev, Gomez et al. 1997) described a direct effect of DNP on potassium currents across the sarcolemma, which was independent of ATP levels. This interesting observation from patch-clamp studies allows for an alternate interpretation of the data presented here, in that direct modulation of sarcolemmal KATP channel activation by DNP independent of metabolic inhibition could have accounted for the observed protective effect.

## CSA

CSA administration, via numerous putative modes of action, is a potent cardioprotective agent. Firstly, Halestrap and coworkers demonstrated that CSA protective effects were linked to the inhibition of the mitochondrial transition pore (Griffiths and Halestrap 1993; Halestrap, Woodfield et al. 1997). Weinbrenner et al showed a protective role of CSA via inhibition of the PP2B phosphatase, calcineurin (Weinbrenner, Liu et al. 1998) which was subsequently found to extend to other phosphatase-inhibitors including FK506 (Cai, Baxter et al. 1998). Importantly, and in addition to its inhibitory effects on the mitochondrial transition pore and on calcineurin activity, CSA directly affects mitochondrial energy metabolism by inhibition of the respiratory chain (Fournier, Ducet et al. 1987; Strzelecki, Kumar et al. 1988) between cytochrome b and c1 (Aupetit, Ghazi et al. 1988). The relevance of these findings in an in vivo setting

was demonstrated in a recent study in rats showing that the metabolic inhibition by CSA is dose dependent (5-25mg/kg/day for 3 days) and correlates with the protection observed following an ischaemia/reperfusion insult (Niemann, Saeed et al. 2002). It appears evident that CSA acts via numerous pathways. The inhibition of mitochondrial respiration is not the best studied. However, the data presented offer an interesting alternate hypothesis on how CSA induces cardioprotection especially when considered in conjunction with the findings involving the mitochondrion protective agent TMZ discussed below.

## TMZ

If perturbation of mitochondrial homeostasis triggers the preconditioning program, then the finding that the antiischaemic, mitochondrial 'protective' (Veitch, Maisin et al. 1995) drug TMZ counteracts DNP, CSA and ischaemic preconditioning is of particular interest. TMZ's mode of action is not fully understood but it may facilitate glucose metabolism in ischaemic hearts, thus improving ATP production (Veitch, Maisin et al. 1995). However, additional actions may exist such as inhibition of ischaemic contracture independently of glycolytic flux (Boucher, Hearse et al. 1994). Moreover, administration of TMZ restored ATP synthesis and resulted in a reduction of CSA-induced increases in mitochondrial  $Ca^{2+}$  (Salducci, Chauvet-Monges et al. 1996; Simon, Tillement et al. 1997). Finally, TMZ partially counteracts CSA-induced mitochondrial swelling (Elimadi, Morin et al. 1997) and reversed ischaemia/reperfusion induced inhibition of mitochondrial function (Elimadi, Settaf et al. 1998). It is therefore conceivable that the attenuation of preconditioning-like cardioprotection by TMZ

may be explained in part by alleviating preconditioning-induced mitochondrial stress.

#### 5-HD

The mitoK<sub>ATP</sub> channel blocker 5-HD abolishes ischaemic preconditioning in numerous models (Auchampach, Grover et al. 1992; Garlid, Paucek et al. 1997; Baines, Liu et al. 1999), thereby mechanistically linking the mitochondrion with preconditioning. In the present study in the isolated rat heart, 5-HD not only reversed the protection afforded by IPC but also abolished CSA-induced cardioprotection. The initial interpretation of these findings was the following: Since the mitoK<sub>ATP</sub> channel is strongly inhibited by ATP a reduction in mitochondrial ATP production - be it via uncoupling with DNP or inhibition of mitochondrial respiration using CSA - would decrease ATP levels, thereby opening the mitoK<sub>ATP</sub> channel. Conversely, TMZ by conserving ATP would tend to keep the mitoK<sub>ATP</sub> channel closed. It is not certain that this proposal also applies to DNP since the combined administration of 5-HD and DNP led to irreversible contracture.

In light of subsequent findings presented in this thesis (see below) questioning the role of the mitoK<sub>ATP</sub> channel this interpretation was replaced by the view favoring a more direct effect of preconditioning triggers on mitochondrial homeostasis.

In summary, the data presented support the novel concept that mitochondrial stress (e.g. via DNP or CSA) can trigger the preconditioning program and that

conversely, mitochondrial protection (e.g. via TMZ) can limit preconditioning-like cardioprotection.

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## **Pharmacological preconditioning directly modulates mitochondrial function**

To further investigate the mitochondrial involvement in preconditioning a cellular model of preconditioning was established. The most important findings of these studies were that in response to the potassium channel opener diazoxide a substrate specific inhibition of mitochondrial respiration is observed resulting in free radical formation, possibly via reverse electron flow through complex I. Interestingly, the medium chain fatty acid 5-HD released the respiratory inhibition caused by diazoxide. Taken together these data further support the concept of mitochondrial involvement in preconditioning and challenge the exclusivity of the mitoK<sub>ATP</sub> channel in this cell-protective phenomenon.

The preponderance of scientific experiments which demonstrate the central role of mitoK<sub>ATP</sub> channel activation in classical cardiac preconditioning has been performed in in-vivo and ex-vivo animal studies (reviewed(Oldenburg, Cohen et al. 2002)). Due to the technical limitations of studying mitochondrial biology in the in-vivo animal or intact heart, the majority of studies to ascertain mitochondrial function have been performed in permeabilized muscle strips, in cell culture and in isolated mitochondria. In these latter systems, the prototypic mK<sub>ATP</sub> channel activator – diazoxide has been used as the pharmacologic trigger to induce the preconditioning phenotype (Garlid, Paucek et al. 1997). These studies focussing on the mitochondrial response to ischaemic and diazoxide preconditioning illustrate the emerging pattern of mitochondrial phenotypic perturbations in response to mK<sub>ATP</sub> channel activation. The biologic perturbations associated with mK<sub>ATP</sub> channel activation have proven to be

dynamic and different mitochondrial effects have been described at temporally distinct periods of observation. To analyze these perturbations it is useful to conceptually divide a preconditioning protocol into 1) the trigger phase, 2) the preconditioned state and 3) the ischaemia/reperfusion phase.

### **Trigger-phase mitochondrial modulation**

The dose-dependent and substrate specific inhibition of mitochondrial respiration observed in the present permeabilized cell model opens a new perspective on the proposed mechanism by which diazoxide exerts its cytoprotective properties. With respect to direct effects of diazoxide on mitochondrial biology Terzic and colleagues have investigated isolated cardiac mitochondria in response to pharmacologic  $mK_{ATP}$  channel activation (Holmuhamedov, Jovanovic et al. 1998). Here numerous perturbations in function including: depolarization of the inner mitochondrial membrane; accelerated respiration; slowed ATP production; release of accumulated mitochondrial  $Ca^{2+}$ ; mitochondrial swelling and the efflux of intermembrane proteins such as adenylate kinase and cytochrome c, have been associated with  $mK_{ATP}$  channel activation (Holmuhamedov, Jovanovic et al. 1998). These data are conflicting in that the collective phenotype mechanistically supports both cell survival and cell death promoting cellular events. However, an emerging concept demonstrates that moderate mitochondrial 'stress', primes the entry of the mitochondria into a 'stress-resistant state' (Minners, van den Bos et al. 2000; Dzeja, Holmuhamedov et al. 2001). The phenotypic response

to  $mK_{ATP}$  channel activation could induce this moderate mitochondrial 'stress'. This concept is further supported where diazoxide, albeit at higher dose than is routine, has been shown to acutely induce transient low conductance opening of the mitochondrial permeability transition pore (MPTP)(Kato, Nishigaki et al. 2002) – a putative alternate 'mitochondrial-stress' that programs the subsequent cell-survival phenotype.

#### Exclusivity of the mitoKATP channel

The exclusivity of  $mK_{ATP}$  channel activation in the mitochondrial perturbations described above has begun to be questioned in numerous studies. Terzic's laboratory has demonstrated this where the ROS attenuating effects of  $mK_{ATP}$  channel openers in the ischemia-reperfusion phase was maintained in a nominally  $K^+$ -free medium (Ozcan, Bienengraeber et al. 2002). These investigators went on to demonstrate that this oxidant stress attenuation was present in the presence or absence of the  $K^+$ -ionophore valinomycin and was mimicked by malonate, a modulator of the mitochondrial redox state (Ozcan, Bienengraeber et al. 2002). These investigators postulate that a  $K^+$  conductance-independent pathway may be activated by the  $mK_{ATP}$  openers to confer mitochondrial protection.

A number of laboratories have supported the concept of  $K^+$  conductance-independent effects of diazoxide (Schafer, Wegener et al. 1969; Ovide-Bordeaux, Ventura-Clapier et al. 2000; Hanley, Mickel et al. 2002). These groups all demonstrate that a cardioprotective dose of diazoxide selectively attenuates succinate oxidation. In the present study these findings were

confirmed and found to be intricately linked to the preconditioning phenotype. Interestingly, Daut and colleagues also go on to demonstrate that pinacidil did not alter succinate oxidation, but selectively inhibited NADH oxidation (Hanley, Mickel et al. 2002). These data support a mechanistic link between partial inhibition of electron transport and preconditioning. Interestingly, partial inhibition of the mitochondrial electron transfer chain complex II with 3-nitropropionic acid confers cardioprotection in rabbits (Ockaili, Bhargava et al. 2001) and we and others have demonstrated cardioprotection with the transient administration of the mitochondrial uncoupler dinitrophenol (Minners, van den Bos et al. 2000; Rodrigo, Lawrence et al. 2002). Finally, the pretreatment of rats with cyclosporine A resulted in a reduction in ATP levels and a subsequent reduction in infarct size (Niemann, Saeed et al. 2002).

## ROS

It has recently been recognized that reactive oxygen species (ROS) play an important role in cellular signaling (Lander 1997; Finkel 1998) and orchestrate cell survival programs (Irani 2000). A putative role of ROS cytoprotective signaling in ischaemic preconditioning was previously demonstrated in the intact rat heart (Chen, Gabel et al. 1995) and in isolated cardiomyocytes (Vanden Hoek, Becker et al. 1998). Subsequently, treatment with diazoxide has been shown to result in ROS generation (Forbes, Steenbergen et al. 2001) and has led to the view that ROS generation resulted from  $mK_{ATP}$  channel activation (reviewed(Oldenburg, Cohen et al. 2002)). The mechanism whereby  $mK_{ATP}$  channel activation generates mitochondrial ROS production is unclear, but this

ROS production is clearly associated with a cytoprotective phenotype in preconditioning (Oldenburg, Cohen et al. 2002). Hence, a feasible mechanism of  $mK_{ATP}$  channel activation induced protection could include cytoprotective ROS signaling during this trigger-phase of preconditioning.

Inhibition of the respiratory chain has been shown to induce ROS (Cai and Jones 1999). In the present study the inhibition of mitochondrial respiration with diazoxide was shown to induce ROS generation, independent of  $mitoK_{ATP}$  channel activation. Respiration via complex I (glutamate/malate supported) was unaffected by diazoxide and diazoxide had no effect on ROS generation in this setting. Secondly, the antioxidant MPG abolished the diazoxide induced increase in ROS but had no effect on respiratory inhibition. Interestingly, 5-HD released respiratory inhibition and abolished ROS generation (see below). The data suggest that the direct effect of diazoxide on succinate-supported respiration is responsible for subsequent ROS generation and resultant cellular protection.

#### Source of ROS

The source of ROS within the mitochondrial respiratory chain has been mapped to complex I and complex III (figure 4). Recently, reverse flow of electrons through complex I has been identified as an important source of ROS generation in cellular systems (Cino and Del Maestro 1989; Paraidathathu, de Groot et al. 1992; Kushnareva, Murphy et al. 2002; Liu, Fiskum et al. 2002). Cino and Maestro (Cino and Del Maestro 1989) showed that succinate supported hydrogen peroxide generation could be inhibited with rotenone in rat

brain mitochondria. Similarly, Kehrer's group (Paraidathathu, de Groot et al. 1992) found rotenone to inhibit succinate-supported ROS generation in mitochondria derived from hypoxic rat hearts, indicating reversed flow of electrons through complex I as the source of ROS under these conditions. In the present study rotenone again abolished diazoxide-induced ROS generation implicating reversed flow of electrons through complex I in free radical generation under these circumstances.

#### Release of metabolic inhibition

In further support for a crucial role of modulation of the mitochondrial respiration in preconditioning Daut and colleagues demonstrated that the classical  $mK_{ATP}$  channel blocker, 5-hydroxydecanoate (5-HD), is converted to 5-HD-CoA (Hanley, Mickel et al. 2002). Here they postulate that  $\beta$ -oxidative metabolites of 5-HD-CoA could enter the electron-transfer chain at the level of ubiquinone, thereby compensating for the partial inhibition of the respiratory chain by diazoxide and pinacidil at complexes II and I respectively. This mitochondrial respiration augmenting effect of the medium chain fatty-acid 5-HD-CoA may explain a mechanism whereby 5-HD inhibits preconditioning. In support of the above findings we found that the respiratory inhibition caused by diazoxide was released by the coadministration of 5-HD. It is tempting to speculate that rather than exerting its effects on the putative  $mitoK_{ATP}$  channel 5-HD abolishes diazoxide-induced protection by releasing the respiratory inhibition thereby preventing the generation of ROS necessary for subsequent cellular signaling.

Collectively, these data support a concept whereby partial respiratory inhibition evokes the preconditioning program via ROS formation and challenge the exclusive role of the mitoK<sub>ATP</sub> channel in diazoxide induced cellular protection.

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## **The preconditioned state is characterized by an uncoupled mitochondrial phenotype**

This time period following the post-ischemic 'trigger' or after the washout of diazoxide represents the cell and mitochondrial 'stress-resistant phase'. The mitochondrial perturbations identified here also reflect mitochondrial function immediately prior to the index ischemia/reperfusion insult. The data presented in the third part of this thesis demonstrate that mitochondria within intact preconditioned cells enter index ischaemia exhibiting features consistent with respiratory uncoupling, reflected by a decrease in inner mitochondrial membrane potential, reduced cellular ATP concentrations, and an increase in oxygen consumption. Thus, cells exhibiting what appears to be a less efficient energetic state immediately prior to prolonged ischaemia are more resistant to this lethal insult. This observation was recently confirmed in the intact rat heart in response to the  $K_{ATP}$  channel opener, P-1075 (Jilkina, Kuzio et al. 2002).

The mitochondrion is known to be a pivotal rheostat in determining cell survival vs. cell death. A multitude of mitochondrial processes including energy production, reactive oxygen species generation/signaling and calcium homeostasis are thought to be major contributors in this determination of cellular fate during cellular stress. How uncoupling of oxidation from phosphorylation by preconditioning can prime these mitochondria to display improved tolerance to ischaemia is the major question generated by the observations demonstrated in this thesis.

The exact effect of uncoupling of oxidative phosphorylation in preconditioning has not been established, however, plausible effects include uncoupling-

induced rapid glucose uptake (Khayat, Tsakiridis et al. 1998), the reduction in ROS generation with a concomitant limitation in oxidative damage (Dzeja, Holmuhamedov et al. 2001), improvement in  $(Ca^{2+})_m$  handling (Holmuhamedov, Jovanovic et al. 1998) or may be linked to maintenance of mitochondrial volume and thereby ATP/CK shuttling (Kowaltowski, Seetharaman et al. 2001).

The initial description of ischaemic preconditioning implicated a role for attenuated ATP depletion during ischaemia as a major factor in delaying ischaemic cell death (Murry, Jennings et al. 1986; Murry, Richard et al. 1990). More recently, Fryer et al (Fryer, Eells et al. 2000) demonstrated that preconditioning maintained mitochondrial ATP biosynthesis following the index ischaemia. Collectively, these and other data suggest that mitochondrial energetics may be central to the preconditioning program. How then could respiratory uncoupling support this hypothesis? One potential mechanism may be via uncoupling induced rapid glucose uptake, as evident in skeletal muscle cells (Khayat, Tsakiridis et al. 1998). The concept of enhanced glucose uptake and glycolytic flux following preconditioning has been demonstrated in the isolated rabbit and rat heart (Janier, Vanoverschelde et al. 1994; Tong, Chen et al. 2000). Thus, hypothetically uncoupling mediated glucose uptake may be a mechanism whereby this mitochondrial phenotype promotes tolerance to ischaemia.

## ROS

The generation of reactive oxygen species (ROS) by the mitochondria to function as signaling intermediates in the preconditioning program has recently been described (Vanden Hoek, Becker et al. 1998; Forbes, Steenbergen et al. 2001; Yao, McPherson et al. 2001). These modulatory effects are probably mediated by a modest induction of ROS (Tritto and Ambrosio 2001). Conversely, a more robust generation of these compounds can have detrimental effects on cellular survival and integrity (Carmody and Cotter 2001; Halmosi, Berente et al. 2001). The role of respiratory uncoupling in the generation of ROS has recently been explored in the context of genetic manipulation of the uncoupling peptides. Upregulation or overexpression of these peptides, have induced an uncoupled phenotype with a concurrent attenuation of the generation of ROS (Cortez-Pinto, Zhi Lin et al. 1999; Yamagishi, Edelstein et al. 2001). Conversely, the genetic ablation of uncoupling peptides (Arsenijevic, Onuma et al. 2000; Vidal-Puig, Grujic et al. 2000) uniformly demonstrate increased respiratory coupling and an accompanying augmentation of ROS production. Collectively these data would be consistent with a potential role for enhanced mitochondrial respiratory uncoupling as a regulatory process that may limit excessive ROS generation in the context of ischaemia.

## Ca<sup>++</sup>

A reduction in mitochondrial calcium-overload, has been demonstrated with preconditioning and is postulated to contribute to ischaemic-tolerance (Fralix, Murphy et al. 1993; Przyklenk, Simkhovich et al. 1999; Wang, Cherednichenko et al. 2001). The role of respiratory uncoupling on mitochondrial calcium homeostasis has not been extensively explored. However, NO-induced protection against cardiomyocyte ischaemia and reoxygenation invokes both a modest and sustained mitochondrial depolarization and a reduction in mitochondrial Ca<sup>2+</sup> uptake (Rakhit, Mojet et al. 2001). Mitochondrial calcium (Ca<sup>2+</sup>)<sub>m</sub> overload in ischemia is detrimental to the cell (Miyata, Lakatta et al. 1992). A reduction in mitochondrial calcium uptake is an attractive hypothesis whereby mK<sub>ATP</sub> channel activation mediates cytoprotection (Holmuhamedov, Jovanovic et al. 1998). In intact adult cardiomyocytes diazoxide does not affect basal (Ca<sup>2+</sup>)<sub>m</sub> (Ishida, Hirota et al. 2001). However, in response to ouabain-induced Ca<sup>2+</sup> overload diazoxide significantly attenuated (Ca<sup>2+</sup>)<sub>m</sub> in these cardiomyocytes over a 30 minute period (Ishida, Hirota et al. 2001). Interestingly, this was associated with a concomitant depolarization of the inner mitochondrial membrane potential ( $\Delta\Psi_m$ ). Mitochondrial depolarization during the preconditioned-phase has been confirmed by numerous investigators (Holmuhamedov, Wang et al. 1999; Minners, Lacerda et al. 2001) and as mitochondrial Ca<sup>2+</sup> uptake via the uniporter is driven, in part by  $\Delta\Psi_m$  (Gunter and Pfeiffer 1990; Holmuhamedov, Wang et al. 1999) a reduction in  $\Delta\Psi_m$  may limit (Ca<sup>2+</sup>)<sub>m</sub>.

### Mitochondrial volume

Garlid and colleagues are skeptical of a direct  $mK_{ATP}$  channel activation effect on mitochondrial coupling and propose that the primary effect of  $mK_{ATP}$  channel activation is to augment mitochondrial matrix volume (Kowaltowski, Seetharaman et al. 2001). These investigators propose that this  $mK_{ATP}$  channel activation augmented mitochondrial matrix volume counteracts matrix contraction that would otherwise occur during increased rates of ATP synthesis. This biochemical scenario could be quite compatible with the preconditioned-phase where an ATP deficit exists (Murry, Richard et al. 1990; Minners, Lacerda et al. 2001).

### Mitochondrial $K_{ATP}$ channel

The role of  $mitoK_{ATP}$  channel activation on the modulation of mitochondrial function in preconditioning is being actively studied and has not yet been fully elucidated (Gross 1995; Grover 1997; Holmuhamedov, Jovanovic et al. 1998; Liu, Sato et al. 1998; Gross and Fryer 1999; Kowaltowski, Seetharaman et al. 2001). The data presented here from two cell lines show that mitochondrial  $K_{ATP}$  activation with diazoxide parallels both ischaemic and adenosine preconditioning by inducing mitochondrial respiratory uncoupling of mitochondria in-situ. Interestingly, the putative specific mitochondrial  $K_{ATP}$  channel antagonist, 5-HD did not reverse all features of uncoupling, but predominantly blunted the membrane depolarization and oxygen consumption. These data preferentially support a role for the modulation of mitochondrial calcium homeostasis (Holmuhamedov, Wang et al. 1999; Rakhit, Mojet et al.

2001; Wang, Cherednichenko et al. 2001) and in the generation of reactive oxygen species (Pain, Yang et al. 2000; Carroll, Gant et al. 2001).

A plausible additional effect of the preconditioning 'trigger' could be the sustained activation of the  $mK_{ATP}$  channel. This scenario has been suggested by data that initiated the debate as to whether  $mK_{ATP}$  channel activation acts as a 'trigger' or a mediator of the preconditioning program (Downey and Cohen 2001). Mechanistic insight as to how the  $mK_{ATP}$  channel could be sustained in the activated/opened state during the preconditioned-phase was illustrated by Li and colleagues where they demonstrated that the reconstituted  $mK_{ATP}$  channel could be activated by superoxide signaling (Zhang, Chen et al. 2001). Moreover, the cytoprotective consequence of diazoxide-sustained  $mK_{ATP}$  channel activation in response to oxidative stress has been demonstrated in cardiomyocytes (Akao, Ohler et al. 2001).

The diverse mitochondrial effects described during the preconditioning-phase are not mutually exclusive and could implicate combinatorial adaptive biology in response to  $mK_{ATP}$  channel activation. As outlined under "Pharmacological preconditioning directly modulates mitochondrial function" data presented in this thesis indicate that the effects of the prototypical  $mitoK_{ATP}$  channel opener diazoxide on mitochondrial biology can be explained without invoking such a putative channel.

### Limitations

Several limitations of the present study have to be acknowledged. Although 5-HD reversed the increase in oxygen consumption in the cell system, it did not

have a significant impact on cellular ATP concentrations. Currently, I cannot offer an explanation for this finding beyond the points made in the discussion above. Secondly, mitochondrial ATP synthesis rather than whole cell ATP levels constitutes a possibly better indicator of mitochondrial function. However, in the intact cellular model used in these studies no meaningful ATP synthesis data could be obtained.

### **Adaptive mitochondrial perturbations during the ischemia-reperfusion phase**

The *sine-quo-non* of preconditioning is the significant diminution of cell death following myocardial ischemia and reperfusion event, above that achieved in the control heart exposed to the same index insult (Murry, Jennings et al. 1986). Mitochondrial function is significantly impaired during ischemia and reperfusion and has been identified as a central rheostat in orchestrating cell death. Hence, a logical extension of preconditioning should include a greater restoration of mitochondrial function in the preconditioned versus control heart following an ischemia/reperfusion insult. In reviewing the literature, the restoration of mitochondrial energetic function does appear to be a common feature in preconditioning across all species examined (Opie and Sack 2002).

The role of  $mK_{ATP}$  channel activation in the restoration mitochondrial function at reperfusion has been demonstrated in numerous laboratories in a variety of different experiments. Marban and colleagues demonstrated that the  $mK_{ATP}$  channel activators diazoxide and pinacidil maintained rat neonatal cardiomyocyte integrity in response to oxidative stress by preventing

mitochondrial depolarization with a concomitant reduction in apoptotic inducing events such as cytochrome c translocation and caspase-3 activation (Akao, Ohler et al. 2001). Complementary data from Garlid and colleagues demonstrate that diazoxide pretreatment preserves the low outer mitochondrial membrane permeability to nucleotides and cytochrome c in response to ischemia and reperfusion in the rat heart (Dos Santos, Kowaltowski et al. 2002). Here it is postulated that  $mK_{ATP}$  channel activation results in a small decrease in intermembrane volume that in turn, increases the half-saturation constant for ADP stimulation of respiration with a resultant reduction in ATP hydrolysis. These effects are proposed to lead to preservation of adenine nucleotides during ischemia with augmented energy transfer upon reperfusion (Dos Santos, Kowaltowski et al. 2002). As an extension to this mechanistic insight, these investigators have also demonstrated that ischemic preconditioning preserves the functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase (Laclau, Boudina et al. 2001). In the direct evaluation of the mitochondrial response to anoxia and reoxygenation following diazoxide administration Terzic and colleagues demonstrate a greater capacity to restore ADP-dependent respiration compared to vehicle treated control mitochondria (Ozcan, Holmuhamedov et al. 2001). In addition, in this same isolated mitochondrial protocol these investigators demonstrated that pre-administration of diazoxide or an alternate  $mK_{ATP}$  channel opener (nicorandil) attenuated ROS generation and cytochrome c release at reoxygenation (Ozcan, Bienengraeber et al. 2002). Finally, diazoxide has also been shown to suppress  $(Ca^{2+})_m$  accumulation during ischemia and reperfusion in rabbit ventricular

cardiomyocytes (Murata, Akao et al. 2001). The role of ischemic preconditioning and diazoxide inhibition of the mitochondrial permeability transition pore in the ischemia-reperfusion phase of preconditioning has also recently been explored (Hausenloy, Maddock et al. 2002; Korge, Honda et al. 2002). However, as  $mK_{ATP}$  channel activation normalizes two of the adverse cellular components required to activate MPTP, (Korge, Honda et al. 2001) i.e.  $(Ca^{2+})_m$  overload and diminished mitochondrial ATP synthesis with a concordant low  $\Delta\Psi_m$ , the primary role of MPT regulation in preconditioning needs to be further delineated. Collectively, however, the consequence of all of these restorative effects of  $mK_{ATP}$  channel activation on post-ischemic mitochondrial function is probably central to the cytoprotective program induced by preconditioning.

## **Working model of the temporal events shaping the preconditioned mitochondria**

Collectively the investigations described can be incorporated into a dynamic model of mitochondrial function that may facilitate the development of the cardioprotective phenotype evident in preconditioning (schematized in figure 22). In brief, the trigger-phase is associated with mitochondrial stress as evident by the multitude of reversible perturbations in mitochondrial function. A probable component of this mitochondrial-stress is the blunting of mitochondrial respiration, which in turn, may generate reactive oxygen species (Lenaz 2001). These, in turn, are postulated to act as cytoprotective signaling intermediates, although downstream targets have not been extensively investigated in classical preconditioning. The preconditioned-phase is typified by mitochondrial uncoupling, optimized mitochondrial architecture to facilitate respiration and diminished mitochondrial  $\text{Ca}^{2+}$  stores.

The preconditioning-induced prosurvival phenotype during post-ischemic reperfusion is characterized by an enhanced capacity to restore ATP production, blunted generation of ROS, maintenance of mitochondrial outer membrane integrity, diminished mitochondrial calcium overload and the possible induction of yet unidentified cytoprotective peptides. In addition, this prosurvival phenotype probably augments the threshold below which MPTP opening facilitates cell death.

The role of the  $\text{mK}_{\text{ATP}}$  channel in preconditioning are still under intense investigation and the further molecular characterization of these channels should ultimately shed additional insight into the mechanistic role of this channel

in preconditioning. However, the investigations into the activation of these channels have, we believe, enabled us to generate this working model of mitochondrial perturbations. The dissection of the regulatory control of the mitochondrial events discussed should facilitate a greater understanding of the role of the mitochondria in orchestrating an ischemia-tolerant cellular milieu.

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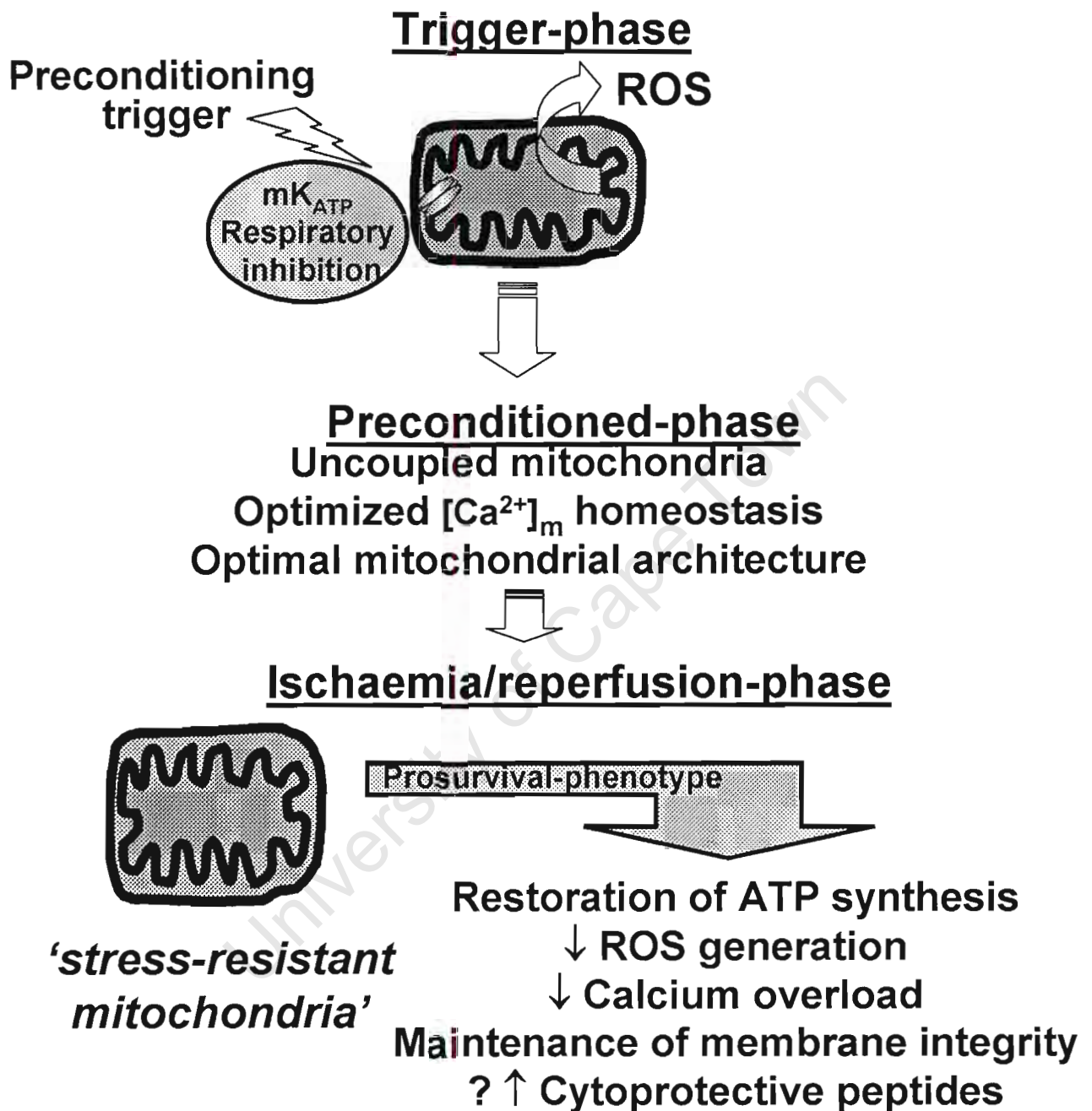


Figure 22 Working model of temporal events shaping the preconditioned mitochondria

In conclusion, the data presented in this thesis demonstrate that sublethal mitochondrial stress plays a pivotal role in triggering the cell protective phenomenon of preconditioning. Respiratory inhibition with subsequent ROS production has been shown to constitute a fundamental early signaling event in diazoxide-mediated cellular protection. Furthermore, the present study has identified the uncoupled mitochondrion as an important component of the cellular phenotype during the preconditioned state. Finally, I believe that the data obtained in these studies have played a significant role in advancing the concept of mitochondrial contribution to the cell-protective phenomenon of preconditioning.

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