Université de Montréal

The cardioprotective effect of a short-term aerobic exercise program and the mitochondrial permeability transition pore of the Rat

par

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Mémoire présenté à la Faculté des études supérieures en vue de l'obtention du grade de Maître ès Sciences (M.Sc.) en sciences de l'activité physique

Décembre 2005

Université de Montréal
Faculté des études supérieures

Ce mémoire intitulé:

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a été évalué par un jury composé des personnes suivantes:

Luc Léger	Président-rapporteur
Yan Burelle	Directeur de recherche
François Peronnet	Membre du jury

Mémoire accepté le .....

Résumé

En 2002, les maladies cardiovasculaires ont constitué la plus grande cause de décès à l'échelle mondiale, devançant le cancer et les maladies pulmonaires. L'infarctus du myocarde (IM) est généralement la première manifestation d'une maladie cardiovasculaire ischémique et il a été démontré que la pratique régulière d'exercices constitue un moyen efficace pour diminuer plusieurs facteurs de risque associés au développement de maladies cardiovasculaires tels l'hypertension, l'hypercholestérolémie, l'obésité et la résistance à l'insuline. De plus, il a été démontré que le taux de survie à l'issue d'un IM est plus élevé chez les sujets actifs comparativement aux sujets sédentaires, indiquant que l'exercice protège le cœur contre ce genre de stress.

Plusieurs études, principalement chez le Rat, ont effectivement démontré qu'un programme d'entraînement en endurance d'une durée variant de trois à cinq jours jusqu'à quatre mois apportait une protection cardiaque contre l'ischémie-reperfusion (I-R). Cette protection se caractérise par une diminution du dommage tissulaire et par une meilleure récupération de la fonction contractile en reperfusion. Cependant, les mécanismes impliqués dans cet effet protecteur demeurent encore mal compris.

Par ailleurs, il est bien connu que la reperfusion d'un tissu ischémique peut engendrer des dommages au niveau des mitochondries. Des dysfonctions mitochondriales qui résultent en une diminution ou une abolition de la production d'ATP engendrent, affectent directement la fonction contractile et mènent éventuellement à la mort cellulaire par nécrose. De plus, il est maintenant reconnu que la mitochondrie joue un rôle important

dans la signalisation de l'apoptose par sa capacité à relâcher plusieurs protéines pro-apoptotiques qui sont normalement séquestrées dans la matrice mitochondriale ou l'espace inter-membranaire. Plusieurs études menées au cours des dernières années ont montré que l'ouverture du pore perméable de transition (PPT), un canal non spécifique à haute conductance de la double membrane mitochondriale, était directement impliqué dans les deux formes de mort cellulaire dans le cœur suite à une période d'I-R.

L'effet de l'entraînement sur la fonction mitochondriale et plus particulièrement sur la régulation du PPT n'a jamais été exploré dans l'optique d'expliquer l'effet cardio-protecteur de l'exercice contre l'I-R. Les travaux effectués au cours de cette Maîtrise avaient donc deux objectifs. Le premier objectif était de mettre sur pied la technique de séquestration mitochondriale du [^{3}H]deoxyglucose ([^{3}H]-DOG), une approche permettant de quantifier l'ouverture du PPT in situ dans le cœur isolé du rat perfusé en mode Langendorff. Le deuxième objectif était d'appliquer cette méthodologie pour déterminer si l'entraînement à court terme (5 jours consécutifs de cours sur tapis roulant à une intensité approximative de 75% du VO_2max) diminue l'ouverture du PPT pendant la reperfusion (40minutes) suite à une épisode ischémique (30minutes).

Les résultats obtenus ont montré que l'entraînement permettait de diminuer le dommage mitochondrial tel que démontré par une moins grande perte du recouvrement de l'enzyme citrate synthase dans la fraction mitochondriale suite à l'I-R. De plus, l'incorporation de [^{3}H]-DOG dans les mitochondries était de 30-40 % inférieure à celle retrouvée dans les mitochondries cardiaques provenant d'animaux contrôles, indiquant une inhibition du

l'ouverture du PPT. Par contre, dans les conditions de perfusion utilisées, l'entraînement n'a pas amélioré la récupération fonctionnelle et la relâche de LDH malgré une réduction significative du dommage mitochondrial induit par le PPT.

Dans l'ensemble, les résultats de ce travail mettent en évidence que l'entraînement à court terme peut atténuer le dommage mitochondrial et l'ouverture du PTP qui survient en reperfusion suite à une période d'ischémie. Par contre, dans les conditions expérimentales utilisées, nous n'avons malheureusement pas été en mesure de déterminer la contribution du PPT comme effet protecteur de l'entraînement à court terme contre les dysfonctions contractiles et le dommage tissulaire, rapportés dans la littérature.

MOTS CLÉS : exercice, ischémie-reperfusion, cardioprotection, perméabilité transitionnelle, deoxyglucose

Summary

Ischemic heart disease claimed more lives worldwide in 2002 than any other single disease. Myocardial infarction is commonly the initial manifestation of ischemic heart disease, and exercise training has been shown to decrease a number of the risk factors for myocardial infarction including hypertension, hyperlipidemia, obesity, and insulin resistance, and has also been reported to improve chance of survival in humans after an ischemic event. Although exercise has proven beneficial in terms of cardioprotection, mechanisms for such an effect have yet to be elucidated.

Recent experimental studies have confirmed that both short-term (days) and long-term (weeks to months) endurance exercises provide myocardial protection against ischemia-reperfusion (I-R) injury in rats. Specifically, short-term exercise (i.e. 1–5 bouts of endurance exercise) reduces cardiac injury and enhances myocardial contractile recovery from an I-R insult as evidenced by an improved recovery of left ventricle developed pressures. While it is widely accepted that exercise improves tolerance against myocardial I-R, the mechanism(s) responsible for this exercise-induced cardioprotection remains elusive.

Reperfusion of the ischemic tissue has been implicated with oxidative modifications and functional impairment of mitochondria. It is for this reason that many studies have focused on the mitochondria as a potential key player in ischemia-reperfusion injury. Indeed, failure to produce ATP as a result of mitochondrial dysfunction is believed to mark the transition toward tissue necrosis and contractile failure during early reperfusion.

In addition, mitochondria are known to play a key role in signalling apoptotic cell death through the release of several pro-apoptotic proteins that are normally confined to the mitochondrial matrix or inter-membrane space. Opening of the permeability transition pore (PTP), a non-specific high conductance channel spanning the double membrane system of the mitochondria is known to trigger both necrosis and apoptosis.

The effect of exercise training on many aspects of mitochondrial function, and particularly on the regulation and behaviour of the PTP, has never been addressed in the context of explaining the cardio-protective effect against I-R. Therefore, the goals of the present Master's thesis were to implement the mitochondrial [^{3}H]2-deoxyglucose entrapment method, a technique that allows to quantify PTP opening *in situ* in the isolated, Langendorff, perfused heart of rats and to apply this methodology to determine whether short-term (5 consecutive days of treadmill running at approximately 75% VO_{2max}) aerobic training reduces the occurrence of PTP opening during reperfusion (40 minutes) following an ischemic episode (30 minutes).

Results have shown that citrate synthase (CS) recovery was significantly greater in the exercise trained compared to control, suggesting a reduction in mitochondrial damage. Furthermore, the incorporation of [^{3}H]-DOG within the mitochondria was shown to be reduced by 30-40% in trained animals, indicating a reduction of PTP opening. However, under the experimental conditions used, training did not result in a significant improvement in functional recovery and LDH release despite a significant reduction in PTP-induced mitochondrial damage, which is in contrast with several other reports.

Taken together the present results provide evidence that short-term training can attenuate mitochondrial damage and PTP opening which normally occurs in the heart following ischemia-reperfusion. However, under the experimental conditions used, the contribution of this mitochondrial protection to the protective effect of short-term training reported could not be established.

KEY WORDS: exercise, ischemia-reperfusion, cardioprotection, permeability transition, deoxyglucose

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Abbreviations

ADP = adenosin di-phosphate
AIF = apoptosis-inducing factor
ANT = adenylate translocator
ATP = adenine tri-phosphate
Ca^{2+} = calcium ion
CO = cardiac output
CS = citrate synthase
CsA = cyclosporin A
CVD = cardiovascular disease
CypD = cyclophilin-D
Da = dalton's
DOG = deoxyglucose
EndoG = endonuclease G
HR = heart rate
HSE = heat shock element
HSF = heat shock factor
HSP = heat shock proteins
HW = hydraulic work
IPC = ischemic pre-conditioning
I-R = ischemia-reperfusion
LCA = left ascending coronary artery
LDH = lactate dehydrogenase
LVDP = left ventricular developed pressure
MPTP = mitochondrial permeability transition pore
P_i = inorganic phosphate
PTP = permeability transition pore
RPP = rate pressure product
SfA = sanglifehrin A
Smac/DIABOLO = second mitochondria-derived activator of caspases-direct inhibitor of apoptosis binding protein with low P_i
SP = systolic pressure
VDAC = voltage-dependent anion channel
WH = isolated working heart preparation

Acknowledgements

First of all I would like to thank Dr. Yan Burelle for his exceptional support as well as his encouragement throughout my studies at the Université de Montréal. I am extremely grateful for his dedication and his availability in order to achieve my success and my time spent with him has rewarded me with an enriching experience. Although we have had many obstacles, his perseverance has given me the power to overcome anything within my path.

I would also like to dedicate this prolonged work to my parents, Gabriel and Solange Ciminelli, for all their support throughout the years. Thanks to their guidance and constant motivation, not only to complete my Master`s but in every facet of my life, I have become what I am today.

I gratefully acknowledge the rest of my family and friends for providing support in their own special ways, and of course my Melina, who has always been there for me, during the good times but especially during the not so good times. She has always listened to me and has helped me in any possible way she could, which has given me the drive needed to accomplish this huge task.

Chapter 1 - Literature Review

1 Introduction

Cardiovascular diseases (heart disease and stroke) are the leading cause of death in Canada (36 % of total mortality). Ischemic heart disease accounts for the greatest percentage of deaths at 20 %, of which half are attributable to complications of acute myocardial infarction (29). The total cost of cardiovascular disease, which is the leading cause of hospitalization for Canadian men and women, was estimated at 19.8 billions $ in 1993, of which more than a third was attributable to coronary heart disease (4.8 billions $)(29). As a result, the socio-economical impact of ischemic heart disease is great, underscoring the importance of primary prevention to reduce both the mortality and costs associated with this disease.

In this regard, there exists a large body of epidemiological studies in humans to support the notion that regular exercise is associated with a reduction in the incidence of cardiovascular disease (CVD). Moreover, the survival rate of heart attack victims is greater in active individuals compared with sedentary ones (29) (71). These beneficial outcomes are at least partly due to systemic adaptations to regular exercise resulting in a reduction of several risk factors for the development of CVD. These adaptations include a reduction in blood pressure, an improvement of the plasma lipid profile, an amelioration of glucose tolerance and insulin sensitivity and improved weight management (29, 46, 90).

In addition to these beneficial systemic effects, a number of studies using animal models have shown that regular exercise is associated with an increased tolerance of the myocardium to ischemia-reperfusion (I-R) injury (7, 22, 40, 64, 80, 85) (10, 21, 41-43, 61, 63, 65-67, 82, 83, 91, 92) and exogenous oxidative stress (91). Indeed both short-term (22, 40-42, 63-65, 67, 80, 92)

and long-term (7, 10, 21, 43, 61, 66, 82, 83, 85, 91) aerobic training was shown to improve functional recovery and reduce tissue damage in isolated ischemic-reperfused hearts (7) (10, 43, 61, 63-67, 80, 91, 92) or in hearts submitted to left ascending coronary artery ligation *in vivo* (21, 22, 40-42, 83, 85) .

The cellular mechanisms underlying this improvement in myocardial tolerance to I-R are not yet well established. Studies performed over the recent years have focused on training-induced adaptations in the expression of heat shock proteins (HSP's) (61, 64, 80) (22, 67), antioxidant defence systems (21, 41, 66, 82), sarcolemmal Ca^{2+} handling (7), myocardial energy metabolism (10) and endothelial function (95). However, there is currently no clear consensus on the respective role, mechanisms of action and relative importance of each of these components in protecting the trained heart against I-R injury.

On the other hand, it is well established that mitochondria play a key role in cell death following I-R in several tissues including the heart. Indeed, failure to produce ATP as a result of mitochondrial dysfunction is believed to mark the transition toward tissue necrosis and contractile failure during early reperfusion (23, 26, 78). In addition, mitochondria are known to play a key role in signalling apoptotic cell death through the release of several pro-apoptotic proteins that are normally confined to the mitochondrial matrix or inter-membrane space (26, 28, 32, 39, 50). Although necrosis is the main form of cell death encountered following I-R, apoptosis has been observed in cells located in periphery of the necrotic zone and which have been less severely affected by I-R (15, 39).

A number of experimental evidence accumulated over the recent years indicate that the opening of the permeability transition pore (PTP), a high conductance non-specific channel spanning the double membrane system of mitochondria is involved in triggering both forms of cell death during reperfusion of the ischemic heart, particularly necrosis (14, 15, 39, 98). Direct pharmacological inhibition of the PTP using cyclosporin A (CsA) and its analogs were shown to improve functional recovery and reduce tissue damage following I-R. Moreover, cardio-protective strategies such as ischemic preconditioning (55), administration of pyruvate (60) and the anti-oxidant anaesthetic propofol (56) were shown to mediate their beneficial effect at least partly through a reduction of PTP opening providing further support for the important role of this phenomenon.

Despite these evidences, the effect of exercise training on many aspects of mitochondrial function, and particularly on the regulation and behavior of the MPTP has never been addressed in the context of explaining the cardio-protective effect against I-R. Therefore, the goals of the present Master's thesis were to implement a new technique that allows to quantify PTP opening *in situ* in the isolated perfused heart and to apply this methodology to determine whether short-term aerobic training reduces the occurrence of PTP opening during reperfusion following an ischemic episode. The mitochondrial entrapment technique developed by Halestrap and colleagues was chosen based on the existing literature in the perfused heart. As for the training model used, it was justified by several studies reporting that short-term training induces a robust cardio-protection characterized by an increased functional recovery and a reduction in tissue damage.

The literature review included in this thesis will be divided in three sections. The first section provides an overview on the mitochondrial PTP and its involvement in ischemia-reperfusion injury in the heart. The second section focuses on the methodologies available to assess PTP opening *in situ* in isolated perfused hearts. Finally, in the third section, the existing literature on the cardio-protective effect of short-term training in rodent models and the possible underlying mechanisms are reviewed.

2 The mitochondrial permeability transition pore and its role in ischemia-reperfusion injury in the heart

2.1 In vitro studies on the mitochondrial permeability transition

2.1.1 Consequences of PTP opening on mitochondria *in vitro*

The mitochondrial permeability transition was initially described in isolated mitochondria in order to explain a sudden increase of the inner membrane permeability to solutes in the presence of a high calcium concentration ($[Ca^{2+}]$) (97). Although initially thought to be due to unspecific membrane damage it is now widely accepted that this phenomenon is actually caused by the opening of the PTP, a non-specific high conductance channel spanning the double membrane system of the mitochondria.

Opening of the PTP causes an immediate collapse of the proton electro-chemical gradient, massive ATP hydrolysis through the reversal of the FoF1ATPase and equilibration of solutes with a molecular weight of less than 1500 Da (97). At least *in vitro*, this phenomenon induces high amplitude swelling of the mitochondrial matrix ultimately leading to the rupture of the

outer-membrane. PTP opening is also associated with the release of several pro-apoptotic proteins usually confined to the inner membrane space including the mobile electron carrier cytrochome c, apoptosis-inducing factor 1 (AIF), Second mitochondria-derived activator of caspases – direct inhibitor of apoptosis binding protein with low P_I (Smac/DIABLO), endonuclease G (EndoG), the serine-protease OMI/HtrA2, and possibly some pro-caspases (26, 31, 32).

These proteins act at different sites and through different mechanisms to initiate a regulated cascade of cellular dismantlement. Cytochrome *c* binds to APAF-1 and allows formation of the apoptosome, leading to cleavage and activation of pro-caspase-9 thus triggering the proteolytic caspase cascade (69). AIF (70) and endonuclease G (49) migrate to the nucleus where they cause chromatin condensation as well as large scale nucleosomal DNA fragmentation. Smac/DIABLO release in the cytosol potentiates some forms of apoptosis by neutralizing one or more members of the inhibitory apoptosis proteins (IAP) (1). Finally OMI/HtrA2, through its interactions with XIAP, is able to potentiate caspase activity.

2.1.2 Molecular identity of the PTP

The actual molecular nature of the PTP is not well established and is a subject of current debate. However it is generally agreed that the PTP is formed by the assembly of a supramolecular complex that spans the double membrane system of mitochondria (97, 98). In addition, the proteins involved in this process usually carry specific physiological roles within mitochondria

and their involvement in PTP formation requires the presence of adverse conditions (15, 39, 48, 62).

The most popular and documented hypothesis regarding the pore composition is that it is formed by three core components (Figure 1) (15): the adenylate translocator (ANT) of the inner membrane, the voltage-dependent anion channel (VDAC) of the outer membrane, and the matrix enzyme cyclophilin-D (CypD). Under normal conditions, these proteins assemble at contact sites between the two membranes where they allow ATP and ADP exchange between the matrix (15). Under adverse conditions such as Ca^{2+} loading and excessive levels of oxidative stress, these proteins would transform into large conductance non-specific pores. This phenomenon would be exacerbated by the binding of CypD to ANT. It should be noted that several proteins are believed to interact with ANT and VDAC to promote or inhibit PTP opening including several kinases (hexokinase, glycerol kinase, mitochondrial creatine kinase), and members of the Bcl-2 family of proteins (bid, bax and bcl-2, bcl_{XL}) (15).

However, an increasing amount of evidence suggests that ANT, VDAC and CypD are not obligatory components of the PTP (48, 62). Indeed, alternate models suggest that the PTP could be formed by aggregates of misfolded proteins within the mitochondrial membranes that would accumulate as a result of damage (48, 62). According to this hypothesis, the PTP could thus be formed by a variety of mitochondrial proteins and not exclusively of ANT, VDAC and CypD. One of the implications of this model is thus that the composition of the PTP could vary according to the tissue studied and the triggering conditions.

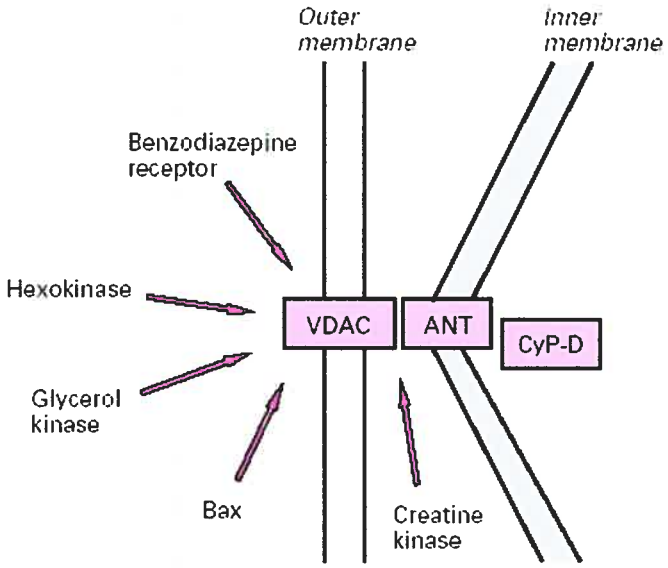

Figure 1: Most popular and documented hypothesis regarding the pore composition. Taken from Crompton, M 1999. The adenylate translocator (ANT) of the inner membrane, the voltage-dependent anion channel (VDAC) of the outer membrane, and the matrix enzyme cyclophilin-D (CypD) . Other proteins associate with the complex as indicated.

2.1.3 Regulatory properties of the PTP

The regulation of PTP opening is mediated by an array of different physiological effectors. Table 1 gives a summary of the factors that play a role in either activation or inhibition of the PTP. Calcium accumulation in the matrix appears to be an absolute requirement for PTP opening (14, 15, 98). In addition, a number of co-activators or antagonists further participate in pore regulation. Cations such as H^+, Mg^{2+}, Sr^{2+}, and Mn^{2+} can compete with Ca^{2+} for binding on the PTP and thus act as inhibitors (3, 14). High levels of adenine nucleotides such as ATP and ADP limit PTP opening, possibly by binding to the ANT (3, 14). In contrast, high levels of inorganic phosphate significantly increase the susceptibility to Ca^{2+}-induced PTP opening. A reduction in membrane potential will also favour permeability transition since the PTP behaves as a voltage-gated channel (14)). It is also well established that oxidation of the pyridine nucleotide pool (NADH and NADPH) favours PTP opening. Finally PTP opening can be inhibited by the immuno-suppressant drug, cyclosporin-A (CsA) and its analogs (24, 35, 55) as well as by the new compound sanglifehrin A (13, 55) that prevent binding of the cyclophilin-D to other putative PTP components.

2.2 Effect of ischemia-reperfusion on the PTP

2.2.1 Overview of the metabolic and ionic consequences of ischemia- reperfusion in the heart

In the heart submitted to ischemia, lack of oxygen and circulating substrates rapidly leads to the abolition of oxidative phosphorylation, a rapid and important decrease in tissue ATP content and accumulation of ADP, P_i and H^+ (37).

Table 1: Regulation of PTP opening by an array of different physiological effectors

Agent/control point	**Open probability**	**Reference**
Matrix Ca^{2+}	Increased	**Hunter & Haworth (1979) Duchen et al. (1993)**
Matrix Mg^{2+}, Sr^{2+}, Mn^{2+}	Decreased	**Hunter & Haworth (1979)**
P_i	Increased	**Hunter & Haworth (1979)**
Oxidants	Increased	**Crompton *et al.* (1987) Crompton & Andreeva (1993)**
ATP	Decreased	**Duchen *et al.* (1993)**
ADP	Decreased	**Crompton & Costi (1990)**
Cyclosporin A (CsA)	Decreased	**Crompton *et al.* (1988)**
Voltage	Increased voltage leads to decreased probability	**Bernardi (1992)**
Matrix pH	Decreased pH leads to decreased probability	**Bernardi *et al.* (1992)**
Surface Potential	More positive leads to decreased probability	**Broekemeier & Pfeiffer (1995)**

Accumulation of ADP will increase its conversion to IMP, adenosine, inosine and xanthine thus leading to a depletion of tissue adenylates. Lack of ATP will bring contraction to a halt and cause a severe disturbance in ionic homeostasis, which is usually maintained by the action of the Na^+/K^+ ATPase pumps. During ischemia, Na^+ and Ca^{2+} will thus accumulate in the cytosol (37).

Re-introduction of oxygen and substrates upon reperfusion of the ischemic region allows a partial or total recovery of electron flow through the respiratory chain and membrane potential (15). In presence of high cytosolic Ca^{2+}, this will rapidly lead to an important accumulation of Ca^{2+} in the mitochondrial matrix (15). In addition, because ischemia causes damage to respiratory chain enzymes, electron leaks through these damaged complexes leads to an increase in the production of reactive oxygen species (ROS) (15).

Therefore, Figure 2 (15) demonstrates that most of the conditions required to trigger PTP *in vitro* in isolated mitochondria prevail in cardiac cells following I-R. Based on this analysis, Crompton (14, 15), Griffiths (34, 35) and Halestrap (39) hypothesized that PTP opening occurred in the heart during reperfusion and was a significant contributor to tissue damage and contractile dysfunction.

2.2.2 Cardio-protective effects of pharmacological PTP inhibitors

In support to this hypothesis, several studies have shown that administration of the PTP inhibitor CsA in perfused hearts or isolated cardiomyocytes protected against reperfusion injury. In isolated perfused hearts, CsA administration was shown to results in an increased recovery of left

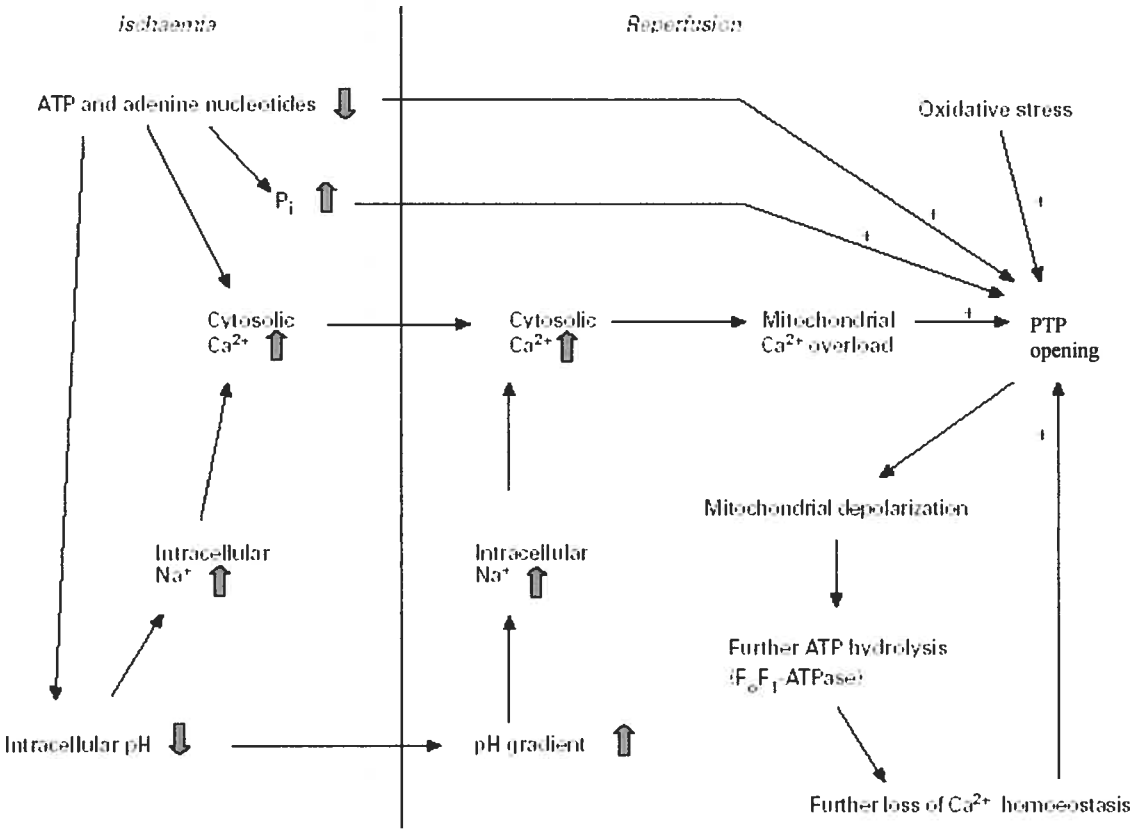

Figure 2: Involvement of the PTP in ischemia-reperfusion –induced cell death. Taken from Crompton, M 1999. Lack of oxygen and circulating substrate rapidly leads to the abolition oxidative phosphorylation and a rapid ATP content and accumulation of P_i and H^+. Upon reperfusion, there is an excessive Ca^{2+} uptake, which, coupled with oxidative stress and prevailing high P_i and low ATP can provoke PTP opening.

ventricular developed pressure (34, 35) (Figure 3), a lower end diastolic pressure (34, 35) (Figure 3), a reduction in infarct size (44) (Figure 4), an improved recovery of adenylates homeostasis (35), a reduction in cytochrome *c* release and activation of apoptosis (6) (Figure 5) and a better preservation of respiratory function in mitochondria isolated following I-R (6). Similarly, in isolated cardiomyocytes, CsA administration during simulated ischemia-reperfusion was shown to improve recovery of normal morphology and contractile activity (36) (25, 77).

While the effect of CsA in isolated mitochondria can unequivocally be attributed to PTP inhibition, its cardio-protective effect when administrated in intact hearts or cardiomyocytes is more difficult to interpret. Indeed, in addition to its effect on the PTP, CsA is a potent inhibitor of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, which has been involved in cell death in the heart (86) (24).

However, studies performed with pharmacological agents that inhibit calcineurin but not the PTP, or solely inhibit the PTP without affecting calcineurin provided results that are compatible with a role of pore opening in I-R injury. Administration of FK-506, a calcineurin inhibitor that does not interact with the PTP (33) was shown to have limited effects on infarct size (44), mitochondrial cytochrome *c* release, caspase activation and DNA fragmentation in hearts submitted to I-R (6) (Figure 5).

In contrast, administration of the CsA analog N-methyl-valine-CsA (24) and the new compound sanglifehrin A (13), which potently inhibit pore opening but have no effect on calcineurin, were shown to improve the recovery of contractile function (13) and reduce tissue damage as measured

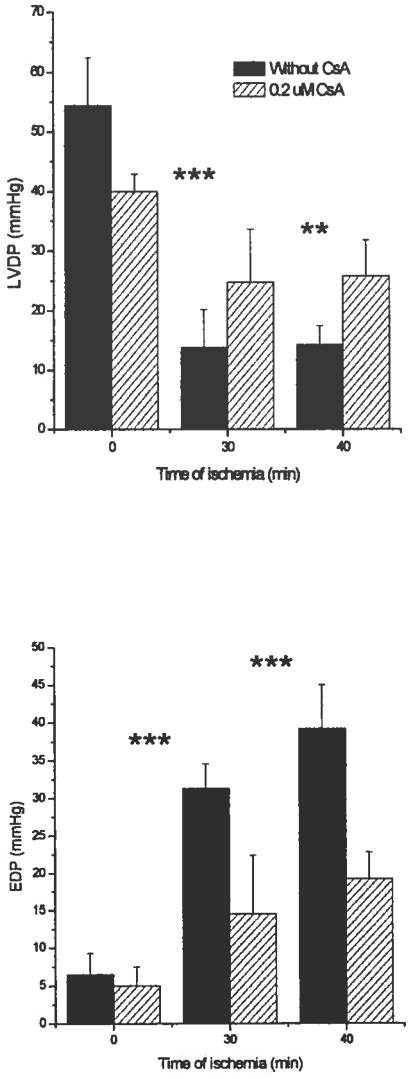

Figure 3: Results of left ventricular developed pressure (LVDP) and end diastolic pressure (EDP) following 15 minutes of reperfusion after 30 or 40 minutes of ischemia with or without CsA. Adapted from Griffiths, 1993 #53. ** P<0.02, *** P<0.01 between reperfused and control hearts.

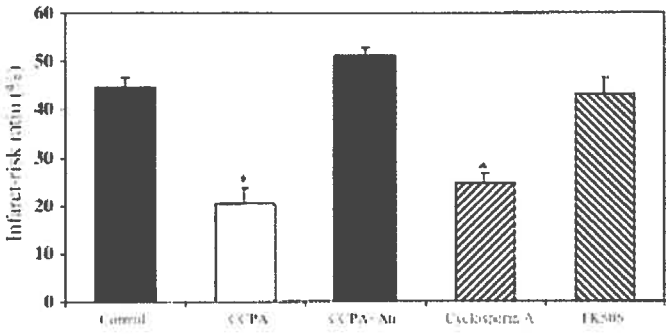

Figure 4: Reduction in the infarct size with the use of CsA. Taken from Hausenloy et al. 2002. Significantly different from control (*P<0.0001).

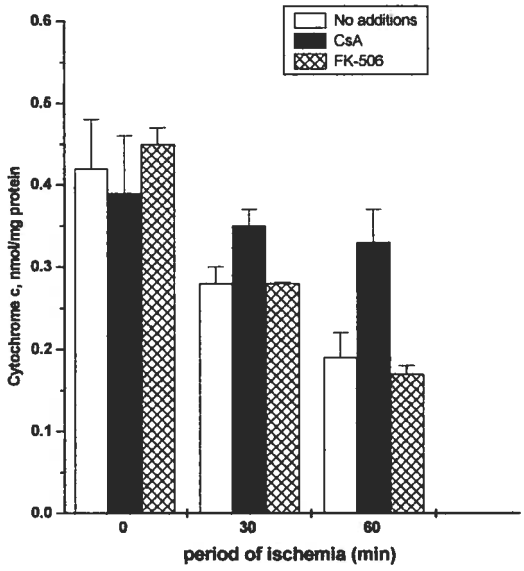

Figure 5: Mitochondrial cytochrome *c* content after a period of ischemia. CsA protects heart mitochondria from loss of cytochrome *c* during ischemia but not FK-506. Taken from Borutaite et al. 2003. *, statistically significant effect of ischemia ($P < 0.01$), if compared to control. #, statistically significant effect of CsA ($P < 0.05$), if compared to treatment without CsA in the same group.

by LDH release in the coronary effluent (13, 24) (Figure 6 & 7). Taken together this pharmacological evidence thus indicates that PTP opening occurs during reperfusion of the ischemic heart and that its inhibition results in a significant improvement in contractile recovery and a reduction in tissue damage.

2.2.3 Assessment of PTP opening *in situ* in the ischemic-reperfused heart

One limitation of the pharmacological approaches used in the above mentioned studies is that the role of pore opening in ischemic injury is only inferred and PTP opening is not directly quantified. Moreover without a direct index of MTP opening, it is not possible to determine whether other cardio-protective strategies could mediate their effects through a reduction in the occurrence of PTP opening. In order to circumvent these limitations, two research groups have developed methods to estimate the extent of PTP opening that are applicable to studies in the intact heart: the measurement of mitochondrial NAD^+ release and the mitochondrial [^{3}H]-deoxyglucose ([^{3}H]-DOG) entrapment technique.

2.2.3.1 Mitochondrial NAD^+ release

NAD^+ is a soluble electron carrier present at high concentration in the mitochondrial matrix. Given its molecular weight, NAD^+ is readily released from the mitochondria upon opening of the PTP (24) (Figure 8). Once permeability transition has occurred, a significant amount of NAD^+ is

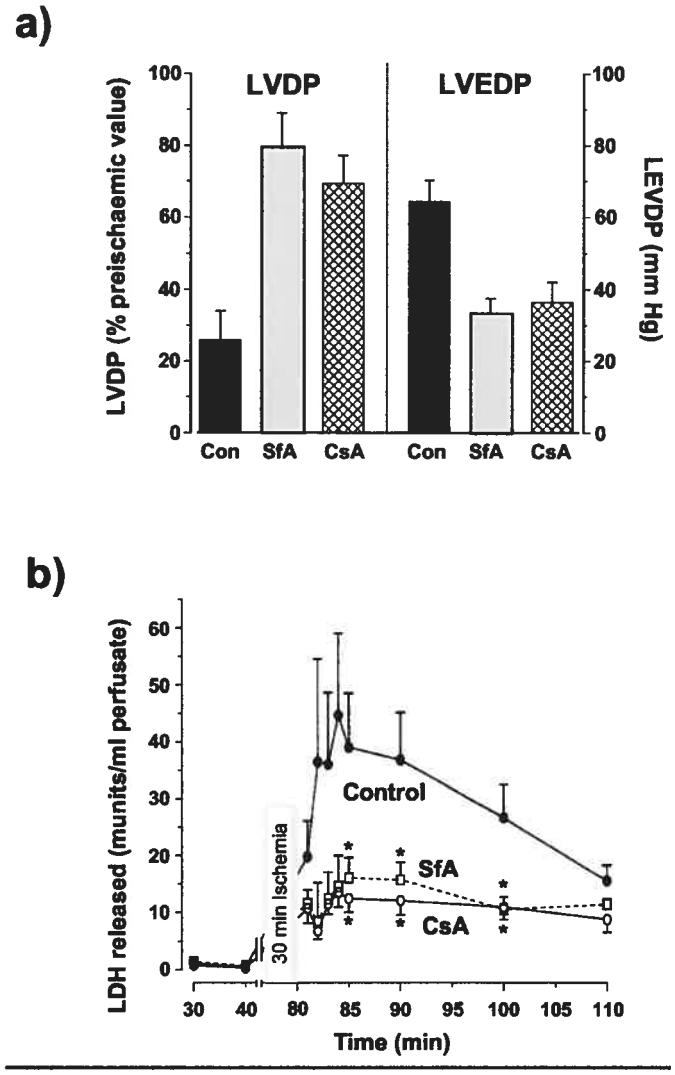

Figure 6 : SfA and CsA protect the ischemic rat heart from reperfusion injury. Taken from Clarke et al. 2002. In *panel a*, greater functional recovery of the SfA- and CsA-treated hearts after a 30-min reperfusion is reflected in higher values for the LVDP and lower values for the LVEDP. Values of the LVDP after reperfusion are presented as a percentage of the pre-ischemic value and were significantly greater ($p < 0.001$) with either SfA or CsA treatment, whereas values for the LVEDP were significantly lower ($p < 0.001$). In *panel b*, the release of lactate dehydrogenase (*LDH*) into the perfusate from the same hearts used in *panel a* was measured as an indicator of necrotic cell death (*, $p < 0.05$ for CsA- or SfA-treated hearts *versus* controls).

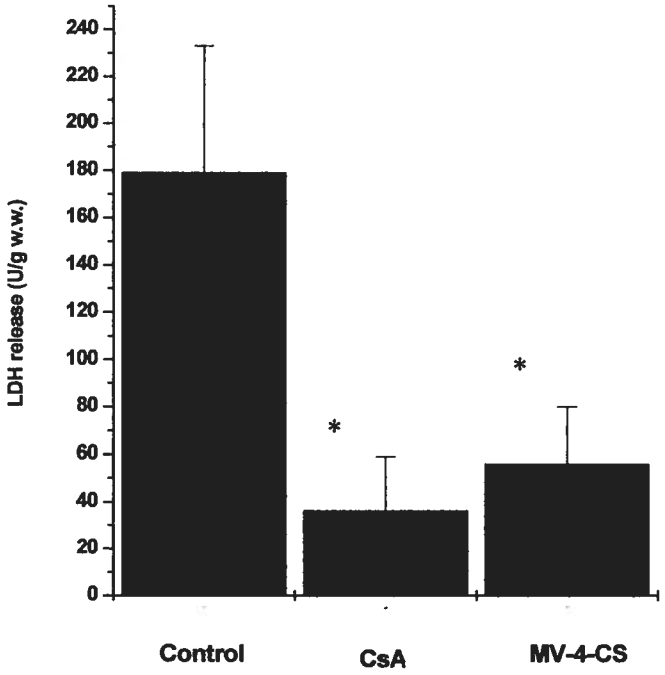

Figure 7: CsA and MV-4-CS reduces the release of LDH in the coronary effluent induced by postischemic reperfusion. Adapted from Di Lisa et al. 2001. *, $P < 0.01$ statistical difference between treated and control hearts.

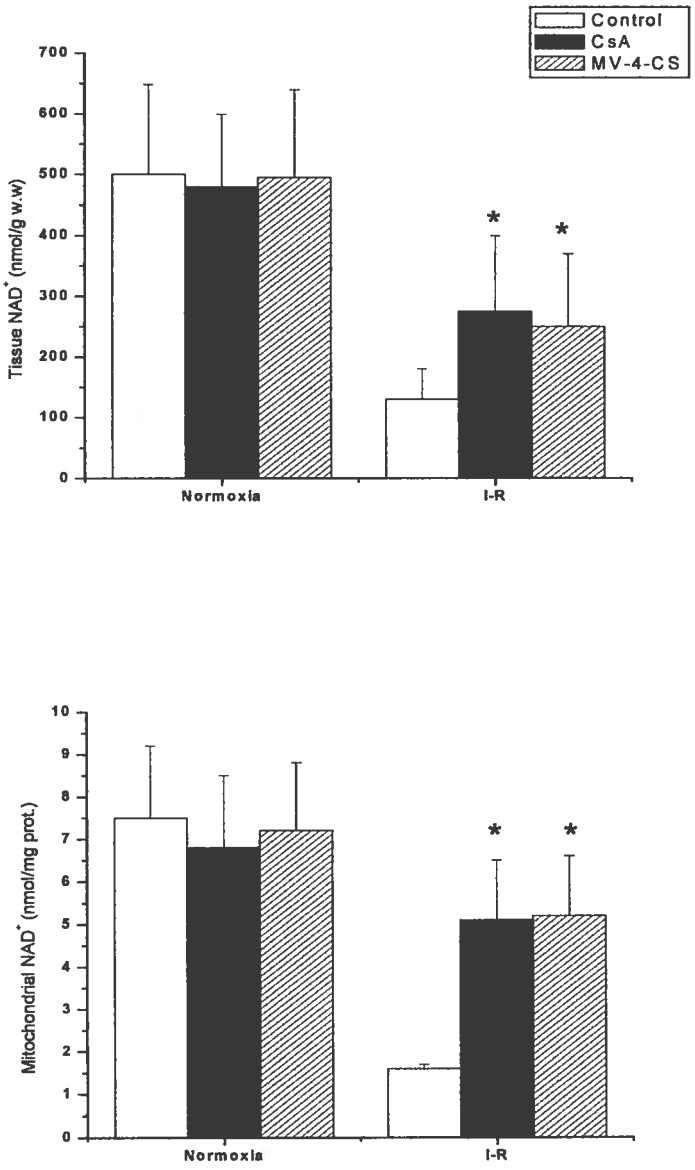

Figure 8: CsA and MV-4-CS reduces the decrease in tissue and mitochondrial NAD+ contents associated with postischemic reperfusion. Taken from Di Lisa et al. 2001. *, $P < 0.01$ statistical difference between treated and control hearts.

released in the outer mitochondrial membrane (24). The remaining NAD^+ that escapes degradation enters the cytosol, and if cellular integrity is altered, will be released in the coronary circulation (24). Only one study has made use of this technique to further document the role of PTP opening in ischemia degraded by NAD^+ glycohydrolase, an enzyme presumably located in the inter-membrane space -reperfusion damage (24). Di Lisa et al. (24) submitted isolated Langendorff-perfused hearts to a period of global ischemia of 30, 60 or 90 min followed by 30 minutes of reperfusion. NAD^+ release in the coronary circulation was measured at regular intervals during reperfusion and at the end of the experiments hearts were homogenized and processed for isolation of mitochondria. NAD^+ content was measured fluorimetrically in the whole homogenate and the mitochondrial fraction using an alcohol dehydrogenase assay. These values were compared to that measured in freshly isolated non-ischemic hearts. In hearts reperfused following 90 minutes of ischemia, mitochondrial and whole tissue NAD^+ contents respectively decreased by $\approx$ 85 and $\approx$ 70 % compared to values obtained in non-ischemic hearts (Figure 8). This was accompanied by a significant leakage of NAD^+ in the coronary effluent. In hearts perfused in the presence of the PTP inhibitors CsA and nmethyl-valine cyclosporin, these phenomena were attenuated (Figure 8). Moreover, a significant correlation between NAD^+ release in the coronary effluent and the extent of tissue damage (as measured by the release of LDH) was observed. Based on these evidences, Di Lisa et al. (24) concluded that PTP opening was a causal event in the death of myocytes following I-R.

On the other hand, DiLisa et al (24) did not report the results obtained in hearts submitted to shorter periods of ischemia (i.e. 30 min) that are more frequently used in the literature. While the authors did not discuss this issue, preliminary work performed in our laboratory suggests that this

might be due to the fact that the method failed to detect significant levels of PTP opening under these conditions. Indeed, we observed that measurement of NAD^+ using fluorescence methods yield variable results due to a low signal/noise ratio. Consequently, in hearts submitted to 30 min ischemia and 40 min of reperfusion, we consistently failed to detect significant and reproducible changes in whole tissue and mitochondrial NAD^+ content. In addition, given the relatively low amount of NAD^+ present in cardiac cells and because 30 min of ischemia is insufficient to cause large scale necrosis, the amount of NAD^+ released in the coronary effluent was well below detection levels.

2.2.3.2 ***Mitochondrial [^{3}H]-deoxyglucose entrapment***

Currently, one of the best strategies to directly assess PTP opening relies on the use of fluorescence microscopy to visualize diffusion within the mitochondrial matrix of exogenous fluorescent probes previously loaded in the cytosol. However, while this approach has provided valuable information about the role of the PTP in cell death, its use is limited to isolated cell models. For this reason, the group of Andrew Halestrap at the University of Bristol have made use of the same general principle to develop a method that can be applied to Langendorff-perfused hearts and which relies on measurements of the incorporation in the mitochondrial compartment of an exogenous radioactive probe previously loaded in cardiomyocytes.

2.2.3.3 ***Principle***

In order to select the appropriate probe to accurately track PTP opening in this setting, several criteria had to be met.

Indeed, the probe:

1. Has to enter cardiomyocytes in relatively large amounts using existing sarcolemmal transporters,
2. has to display a relatively low level of toxicity to the cell,
3. has to exert a minimal effect on cellular metabolism,
4. has to remain relatively stable once within the cell i.e. its degradation by metabolic pathway has to be minimal within the time frame of the study, and has
5. to enter the mitochondrial compartment only when the PTP opens and to stay within mitochondria thereafter.

Based on these criteria, [^{3}H]-DOG was selected as the probe of choice. Indeed, as a non-metabolizable glucose analog, [^{3}H]-DOG rapidly enters cardiomyocytes through glucose transporters and is phosphorylated in [^{3}H]-DOG-6P in a step catalyzed by hexokinase. Because [^{3}H]-DOG-6P degradation through non-specific dephosphorylation occurs slowly compared to its uptake (54), [^{3}H]-DOG-6P can accumulate in significant amounts in the cytosol (Figure 9). This is a key factor in order to obtain a measure of PTP opening that offers a reasonable signal/noise ratio. On the other hand, because [^{3}H]-DOG phosphorylation results in Pi trapping, accumulation of great amounts of [^{3}H]-DOG-6P can result in a rapid depletion of myocardial ATP and PCr stores. However, this unwanted effect could be avoided if the [^{3}H]-DOG concentration used is maintained below 2 mM (52, 54). The concentration that has typically been used for the measurement of mitochondrial [^{3}H]-DOG entrapment is of 0.5 mM (34, 37, 54-56, 60), well below the concentration that will compromise energy homeostasis (52, 54).

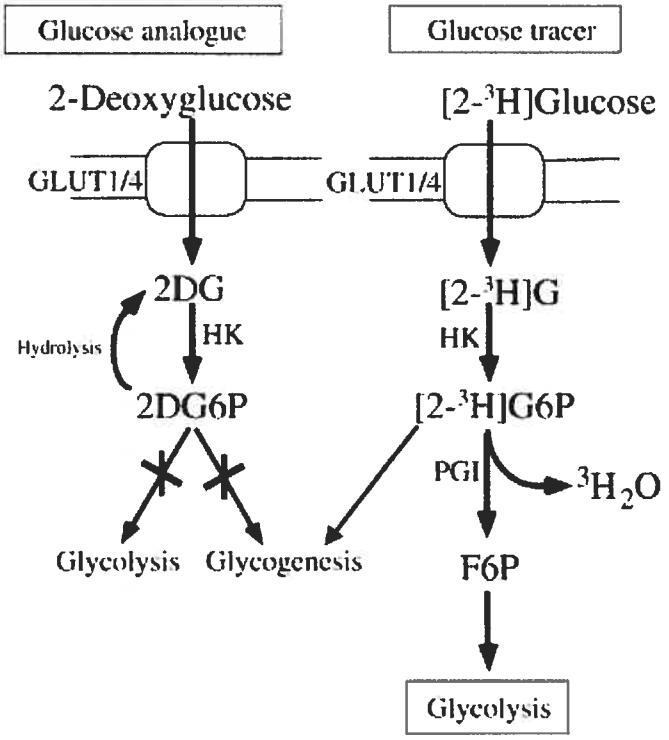

Figure 9: [^{3}H]-DOG-6P degradation through non-specific dephosphorylation occurs slowly compared to its uptake, [^{3}H]-DOG-6P can accumulate in significant amounts in the cytosol. Taken from Hopkins et al. 2004.

Regarding the selectivity of [^{3}H]-DOG-6P as probes for PTP opening, studies have shown that mitochondrial membranes have a low permeability for sugars in general. Indeed, when mitochondria are isolated in presence of ^{14}C mannitol, only a small fraction of ^{14}C becomes permanently associated with mitochondrial membranes. This amount represents 10-15 % of that expected for ^{14}C-mannitol uptake and complete equilibration (94). On the other hand, PTP opening allows equilibration of solutes with a molecular weight of less than 1500 Da across mitochondrial membranes. Given this property, one of the assays commonly used to monitor PTP opening *in vitro* is to incubate mitochondria in a sucrose-based medium and to measure mitochondrial swelling secondary to the diffusion of sucrose and H_2O in the mitochondrial matrix. Therefore, these properties insure that PTP opening *in vivo* will rapidly increase [^{3}H]-DOG-6P entrapment in mitochondria and that [^{3}H]-DOG-6P incorporation will be comparatively much less important in the absence of PTP opening (Figure 10).

Another key aspect regarding the accuracy of the method is that [^{3}H]-DOG-6P has to remain within mitochondria once PTP opening has occurred. In addition, accidental PTP opening during the mitochondrial isolation procedure must be minimal. In order to limit the potential problems associated with these phenomena, Halestrap (34) proposed to homogenize heart tissues and isolate mitochondria using a rapid method and to supplement the buffers with a high concentration of EGTA (2 mM). Indeed, studies on isolated mitochondria have shown that once PTP opening has occurred, addition of EGTA to chelate Ca^{2+} is able to close open pores and allow mitochondrial recovery (16).

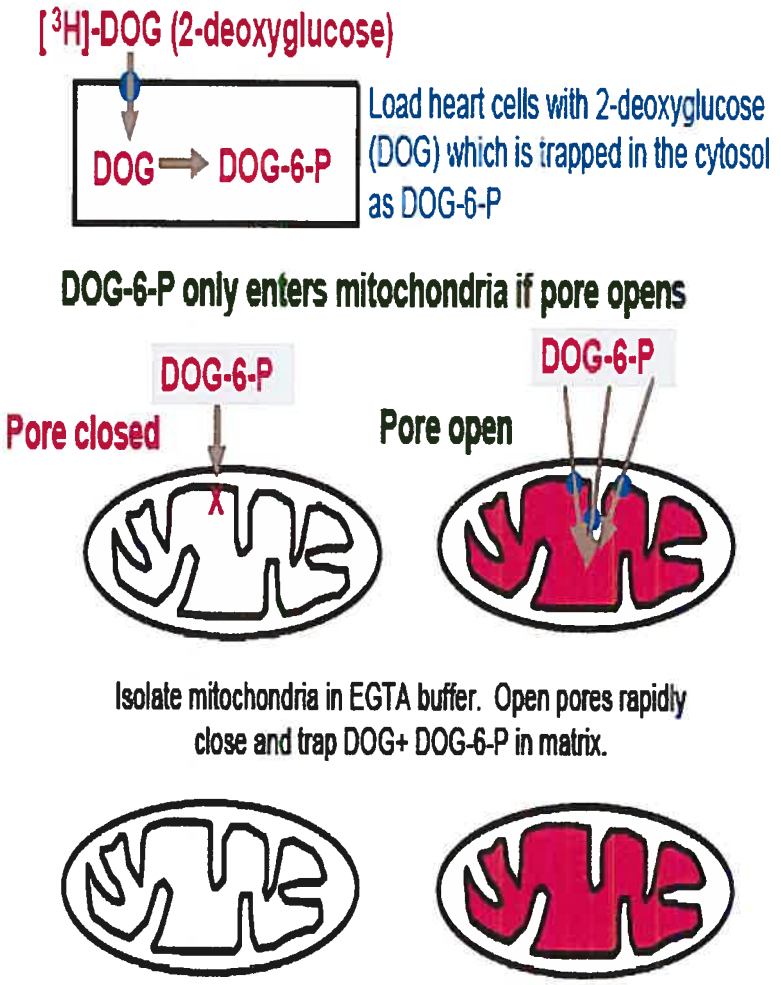

Figure 10: Typical experimental protocol used for the assessment of PTP opening using the [^{3}H]-DOG entrapment method. Taken from Halestrap et al. 2004.

2.2.4 Experimental protocol:

Figure 10 presents the typical experimental protocol used for the assessment of PTP opening using the [^{3}H]-DOG method. In practical terms, hearts are placed on the perfusion apparatus, instrumented for measurement of hemodynamic parameters and perfused in the non-recirculating mode with a normal Krebs-Henseleit (K-H) buffer. Following a period of stabilization, hearts are perfused for 20 min with the same buffer supplemented with 0.5 mM of [^{3}H]-DOG (0.1 μCi/ml). In order to maximize uptake of the tracer, the perfusion is performed in the re-circulating mode. Following this loading period, perfusion is switched back to the non-circulating mode with the [^{3}H]-DOG-free K-H buffer in order to washout extracellular [^{3}H]-DOG. Ischemia is then initiated and following reperfusion, hearts are rapidly processed for the isolation of mitochondria and measurement of mitochondrial [^{3}H]-DOG entrapment.

2.2.4.1 Calculation of the [^{3}H]-DOG index:

In order to achieve an accurate measurement of PTP opening, calculation of mitochondrial [^{3}H]-DOG entrapment has to take into account several confounding factors.

The first factor is that total tissue uptake of [^{3}H]-DOG through GLUT-mediated transport can vary between experiments, thus resulting in various levels of cytosolic [^{3}H]-DOG-6P accumulation. Similarly, depending on the severity of the damage induced by I-R, variable amounts of [^{3}H]-DOG-6P could leak out of cardiomyocytes that have loss membrane integrity.

Failure to take this into account can lead to variations in the amount of mitochondrial [^{3}H]-DOG incorporation that are not related do differences in PTP opening.

The second factor is that the amount of mitochondria recovered following isolation varies between days and according to the severity of I-R injury. Therefore, the total amount of [^{3}H]-DOG recovered will be influenced by the amount of mitochondria isolated. For these reasons, the PTP opening index (termed DOG index) is calculated as follows:

$$\text{DOG index} = \frac{10^5 \text{ x mito } [^3\text{H}]\text{-DOG-6P per unit CS}}{\text{tissue } [^3\text{H}]\text{-DOG-6P per g wet weight}}$$

In this calculation, mitochondrial [^{3}H]-DOG-6P measured in d.p.m. is expressed per unit of citrate synthase. This allows normalization for the amount of mitochondria recovered following isolation. In addition, in order to account for variations in the amount of cytosolic [^{3}H]-DOG-6P present, mitochondrial [^{3}H]-DOG-6P entrapment is expressed relative to tissue [^{3}H]-DOG-6P per g wet weight. Therefore, by expressing the DOG index as a ratio, the measure is not influenced by loss of cytosolic [^{3}H]-DOG-6P.

On the other hand, one of the limits of the method is that PTP opening can only be measured in mitochondria that retained sufficient integrity to survive the isolation procedure (55) (37). Indeed, as discussed in our study, I-R induces a significant loss in the recovery of intact mitochondria following isolation and the DOG index fails to consider PTP opening that occurred in mitochondria that were lost. In order to take this phenomenon into account, recent studies have

thus normalized the DOG index by the total amount of citrate synthase recovered in the mitochondrial fraction by gram of heart (55,37).

2.2.4.2 ***Role of the PTP inhibition in cardio-protection:***

This section will provide an overview of the results published in studies that have used the mitochondrial [^{3}H]-DOG method to investigate various cardio-protective strategies.

2.2.4.2.1 Cyclosporin A and Sanglifehrin A:

The effect of administering CsA and SfA, two direct inhibitors of the PTP, to perfused hearts submitted to I-R has been investigated in two studies (34,55). Javadov et al. (55) have shown that administration of CsA during ischemia and early reperfusion was associated with a ≈ 20% reduction in the DOG index (Figure 11 Panel A). As pointed out by the authors (37,34,55), this relatively small effect of CsA on PTP opening is somewhat surprising given the strong potency of CsA at inhibiting pore opening in isolated mitochondria.

However, as mentioned above (p 23), one limitation of the DOG index is that it fails to consider PTP opening in mitochondria that were lost during isolation due to excessive damage, thus underestimating true levels of pore opening. Because CsA allows to significantly attenuate the

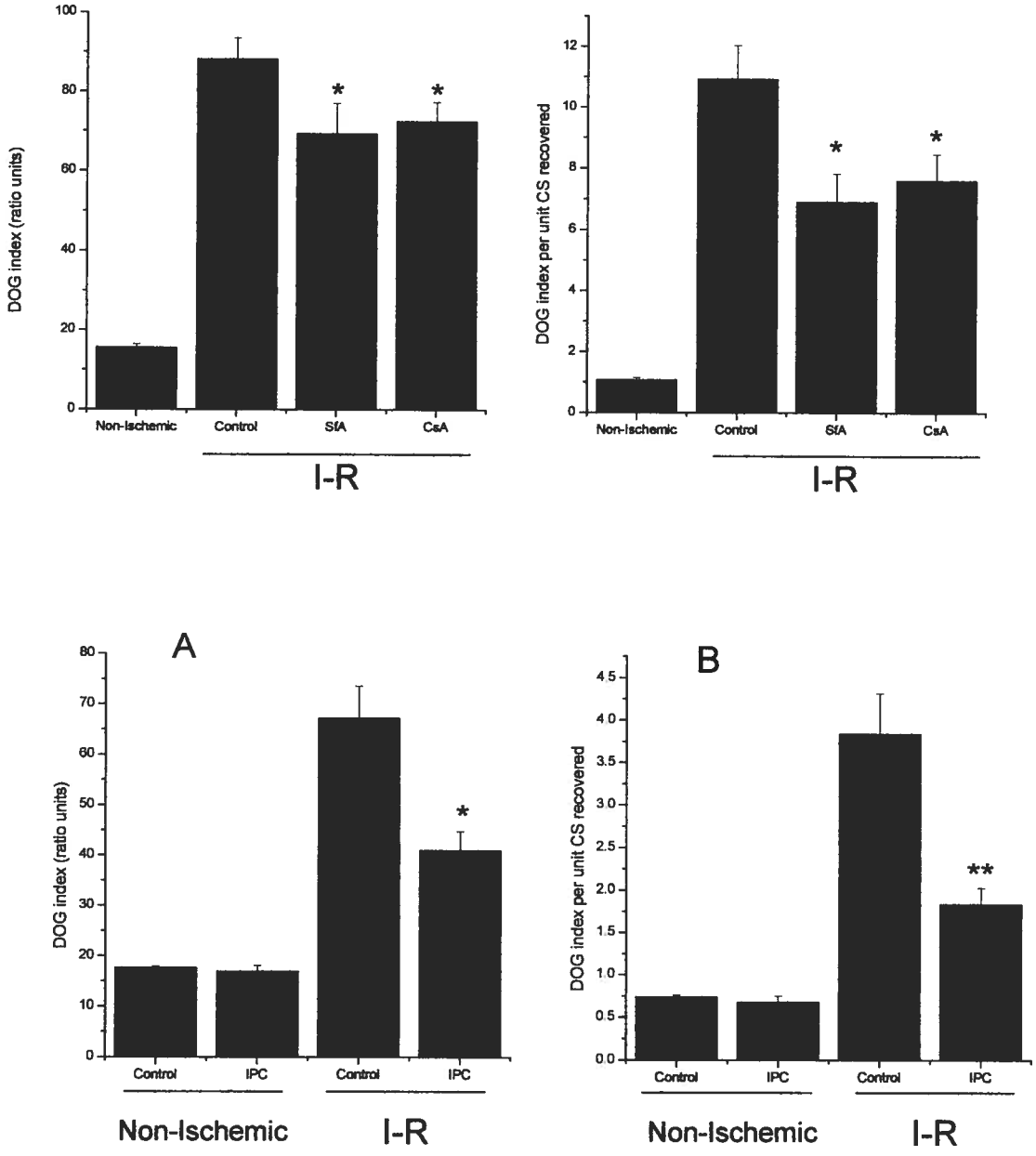

Figure 11: Result of DOG indexes either before 30 minutes of global ischemia or after ischemia and 25 minutes of reperfusion. Adapted from Javadov et al. 2003. TOP 2 graphs, statistical significance of SfA or CsA versus control hearts (*P<0.05;**P<0.01). BOTTOM 2 graphs, statistical significance of IPC versus control hearts (*P<0.05;**P<0.01). IPC, ischemic pre-conditioning; CsA, cyclosporin A; SfA, sanglifehrin A; CS, citrate synthase.

loss of intact mitochondria observed in response to I-R, this underestimation is attenuated. This phenomenon thus contributes to explain the apparently small effect of this drug on PTP opening. When this difference is taken into account by normalizing the DOG index values by the amount of citrate synthase recovered, the inhibitory effect of CsA on mitochondrial [^{3}H]-DOG entrapment is more compatible with the known potency of this drug at inhibiting pore opening (Figure 11 panel B). Javadov et al. (55) have also shown that Sanglifehrin A (Sfa), a new agents that inhibits the PTP by binding to cyclophilin D, also reduced PTP opening in ischemic-reperfused hearts (Figure 11).

2.2.4.2.2 Pyruvate and Propofol

Pyruvate supplementation was repeatedly shown to improve the functional recovery of the heart following ischemia-reperfusion (9, 11) (20). Kerr et al. (60) investigated whether the protective effect of pyruvate could be mediated by an attenuation of PTP opening. These authors reported that administration of 10 mM pyruvate during ischemia and reperfusion reduced mitochondrial [^{3}H]-DOG entrapment by ≈ 38% (Figure 12) and significantly retarded time to ischemic contracture, improved the recovery of left ventricular developed pressure (Figure 12), and lowered end diastolic pressure. However, in this study no attempt was made to correct for differences in the recovery of intact mitochondria.

Three main hypotheses have been suggested to explain how pyruvate could inhibit PTP opening during reperfusion. The first hypothesis involves the fact that pyruvate allows maintenance of a more acidic intracellular pH during reperfusion. The second hypothesis is that pyruvate, by virtue

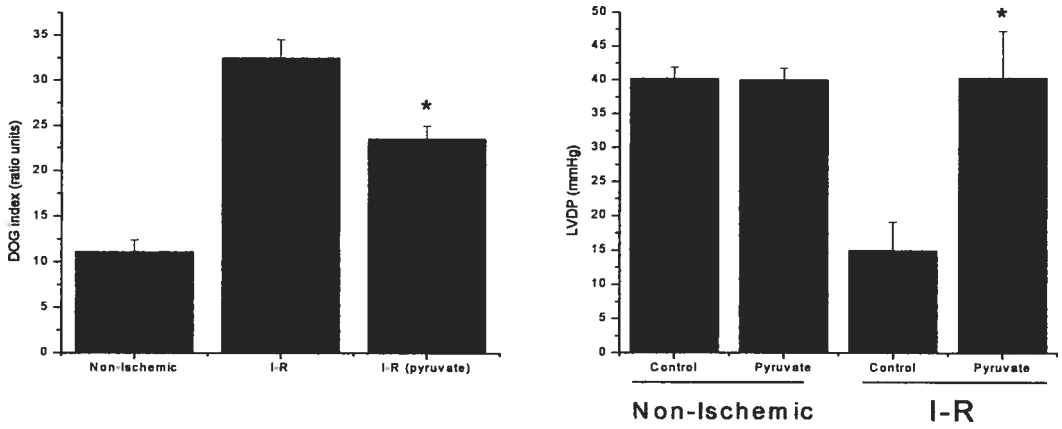

Figure 12: Effect of pyruvate treatment on the mitochondrial permeability transition and left ventricular developed pressure (LVDP) during ischemia reperfusion. Adapted from Kerr et al. 1999. Significant differences between control and pyruvate-treated hearts (*P<0.05)

of its antioxidant properties (4, 20) could scavenge free radicals produced during reperfusion. Finally, the third hypothesis is that pyruvate oxidation in the mitochondria could favour a better recovery of mitochondrial membrane potential ($\Delta\Psi$). Indeed, as described in section 2.1.3, an acidic pH, low levels of oxidative stress and an increase in $\Delta\Psi$ all favour the maintenance of the PTP in the close conformation.

Similarly, the anaesthetic agent propofol (DIPRIVAN©, Zeneca Pharma), which is known for its capacity to scavenge free radicals (27, 73), was shown to reduce mitochondrial [^{3}H]-DOG entrapment by ≈ 26% and to improve functional recovery following I-R (Figure 13) (56). This inhibition of PTP opening *in situ* was at least partly due to a direct effect of mitochondria rather than on intracellular modulators. Indeed, swelling assays performed on mitochondria isolated from non-ischemic hearts treated with propofol showed that significantly more Ca^{2+} was required to open the PTP compared to that measured in mitochondria from control hearts.

Taken together, the studies on the cardio-protective effect of pyruvate and propofol thus suggest that several pharmacological agents that are not directly targeting the PTP structure could exert their protective effects at least partly by affecting modulators of pore opening thus making the PTP a central target for cardio-protective strategies.

2.2.4.2.3 Ischemic pre-conditioning

Ischemic pre-conditioning (IPC) is one of the most powerful physiological stress that can induce

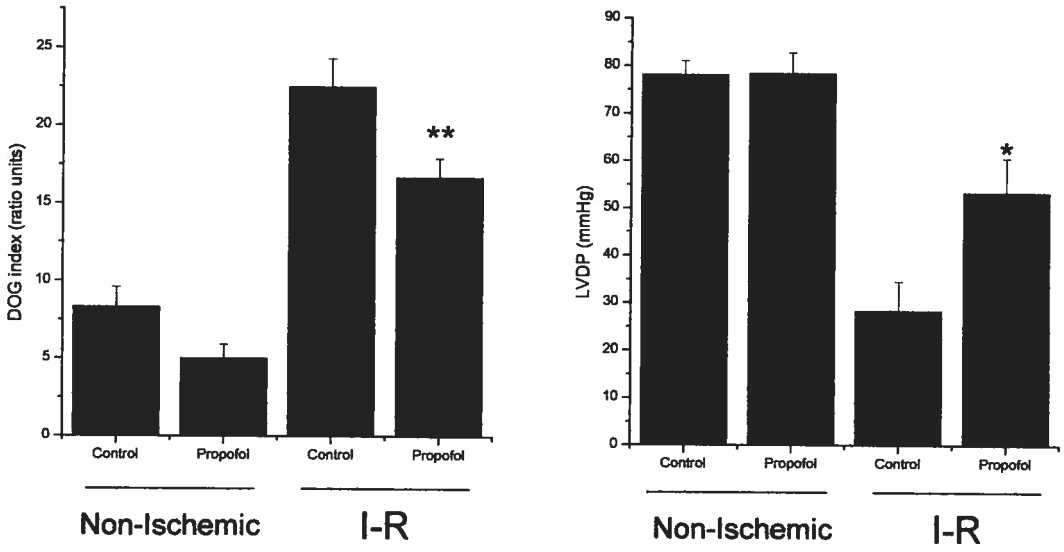

Figure 13: Effect of propofol treatment on the mitochondrial permeability transition and left ventricular developed pressure (LVDP) during ischemia reperfusion. Adapted from Javadov et al. 2000. Significant differences between control and propofol-treated hearts (**P<0.025)

endogenous mechanisms that protect the heart against subsequent periods of prolonged ischemia. Typically, two episodes of brief ischemia (5 min) intercalated with 5 min of reperfusion significantly attenuates tissue damage and improves the recovery of contractile function during reperfusion following periods of ischemia ranging between 20 to 30 min (44). Using the [^{3}H]-DOG technique, Javadov et al. (55) reported that IPC was associated with a significant reduction in the DOG index and an increase in the recovery of intact mitochondria following I-R (Figure 12). This inhibition of PTP opening by IPC was also reported by other groups in perfused hearts (2), isolated cardiomyocytes (45) and mitochondria (2) using other methodologies.

The mechanisms by which IPC results in the inhibition of PTP opening are not yet defined and a detailed discussion on this issue is beyond the scope of this thesis. Inhibition of PTP opening could occur through an indirect mechanism by beneficially altering the intracellular milieu e.g. by attenuating cellular Ca^{2+} overload (72, 87), and ROS production (75, 79). In addition IPC could directly act at the level of mitochondria by beneficially altering factors that regulate PTP opening such as Ca^{2+} loading, ROS production, membrane potential, matrix pH and matrix adenylates (2). Finally, opening of mitochondrial K_{ATP} channels, which are involved in the IPC signalling cascade, could underlie some of these effects (53, 72).

3 Cardio-protection induced by short-term training

A number of studies using rodent models have provided strong evidence indicating that hearts from trained animals are better protected against contractile dysfunction and tissue injury induced by periods of ischemia ranging between 15 and 40 min followed by 15-30 min of reperfusion (22, 40-42, 63-65, 67, 80, 92). While this beneficial effect has been demonstrated using training

programs of various durations ranging from a few days to several weeks, the present review will focus on short-term training. In a first section, the effect of this type of training on the recovery of contractile function and tissue damage measured using various experimental models is presented. Finally, in a second section, the main hypotheses concerning the mechanisms involved in the cardio-protective effect of short-term training are analyzed with an emphasis on how these could be linked to the regulation of the PTP.

3.1 Animal models and training paradigms

Table 2 provides an overview of the type of animals, training paradigms and experimental models used in the eight studies that have investigated the effect of short-term training on myocardial protection against I-R (40-42, 63-65, 67, 92). In general, all studies used treadmill running as the training modality. Most protocols involved 1 to 5 consecutive days of running for a duration of 60 min at a speed 30 m/min with a slope of 0%. The only two exceptions are the studies by Taylor et al. (92) and Lennon et al. (64) in which longer running periods (100 min) and/or lower speeds were used (18-20 m/min). Experiments were generally performed 24 h following the last bout of exercise, except in the study by Lennon et al. (63) in which experiments were performed up to 18 days after training cessation. Both males and females have been studied and the two genders appear to benefit from cardio-protection in response to this type of training. It should however be noted that one controversial study reported that a single bout of exercise was sufficient to induce cardio-protection against I-R in males but not in females (80), however this

Table 2 : Summary of short-term training studies

Authors	Animals	Training model	Exp. model
Hamilton et al. 2001	Female Sprague-dawley (SD)-4 months	3-5d, 60 min, 30 m./min, 0% 1wk habituation at these setting (5 min +10-15 min increase daily)	In vivo occl. 24 h post exercise
Hamilton et al. 2003	Female SD- 3 months	5d, 60 min, 30 m./min, 0% 1wk habituation at these setting (10 min +10-15 min increase daily)	In vivo occl. 24 h post exercise
Hamilton et al. 2004	Male SD- 4 months, 300-350g	3d 60 min, 30 m./min, 0% 1wk habituation at these setting + 2 d of rest	In vivo occl. 24 h post exercise
Lennon et al. (2004)	Male SD- 4 months, 300-350g	3d 60 min, 30 m./min, 0% 1wk habituation at these setting + 2 d of rest	WH 24 h post exercise
Lennon et al. (2004a)	Male SD- 6 months, 430-470g	3d 60 min, 30 m./min or 18 m./min, 0% 1wk habituation at these setting + 2 d of rest	WH 24 h post exercise
Lennon et al. (2004)	Male SD- 4 months, 370-400g	3d 60 min, 30m/min, 0% 5 d habituation (begin 10min at 30m/min, with daily increases of 10 min until 50 min/day were achieved	WH
Taylor et al. (1999)	Female SD- 5-7 months	1 or 3d, 100min, 20m./min, 6% no habituation	WH 24 h post exercise
Locke et al (1995)	Male SD- 250-300g	Male SD rats (250-300 g) 1 or 3 d 60 min, 30 m.min-1, 0% no habituation described	Langendorff 24 h post exercise

study had received many comments and critiques (96). Finally, the studies available can be distinguished according to the experimental model used to document cardio-protection. The two models that have been most frequently used are the ligation of the left ascending coronary artery (LCA) *in vivo* (40-42) and the isolated working heart preparation (63-65, 92). As for the Langendorff perfusion, it has only used once by Locke et al. (67).

3.2 Effect of short-term training on myocardial function after ischemia and on markers of tissue damage

Studies using *in vivo* LCA occlusion, a model of regional ischemia-reperfusion, have shown that short-term training results in a better preservation of LVDP during ischemia and reperfusion, a reduction in the occurrence of arrhythmia (Figure 14) during reperfusion and a reduction in the % of risk area infarcted (Figure 15) (40) (41) (42).

A significant level of cardio-protection was also obtained with the isolated working heart preparation (63-65, 92). This model allows to study cardiac performance without the confounding effects of other organs systems, the systemic circulation, and a host of peripheral complications (88). Another advantage over other isolated heart models such as the Langendorff preparation is that it permits cardiac pump function to be measured while controlling cardiac filling pressure and afterload. Under these conditions, short-term training was found to increase the recovery of cardiac output (CO) and hydraulic work (HW = cardiac output x peak systolic pressure) by 24 to 57 % compared to that measured in hearts from sedentary control animals (Table 2) (63-65, 92). Lennon et al. (63, 64) also reported a 50-62 % reduction in the release of LDH in the coronary effluent, indicative of a better preservation of sarcolemmal integrity.

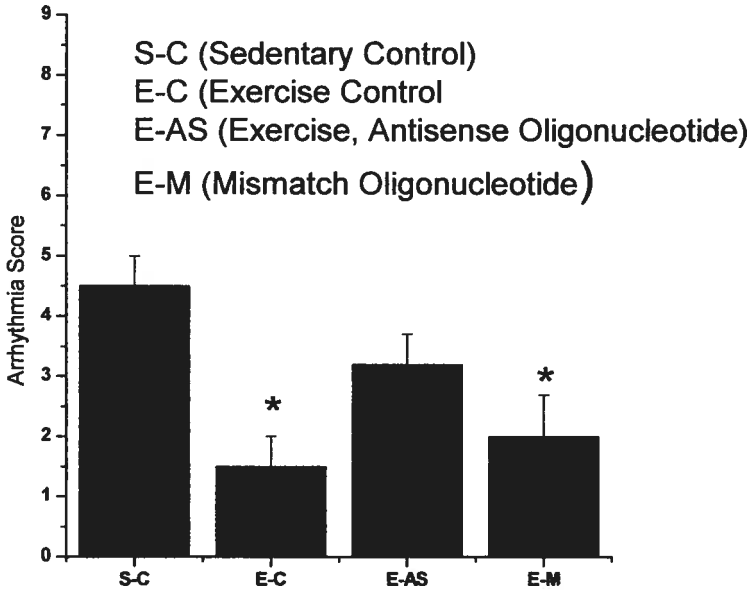

Figure 14: Effect of in vivo ischemia-reperfusion on arrhythmia scores. Taken from Hamilton et al. 2004. Significantly different from S-C ($P < 0.05$).

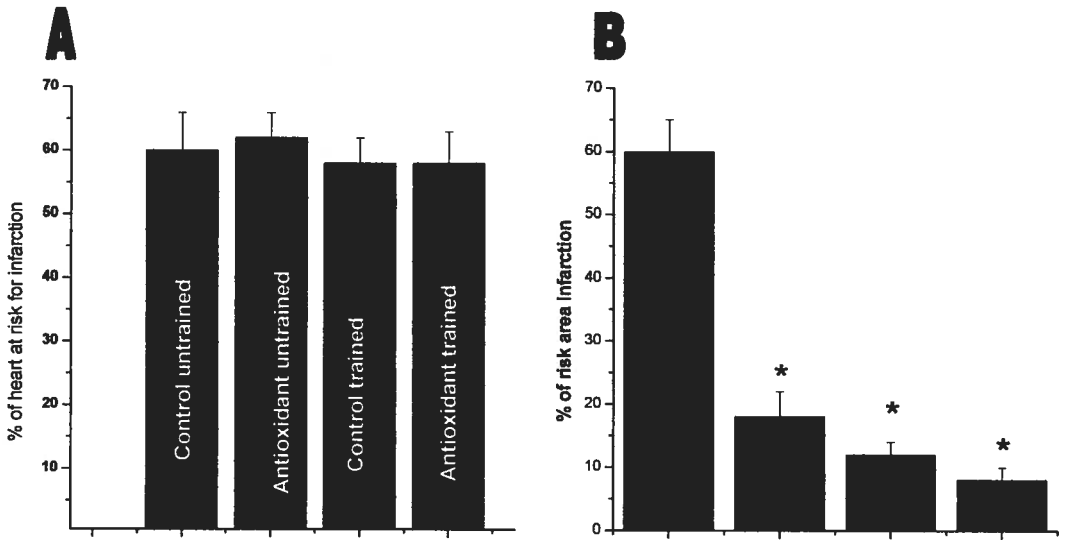

Figure 15: Analysis of the effect of long-duration I-R (60 min ischemia-120 min reperfusion) on the risk of infarction. Taken from Hamilton et al. 2003. A) Risk area expressed as the percentage of the heart. B) Infarction area expressed as a percentage of the area at risk. * less than control diet/untrained ($P<0.05$)

Importantly, Lennon et al. (64) showed that an equal degree of cardio-protection could be obtained using high (75 % VO_2max) and low (55 % VO_2max) training intensities. Moreover, the same authors (63) have also showed that the cardio-protective effect induced by short-term training lasted up to nine days after training cessation, indicating that this was not due to acute changes induced by the last training session.

Interestingly, studies using the working heart model also reveal that the degree of cardio-protection provided by short-term training depends on the type of hemodynamic parameter investigated. Indeed, while short-term training substantially increased the recovery of cardiac output and hydraulic work during reperfusion, it had either marginal or no effects on the recovery of heart rate (HR), systolic pressure (SP) and rate pressure product (RPP = HR x SP), which are parameters that are typically measured in Langendorff heart preparation (Table 2). However, the only study in which the Langendorff model was used reported that three consecutive days of training resulted in a significant improvement in the recovery of LVDP and rates of pressure development and relaxation (dp/dt max and min) (67) (Figure 16). However, Locke et al. (67) failed to find a cardio-protective effect following one day of training while Taylor et al. (92), using the working heart model, were able to show a significant cardio-protection at this early stage. Taylor et al. (92) suggested that parameters such as CO and HW, which depend on ventricular filling, could be more sensitive to training adaptation compared to HR, SP and RPP.

Taken together, these studies thus indicate that training for short periods of time ranging between 1 and 5 days can confer a significant degree of cardio-protection against I-R, which can last up to nine days after exercise cessation. This protection is characterized by an improved ability to

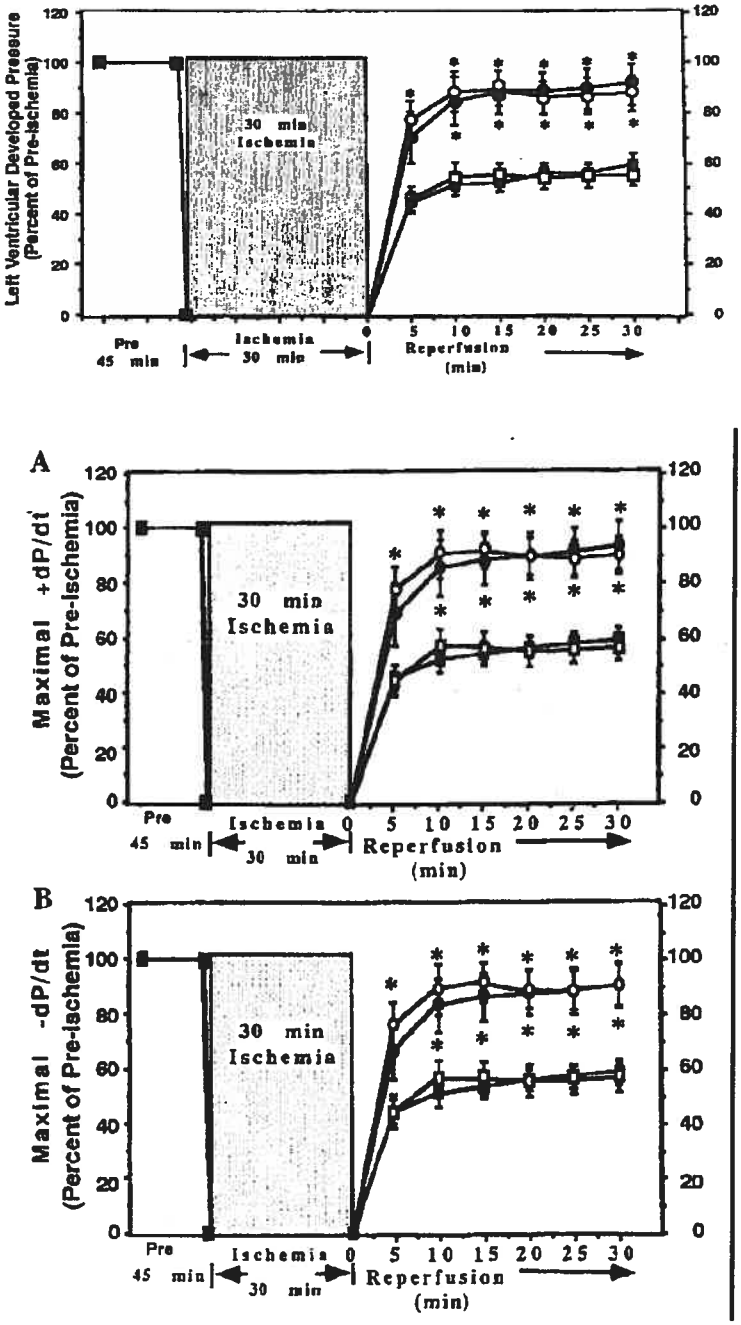

Figure 16: Postischemic recovery of left ventricular pressure and rates of contraction (+dP/d*t*) and relaxation (-dP/d*t*). Taken from Locke et al. 1995. Heat shock (●) and 3 bouts of exercise (○). *P<0.05 compared with controls.

recover contractile function, by a reduction in the occurrence of arrhythmias and a reduction in tissue damage.

3.3 Mechanisms underlying the cardio-protective effect of short-term exercise training

Currently, two general mechanisms were proposed to explain the protective effect of short-term training against I-R induced contractile dysfunction and tissue injury. The first mechanism is related to an up-regulation in the expression of heat-shock proteins, particularly those of the 70-kDa family, while the second mechanism involves the upregulation of one or many antioxidant defence systems.

3.3.1 Heat-shock proteins

3.3.1.1 Overview of the heat-shock family of proteins

Stress proteins are synthesized in response to variety of stressful conditions including elevated temperature and oxidative stress (84). Although there are two different classes of stress proteins, heat shock proteins and glucose-regulated proteins, experimental work related to exercise-induced cardio-protection has only focused on the former.

Heat shock proteins (HSPs) are expressed in both prokaryotes and eukaryotes and can be separated in a number of different groups based on their molecular weight: 1) small HSPs (8-32 kDa); 2) 40- to 60 kDa HSPs; 3) 70 kDa HSPs; 4) 90 kDa HSPs; and 5) 100- to 110 kDa HSPs (84)(Table 3). The primary functions of these stress proteins are to control protein folding, to

prevent the denaturation and aggregation of intracellular proteins during stress, to accelerate the breakdown of damaged proteins and to function as molecular chaperones based upon their molecular weights.

Expression of HSPs is generally believed to be regulated at the level of transcription although translational steps may also be important (84). The promoter regions of HSP genes have been sequenced and shown to contain a highly conserved cis-acting element, termed the heat shock element (HSE). This HSE is required to transduce the effects of cellular stress to the transcriptional factors (HSFs).

There exist at least two distinct HSFs (HSF1 and HSF2) in mammals. HSF1 is activated by heat, heavy metals, reactive oxygen species, and other factors that denature proteins. HSF2 is activated by hemin, used in heme-binding groups of myoglobin and catalase.

Stress-induced regulation of HSP transcription is mediated by HSF1 binding to HSE. Activation of HSF is a multi-step process including oligomerization of inactive monomers to trimers, nuclear localization, HSF-DNA binding at the promoter region of the gene, final modulation of HSF leading to transcriptional competency.

3.3.1.2 HSP-mediated cardio-protection:

The first evidences suggesting a role for heart shock proteins as a cardio-protective mechanism

Table 3: Summary of different HSP groups and their primary functions

Name of stress protein	Cellular location	Example(s) of cellular function	Comments
Ubiquitin (member of small HSPs)	Cytosol	Damaged proteins are conjugated to ubiquitin and targeted for degradation	Ubiquitin levels in cells increase following cellular injury
HSP40	Cytosol	Molecular chaperones and regulators of HSP70 ATPase activity	HSP40 family of stress proteins contains at least 20 different proteins
HSP72	Cytosol and nucleus	Molecular chaperone, prevention of protein aggregation, and refolding damaged proteins	Postulated to play an important role in myocardial protection against I-R injury
HSP73	Cytosol and nucleus	Molecular chaperone, prevention of protein aggregation, and refolding damaged proteins	Importance of HSP73 in protection against I-R injury is unknown
HSP90	Cytosol	May function as a molecular chaperone during maturation of steroid receptor: assists in the folding of newly synthesized peptides	At least two isoforms of HSP90 exist

has come from isolated hearts studies in which animals were submitted to whole body thermal stress (15 min at 42 ^{0}C) 24-72 hours prior to the experiments. This type of maneuver induces an important up-regulation in the expression of myocardial HSP's particularly HSP 72 (19, 67, 89). Several studies have shown that hearts from heat-shocked animals display an improved recovery of contractile function and a reduction in tissue damage following I-R (19, 67).

Further support for the cardio-protective property of HSP-70 has come from studies in which this protein was over-expressed in the heart using in vivo gene transfer technology (57, 58, 89). These studies have shown that isolated perfused hearts from transfected animals had a significantly greater recovery of LVDP and dt/dt min and max as well as a reduction in tissue leakage of creatine kinase indicative of less tissue damage.

3.3.1.3 Mechanisms underlying HSP-mediated cardio-protection:

Jayakumar et al. (58) reported that HSP-70 overexpression in the heart was associated with a significant protection of mitochondrial function against I-R injury. Indeed, oxidative phosphorylation capacity in mitochondria isolated from post ischemic hearts was several fold greater in HSP-70 transfected animals compared to control. Heat shock-induced mitochondrial protection has also been reported in liver submitted to I-R (5). Interestingly, He and Lemasters (47) recently observed that whole body heat shock was able to suppress PTP opening in isolated liver mitochondria presumably by increasing the expression of HSP 25, a mitochondrial chapronin that could interact with denaturised proteins forming the pore structure. By preventing mitochondrial damage and PTP opening, HSP's could thus favor the recovery of normal ATP

production following I-R and/or attenuate the release of mitochondrial proteins involved in apoptosis signaling. In this regards, it is interesting to note that HSP-70 also seems to have the capability to bind and reduce cytochrome *c*, which could inhibit its release in the cytosol and the subsequent activation of the caspase pathway. Similarly, Garrido et al. (30) have also shown that HSP-70 can directly bind AIF, another mitochondrial protein involved in the activation of apoptosis.

HSP-72 is also involved in protecting ion channels and pumps from dysfunction. At the level of the sarcolemma, opening of K_{ATP} channels was shown to have a negative impact on the recovery of the heart from I-R and HSP-72-associated cardioprotection is believed to be at least partly due to inhibition of K_{ATP} channel opening (51, 59). Recently, HSP-72 was also found to prevent inactivation of sarcoplasmic reticulum Ca^{2+} ATPase following heat stress by stabilizing the nucleotide binding domain (93). However, protection against I-R was not investigated in this study.

Taken together these results thus indicate that the protective action of HSP's against I-R injury can probably be exerted at various sites within cardiomyocytes which will impact on the maintenance of normal excitation-contraction coupling and energy production and contribute to prevent activation of the mitochondrial cell death pathways.

3.3.1.4 Implication of HSP's in training-induced cardioprotection

Locke et al. (67) were the first to suggest that HSP's could be involved in the cardio-protective effect of short-term training. These authors have shown that three consecutive days of running

induced a robust expression of HSP 72 that was comparable to that obtained following whole body thermal stress. Moreover, the authors reported a significant correlation between HSP-72 expression levels and the improvement of functional recovery in isolated hearts submitted to I-R.

Several studies have confirmed this early finding since then (63, 64, 92). Lennon et al. (64) also observed that the expression of HSP-72 at the mRNA and protein level was greater as the training increased from a moderate (55 % VO_2max) to a high intensity (75 % VO_2max). However, the higher levels of HSP-72 expression following high-intensity training did not confer a stronger cardio-protection. Indeed, the recovery of cardiac work and LDH release was similar in rats trained at both intensities. These results thus suggested that the degree of HSP-72 expression is not necessarily a good indicator of the cardio-protective potential of a training program.

Consistent with this idea, Lennon et al (63) later reported that the effect of short-term training on HSP-72 expression lasted up to three days following training cessation while the protection against I-R injury lasted up to nine days. Moreover, Taylor et al. (92) reported that running animals in a cold environment could abolish the increased expression of HSP-72 following one day of training. However, despite the absence of HSP-72 induction, hearts from exercised animals displayed an improved recovery of contractile function following I-R. Taken together these data thus indicated that while an increased expression of HSP-72 is cardio-protective per se, it does not appear to be essential to observe a cardio-protective effect following short-term training. As mentioned by these authors, one of the corollaries is that training probably induces multiple response that can result in protection against I-R injury and that when one response is blocked the other ones can take over.

3.3.2 Oxidative stress and antioxidant responses

Another longstanding hypotheses on the underlying mechanisms involved in training-induced cardio-protection is that regular exercise results in an improved capacity of enzymatic and non-enzymatic antioxidant systems (12, 21, 27, 41). This adaptation would result in a reduction in oxidative stress and resulting damage to key proteins, membrane lipids and DNA. With regard to short-term training, several studies have reported a reduction in oxidative damage to membrane lipids as indicated by lower levels of 8 isopostane and lipid hydroperoxydes following I-R in hearts from animals that were trained for 3-5 days (40, 42). Hamilton et al. (42) also reported lower levels of protein carbonylation and nitrosylation another evidence supporting the notion that short-term training attenuates oxidative damage in response to I-R.

Currently, the mechanisms responsible for the lower level of oxidative damage in the trained heart are largely unknown. In cardiomyocytes, several sources of ROS exist. Moreover, there are several enzymatic and non-enzymatic systems involved in their degradation, and at any time the level of oxidative damage depends on the rate at which ROS are produced and eliminated by these various pathways.

Regarding pathways for ROS elimination, the studies available indicate that short-term training increases the activity of some antioxidant systems. The most systematically reported adaptation is an increase in the activity of superoxide dismutase (SOD), an enzyme responsible for the dismutation of the highly toxic superoxide anion in H_2O_2, a relatively less noxious molecule (41). Two isoforms of this enzyme exist, the cytosolic Cu/Zn SOD and the mitochondrial Mn-SOD.

The studies available indicate that short-term training only affects the mitochondrial isoform (40-42, 64), which could result in a reduction in oxidative damage to this organelle.

Interestingly, a recent study by Hamilton et al. (41) have shown that blunting training-induced Mn-SOD upregulation using silencing RNA technology resulted in a significant reduction in the protective effect of training on reperfusion arrhythmias. This is the only study in which a causal link between an increase in anti-oxidant capacity and training-induced cardio-protection is demonstrated.

On the other hand, short-term training has little to no effect on the activity of other antioxidant enzymes. Catalase, an enzyme converting H_2O_2 into H_2O, was shown to be slightly up regulated (63, 64) or unchanged (40-42) following short-term training. As for glutathione peroxidase and the glutathione pool, which are also involved in converting H_2O_2 to H_2O, they are apparently not affected by training (40-42). One question that remains unanswered is whether short-term training can attenuate the rate of ROS production independent of changes in anti-oxidant capacities.

3.4 Is the PTP involved in the protective effect of short-term training?

Although the effect of short-term training on the mitochondria in general and the PTP in particular has never been assessed, we believe that some of the experimental evidence reviewed in this thesis suggest that they could have a role in protecting the heart against I-R injury and contractile dysfunction.

A reduction in ROS production, especially at the mitochondrial level (40-42, 63, 64) could directly favour the maintenance of the PTP in the close conformation as mentioned in table 1 of section 2.1.3 as well as the article by Zoratti and Szabo. (97). In addition, by attenuating oxidative damage to components of the respiratory chain, a reduction in ROS production could favour a better recovery of $\Delta\Psi$, which will also reduce the likelihood of pore opening (97).

In addition to ROS, an increased expression of HSP's could also result in the inhibition of the PTP. Indeed, mitochondria were shown to be a direct target for HSP-70 mediated protection against I-R injury (5, 57, 58, 89) and induction of HSP expression was recently found to inhibit PTP opening in isolated mitochondria (47). By protecting against ionic channel and pump dysfunction (79,72,53,51), HSP overexpression in the trained heart could also indirectly contribute to inhibit PTP opening by reducing disturbance in ion homeostasis and thus Ca^{2+} overload.

The experimental work performed in this thesis directly aimed to determine whether following short-term training PTP opening was reduced in the heart submitted to ischemia-reperfusion.

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Chapter 2- The cardioprotective effect of a short-term aerobic exercise program and the mitochondrial permeability transition pore of the rat

Abstract

Opening of the mitochondrial permeability transition pore (PTP) is known to occur during reperfusion of the ischemic heart and to contribute to contractile dysfunction and tissue injury. The purpose of the present study was to determine whether short-term training (treadmill running for 5 days, 30 $m.min^{-1}$, 0%) in male SD rats reduces the occurrence of PTP opening in the ischemic-reperfused heart. Hearts from control (C) and trained (T) rats perfused in the Langendorff mode were submitted to ischemia-reperfusion (I-R: 30 and 40 min respectively). In situ PTP opening was quantified using the mitochondrial 2-deoxy [^{3}H]glucose ([^{3}H]DOG) entrapment method. Following I-R, the recovery of intact mitochondria upon isolation was significantly greater in T vs C hearts (11.7 ± 0.5 vs 9.1 ± 0.4 mU citrate synthase/g^{-1} wet ventricles, $P \leq 0.01$). Training also reduced the entrapment of mitochondrial [^{3}H]DOG normalized for the loss of intact mitochondria (14.4 ± 1.4 vs 9.6 ± 0.8 [^{3}H]DOG units, $P \leq 0.01$). However, under the experimental conditions used the recovery of contractile function, coronary flow and release of LDH in the coronary effluent were similar in both experimental groups. Taken together, these results suggest that short-term training can confer mitochondrial protection and reduce PTP opening.

KEY WORDS

Ischemia-reperfusion injury

Isolated heart perfusion

2-deoxyglucose entrapment

MPTP

Exercise

INTRODUCTION

The mitochondrial permeability transition pore was initially described in isolated mitochondria as a sudden increase in the permeability to solutes of < 1500 Da (33). It is now largely recognized that this phenomenon is caused by the opening of a non-specific high conductance channel of the inner membrane presumably formed by the association of cyclophilin D with the adenine nucleotide translocator (ANT) and porin (VDAC) as the core of the complex (33,34). Opening of the PTP induces the loss of mitochondrial membrane potential (33), uncoupling of oxidative phosphorylation, high amplitude swelling of the matrix and the release of several pro-apoptotic factors that are normally sequestered in mitochondria such as cytochrome c, AIF, Smac/Diablo, endonuclease G and Omi/HtrA2 (14,26,27,33). For this reason, PTP opening has been implicated in several models of necrotic and apoptotic cell death in various tissues (14,26,27).

Accumulation of Ca^{2+} in the matrix is the most important stimulator of PTP opening (33). However, the sensitivity of the PTP to Ca^{2+} is increased by a variety of factors including elevated matrix [P_i] and pH, low [adenylates] and membrane potential (33), as well as an increased oxidative stress and oxidation of the pyridine nucleotide pool. PTP opening was also shown to be influenced by members of the bcl-2 family of proteins, with the pro-apoptotic bid and bax and the antiapoptotic bcl-2 and bcl_{XL} acting as facilitators and repressors of PTP opening respectively (8, 28, 29, 30).

In the heart PTP opening was suspected to occur following ischemia-reperfusion (I-R) based on the fact that several of the conditions required to observe PTP opening *in vitro* prevail in cardiac cells, particularly during early reperfusion (17, 18). Development of *in situ* methods applicable

for the measurement of PTP opening in the perfused heart allowed confirmation of this hypothesis. Indeed, measurement of mitochondrial entrapment of 2-deoxy [^{3}H]glucose ([^{3}H]DOG) and release of NAD^+ (reviewed in (6)) has clearly shown that PTP opening occurs during reperfusion following ischemia and can have deleterious effects on myocardial recovery.

Recently, ischemic preconditioning (IPC) was reported to inhibit PTP opening following I-R (2, 12,16). However, whether exercise training, another physiological stress capable of inducing cardio-protection can reduce the occurrence of PTP opening has not been investigated. Therefore, in the present study the mitochondrial [^{3}H]DOG entrapment method was used to determine if short-term training reduced the occurrence of PTP opening in ischemic-reperfused hearts and whether this was associated with cardio-protection.

METHODS

Animal care

All experiments were conducted according to the directives of the Canadian Council on Animal Care. Male Sprague-Dawley rats (Charles River, St-Constant, PQ, Canada) weighing approximately 250 g were housed by pair and kept in a temperature, humidity, and light controlled (12:12h light-dark cycle) environment. The animals were fed standard rat chow and provided water *ad libitum*.

Exercise protocol

Following 2-3 days of habituation on the rodent treadmill, animals ran 60 min/d for 5 consecutive days (30 $m.min^{-1}$ and 0 % slope) at an intensity corresponding to ~75 % of VO_2 max (24). Air puffs and mild electrical shocks were sporadically used in order to maximize running time. Control animals consisted in age-, sex- and cage-matched sedentary rats.

Langendorff perfusion

All experiments were performed 48 hours after the last training session. Hearts from ketamine-xylazine (62:8 mg/kg) anesthetized rats were rapidly excised and immersed in ice-cold Henseleit (KH) buffer (in mM: NaCl 119, KCl 4.8, $MgSO_4$ 1.2, $NaHCO_3$, 24 KH_2PO_4 1.2, $CaCl_2$ 1.3, glucose 11, pH 7.4). The aorta was cannulated and the coronary arteries perfused with KH buffer in the Langendorff mode at a constant pressure of 70 mm Hg. The perfusion solution was oxygenated with 95% O_2 – 5% CO_2 and maintained at 37 °C throughout the perfusion. Perfusion pressure was monitored by use of an in-line pressure transducer connected to an in line data acquisition system (Powerlab 8/30, ADInstruments, Colorado Springs, CO). Ventricular pressure

was monitored via a separate pressure transducer connected to a fluid-filled latex balloon inserted in the left ventricle via the left atrium. The balloon was inflated to provide an end-diastolic pressure of 5-8 mm Hg. Hearts were maintained in a water-jacketed chamber maintained at 37 °C and global isothermic ischemia was induced by clamping the aortic line. Following 30 min of ischemia, flow was re-established for 40 min. Coronary effluent was collected regularly throughout the perfusion for measurements of coronary flow and determination of LDH release which was expressed in $mU.min^{-1}.g^{-1}$ of wet tissue.

Measurement of MPTP opening in situ with 2-deoxy [^{3}H]glucose

Measurement of mitochondrial [^{3}H]DOG entrapment was performed as described in Griffiths and Halestrap (6) with minor modification. After a 20-min period of stabilization in the flow-through mode, hearts were perfused for 30 min in the re-circulating mode with 100 mL of KH buffer supplemented with 0.5 mM 2-deoxy [^{3}H]glucose (0.1 $\mu Ci.mL^{-1}$). During this period, perfusion was performed at a constant flow of 10 $ml.min^{-1}$ in order to minimize differences in [^{3}H]DOG uptake between experiments. Perfusion was then returned to the constant pressure flow-through mode with normal KH buffer for 15 min in order to wash-out extra-cellular [^{3}H]DOG. Hearts were then submitted to ischemia-reperfusion as described above or immediately processed for isolation of mitochondria and determination of baseline [^{3}H]DOG entrapment.

At the end of perfusion hearts were removed, weighted and homogenized in 5 mL of ice-cold sucrose buffer (in mM: sucrose 300, Tris-HCl 10, EGTA 2, BSA 5 mg/mL, pH 7.4) with a polytron homogenizer (setting 3 for 5 sec) and volume was then completed to 40 mL. An aliquot of the crude homogenate were retained for measurements of [^{3}H]DOG and the remainder was

immediately centrifuged at 800 g for 2 min to remove cellular debris. The surpernatant was recovered and centrifuged at 10 000 g for 10 min. The mitochondrial pellet obtained was washed in 30 mL of sucrose buffer containing no BSA and centrifuged at 10 000 g for 10 min. The final mitochondrial pellet was re-suspended in 0.5 mL of sucrose buffer without BSA. 100 µL of this mitochondrial suspension was retained for the measurement of citrate synthase activity (CS) and the remainder used for the determination of [^{3}H]DOG. In all buffers a high concentration of EGTA was used in order to favour rapid PTP closure and entrapment of [^{3}H]DOG (34). For the measurement of [^{3}H]DOG, crude homogenate and mitochondrial samples were mixed with an equal volume of 5% (w/v) perchloric acid and centrifuged at 10 000 g for 2 min. Radioactivity of the supernatant (500 µL samples) was counted in 10 mL of scintillant.

Mitochondrial [^{3}H]DOG entrapment

Calculation of the DOG index was performed as described in Javadov et al. (16). This index was expressed as follows:

$$\text{DOG index} = 10^5 \times \text{mitochondrial [}^3\text{H]DOG / tissue [}^3\text{H]DOG.}$$

where mitochondrial and tissue [^{3}H]DOG are expressed in d.p.m per unit of CS and d.p.m per g of wet ventricular tissue respectively. This calculation thus allows mitochondrial [^{3}H]DOG entrapment to be normalized for the concentration of mitochondria present in the mitochondrial fraction and for possible differences in the tissue uptake of the tracer. In addition, this index was corrected by the amount of CS recovered per gram of ventricle, which is an indicator of mitochondrial yield (16). This correction provides a more valid index of PTP opening since it

takes into account [^{3}H]DOG entrapment in mitochondria that become totally disrupted as a result of I-R and are not recovered during isolation (7, 16).

Statistical analyses

Results are expressed as means ± S.E.M. Difference between trained and control groups before and after ischemia were analyzed by means of a two way ANOVA. Tuckey post hoc tests were performed to identify the location of significant differences when the ANOVA yielded a significant F ratio. The Bonferonni correction was applied to the P value obtained to correct for multiple comparisons. A corrected P value < 0.05 was considered significant.

RESULTS

Heart Perfusion:

Figure 1 shows the evolution of heart rate (HR) and left ventricular developed pressure (LVDP) throughout the perfusion protocol in hearts submitted to ischemia-reperfusion. At the end of the initial 20-min period of stabilization HR and LVDP were similar in both experimental groups. Loading of hearts with [^{3}H]DOG and subsequent washout resulted in a small decline in LVDP, which reached statistical significance only in the control group. Similar results were obtained in the hearts that were not submitted to ischemia-reperfusion and which were used for the measurement of baseline mitochondrial [^{3}H]DOG entrapment (results not shown). Consistent with previously published data using the [^{3}H]DOG method (6,19) preliminary experiments showed that recirculation of the buffer more than the presence of DOG *per se* was responsible for this slight reduction of function.

Reperfusion following 30 min of global ischemia led to a progressive increase in contractile function. However, no significant differences between control and trained hearts were observed in the recovery of heart rate, LVDP, maximal and minimal dp/dt, and coronary flow (Table 1). Similarly, no significant difference was observed in the release of tissue LDH in the perfusate (Figure 2).

Measurement of mitochondrial recovery and PTP opening:

Figure 3 shows the activity of citrate synthase recovered in the mitochondrial fraction per gram of ventricular tissue. In hearts loaded with [^{3}H]DOG but not submitted to I-R, the CS activity recovered ranged between 12-15 $mU.g^{-1}$ of wet ventricle and no significant difference was

observed between the two experimental groups. Compared to non-ischemic hearts, the recovery of CS was significantly reduced by 40 % in the control group submitted to I-R. In contrast the loss of CS caused by I-R was only of 18% in the trained group.

It was previously shown that in hearts that are not submitted to I-R, [^{3}H]DOG incorporation in mitochondria is low and probably represents a combination of a slow PTP-independent uptake of [^{3}H]DOG into mitochondria and contaminant vesicular components in the mitochondrial fraction (6). Consistent with these results, the mitochondrial DOG index was low in non-ischemic hearts (Figure 4A). Moreover, no significant differences were observed between the two experimental groups (11.8 ± 1.6 and 13.5 ± 1.6 DOG ratio units in C and T respectively, P = NS).

In hearts from control animals, I-R increased the DOG index 11 fold above baseline normoxic values reaching 130 ± 12 DOG ratio units (Figure 4A). In the trained group the DOG index measured following I-R (110 ± 7 DOG ratio units) was 16 % lower compared to the control group, although this difference did not reach statistical significance (P=0.1). However, this index does not take into account [^{3}H]DOG entrapment in mitochondria that became totally disrupted as a result of I-R, therefore leading to an underestimation of the true extent of PTP opening (6,16). Figure 4 B shows that when the difference in mitochondrial recovery (Figure 3) was taken in to account, the inhibitory effect of training on mitochondrial [^{3}H]DOG entrapment increased to 33 % (9.6 ± 0.8 *vs* 14.4 ± 1.4 DOG units respectively $P < 0.05$).

DISCUSSION

Results from the present study provide evidence that short-term training can provide mitochondrial protection against injury as evidenced by an enhanced recovery of intact mitochondria following I-R. In addition, the [^{3}H]DOG entrapment data indicates that mitochondrial PTP opening during reperfusion is reduced in the heart of trained animals. However, under our experimental conditions these beneficial changes were not accompanied by an improved recovery of contractile function and LDH release.

Mitochondrial protection:

Several studies using the [^{3}H]DOG method indicate that an ischemia-reperfusion protocol similar to the one used in the present study is sufficient to induce a significant alteration in mitochondrial recovery and PTP opening (6, 8, 16, 17, 19). Indeed, the recovery of intact mitochondria assessed by CS activity was shown to be reduced by 22-34 % in response to 30 min of ischemia followed by 15-30 min of reperfusion (6, 16). This loss of mitochondria was accompanied by PTP opening, as evidenced by a 3.5-5.6 fold increase in the mitochondrial [^{3}H]DOG entrapment (6, 16). Data from the present experiments obtained in hearts of control animals are in line with these results. Indeed, following I-R the recovery of CS was reduced by 40 % compared to values obtained in non-ischemic hearts. In addition, this was accompanied by a substantial increase in mitochondrial [^{3}H]DOG entrapment (Figure 4).

To our knowledge, the effect of exercise training on mitochondrial integrity and PTP opening in the ischemic-reperfused heart has not been investigated before. However, the effect of other physiological stresses such as ischemic-preconditioning (IPC) has recently been studied by several research groups (1, 12, 16). Javadov et al. (16) reported that IPC abolished the loss of CS

activity following I-R and reduced by 52 % mitochondrial [^{3}H]DOG entrapment corrected for CS recovery. These data obtained in perfused hearts are consistent with results from studies on isolated heart mitochondria (1) and cardiomyocytes (12) indicating that the susceptibility of the PTP to Ca^{2+} and oxidative stress is reduced following IPC.

In the present study, 5 consecutive days of running was able to attenuate by 2 fold the loss of CS recovery following I-R. This improvement in mitochondrial yield was accompanied by a modest 16 % reduction in mitochondrial [^{3}H]DOG entrapment, which did not reach statistical significance. However, this reduction reached 33 % when the better recovery of intact mitochondria was taken into account. This phenomenon is similar to that observed following administration of the PTP inhibitors cyclosporin A (6, 16) and sanglifehrin A (16) in perfused hearts. Indeed, both inhibitors only reduce mitochondrial [^{3}H]DOG entrapment by 17-21 %. However, when the large improvement in the recovery of intact mitochondria observed with these agents is taken into account, the reduction in mitochondrial [^{3}H]DOG entrapment reaches 35-50 % (7).

The mechanisms by which training could inhibit PTP opening during reperfusion were not investigated in the present study. A reduction in PTP opening could be due to training adaptations that beneficially effect the concentration of intracellular modulators of PTP opening and/or alter its regulatory properties directly at the mitochondrial level. The reduction in oxidative stress (9-11, 21-23) and the increased expression of heat shock proteins (9, 11, 22, 23, 25, 32) reported following short-term training could be a candidate mechanism. Indeed, it is well documented that oxidative stress favours PTP opening in isolated mitochondria (33) and that treatment with agents

displaying anti-oxidant properties inhibit PTP opening in perfused hearts (17, 19, 31). Similarly, increased expression of heat shock proteins decrease PTP opening in isolated liver mitochondria (13) and attenuate I-R (18) and H_2O_2 (2) induced mitochondrial dysfunction in the heart. This hypothesis however remains to be ascertained.

Functional recovery and tissue damage:

In the present study, training did not result in a significant improvement in functional recovery and LDH release despite a significant reduction in PTP-induced mitochondrial damage. This observation contrasts with several studies (9-11, 21-23, 25, 32) showing that 3 to 5 consecutive days of treadmill running at an intensity similar to that used in the present study improves functional recovery and reduce tissue damage following a period of ischemia ranging between 20 and 30 min.

Although the reason for this discrepancy remains unclear, one possibility is that this is related to loading of the hearts with [^{3}H]DOG. 2-deoxyglucose is a non-metabolizable glucose analog which, following phosphorylation by hexokinase, accumulates in cells as DOG-6P. Accumulation of DOG-6P is known to result in a rapid depletion of myocardial ATP when DOG is used at concentration higher than 2 mM (15). In the present study, the [DOG] used was well below 2 mM (0.5 mM) and the accumulation of DOG-6P was not sufficient to induce significant contractile dysfunction prior to ischemia. However, during reperfusion, it cannot be excluded that the presence of DOG-6P affected myocardial recovery to a greater extent in trained compared to control animals.

In addition to the possible effect of DOG, examination of the literature suggests that the Langendorff preparation *per se* might not be optimal for the observation of exercise induced cardio-protection. Indeed, most (9-11, 21-23, 32), but not all (25) studies on the cardio-protective of short-term training, used either *in vivo* left coronary occlusion or the isolated working heart preparation which more closely mimics normal loading conditions. In addition, it is known that the contractile parameters measured in Langendorff perfusions are not as sensitive as the ones that can be obtained with the working heart preparation (32). Indeed, in studies using the working heart model (21-23, 32) the recovery of cardiac output and hydraulic work is substantially increased by short-term training (+26-57 % vs control) while heart rate, systolic pressure and rate pressure product, which are the contractile indexes measured in the Langendorff perfusion, are either unchanged or marginally improved (+0-10% vs control). In line with this observation, Lennon et al. (22) observed that 5 days of treadmill running at low intensity (18 $m.min^{-1}$, 0% slope) was able to increase the recovery of cardiac output and hydraulic work by 45 and 57 % respectively and to reduce the release of LDH by 62 %. In contrast, Libonati et al. (24) reported that a similar training program (60 min per day, 20 m.min-1, 0%) lasting 6 weeks had no effect of the recovery of LDVP, rates of pressure development and LDH release in Langendroff perfused hearts. A similar phenomenon could therefore account for the lack of effect of training on the recovery of contractile function and LDH release observed in the present study.

Taken together the present results provide evidence that short-term training can attenuate mitochondrial damage and PTP opening which normally occurs in the heart following ischemia-reperfusion. However, under the experimental conditions used, the contribution of this

mitochondrial protection to the protective effect of short-term training reported (9-11, 21-23, 25, 32) could not be established.

AKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Institute of Health Research. YB is a Junior Investigator of the Fonds de Recherche en Santé du Québec (FRSQ).

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Figure legends

Figure 1: Contractile function in [^{3}H]DOG-loaded heart before ischemia and during reperfusion. The figure shows the evolution of heart rate (panel A) and left ventricular developed pressure (LDVP: panel B) in control and trained hearts that were submitted to ischemia-reperfusion. For sake of clarity, the values obtained in hearts that were not submitted to I-R and were used for measurement of baseline [^{3}H]DOG entrapment are not shown. Following the initial 20-min period of stabilization in the flow-through mode, hearts were re-circulated with [^{3}H]DOG as indicated. Perfusion was switched back to the normal flow-through mode for 15 min prior to ischemia in order to washout extra-cellular [^{3}H]DOG. a: Significantly different ($P< 0.05$) from pre-ischemic value at T= 60 min within the same experimental group.

Figure 2: Release of LDH in the perfusate before ischemia and during reperfusion. The figure shows the release of LDH during reperfusion expressed relative to baseline pre-ischemic (PI) values. The release of LDH was calculated by multiplying time-matched values of coronary flow (in $mL.min^{-1}.g^{-1}$ wet weight) and LDH concentration measured in the perfusate ($mU.mL^{-1}$). Pre-ischemic (PI) release of LDH was low (<10 $mU.min^{-1}.g^{-1}$ wet tissue) and not significantly different in the control and trained groups. a: Significantly different ($P< 0.05$) from pre-ischemic value within the same experimental group.

Figure 3: Effects of I-R and short-term training on the mitochondrial isolation yield. The figure shows the recovery of citrate synthase (CS) in the mitochondrial fraction per

gram of ventricular tissue in non-ischemic hearts (NI: n= 4 in each experimental group) and hearts submitted to I-R (I-R: n of 11 and 9 in the control and trained groups respectively). a: Significantly different (P< 0.05) from non-ischemic hearts within the same experimental group. b: Significantly different (P< 0.05) from control.

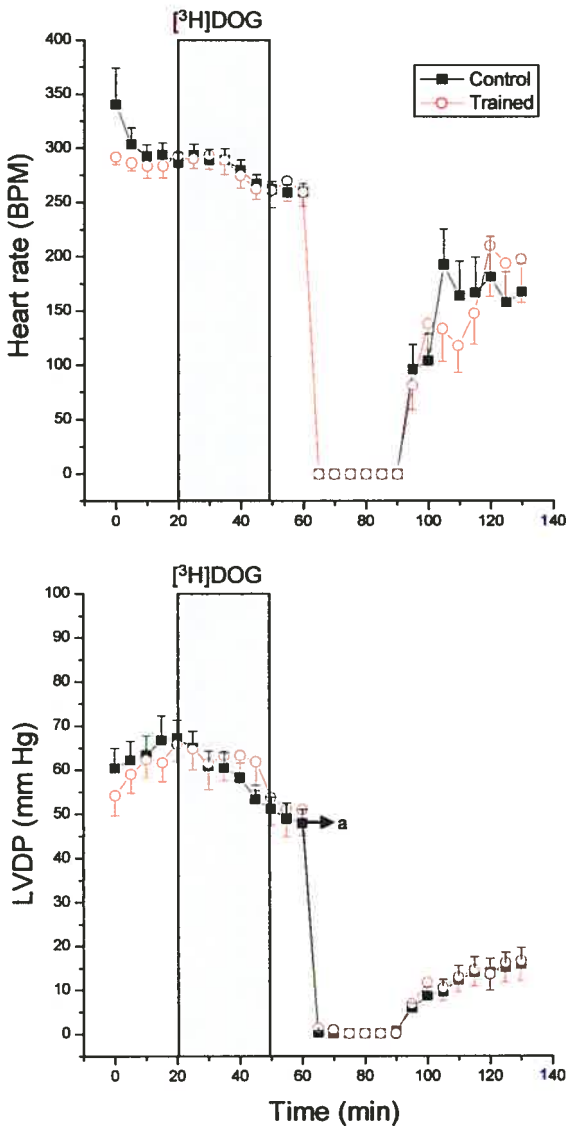
Figure 4: Effect of I-R and short-term training on mitochondrial [^{3}H]DOG entrapment. Panel A shows the DOG index expressed as the ratio mitochondrial d.p.m. / tissue d.p.m (see methods for further details) in non-ischemic hearts (NI: n= 4 in each experimental group) and hearts submitted to I-R (I-R: n of 11 and 9 in the control and trained groups respectively). Panel B shows the same DOG index normalized for the activity of CS recovered in the mitochondrial fraction per g of ventricular tissue (see figure 3). a: Significantly different (P< 0.05) from non-ischemic hearts within the same experimental group. b: Significantly different (P< 0.05) from control.

Tables legends

Table 1: Myocardial function before ischemia and at the end of reperfusion in hearts from control and trained rats. Data are presented as means ± SEM for a *n* of 11 and 9 hearts in the control and trained group respectively. a: Significantly different ($P < 0.05$) from pre-ischemic values within the same experimental group.

Figures

Fig.1


[3H]DOG
Control
Trained
Heart rate (BPM)
400
350
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200
150
100
50
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0
20
40
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80
100
120
140
[3H]DOG
LVDP (mm Hg)
100
90
80
70
60
50
40
30
20
10
0
a
Time (min)

Fig.2

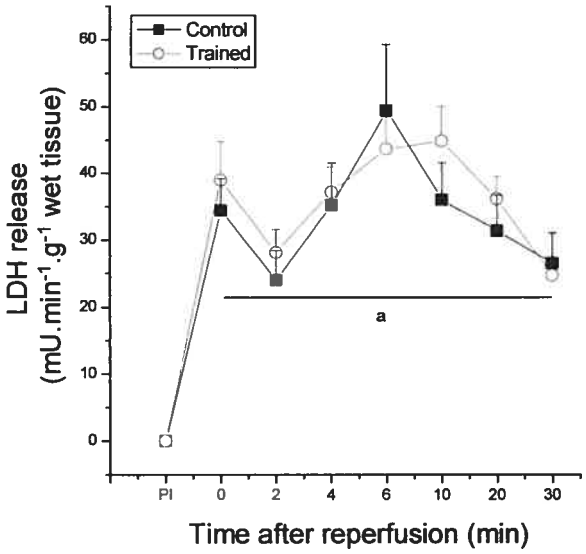
Control
Trained
LDH release
(mU.min-1.g-1 wet tissue)
60
50
40
30
20
10
0
a
PI
0
2
4
6
10
20
30
Time after reperfusion (min)

Fig.3

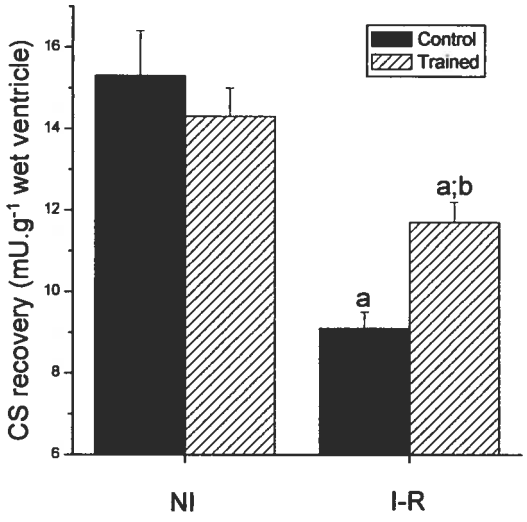
Control
Trained
CS recovery (mU.g-1 wet ventricle)
16
14
12
10
8
6
a
a;b
NI
I-R

Fig.4

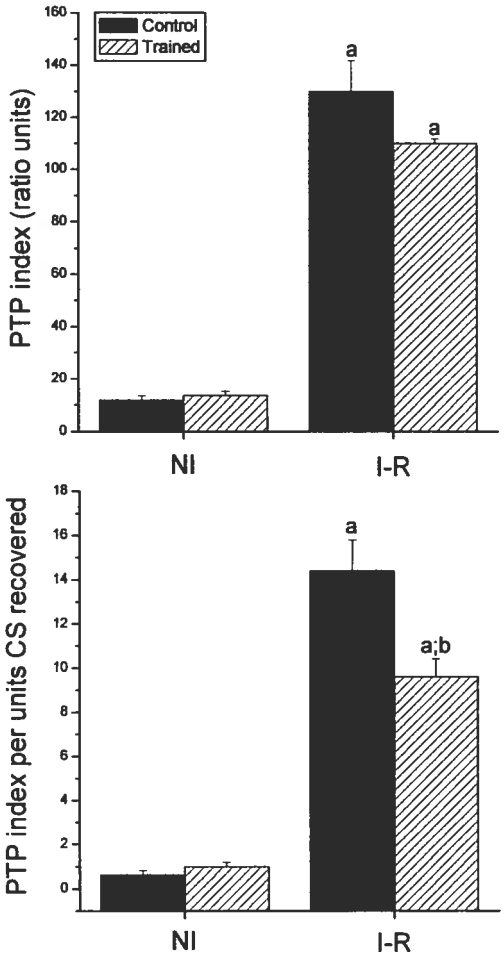
Control
Trained
PTP index (ratio units)
160
140
120
100
80
60
40
20
0
a
a
NI
I-R
PTP index per units CS recovered
18
16
14
12
10
8
6
4
2
0
a
a;b
NI
I-R

Table 1 : Myocardial function before ischemia and at the end of reperfusion in hearts from control and trained rats

	Pre-ischemic	Reperfusion	Recovery (%)
Heart rate (bmp)			
C	260 ± 8	168 ± 26[a]	65 ± 10
T	260 ± 13	198 ± 39[a]	74 ± 10
LVDP (mm Hg)			
C	48 ± 3	16 ± 4[a]	32 ± 7
T	51 ± 6	16 ± 4[a]	34 ± 8
max dp/dt (mm Hg^{-1})			
C	1886 ± 247	498 ± 100[a]	28 ± 6
T	1719 ± 175	517 ± 133[a]	31 ± 7
Min dp/dt (mm Hg^{-1})			
C	-961 ± 97	-294 ± 56[a]	33 ± 6
T	-923 ± 93	-297 ± 72[a]	33 ± 7
Coronary flow ($mL.min^{-1}.g^{-1}$ wet weight)			
C	7.9 ± 0.5	5.0 ± 0.7[a]	63 ± 8
T	8.0 ± 0.2	4.1 ± 0.3[a]	51 ± 4

Data are presented as means ± SEM for a *n* of 11 and 9 hearts in the control and trained group respectively. a: Significantly different (P< 0.05) from pre-ischemic values within the same experimental group.