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Endurance training-induced cardioprotection against oxidative-based insults

Biochemical, morphological and functional evidences in whole tissue and isolated mitochondria of rodents

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THEORETICAL BACKGROUND

1.

Ascensão, A., Magalhães, J., Soares, J., Oliveira, J., Duarte, J. (2003). Exercise and cardiac oxidative stress. ***Rev. Port. Cardiol***. 22 (5): 651-678.

2.

Ascensão, A., Magalhães, J., Soares, J., Ferreira, R., Neuparth, M., Appell, H., Duarte, J. (2005). Cardiac mitochondrial respiratory function and oxidative stress: the role of exercise. ***Int. J. Sports Med.*** (*in press*).

EXPERIMENTAL WORK

I.

Ascensão, A., Magalhães, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., Oliveira, J., Duarte, J. (2005). Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. ***Int. J. Cardiol.*** (*in press*).

II.

Ascensão, A., Magalhães, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., Duarte, J. (2005). Endurance exercise training attenuates morphological signs of cardiac muscle damage induced by doxorubicin in mice. Submitted for publication to ***Basic Appl. Myol.***

III.

Ascensão, A., Magalhães, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., Oliveira, P., Duarte, J. (2005). Moderate endurance training prevents doxorubicin-induced *in vivo* mitochondriopathy and reduces the development of cardiac apoptosis. Under review for publication in ***Am. J. Physiol. Heart and Circ. Physiol.***

IV.

Ascensão, A., Magalhães, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., Oliveira, P., Duarte, J. (2005). Endurance training limits the functional alterations of heart rat mitochondria submitted to *in vitro* anoxia-reoxygenation. Submitted for publication to ***Int. J. Cardiol.***

ABSTRACT

Endurance exercise training is postulated as one of the major non-pharmacological countermeasures against myocardial damage. Nevertheless, the cellular basis for training-induced cardioprotection has not been identified, although various candidate adaptations have been proposed. This dissertation, comprising four experimental studies, intended to analyze the impact of cardiac deleterious stimuli and training on markers of oxidative stress and damage, apoptosis and respiratory function either on overall cardiac homogenate or on isolated mitochondria.

The studies I and II aimed to investigate the effects of 14-wks of endurance training on the biochemical and histological deleterious alterations induced by *in vivo* administration of doxorubicin (DOX) on plasma cardiac troponin I (cTnI) and on the whole cardiac muscle through the analysis of the following parameters: contents of reduced and oxidized glutathione, thiobarbituric acid reactive substances, protein sulfhydryl (-SH) and carbonyl groups, heat shock proteins 60 Kda (HSP60) and 70 KDa (HSP70) expression. Total antioxidant status and the enzymatic activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase were also measured. Morphological damage scores of cardiac tissue and specifically, of mitochondrial structure, were obtained using light and/or electron micrographs. The protective effects of endurance training against *in vivo* DOX (III) and *in vitro* anoxia-reoxygenation (IV) were tested regarding mitochondrial respiratory function and oxidative damage (III and IV) and the triggering of apoptosis (III). To reach these purposes, the used biochemical markers included the contents of plasma cTnI, mitochondrial –SH and carbonyl groups, malondialdehyde, Bax, Blc2, mitochondrial HSP60 and tissue HSP70 expression, mitochondrial aconitase and tissue caspase 3 activities. Mitochondrial respiratory rates were determined in state 3, state 4, in the presence of oligomycin, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), guanosine diphosphate (GDP) and calcium. Respiratory control and ADP/O ratios were also calculated.

Previous endurance training attenuated the rise in cardiac and/or mitochondrial biochemical, morphological and functional impairments caused by the oxidant-based stimuli used in the studies. Taken together, these data provided additional contribution to understand the training-induced cardioprotection phenomenon against oxidative-related damage induced by *in vivo* DOX at different levels of cellular organization and by *in vitro* mitochondrial anoxia-reoxygenation.

***Keywords*:** Chronic exercise training, heart, mitochondria, cross-tolerance, doxorubicin, anoxia-reoxygenation, oxidative stress and damage, respiration, animals

RESUMO

O treino de resistência tem sido referido como uma das principais medidas não farmacológicas aplicadas no combate a situações de lesão e disfunção do miocárdio. Contudo, os mecanismos celulares associados à protecção cardíaca mediados pelo treino encontram-se ainda pouco esclarecidos, embora tenham sido propostas várias explicações para o referido efeito. A presente dissertação, composta por quatro estudos experimentais, pretende estudar o impacto do treino e de estímulos cardíacos nefastos nos marcadores de lesão e stress oxidativo, apoptose e funcionalidade respiratória, quer no homogeneizado cardíaco quer em mitocôndrias isoladas.

Os estudos I e II pretenderam estudar os efeitos de 14 semanas de treino de resistência nas alterações bioquímicas e histológicas teciduais cardíacas induzidas pela administração *in vivo* de doxorubicina (DOX), através da análise dos seguintes parâmetros: concentrações de glutationa reduzida e oxidada, substâncias reactivas ao ácido tiobarbitúrico, grupos sulfidril (-SH) e carbonilo, troponina cardíaca plasmática (cTnI) e expressão de proteínas de choque térmico 60 Kda (HSP60) e 70 Kda (HSP70). O *status* antioxidante e as actividades enzimáticas da superóxido dismutase, catalase, glutationa redutase e glutationa peroxidase foram igualmente determinadas. Os níveis de lesão tecidual cardíaca e especificamente, da ultraestrutura mitocondrial, foram obtidos através da análise de fotografias de microscopia óptica e/ou electrónica. Nos estudos III e IV, foram testados os efeitos do treino de resistência na função respiratória e nos níveis de lesão oxidativa em mitocôndrias isoladas de ratos tratados *in vivo* com DOX (III) e em mitocôndrias submetidas a anóxia-reoxigenação *in vitro* (IV). No estudo III foram avaliados marcadores de activação de morte celular por apoptose. Para atingir estes objectivos, vários parâmetros bioquímicos foram utilizados, tais como concentração plasmática de cTnI, conteúdos mitocondriais de grupos –SH e carbonilos, malondialdeido, Bax, Bcl2, expressão de HSP60 mitocondrial e de HSP70 tecidual, actividade mitocondrial de aconitase e tecidual de caspase 3. As taxas respiratórias mitocondriais foram determinadas em estado 3, estado 4, na presença de oligomicina, carbonil cianide *m*-clorofenil-hidrazona (CCCP), guanosina difosfato (GDP) e cálcio. O índice de controlo respiratório e o *ratio* ADP/O foram também calculados.

A realização de treino de resistência induziu atenuação dos efeitos deletérios teciduais e/ou mitocondriais causados pelos estímulos-indutores de lesão e disfunção com etiologia oxidativa utilizados nestes estudos, observados ao nível bioquímico, morfológico e respiratório. Em conclusão, os nossos resultados representam uma contribuição adicional para a compreensão dos mecanismos relacionados com a protecção cardíaca mediada pelo treino de resistência, perante situações de lesão oxidativa induzida pela administração *in vivo* de DOX em diferentes níveis de organização celular, bem como por anóxia-reoxigenação mitocondrial *in vitro*.

***Palavras chave*:** Exercício crónico, Treino de resistência, coração, mitocôndrias, tolerância cruzada, doxorubicina, anoxia-reoxigenação, stress e lesão oxidativa, respiração, animais

RÉSUMÉ

L'entraînement d'endurance est référé comme une des principales mesures non pharmacologiques contre les situations de lésion et de dysfonctions cardiaques. Cependant, les mécanismes cellulaires associés à la protection cardiaque mesurés par l'entraînement sort encore peu connus, même si plusieurs explications ont été proposées, à ce sujet. La présent dissertation, comportant quatre études expérimentales voudrait analyse les impacts de l'entraînement et de stimulus cardiaques délétères dans les marqueurs de lésion et de stress oxydants, l'apoptosis et la fonction respiratoire, soit dans l´homogénat cardiaque soit dans des mitochondries isolées. Les études I et II prétendent étudier les effets de 14 semaines dans lu changements biochimiques et histologiques des tissus cardiaques produites par l'administration *in vivo* de doxorubicin (DOX) dans l'ensemble du muscle cardiaque à travers de paramètres suivants : des concentrations de glutathion réduite et oxydée, des substances réactives à l'acide thiobarbiturique, des groupes sulfhydryle (-SH) et carbonyliques, la troponin cardiaque de plasma (cTnI) et l'expression de protéines de choc thermique 60 Kda (HSP60) et 70 KDa (HSP70). Le status antioxydant et les activités enzymatiques de la dismutase de superoxyde, de la catalase, de la réductase de glutathion et de la péroxydase de glutathion ont été mesurés également. Les niveaux de lésion du tissu cardiaque et, notamment, de la structure mitochondrique ont été obtenus à l'aide de la lumière et/ou de micrographes électroniques. Dans les études III et IV, on a testé les effets de l'entraînement d'endurance sur la fonction respiratoire et sur les niveaux de lésion oxydant dans mitochondries isolées de rats traités *in vivo* avec DOX (III) et dans des mitochondries soumises à l'anoxie-réoxygenation in vitro (IV). Dans l'étude III on a évalué des marqueurs d'activation de mort cellulaire par apoptosis. Pour atteindre ces objectifs, on a utilisé plusieurs paramètres biochimiques, comme par exemple, des contenus de plasma cTnI, concentration mitochondriques de groupes SH et carbonyliques, le malondialdehyde, le Bax, le Blc2, l'expression mitochondrique de HSP60 et de tissu HSP70, l'aconitase mitochondrique et le caspase 3 de tissu d'activités. Des taux respiratoires mitochondriques ont été déterminés dans l'état 3, l'état 4, en présence de l'oligomycin, de l'm-chlorophénylique-hydrazone de cyanure de carbonylique (CCCP), du diphosphate de guanosine (GDP) et du calcium. Le contrôle respiratoire et les rapports d'ADP/O ont été calculés également. L'entraînement d'endurance a atténué l'élévation de cardiaque et/ou les affaiblissements biochimiques, morphologiques et fonctionnels mitochondriques causé par les stimulus oxydant-basés utilisés dans les études. Considérés dans leur ensemble, ces données ont fourni la contribution additionnelle pour comprendre le phénomène d'entraînement d'endurance en ce qui concerne la cardioprotection en face des situations de lésion oxydante provoquée par l'administration *in vivo* de DOX dans différents niveaux d'organisation cellulaire et aussi par l'anoxie-réoxygénation mitochondrique *in vitro*.

Mots-clés: entraînement d'endurance, coeur, mitochondries, tolérance croisée, doxorubicin, anoxie-réoxygénation, stress et dommages oxydant, respiration, animaux de rongeur

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

It is estimated that approximately 100 million of citizens from the so-called western industrialized and developed countries evidence signs of cardiovascular disease (CVD), which accounts for a great percentage of all deaths in these countries, with significant impact on health-related coasts (Control 2001; American-Heart-Association 2003). For example, nearly 14 million of US citizens suffer from coronary artery disease (CAD), and treatment-related coasts for these diseases, including emergency room visits, cardiac catheterisations, coronary angioplasties, and bypass surgery, exceed 95 billion dollars per year (Control 2001; American-Heart-Association 2003). Indeed, cardiac dysfunctions resulting from CAD, like those related to ischemic heart are also devastating with frequent development of myocardial infarcts and consequent thousands of deaths (American-Heart-Association 2003).

A primary pathological manifestation of CAD is myocardial damage. The level of myocardial injury can range from small insult, resulting in limited damage, to a large insult, culminating in major cardiac electrical, metabolic and mechanical dysfunction. Major injury to the heart can even result in permanent disability or death. Although the pathogenesis of myocardial damage-induced dysfunction has not been definitively established, due to the complexity of the responsible cellular mechanisms, essential hypotheses have been delineated in recent years. Experimental evidence indicates that several interrelated factors including the generation of oxygen-derived free radicals, transient calcium overload, protein breakdown, cellular membrane and DNA damage contribute to heart injury (Ferrari 1996; Bolli and Marban 1999; Monteiro et al. 2003b; Becker 2004; Halestrap et al. 2004). In fact, the cellular mechanisms involved in the pathogenesis of myocardial injury are complex and involve the interaction of a number of cell types, including capillary endothelial cells, circulating blood neutrophils, and cardiomyocytes, most of which are capable of generating ROS (Vanden Hoek et al. 1997a; Vanden Hoek et al. 1998; Becker et al. 1999; Cappola et al. 2001). Collectively, these oxidant sources promote cellular injury, which can lead to cardiac myocytes death under conditions of increased loss of homeostasis (for refs. see Ferrari 1995; Colucci 1997; Lefer and Granger 2000).

Despite all the ROS generating systems have recognized importance in myocardium injury, cardiac mitochondria have assumed special interest during exposure to stress conditions (Ferrari 1996; Vanden Hoek et al. 1997b; Lucas and Szweda 1998; Vanden Hoek et al. 1998; Becker et al. 1999; Lucas and Szweda 1999; Paradies et al. 2000; Jassem et al. 2002; Paradies et al. 2002; Paradies et al. 2004). The balance between mitochondria oxidants and antioxidants is essential for the regulation of biological processes such as calcium cycling, enzymatic activity, membrane permeability, signal transduction pathways, and regulation of gene transcription (reviewed in Dalton et al. 1999; Thannickal and Fanburg 2000; Cadenas 2004). Moreover, mitochondria are increasingly recognized as key players in cell survival, not only because of their traditional role as energy providers for vital cellular processes, but also because their critical involvement in the modulation of programmed cell death via apoptosis (reviewed in Ozawa 1997; Wallace et al. 1997;

Kroemer et al. 1998; Skulachev 1998; Crompton 1999; Skulachev 2000; Pollack and Leeuwenburgh 2001; Primeau et al. 2002; Borutaite and Brown 2003; Gottlieb 2003; Smaili et al. 2003).

It is therefore assumed that the above-referred injury mechanisms often led to a series of structural, biochemical and functional alterations verified at different levels of cellular organization. These comprise changes in the morphological appearance of cardiac tissue, seen under light and electronic microscopy and modifications in several biochemical markers associated with damage and dysfunction. However, myocardial cells exposed to a variety of light non-lethal insults have the capacity to re-establish cellular homeostasis, orquestrating a cardioprotective response against more intense stimuli (Ferrari 1995; Colucci 1997; Swynghedauw 1999; Dhalla et al. 2000; Lefer and Granger 2000; Wakatsuki et al. 2004). Actually, the plasticity of heart tissue allows physicians and researchers to persistently develop appropriate strategies in order to counteract and/or attenuate the devastating effects of stress stimuli inflicted to this vital organ (Sen et al. 1994; Ji 2000; Pryor 2000; Powers et al. 2001; Penckofer et al. 2002; Powers et al. 2002; Powers et al. 2004; Siu et al. 2004; Wakatsuki et al. 2004).

The last two decades have witnessed major strides in the prevention and treatment of CAD through modification of its risk factors and counteracting its effects. The American Heart Association and the American College of Cardiology have published joint recommendations for interventions in patients with and without established CAD (Pearson et al. 2002). Several pharmacological and non-pharmacological measures have been advanced to counteract these pathologies, and exercise is included among the most referred non-pharmacological interventions. Numerous epidemiological studies indicate that regular physical activity reduces the risk of cardiovascular mortality independent of other lifestyle modifications such as diet or smoking (Paffenbarger et al. 1978; Paffenbarger et al. 1986a; Paffenbarger et al. 1986b). Moreover, exercise-related protection against CVD follows a dose-response relationship, i.e., the risk of death from CVD becomes progressively lower as the total energy expenditure due to physical activity increases from 500 to 3500 kcal/week (Paffenbarger et al. 1986b). These data strongly support the notion that increased physical activity is protective against several forms of CVD, including myocardial damage. In fact, regular physical exercise using large muscle groups, such as walking, running, or swimming, produces cardiovascular adaptations that increase exercise capacity in both healthy persons and those with CVD, including heart failure (see Fletcher et al. 1996). This improvement is the result of increased ability to use oxygen to drive energy for work, and is accompanied by beneficial changes in cardiovascular, hormonal, metabolic, neurological and respiratory functions (Fletcher et al. 1996; Thompson et al. 2003). Habitual physical activity also prevents the development of CAD and reduces symptoms in patients with established CVD (Pearson et al. 2002). There is also evidence that exercise reduces the risk of other chronic diseases, including type 2 diabetes, obesity, lipid abnormalities, osteoporosis, depression and cancer of breast and colon (Fletcher et al. 1996; Thompson et al. 2003). In fact, given the worldwide of CAD and the associated myocardial injury, developing a specific countermeasure to protect heart is important. In the same way to other types of CVD, a pragmatic intervention to counteract myocardial dysfunction is physical exercise (Paffenbarger et al. 1986a; Paffenbarger et al. 1986b). Although not fully understood, the cardioprotection by regular exercise may be exerted synergistically through improvement in many risk factors associated with CVD.

Actually, the cellular basis for training-induced cardioprotective effect has not been identified, although various candidate adaptations have been proposed. Cardioprotective countermeasures against myocardial injury may include the induction of myocardial heat shock proteins (HSP) and improved cardiac antioxidant capacity (Ji 1995, 1996; Ji and Leichtweis 1997; Ji et al. 1998; Ji 1999; Ji 2000; Powers et al. 2002; Powers et al. 2004). Both types of adaptations have been strongly correlated to the increased resistance of the trained heart against the harmful functional consequences of the challenges induced by insults. Improving our understanding of the molecular basis for exercise-induced cardioprotection will play an important role in developing optimal exercise interventions to protect the heart from injury-mediated dysfunction. Likewise, numerous experimental studies reveal that regular endurance exercise protects the heart from injury during a deleterious insult (Powers et al. 1993; Ji et al. 1994; Demirel et al. 1998; Powers et al. 1998; Demirel et al. 2001; Hamilton et al. 2001; Ramires and Ji 2001; Powers et al. 2002; Demirel et al. 2003; Hamilton et al. 2003). This topic has also been widely addressed in extensive reviews (Ji 1995, 1996; Ji and Leichtweis 1997; Atalay and Sen 1999; Ji 1999; Ji 2000; Powers et al. 2001; Powers et al. 2002; Ascensao et al. 2003). The phenomenon, usually known as cross-tolerance of regular exercise (in this particular case) against heart dysfunction, have also been studied using acute severe exercise (Venditti and Di Meo 1996, 1997) and pharmacological treatments (Kanter et al. 1985) as models of cardiotoxicity.

Actually, high levels of cellular organization need a constant remodelling, being chaperones ideal candidates for being a major constituent of cellular meshwork actively participating in the organization of cytoplasmatic proteins and RNAs (Latchman 2001; Papp et al. 2003; Soti et al. 2003; Chi and Karliner 2004; Sreedhar and Csermely 2004). The network of molecular chaperones includes HSPs, which are ubiquitous, highly conserved proteins that help other proteins and macromolecules to fold, unfold or re-fold and reach their final native conformation, ensuring the correct assembly of multimeric complexes and organelles (Papp et al. 2003). Because of their function, myocardial research has been focused on the role of HSP as protective molecules transiently expressed (inducible stress proteins) following cellular stress stimuli (Powers et al. 2001; Snoeckx et al. 2001; Mogk et al. 2002), including that induced by physical exercise (Noble 2002a; Powers et al. 2002; Powers et al. 2004).

A crucial element of the myocardial cell involved in both trigger and mediation of cardioprotective response is the mitochondria, whose primary role is producing and regulating the cellular bioenergetic supply in the form of adenosine triphosphate (ATP). As referred, mitochondria house and regulate pivotal early events in the apoptotic pathway, functioning as both a target and a player in myocardial signal transducing events and in the generation of ROS in response to a variety of stress insults, and serve a major role in providing an appropriate antioxidant response (Duchen 1999; Monteiro et al. 2003b; Duchen 2004; Halestrap et al. 2004). Due to the well-recognized relevance of these organelles in many important cellular functions, several researchers have focused on the role of mitochondria in cardioprotection during several pathophysiological conditions (Jayakumar et al. 2001; Laclau et al. 2001; Monteiro et al. 2003a; Monteiro et al. 2003b; Weiss et al. 2003; Duchen 2004; Halestrap et al. 2004; Marin-Garcia and Goldenthal 2004; McLeod et al. 2004a; McLeod et al. 2004b; Piper et al. 2004).

One classical method to study mitochondrial bioenergetics is based on measurements of mitochondrial oxygen consumption using an oxygen sensitive electrode. Respiratory trials allow, among others, to test qualitative standard parameters in order to test the coupling levels of mitochondrial respiration and the efficiency of mitochondria oxidative phosphorylation in different experimental *in vitro* conditions. Although our understanding concerning exercise-induced cytoprotection has increased substantially within the last years, the precise mechanisms mediating mitochondrial involvement in cardioprotection remain to be delineated. For instance, cardiac mitochondria produce ROS during basal respiration, i.e., when the transmembrane potential ($\Delta\Psi$) is above a determined critical threshold value. In fact, Korshunov et al. (1997) showed that small decreases in $\Delta\Psi$ resulted in a strong inhibition of ROS generation in a mechanisms named by the authors as mild uncoupling. This mechanism would keep the $\Delta\Psi$ below the critical threshold for augmented ROS production. This membrane-linked "antioxidant" mechanism of mild uncoupling has been used as an argument to explain, at least partially, the improvements in mitochondria functions mediated by pharmacological agents with protonophoric properties such as carvedilol (Oliveira et al. 2000; Oliveira et al. 2001). Moreover, several experiments have demonstrated the role of different mild membrane pathways (opening of ATP-sensitive K^+ channels and ATP/ADP antiporters) as mediators of lower mitochondrial-generated ROS (Simonyan and Skulachev 1998; Ozcan et al. 2002; Ferranti et al. 2003). The protective outcome of this uncoupling-mediated effect was also demonstrated by the addition of small concentrations of fatty acids with concomitant decrease in ROS formation and by the increased stimulation of H_2O_2 production in the presence of GDP in mitochondria containing UCP (Negre-Salvayre et al. 1997; Korshunov et al. 1998; Kowaltowski et al. 1998). Thus, the role of exercise in the mediation of this mitochondrial protective mechanism is yet unknown, despite Boss et al. (1998) had observed a decreased in heart UCP2, UCP3 expression and RNAm after 8wks of endurance training and suggested that these alterations result in a higher need of metabolic efficiency, diminishing the levels of energy dissipation. The question arises from whether or not some of these changes can really occur in response to an endurance-training program and which are the effects on mitochondrial respiratory function. In fact, studies analyzing the possible changes in mitochondria respiratory function in response to a moderate endurance-training program are missing and most of the times, distinct mitochondrial respiratory responses can only be uncovered by submitting these isolated organelles to additional stress conditions.

Moreover, far less is known about the exercise-related effect has on stress-activated cell and mitochondrial pathways such as the chaperone defense mechanism, which plays fundamental roles in reducing mitochondria oxidative damage caused by many deleterious stimuli. The relationship of heart mitochondria respiratory function and the expression of some HSP was previously demonstrated (Lin et al. 2001; Sammut et al. 2001; Suzuki et al. 2002; Voos and Rottgers 2002; Sammut and Harrison 2003). Because moderate exercise represents an important heart mitochondrial stress-induced stimuli, it is likely that the structural and biochemical adaptations caused by training in these organelles, such as increased volume density, HSP expression and improvements in antioxidant capacity, could be associated to the training-induced cardiac cross tolerance effect (Frenzel et al. 1988; Powers et al. 1998; Ji 2002). Hence, one may

suppose that these biochemical and structural adaptations parallel with improvements in several features of mitochondria functionality, including the respiratory function (Tonkonogi and Sahlin 2002).

Due to its applicability and clinical usefulness, the majority of researches in the field have been widely carried out on I-R as a model of cardiac oxidative damage and dysfunction (for refs see Ji 1995; Atalay and Sen 1999; Ji 2000; Powers et al. 2001; Powers et al. 2002; Ascensao et al. 2003; Powers et al. 2004). Nevertheless, the experimental approaches to study the cross-tolerance effect of endurance training against other stimuli rather than I-R must be considered as well, with the purpose of increasing the spectrum of training cross-tolerance applications. Therefore, the *in vivo* administration of Doxorubicin (DOX), also known as Adriamycin, can be seen as an appropriate model to accomplish the above referred features and thus to test training-induced cardiac cross-tolerance, since DOX induces a dose-related and potentially lethal cardiotoxicity that may be in part due to increased oxidative stress (Horenstein et al. 2000; Hrdina et al. 2000). Among the several hypothesis that have been advanced to explain the toxic effects of DOX, the most frequently referred involves the generation of reactive species in heart mitochondria (Davies and Doroshow 1986; Doroshow and Davies 1986). Specifically, the initial step of DOX *redox* cycling is the univalent reduction of its quinone form to the corresponding semiquinone free radical or by forming a DOX Fe^{3+} complex (Horenstein et al. 2000). These highly unstable intermediates rapidly transfer the unpaired electron to a more favorable electron acceptor that includes glutathione and related thiols, haem proteins, α-tocopherol, ascorbic acid and molecular oxygen and generates secondary more stable free radical species (Wallace 2003). In fact, under aerobic conditions, this futile *redox* cycling of the drug produces superoxide anion at the level of mitochondria complex I, namely NADH-dehydrogenase (Davies and Doroshow 1986; Doroshow and Davies 1986), which is converted to other forms of more deleterious ROS, such as hydrogen peroxide and hydroxyl radical (Figure 1).

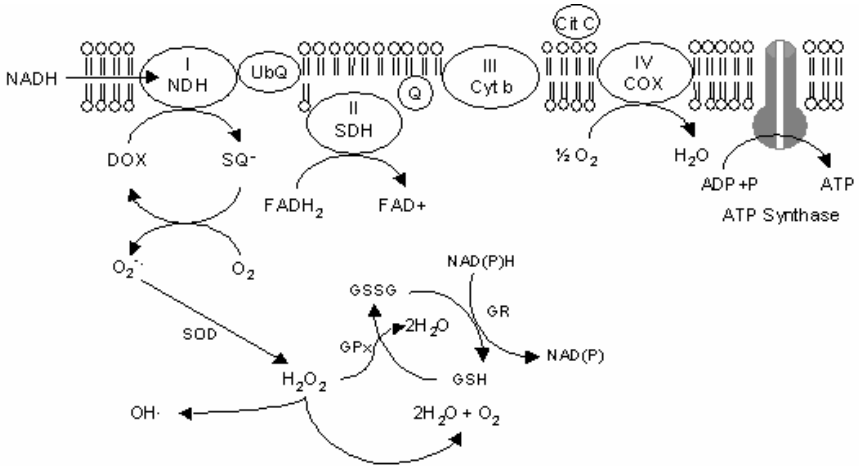

Figure 1. Representation of the site-specific redox cycling of DOX on complex I of the mitochondrial electron transport chain with the formation and the reduction of the main ROS. Abbreviations: semiquinone free radical of DOX ($SQ^{\cdot}$), NADH dehydrogenase (NDH); ubiquinode (UbQ); succinate dehydrogenase (SDH); cytocrome oxidase (COX); superoxide radical (O_2-.); hydrogen peroxide (H_2O_2); hydroxyl radical (OH-.); superoxide dismutase (SOD); glutathione reductase (GR), glutathione peroxidase (GPx), reduced glutathione (GSH), oxidized glutathione (GSSG).

Studies dealing with the effect of endurance training in DOX treated hearts are lacking (Kanter et al. 1985). In the referred study, it was concluded by the authors that exercise ameliorated severe toxic damage caused by the drug, seen in histological light evaluation, possibly by increasing cardiac antioxidant enzymes activity. However, there is a clear diversity and variability in the effects of endurance training in modulating the various enzyme systems of cardiac antioxidant defence (for refs see Ji 1995; Atalay and Sen 1999; Ascensao et al. 2003). In addition, other systems rather than antioxidant enzymes may also be involved in the protection of cardiac tissue, like glutathione and HSP (Ji and Leeuwenburgh 1996; Powers et al. 2002). Moreover, qualitative and semi-quantitative information obtained by means of electron microscopy may provide additional contribution to better understand the protective effect of previous endurance training on the toxicity caused by DOX. These contributions would consist in the identification of the specific cardiomyocyte compartments altered by the coordinated effects of endurance training and DOX treatment. The data provided by both histopathological parameters concomitantly with systemic markers of cardiomyocyte injury, such as the plasma content of the cardiac specific troponin I, together with damage markers and heart-protecting molecules, may afford additional contribution to understand the systems by which endurance training is hypothetically cardioprotective against DOX.

Bearing in mind the above-mentioned considerations regarding possible training cross-tolerance effect against oxidative-based insults, the main purpose of this work was to analyze the impact of cardiac deleterious stimuli and training on markers of oxidative stress and damage, apoptosis and respiratory function either on overall cardiac homogenate or on isolated mitochondria, contributing to better understand the exercise-induced cardioprotection phenomenon.

Some answers to the questions that derived from this general objective supported the specific goals of the original articles corresponding to the four chapters of the experimental work of this thesis, as follows:

Paper I

To analyze:

(i) the effect of 14-wk swimming endurance training in cardiac muscle tolerance to *in vivo* DOX-induced oxidative stress and damage;

(ii) the expression of heat shock proteins 70 (HSP70) and 60 (HSP60) as important cardioprotective molecular chaperones as well as markers of cardiac cell stress;

(iii) the contribution of some cardiac gene-modulated antioxidant enzymes and of glutathione system.

Paper II

To analyze:

(i) the effect of endurance training on cardiac muscle tolerance to *in vivo* DOX-induced damage and dysfunction through histo-morphological quantitative and qualitative examination;

(ii) the levels of heart mitochondrial damage in mouse hearts using semiquantitative procedures.

Paper III

To study:

(i) the effect of endurance training on the well-established heart mitochondria respiratory malfunction mediated by DOX;

(ii) the effect of training and DOX on the levels of mitochondrial superoxide radical production and oxidative damage;

(iv) the contribution of tissue heat shock proteins 70 (HSP70) and mitochondrial HSP60, important cardioprotective molecular chaperones, for the hypothetical enhanced respiratory function of DOX-trained mitochondria;

(iii) the effect of training and DOX on the susceptibility of mitochondria to calcium-induced stimulation of respiration;

(iv) the effect of training on DOX-induced apoptosis.

Paper IV

(i) to analyze the effect of moderate endurance treadmill training on the heart mitochondrial respiratory susceptibility to *in vitro* damage caused by anoxia-reoxygenation (A-R);

(ii) to relate the findings with the levels of mitochondrial oxidative damage;

(iii) to investigate whether or not training-induced HSP overexpression would correspond to mitochondrial respiratory protection.

THEORETICAL BACKGROUND

Review Article

Exercise and Cardiac Oxidative Stress

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A B S T R A C T

Cardiac muscle is frequently affected by many stimuli responsible for loss of cell homeostasis, including physical exercise. While exercise has been presented as a recommended activity for health reasons, it also provides favorable conditions for additional production of reactive oxygen species. These compounds are associated with fundamental mechanisms of cell metabolism but are also related to the etiology and pathophysiology of some cardiac diseases. Cardiac muscle tissue has a high oxidative metabolic rate and relatively low activity of the main antioxidant enzymes, which could enhance its susceptibility to oxidative injury after acute exercise. However, physical training could be considered an important stimulus for the different antioxidant systems as glutathione and those related to the activity of some important antioxidant enzymes in myocardial protection such as superoxide dismutase and glutathione peroxidase. Endurance training seems to induce up-regulation in some antioxidant defenses, protecting cardiac muscle in potentially harmful situations that induce additional oxidative stress. Nevertheless, the mechanisms related to this cross-tolerance effect of training are not yet well understood.

Key words

OXIDATIVE STRESS; CARDIAC MUSCLE; ACUTE EXERCISE; TRAINING; TOLERANCE

INTRODUCTION

In recent years, there has been a massive expansion in physical exercise for different purposes, from improvement of competitive performance to disease prevention and promotion of a healthier lifestyle (Paffenbarger et al. 1986a; Paffenbarger et al. 1986b; Penckofer et al. 2002). Indeed, there is now clear evidence to support the importance of regular exercise in the prevention and/or control of certain chronic diseases (Pearson et al. 2002; Thompson et al. 2003). However, an inevitable consequence is that the increased oxygen (O_2) consumption induced by exercise produces favorable conditions for increased generation of reactive oxygen species (ROS) and apparently an increase in oxidative stress at the cell, tissue and organic level (Jenkins 1988; Ji and Leichtweis 1997). Since around 2-5 % of O_2 consumed can result in ROS generation, an increased cardiac oxidative metabolic rate arising from physical exercise becomes a predisposing factor for increased ROS production at the mitochondrial level, leading to alterations in the cell's redox state and increases in the direct and indirect markers of tissue oxidative injury (Halliwell and Gutteridge 1999; Jackson 2000). However, if this situation is repeated over time, it may have a strong modulating effect on various cardiac antioxidant systems (Ji 1995; Atalay and Sen 1999; Ji 1999; Ji 2000).

There appears to be agreement that acute exercise promotes an increase in markers of cell damage from cardiac oxidative stress, as well as chronic alterations in the antioxidant defense system when practiced regularly (for refs. see Ji 1995; Atalay and Sen 1999; Powers et al. 2002). On the other hand, and since regular exercise has been used as a therapeutic measure in patients with chronic heart disease (Shern-Brewer et al. 2000; Thompson et al. 2003), it is important to analyze its clinical utility and the associated potential cardiac adaptations. Some authors have suggested that endurance training may lead to increased tolerance to adverse situations that greatly exacerbate cardiac oxidative stress (Lew and Quintanilha 1991; Ji et al. 1994; Somani et al. 1995; Venditti and Di Meo 1996; Demirel et al. 1998; Powers et al. 1998; Ramires and Ji 2001). Thus, in view of its potential clinical applications, the aim of this review was to analyze the works in the literature on the acute and chronic cardiac effects of exercise, as well as on the cross-tolerance effect produced by exercise on certain agents responsible for changes in cardiac homeostasis.

CELLULAR OXIDATIVE STRESS

There appears to be consensus nowadays that free radicals are involved in many biological processes, often playing a crucial role (Finkel 2001; Droge 2002). These compounds appear to be responsible for numerous physiological processes at the general cell level, including signal transduction and, particularly the in cardiovascular system, regulating vascular tone and platelet aggregation (Azzi et al. 2000; Han et al. 2000; Lefer and Granger 2000; Sen 2000). However, it is in the pathophysiology of a wide range of diseases, particularly degenerative disorders, that free radicals assume a major role. A growing number of studies on this topic have been carried out in various fields, from basic biochemistry to clinical and applied areas (for refs see Asmus and Bonifacic 2000).

A free radical is defined as a molecule or atom carrying an unpaired electron in one of its orbits, which leads to instability and biochemical reactivity

(Sen 1995; Asmus and Bonifacic 2000; Clarkson and Thompson 2000). Free radicals acquire chemical stability by interacting with non-radical substances, by either giving up the unpaired electron to a stable molecule and forming a reduced radical, or acquiring an electron from a stable molecule, forming an oxidized radical. The various radicals found in biological systems are usually associated with four elements, namely carbon (C), sulfur (S), nitrogen (N) and oxygen (O) (Halliwell and Gutteridge 1999; Sies 2000). Depending on the atom involved in the formation of radicals, they are generally called C, S, N or O radicals. However, other cellular compounds although not strictly considered free radicals, since they do not have any unpaired electrons in their chemical structure, are extremely reactive and may generate free radicals. In this case, it is usual to employ the wider term 'reactive species' (RS) to cover both free radicals and the non-radical compounds that are their precursors. Since during exercise increased O_2 consumption seems to be a predisposing factor for RS generation, particular attention will be given to reactive oxygen species (ROS). Of these, those most often referred to in the literature are the superoxide radical (O_2•-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$).

An imbalance between ROS production and neutralization mechanisms is generally known as oxidative stress, a natural process that results from the inability of antioxidant systems to cope with increased generation of ROS (Atalay and Sen 1999; Sen and Goldfarb 2000).

There are various cellular sources capable of producing ROS, the most frequently cited being mitochondria and the mechanisms associated with the enzymes xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (for refs see Ji and Leichtweis 1997; Ji et al. 1998; Ji 1999; Leeuwenburgh and Heinecke 2001).

Although the main function of mitochondria is to produce energy, there is evidence that points to their being a source of ROS during the reactions that precede oxidative phosphorylation (Vanden Hoek et al. 1997; Vanden Hoek et al. 1998; Becker et al. 1999; Di Meo and Venditti 2001). The formation of intermediates from the tetravalent reduction of oxygen, and the loss of electrons to O_2 from the electron transport chain (ETC), particularly from complexes I and III, are the mechanisms suggested as being responsible for ROS production in the mitochondrion (Figure 1) (Kowaltowski and Vercesi 1999; Chandel and Schumacker 2000; Di Meo and Venditti 2001).

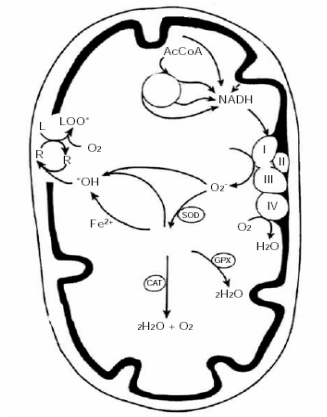

Figure 1. ROS production in the mitochondrion. CAT - catalase; GPX - glutathione peroxidase; SOD - superoxide dismutase; LOO• - lipid peroxyl radical; QH• - semiquinone. Adapted from Ji and Leichtweis (1997).

The detection and direct quantification of ROS require complex experimental procedures since they are short-lived. The facility with which they rapidly give up or capture electrons from other

compounds gives them high biochemical instability and consequently extremely short lifetimes, making it difficult to determine their concentrations accurately (for refs see Ji and Leichtweis 1997; Kowaltowski and Vercesi 1999). However, experimental studies on isolated mitochondria have used various methods to determine both ROS production at different metabolic stages and the different quantities of O_2•- and H_2O_2 produced (Barja 2002). For example, it has been estimated that cardiac muscle mitochondria generate 0.3-0.6 nmol O_2•-/min/mg of protein, which represents around 2% of total O_2 consumed (see Ji and Leichtweis 1997).

The hypothesis that the mitochondrion is in fact one of the main sources of ROS, thereby contributing to oxidative stress, would also seem to be borne out by indirect evidence of mitochondrial damage. The presence of increased markers of lipid peroxidation, notably malondialdehyde (MDA) (Liu et al. 2000) and thiobarbituric acid reactive substances (TBARS) (Ide et al. 1999), together with the high oxidation rates of dichlorofluorescin (DCFH) shown by isolated mitochondria from different tissues subjected to situations that induce changes in cell homeostasis (Vanden Hoek et al. 1997; Bejma and Ji 1999; Bejma et al. 2000), suggest that this organelle is simultaneously a production site for ROS and their target. Moreover, changes in the activity of certain mitochondrial antioxidant enzymes after application of chronic stimuli, as well as disturbances in the glutathione *redox* state in the mitochondria of different tissues following acute stimulation, support the theory of mitochondrial ROS production (for refs see Ji and Leichtweis 1997).

Besides ROS, the mitochondrion also appears to be a site for the production of other RS, such as nitrogen RS, of which one example is the nitric oxide radical (NO) (Leeuwenburgh and Heinecke 2001; Droge 2002). The results of various studies suggest that nitric oxide synthase is found in mitochondria. This enzyme, initially studied in vascular endothelium, is responsible for the synthesis of nitric oxide from its precursor L-arginine, there being a strong correlation between endothelial levels of expression of the enzyme and its mitochondrial content in cardiac and skeletal muscle, the kidneys and the brain (Leeuwenburgh and Heinecke 2001). NO and O_2•- can react together to form peroxynitrite (ONOO-). This highly potent oxidant is another important aggressive agent against various cell structures (for refs see Pollack and Leeuwenburgh 2000; Leeuwenburgh and Heinecke 2001).

Although the mitochondrion is in general the principal production site of ROS, other cellular sources may be involved in their generation. Some are active in normal physiological conditions, while others are activated only in particular circumstances such as ischemia/reperfusion (I/R), administration of drugs and intense exercise (Ji 2000).

One of the main extra-mitochondrial sources of ROS production is xanthine oxidase, the enzyme responsible for the conversion of hypoxanthine (Hx) to xanthine and hence to uric acid (Ji 2000). The production of ROS by this enzyme has been linked to I/R in particular, as well as to situations of marked degradation of adenine nucleotides, notably ATP, with the formation of AMP, IMP, inosine and Hx, arising from a higher rate of ATP consumption than its regeneration in tissues (Jackson 1996; Hellsten et al. 1999; Hellsten 2000).

Xanthine oxidase, mainly found in the vascular endothelium of most tissues, including skeletal and

cardiac muscle, may be in the form of xanthine dehydrogenase (XDH), which uses nicotinamide adenine dinucleotide (NAD^+) as the H^+ receptor, or in the form of xanthine oxidase (XO), using O_2 as the electron receptor, with the consequent formation of O_2•- (Hellsten et al. 1999). Two essential conditions for the conversion of XDH to XO are the availability of high levels of Hx as substrate, and increased intracellular concentrations of calcium (Ca^{2+}) by the activation of cytoplasmic proteases (Hellsten 2000). At rest and in the absence of any stimulus that might induce tissue ischemia, this enzyme acts as a dehydrogenase. However, in situations of intense exercise, in which the degradation rate of adenine nucleotides is high, or in conditions of reduced blood flow and oxygen supply to tissues, such as during certain types of surgical procedure, XDH is converted to XO (Hellsten 2000). When the oxygen supply is re-established during reperfusion, XO oxidizes the Hx and uses O_2 as the electron acceptor, forming O_2•- radicals (Figure 2).

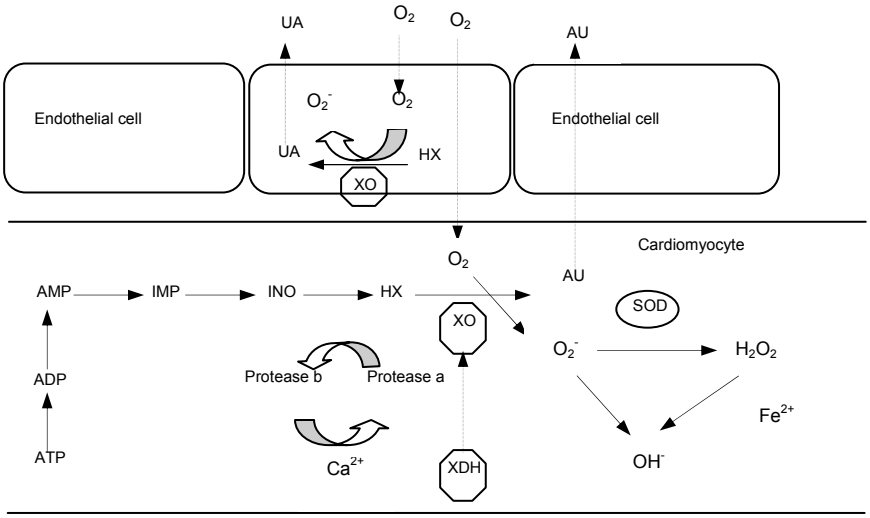

Figure 2. Role of xanthine oxidase (XO) in ROS production in cardiac muscle tissue and endothelial cells. Hx - hypoxanthine; UA - uric acid; XDH – xanthine dehydrogenase; INO - inosine; IMP - inosine monophosphate; AMP, ADP, ATP – adenosine mono-, di- and triphosphate. Adapted from Ji and Leichtweis (1997).

ROS production by polymorphonuclear neutrophils (PMNs) is another important mechanism in increasing oxidative stress after tissue aggression (for refs see Ji 2001; Leeuwenburgh and Heinecke 2001; Kaminski et al. 2002). During the acute phase response, neutrophils are attracted to the damaged region and release lysosomic enzymes and ROS (Fielding et al. 1993; Kaminski et al. 2002). In the presence of agents that destabilize the neutrophil plasma membrane, the production of O_2•- radicals is stimulated by the action of the enzyme NADPH oxidase, which uses NADPH as cofactor, directly reducing O_2 (Halliwell and Gutteridge 1999). Besides O_2•-, neutrophils have also been reported as the source of a series of other RS, including ON, H_2O_2 and hypochlorous

acid (HOCl), to combat invading pathogens (Figure 3). In fact, whereas this inflammatory response is considered of crucial importance in the repair and regeneration of damaged tissue, the reactive species produced by neutrophils can cause secondary damage by degrading the surrounding tissue and thus aggravating the injury (Halliwell and Gutteridge 1999).

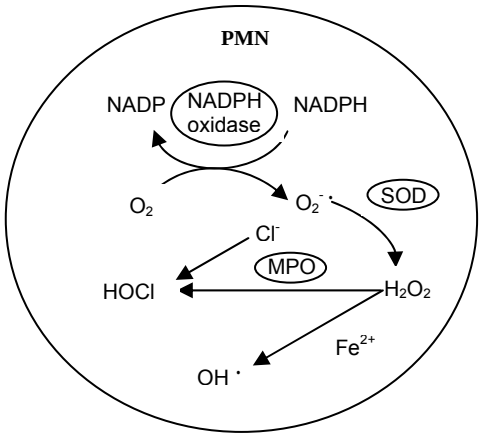

Figure 3. ROS formation in polymorphonuclear neutrophils (PMNs). NADPH Ox – NADPH oxidase; MPO - myeloperoxidase; SOD - superoxide dismutase. Adapted from Ji and Leichtweis (1997).

The cells making up different types of tissue have antioxidant enzymes that play a crucial role in protective mechanisms against oxidative stress, of which superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are the most commonly cited. Each of these enzymes is able to catalyze reactions that lead to the production of less reactive species or the neutralization of ROS (Sen 1995; Powers et al. 1999; Sen and Goldfarb 2000). In addition to these enzymes, there are other nonenzyme antioxidant substances, both endogenous and exogenous, such as glutathione (GSH), vitamins C and E, and lipoic acid, that play an important role in the neutralization or attenuation of the effects caused by increased ROS production (Janero 1991; Dhalla et al. 2000; Lefer and Granger 2000; Pryor 2000; Sen and Goldfarb 2000; Sen and Packer 2000; Sen 2001).

SOD promotes the dismutation of the O_2•- radical, forming H_2O_2 and O_2. There are two isoforms of this enzyme in mammals, which differ in their cell location, as well as in the metal ion that is linked to its active site. These isoforms catalyze the dismutation of the O_2•- anion with similar efficiency (Suzuki et al. 2000). Cu/Zn-SOD is mainly found in the cytosol, whereas Mn-SOD is found in larger quantities in the mitochondrial matrix.

CAT, whose main function is to catalyze the decomposition of H_2O_2 into water and O_2, is present in greater concentrations in peroxisomes than in mitochondria and is more abundant in predominantly oxidative tissues (Halliwell and Gutteridge 1999). Although the functions of CAT and GPX overlap to some extent, the two enzymes differ in their affinity for H_2O_2 as substrate (Halliwell and Gutteridge 1999). Mammalian GPX has a much greater affinity for H_2O_2 than CAT (GPX Km=1 μM vs. CAT Km= 1mM), which means that with low H_2O_2 concentrations, GPX has a more active role in its removal from cells. GPX, which depends on the presence of selenium, catalyzes the reduction of H_2O_2 to water or hydroperoxides to aldehydes, using reduced glutathione (GSH) as electron donor. Located in either the cytosol or the mitochondria, with around 45 % and 55 % of its total activity respectively in each of these cell compartments, this enzyme functions as one of the main neutralizing mechanisms of hydroperoxides from different cell sources (Halliwell and Gutteridge 1999).

As GSH is oxidized by GPX and forms glutathione disulfide (GSSG), cells must have a way of regenerating GSH. This reaction is catalyzed by the enzyme glutathione reductase (GR), which uses NADPH as cofactor and reducing agent, converting GSSG back into GSH (Figure 4).

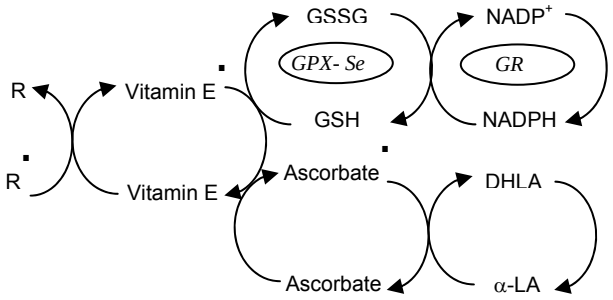

Figure 4 Illustration of interaction between α-lipoic acid (α-LA), glutathione (GSH), and vitamin C (ascorbate) in the recycling of vitamin E. Ascorbate • (ascorbate radical); Vitamin E • (vitamin E radical), DHLA (dihydrolipoic acid); R (free radicals); GSSG (oxidized glutathione). Adapted from Ji (1995).

Intracellular GSH levels depend on the effects of the enzymes GPX and GR on the cell's redox GSH/GSSG balance, as well as on mechanisms of intracellular GSH resynthesis and uptake from the blood (Sen and Packer 2000; Sen 2001). Although this compound can also be produced in other tissues, most *de novo* GSH synthesis takes place in the liver. Particularly in situations of increased oxidative stress, the liver releases higher quantities of GSH into the blood, most of the remaining tissues needing to import GSH from the circulation via the γ-glutamyl cycle, a process for which certain enzyme complexes are crucial (Sen and Goldfarb 2000). Due to the difficulty in transporting this tripeptide across the plasma membrane while preserving its original structure, it must be cleaved into its various component amino acids to enable subsequent transmembrane transport, a step that is catalyzed by the γ-glutamyl transpeptidase (γGT). *De novo* synthesis of GSH at the intracellular level is in turn re-initiated by γ -glutamylcysteine synthetase (γGCS), the catalyst for the formation of the initial peptide of the cycle, by associating glutamate and cysteine. This step is a limiting factor in the rate of GSH production and is controlled by negative feedback from GSH.

The final reaction in GSH synthesis, the linking of glycine to the above dipeptide, is catalyzed by the enzyme GSH synthetase (GS). The rate of intracellular GSH production in each type of tissue depends on the activity of these synthesizing enzymes (for detailed review see Sen and Packer 2000; Sen 2001).

OXIDATIVE STRESS AND HEART DISEASE

Besides the many recent studies that confirm the crucial importance of ROS in the stimulation and activation in the heart of signaling mechanisms that determine cell survival and adaptation (Nishizawa et al. 1999; Talmor et al. 2000; Yet et al. 2001), the consequences of marked increases in their production, together with variations in the levels of certain antioxidant compounds, have been investigated in the context of heart disease (Ferrari 1995; Bolli and Marban 1999; Lefer and Granger 2000). Cardiac pathologies, particularly those with an ischemic and/or I/R component (e.g. myocardial infarction), have been the subject of numerous extensive studies, although the mechanisms underlying their pathophysiology are still poorly understood (Colucci 1997; Dhalla et al. 2000; Lefer and Granger 2000). Nevertheless, there is a good deal of scientific evidence that ROS production

systems and antioxidant systems are associated with the genesis, manifestation and development of these pathologies (Colucci 1997; Dhalla et al. 2000; Lefer and Granger 2000).

Episodes of myocardial I/R are a major clinical problem, being associated for example with angioplasty and coronary bypass surgery. Cardiac muscle injury caused by I/R can lead to contractile dysfunction and arrhythmias, as well as irreversible damage to cardiomyocytes (Lefer and Granger 2000). It is thought that these alterations can be related to situations of cellular oxidative stress (for refs see Colucci 1997; Dhalla et al. 2000; Lefer and Granger 2000). Although it remains an open question whether oxidative stress occurs in parallel with or as a consequence of the development of pathologies with an I/R component, there is experimental evidence that ROS production is closely linked to the genesis of these pathologies and cardiac dysfunctions (Ide et al. 1999; Dhalla et al. 2000; Cappola et al. 2001; Clermont et al. 2002). Analysis of changes in markers of oxidative injury in certain cardiac cell structures confirms the role of increased ROS production in the origin and development of this type of pathology and dysfunction. Increases in protein oxidation markers resulting from I/R support the hypothesis that oxidative injury to myofilaments may be one possible explanation for changes in myocardial contractile function arising from exposure to this type of stimulus (Powell et al. 2001). Furthermore, significant alterations in membrane phospholipid elements (e.g. cardiolipin) have been described in cardiac mitochondria subjected to I/R, thus compromising their selective permeability (Paradies et al. 1998; Paradies et al. 1999; Paradies et al. 2000; Petrosillo et al. 2001; Paradies et al. 2002).

Another indirect experimental model used to assess the importance of oxidative stress in the etiology and pathophysiology of heart failure and myocardial infarction is the analysis of tissue and plasma expression of certain antioxidant compounds (Dhalla et al. 2000). Studies have demonstrated a clear tendency for reduced antioxidant capacity immediately following myocardial infarction (Cherubini et al. 2000; Gariballa et al. 2002; Polidori et al. 2002). Chamorro et al. (Chamorro et al. 2002) have shown the important antioxidant role of serum uric acid in reducing the severity of ischemic infarction, observing a 12 % increase in the odds ratio for a favorable clinical outcome for each mg/dl increase in serum levels of this antioxidant.

Although some experimental studies have failed to demonstrate the cardioprotective effect of antioxidant supplements (Dhalla et al. 2000; Vergely et al. 2001), there is a large body of work that suggests a relationship between cardiac levels of certain antioxidants and the extent of cardiac damage and dysfunction through I/R. For example, circadian variations in melatonin (Dominguez-Rodriguez et al. 2002) and concentrations of lipoic acid (Freisleben 2000) and vitamin E (Venditti et al. 1999), as well as supplements of antioxidant combinations (β- carotene, vitamins C and E) (Shite et al. 2001), appear to influence the response of cardiac muscle tissue subjected to I/R. *In vitro* studies have revealed the protective effect of metallothionein, an important antioxidant due to its uptake of iron ions and consequent reduction in the formation of OH• radicals, against the toxic action of H_2O_2 in cardiomyocytes (Wang et al. 1999). The paucity of studies analyzing this relationship in humans reflects the understandable difficulty in

obtaining samples of cardiac tissue in order to analyze markers of oxidative stress and injury.

Against this background of direct and indirect evidence, in both humans and animal models, there appears to be a clear relation between oxidative stress and cardiac pathologies resulting from I/R. Thus, from the clinical point of view, any measures designed to attenuate or minimize the harmful effects of oxidative stress should be considered. Together with other strategies, exercise has been used as a preventive and therapeutic measure in certain clinical areas, particularly in cardiovascular medicine. However, as with situations of I/R, acute exercise can also be a vehicle for oxidative stress due to the changes it causes in cardiac metabolism, and should therefore be recommended only under controlled circumstances for patients with heart disease (Starnes and Bowles 1995; Swain and Franklin 2002).

ACUTE EXERCISE AND CARDIAC OXIDATIVE STRESS

Post-neonatal heart normally has a low rate of cell growth and slow protein turnover (Halliwell and Gutteridge 1999). These functional characteristics suggest that the myocardium may have limited ability to adapt to acute and/or chronic oxidative stress (Ji 2000). Since the myocardium consists of predominantly aerobic tissue, it has a higher activity in absolute terms of most antioxidant enzymes compared to skeletal muscle (for refs see Ji 2000). Even so, given the high rates of oxygen consumption and ROS production, cardiac muscle's ability to neutralize ROS appears to be limited. Using the activity of the mitochondrial enzyme citrate synthase (CS) as the criterion to estimate the oxidative capacity of the myocardium, the activity ratios of various antioxidant enzymes to CS would suggest that this tissue has limited antioxidant potential compared, for example, with the liver or the vastus lateralis and soleus muscles (the latter possessing similar oxidative metabolic characteristics to the myocardium (Table I).

Table 1. Comparison of antioxidant enzyme activity in myocardium, liver and skeletal muscle

Tissue	SOD		GPX		CAT	GR	GST	CS
	Cu/Zn	Mn	Cit.	Mit.				
Liver	500	50	550	430	670	44	940	18
Heart	65	21	150	70	39	1.3	2.1	72
Soleus	ND	ND	ND	ND	37	1.3	1.1	40
Vastus lateralis	21	8	23	17	12	0.6	0.5	8

Note: Cu/Zn and Mn SOD activity, units/mg protein; cytosolic and mitochondrial GPX, nmol/min/mg protein; CAT, K x 10-2/g wet muscle; unit for the expression of the activity of the remaining enzymes, µmol/min/g wet muscle; ND, no data. Adapted from Ji (1995).

This relatively low antioxidant enzyme activity in the heart is attenuated during heart surgery by frequent administration of SOD and CAT supplements in order to increase tissue resistance to exposure to ROS resulting from I/R (Ji 2000). In the light of the above, cardiac muscle would indeed appear to be extremely susceptible to oxidative stress.

The ROS sources that appear to play a more important role in the heart are the mitochondrion and mechanisms associated with XO. However, catecholamines, peroxisomes and PMNs are also reported as ROS generation sites. The increase in the cardiac mitochondrial/ cytosolic activity ratio of the antioxidant enzymes SOD, CAT and GPX following an endurance training program highlights the role of the mitochondrion as both agent and target of oxidative stress (Somani et al. 1995). There are two characteristics of cardiac metabolism that greatly influence ROS production: *i*) the high volume and density of mitochondria, which means a larger number of ROS sources; and *ii*) the equally high rate of O_2 supply to mitochondria, which may increase the rate of electron leakage from the ETC, with the consequent formation of O_2•- radicals.

Even at rest, the consumption of O_2/g of cardiac muscle is higher than that of skeletal muscle during intense exercise (Atalay and Sen 1999). During exercise, coronary blood flow increases more than four-fold, and the myocardium has a remarkable capacity to extract O_2 from the blood. While O_2 is essential for the aerobic metabolism of cardiac muscle, a high cardiac oxidative metabolic rate resulting from physical exercise may be a predisposing factor for increased production of partially reduced forms of O_2 and its reactive derivatives (Ji 1996; Ji and Leichtweis 1997; Ji 1999).

During and after exercise, various mechanisms are activated in different organs and systems in order to maintain or restore cell homeostasis. Alterations in intracellular concentrations of ATP with increases in ADP and AMP levels, reductions in glycogen reserves, changes in temperature and pH, loss of Ca^{2+} ion homeostasis and uncoupled mitochondrial respiration, among others, can be important stimuli for the increased formation of ROS in the myocardium during and after acute exercise (Noble 2002). Besides the mitochondria, these changes can also affect the conversion of XDH into XO. It has been suggested that XO activity in cardiac tissue is species-specific. For example, rat, pig and dog hearts appear to present some XO activity, unlike rabbit myocardium. However, in humans, this enzyme has been reported as playing a crucial role in cardiac muscle damage resulting from I/R (reviewed in Ji 2000).

There is direct evidence that acute exercise can induce increased ROS generation in the myocardium, as confirmed by the results of a study by Ohkuwa et al. (Ohkuwa et al. 1997), who found an increase in cardiac levels of the $OH^{\cdot}$ radical. The increase in tissue metabolic rate obviously represents a major aggression against cardiac muscle. Moreover, alterations in antioxidant systems have been reported, as well as in myocardial injury markers, following acute exercise. Various authors (Ohkuwa et al. 1997; Liu et al. 2000) have observed an increase in cardiac concentrations of GSH in young rats subjected to a short period of intense exercise. This rise may be the result of an increased rate of GSH outflow from the liver during exercise and a concomitant increase in γGT activity.

Venditti and Di Meo (1996) found an increase in cardiac markers of lipid peroxidation in rats after a period of exhausting swimming with a load equal to 2% of the animals' weight. This exercise induced an increase in myocardial lipid peroxidation products, MDA and hydroperoxides, which supports the hypothesis that it increases oxidative stress and that this in turn, causes structural damage to cardiomyocytes. In the same study, a reduction in the integrity of plasma membranes and the sarcoplasmic reticulum was also evident following exercise.

GSH is a non-enzyme compound, with a protein structure, which is crucial to tissue antioxidant activity in general, and the myocardium in particular. Concentrations of this tripeptide in cardiac muscle are approximately 1-2 mM, well below those found in other tissue, notably oxidative skeletal muscle (3 mM), the liver (6-8 mM) and the kidneys (3-4 mM) (Powers et al. 1999; Powers and Sen 2000). Nevertheless, since the activity of the main antioxidant enzymes of the myocardium appears to be relatively low, GSH is the most important antioxidant found in cardiac muscle (Ji and Leeuwenburgh 1996).

The potential protective role of GSH in against cardiac oxidative stress induced by acute exercise has been studied in animals subjected to the drug buthionine sulfoximine (BSO), an inhibitor of the enzyme γGCS and therefore a depletor of intracellular GSH (Sen et al. 1994; Atalay et al. 1996a). Data from these studies demonstrated that in contrast to the control group, the rats with GSH deficiency did not show a further reduction in cardiac concentrations of total glutathione with acute exercise, presenting a 50 % lower capacity to withstand exhausting exercise (Sen et al. 1994). A recent study by Khanna et al. (1999) showed that the systematic administration (150 mg/kg/day) of α-lipoic acid, an important compound in the resynthesis of ascorbate from its radical semidehydroascorbate, protects cardiac tissue from the GSH-depleting effect of acute exercise. Furthermore, compared to the animals in the control group, the hearts supplemented with α-lipoic acid had lower levels of lipid peroxidation, as assessed by the concentration of TBARS in the cardiac homogenate.

Although GSH can be considered the most efficacious cardiac antioxidant compound, the role of exogenous antioxidants in protecting cardiac tissue during and after intense exercise has also been studied. Benderitter et al. (1996) analyzed the relationship in rats between the variation in cardiac concentrations of vitamin E and markers of lipid peroxidation (TBARS) induced by exercise. The study found a fall in vitamin E levels in animals subjected to exhausting exercise compared to those not exercised, with no alterations in TBARS levels in the cardiac homogenate of the two groups, a fact that would seem to reflect the contribution of vitamin E in combating exercise-induced cardiac lipid peroxidation.

Another response of cardiac muscle to stress induced by various stimuli, including exercise, is elevation of heat shock proteins (HSP). Several studies (reviewed in Noble 2002; Starnes 2002) have demonstrated that acute intense exercise appears to promote increased production of these proteins in cardiomyocytes, particularly those of the 70 kDa family, HSP 70.

It therefore seems clear that cardiac muscle tissue, when stimulated by acute exercise, presents increases in markers of cell damage due to

oxidative stress, notably lipid peroxidation and protein and DNA oxidation. Despite direct evidence of oxidative stress and lipid peroxidation, the GSH and vitamin E systems would appear to play a crucial role in protecting the heart in harmful situations arising from acute exercise.

On the other hand, the potential adaptability of the myocardium faced with systematic aggression presents a paradox, which appears to be O_2-dependent, and therefore leads us to carefully analyze the effect of training, particularly endurance training, on oxidative stress in this major organ.

TRAINING AND THE CARDIAC ANTIOXIDANT SYSTEM

There are a growing number of experimental studies that associate certain types of intervention or stimulus, such as long-term manipulation of diet, genetic overexpression of enzymes and antioxidant compounds, and supplementation and/or deficiency of antioxidants, with a greater or lesser susceptibility to cardiac tissue damage when it is subjected to situations that induce oxidative stress. Kim et al. (1996) demonstrated in rats that chronic calorie restriction over a period of 18.5 months can alter antioxidant metabolism at the cell level. At the end of this period, the authors found an increase in the activity of the cytosolic antioxidant enzymes SOD, GPX and GSH S-transferase, but no significant increase in CAT activity. These data may point to an effect of this intervention on the heart's antioxidant systems, and appear to demonstrate that prolonged systematic reduction of energy input, paradoxically, improves the efficacy of the mechanisms that neutralize agents causing cardiac tissue damage through oxidative stress. However, the mechanisms associated with increased efficacy of antioxidant defences after periods of restricted calorie intake are still not completely understood (Kim et al. 1996). In addition, some studies have demonstrated that inducing endogenous overexpression of antioxidant compounds appears to produce a protective effect in both the myocardium (Suzuki et al. 1999) and isolated cardiomyocytes (Wang et al. 1999) when subjected to intrinsic or extrinsic factors that trigger oxidative injury. Cardiac dysfunction and oxidative stress caused by I/R (Leichtweis and Ji 2001), as well as severe hypertension (Vaziri et al. 2000), are significantly intensified in rats following administration of BSO, with a consequent reduction in levels of GSH synthesis. Thus, since cardiac muscle tissue appears capable of a series of adaptations when submitted to certain chronic stimuli, the role of regular endurance training in increasing cardiac tolerance to oxidative stress is a subject worthy of investigation.

In general, most studies designed to investigate the influence of regular exercise on cardiac antioxidant activity focus on endurance training programs, and there is little information available on the effect of sprint training in regulating the heart's antioxidant defenses. In one study, a predominantly anaerobic training program (with significant increase in activity of the glycolytic enzyme lactate dehydrogenase in all the skeletal muscles analyzed except the soleus) caused an increase in the cardiac activity of the GPX and GR enzymes of the glutathione *redox* cycle, without however inducing any changes in the activity of GSH S transferase, SOD or GSH levels in the myocardium (Atalay et al. 1996b).

The number of studies related to the effect of endurance training on the antioxidant system and damage markers of cardiac oxidative stress is, on the other hand, much greater (Table II).

Table 2. Effect of regular training on antioxidant defenses and cardiac markers of oxidative damage

Study	Model	Type of training	Effect
(Hong and Johnson 1995)	Rats	Running - 10 weeks	SOD ↑ CAT ↓ GPX ↓ GR ↓ TBARS ↑
(Ramires and Ji 2001)	Rats submitted to I/R and GSH supplement (GSHs)	Running - 10 weeks	SOD ↑ CAT → GPX ↑ GR ↑ γGT ↑ GSH ↑ (GSHs) GSH/GSSG ↑ (GSHs)
(Demirel et al. 1998)	Rats	Running - 10 weeks	SOD → GPX → MDA ↓ HSP 72 ↑
(Leichtweis et al. 1997)	Rats submitted to I/R	Rigorous swimming 8-9 weeks	γGT ↑ GSH ↓ VO_2 mitocondrial ↓
(Ji et al. 1994)	Rats submitted to I/R	Swimming/running 5 weeks	GSH ↑ GPX ↓ GR ↓
(Powers et al. 1993)	Rats	Running - 10 weeks 30, 60, 90 min/day Low, moderate and high intensity	SOD ↑ (high intensity for all durations) CAT → GPX →
(Gore et al. 1998)	Rats	Running - 10 weeks	CS → Mn SOD → Cu-Zn SOD → GPX → Cu-Zn SOD RNAm ↑
(Leeuwenburgh et al. 1997)	Rats	Running - 10 weeks	CS → GPX → GR → Mn SOD → Cu-Zn SOD → MDA → GSH → GSSG ↓ GSH/GSSG ↓
(Tiidus and Houston 1994)	Rats	Running - 8 weeks	SOD → CAT → GPX →
(Lew and Quintanilha 1991)	Rats	Running - 10 weeks	GPX ↑ GR → G6PDH ↑ CAT ↑
(Somani et al. 1995)	Rats	Running - 10 weeks	SOD ↑ CAT ↑ GPX ↑ MDA mitocondrial ↓
(Kim et al. 1996)	Rats	Running – 18.5 months	CAT ↑ MDA mitocondrial ↓
(Venditti and Di Meo 1996)	Rats	Running - 10 weeks	MDA ↓ GPX ↑ GR ↑ RCI →
(Kihlstrom 1992)	Rats e mice	Swimming and running– 149-159 h/animal	GSH → TBARS →
(Kihlstrom 1990)	Rats	219-229 h/animal	TBARS ↓

	H-R TBARS ↓ H-R CK *leakage* ↓ GSH ↑ GSSG → Ventrículo direito G6PDH ↑ SOD → CAT ↓ GR ↓

As shown by Table II, there is clearly great diversity and variability in the effects of endurance training in modulating the various enzyme systems of cardiac antioxidant defence. Direct comparison of the results is hampered by methodological differences, of which the following are the most important: i) characteristics of the training protocols used; ii) biochemical analytical techniques used; iii) tissue sub-fractions studied; iv) time between the end of the training program and sacrifice of the animals; and v) type of markers and enzymes studied.

Since cardiac antioxidant enzymes appear to have a relatively low level of activity, the importance suggested by *Table II* of these enzymes in cardiac protection should be the subject of more detailed analysis, given the importance of GSH metabolism, the possible role of other cellular protection mechanisms (for example HSP), and the possible decrease in ROS production resulting from endurance training.

The idea that regular exercise can have beneficial effects, by preserving cardiac structure and function through its positive influence on antioxidant defenses, can be confirmed by analysis of the results of various studies in which endurance training: i) induced increases in the activity of GPX, GR (Lew and Quintanilha 1991; Somani et al. 1995; Venditti and Di Meo 1996; Ramires and Ji 2001), CAT (Lew and Quintanilha 1991; Somani et al. 1995; Kim et al. 1996), SOD and γGT (Ramires and Ji 2001) and reduced levels of lipid peroxidation, as determined by MDA concentrations in tissue (Venditti and Di Meo 1996) and mitochondria (Somani et al. 1995; Kim et al. 1996); and ii) led to increases in cytosolic GSH levels (Somani et al. 1995) and in the activity of glucose-6-phosphate dehydrogenase (G6PDH) (Lew and Quintanilha 1991), an essential enzyme in the pentose cycle, one of the main cellular sources of NADPH.

There is evidence that the intensity of endurance training and the duration of sessions influence the antioxidant system's ability to adapt. Powers et al. (1993) observed that different combinations of intensity (low, moderate and high) and duration (30, 60 and 90 minutes/day) produced different effects on the regulation of the antioxidant enzymes SOD, CAT and GPX in the left ventricle. None of the above combinations induced increases in CAT and GPX activity in both ventricles, with SOD increasing after high intensity training for all durations (right and left ventricle) and after moderate intensity for 90 minutes/day (right ventricle).

The adaptive response of the antioxidant system, and consequently of the expression of the various indirect markers of oxidative tissue damage, would also appear to be specific, to either the type of tissue (Leeuwenburgh et al. 1997; Liu et al. 2000), or the different antioxidant systems involved (Hong and Johnson 1995).

Some studies have demonstrated that endurance training can cause deregulation of certain antioxidant systems or maintain them unchanged. For example, Hong and Johnson (1995), despite observing an increase in cardiac SOD activity in

trained animals, also found an unexpected increase in TBARS concentrations and reduced CAT, GPX and GR activity. This finding appears to be in agreement with the results of other studies in which the cardiac activity of the main antioxidant enzymes did not in general undergo any significant change with endurance training (Tiidus and Houston 1994; Leeuwenburgh et al. 1997; Gore et al. 1998). It should also be noted that in the work of Leeuwenburgh et al. (1997), training induced a significant decrease in the GSH/GSSG ratio due to a greater increase in GSSG concentrations in the trained group. Gore et al. (1998) found that although endurance training did not produce any change in antioxidant enzyme activity, it did raise the cardiac expression of the mRNA of Cu-Zn SOD by 112 %, which would suggest post-translation alterations.

The role of endurance training has also been studied as a possible factor in increasing the heart's tolerance to certain pathologies, in particular the cross-tolerance effect of training in various models that are generally used to induce cardiac muscle injury, of which one example is I/R.

With the aim of studying the effect of endurance training on the biochemical and physiological response of myocardium subjected to I/R, Ji et al. (1994) observed that in the trained group, I/R did not produce a decrease in GSH levels or in the GSH/GSSG ratio, as had been found in the myocardium of untrained animals. In addition, the activity of the cardiac enzymes GPX and GR was lower in the trained group than in the control group, although GSH levels in the trained hearts were significantly higher than in those of the untrained animals when subjected to I/R. In the study of Demirel et al. (Demirel et al. 1998), although endurance training did not induce any change in SOD or GPX activity, the authors found that the trained rats were less susceptible than untrained animals to I/R-induced lipid peroxidation as assessed by left ventricular MDA concentrations. However, although ventricular GSH content was not determined, the authors propose that the hypothetical increase of this important polypeptide with training, confirmed by other studies (Ji et al. 1994; Somani et al. 1995), together with elevated levels of cardiac HSP, in this case HSP 72 (Su et al. 1996; Noble 2002; Starnes 2002), may explain the decrease in lipid peroxidation in the myocardium following I/R.

This tolerance to I/R injury in animals subjected to endurance training or a GSH-rich diet has also been studied by Ramires and Ji (2001). The authors concluded that endurance training combined with administration of a GSH supplement during the last 17 days of training could improve the tolerance of cardiac muscle to I/R-induced injury. This important adaptation may be the result of preserved homeostasis of cardiac GSH metabolism associated with an increase in antioxidant activity brought about by training.

Leichtweis et al. (1997) studied the effect of a rigorous swim training program of 8-9 weeks' duration on mitochondrial function in the hearts of rats subjected to I/R. The results demonstrated that training produced a decrease in the cytosolic and mitochondrial content of GSH and total GSH (TGSH), although it improved the ability of cardiac tissue to import extracellular GSH through increased γGT activity. Furthermore, this study showed that intensive training regimes may diminish mitochondrial function following *in vitro* stimulation, as shown by the decrease in the rate of state 3 and 4 mitochondrial respiration of the

trained animals compared to the control group. Whereas reduced state 4 respiration may not necessarily be an indication of dysfunction, the dramatic reduction in respiratory state 3 would appear to demonstrate clearly that the mitochondria of the trained hearts presented a reduced capacity for oxidative phosphorylation (Leichtweis et al. 1997). The authors state that, since the production of reduced equivalents from the tricarboxylic acid cycle did not diminish with this type of rigorous training, as shown by increases in cardiac CS activity in the trained rats, the low response of O_2 consumption in state 3 may be attributable to the possible decrease in the activity of NADH dehydrogenase (complex I) and/or of other ETC enzyme complexes. These changes in mitochondrial function seem very different from those observed by Ji and Mitchell (1994), in which the fall in the respiratory control index was attributed to increased state 4 respiration and not to reduced state 3 respiration. In this study, the increased O_2 consumption in state 4 was interpreted as evidence of damage to the inner mitochondrial membrane, with a consequent decrease in the proton gradient, which may suggest different mechanisms associated with dysfunction of cardiac mitochondrial respiration arising from different types of exercise (Leichtweis et al. 1997). The authors concluded that intense endurance training has a negative effect on cardiac mitochondrial respiratory function, making mitochondria more susceptible to oxidative stress, and that this effect may be associated with lower tissue reserves of GSH.

The effect of endurance training on the heart's susceptibility to lipid peroxidation induced by perfusion with the oxidizing substance cumene hydroperoxide (CumOOH) was studied by Kihlstrom (1992). The results suggest that training reduces the susceptibility of rat and mouse myocardium to lipid peroxidation induced by hydroperoxides. Another study conducted by Kihlstrom (1990) suggests GSH as an important mediator in the cardiac protective effect of training in situations of tissue damage arising from anoxia/reoxygenation (A/R). In this study, despite decreases in the activity of enzymes such as CAT and GR and the absence of change in cardiac SOD activity with training, increases in GSH levels and G6PDH activity in the myocardium, together with reduced release of creatine kinase following A/R, would suggest that endurance training has a protective effect in situations of A/R. This interpretation of the data was confirmed by the fall in tissue TBARS levels in the trained hearts subjected to A/R when compared to the control group (Kihlstrom 1990).

CONCLUSION

Despite some disagreement in the literature, regular exercise appears to have a positive influence on cardiac antioxidant systems and to promote greater tolerance in the myocardium, with a consequent general improvement in its function, both at rest and when subjected to stimuli that produce additional oxidative stress. The GSH system, reduced ROS production, and the synthesis of other potential protective agents in situations of stress (such as HSP) appear to be the mechanisms in which systematic endurance training has the greatest modulating effect. Nevertheless, some of the mechanisms associated with the cross-tolerance effect of endurance training in relation to different stimuli that cause cardiac toxicity are not yet well understood.

LIST OF ABBREVIATIONS

ROS - Reactive oxygen species
DCFH - Dichlorofluorescin
GSH - Reduced glutathione
NO• - Nitric oxide radical
GSSG - Oxidized glutathione
ONOO - Peroxynitrite
TGSH - Total glutathione
I/R - Ischemia/reperfusion
SOD - Superoxide dismutase
Hx - Hypoxanthine
CAT - Catalase
ATP - Adenosine triphosphate
GPX - Glutathione peroxidase
ADP - Adenosine diphosphate
GR - Glutathione reductase
AMP - Adenosine monophosphate
O_2 - Molecular oxygen
IMP - Inosine monophosphate
O_2-• - Superoxide radical
XDH - Xanthine dehydrogenase
H_2O_2 - Hydrogen peroxide
XO - Xanthine oxidase
OH• - Hydroxyl radical
Ca^{2+} - Calcium ion
C - Carbon
HOCl - Hypochlorous acid
S - Sulfur
γGT - γ-glutamyl transpeptidase
N - Nitrogen
γGCS - γ-glutamylcysteine synthetase
NAD - Nicotinamide adenine dinucleotide
GS - Glutathione synthetase
NADP - Nicotinamide adenine dinucleotide phosphate
HSP - Heat shock proteins
ETC - Electron transport chain
BSO - Buthionine sulfoximine
MDA - Malondialdehyde
mRNA - Messenger RNA
TBARS - Thiobarbituric acid reactive substances

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Review Article

Cardiac mitochondrial respiratory function and oxidative stress: the role of exercise

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A B S T R A C T

Investigations on the mechanisms capable to influence heart mitochondrial function constitute a central contribution to understand cardiac bioenergetics. In contrast to the conventional idea that reactive oxygen species (ROS) mostly act as a trigger for oxidative damage of biological structures, in low physiological concentrations they can regulate a variety of important molecular mechanisms, including those related to mitochondrial respiratory function. Among others, moderate physical exercise seems to be an important agent to induce cellular and mitochondrial environmental *redox* modifications and it is possible that these alterations could mediate cardiac mitochondrial respiration patterns. This brief review concentrates some current knowledge on mitochondrial respiratory pathways and focuses on data provided by studies dealing with exercise and cardiac respiratory mechanisms. It is emphasized the need of further experimental studies that analyze the association between physical exercise, particularly endurance training, and several mechanisms hypothetically related to the improvement of mitochondrial function, such as the overexpression of some important chaperone machinery and the up-regulation of both cellular and mitochondrial antioxidants. The influence of chronic moderate exercise on the functionality of some inner membrane components and on mitochondrial calcium loading capacity remains to be established.

Key words

RESPIRATION, OXYGEN CONSUMPTION, HEART, TRAINING, REACTIVE OXYGEN SPECIES

INTRODUCTION

As the central organ of the cardiovascular system, the heart is of post-mitotic origin and is highly susceptible to deleterious stimuli, such as those associated with cellular *redox* disturbances (Ji 2002). In fact, the condition of oxidative stress, i.e., the additional production of reactive oxygen species (ROS) with an insufficient response of the antioxidant systems, is involved in many dysfunctions that affect cardiac muscle tissue (Colucci 1997; Shern-Brewer et al. 1998; Dhalla et al. 2000; Lefer and Granger 2000). Many preventive and therapeutic strategies have been suggested for those *redox* related pathologies, including diet, pharmacological therapeutics, and exercise (Paffenbarger et al. 1986). It is well known that regular moderate exercise has many beneficial effects, whereas acute and severe exercise can cause significant damage in many tissues including heart (Venditti and Di Meo 1996; Atalay and Sen 1999; Ascensao et al. 2003). It also appears evident that the extent of tissue damage mainly depends on the duration and the intensity of the stimuli associated with exercise (Ji 1995, 1999).

Despite the scarcity of direct evidences supporting an increased ROS production during exercise, there is abundant data providing indirect proofs that oxidative stress does really occur (reviewed in Di Meo and Venditti 2001). During exercise, several possible cellular sources for ROS have recently been debated, although the most frequently considered are those related to the enhanced activity of xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and to the mitochondrial respiratory electron transport chain (Halliwell and Gutteridge 1999). It appears evident that investigations on the mechanisms capable to influence mitochondrial function constitute a central contribution for the understanding of cardiac bioenergetics. A large number of studies have associated mitochondrial respiratory dysfunction with the formation of ROS (Davies and Doroshow 1986; Du et al. 1998; Ide et al. 1999; Paradies et al. 1999; Tonkonogi et al. 2000b).

Mitochondria are the cells' powerhouses, being the major site of ATP production for tissue survival and functionality. Coupled with ATP synthesis, mitochondria are also critical organelles involved in the modulation of osmotic regulation, cell *redox* status and pH control, signal transduction, and for the establishment of Ca^{2+} homeostasis (Wallace et al. 1997). Considering cardiac work as a process with high energetic costs, it requires a constant mitochondrial ATP production, which could be severely affected by altered mitocondrial integrity, with the consequent functional reduction. The disruption of the lipid and protein constituents as well as consequently compromised mitochondrial bioenergetics have been recognized to participate in cardiac cell injury induced by many stimuli, including exercise (Phaneuf and Leeuwenburgh 2001; Primeau et al. 2002). It is widely assumed that during exercise, and because of enhanced ATP requirements, the increased electron flow through the mitochondria electron transport chain constitutes a prompt condition for electron leakage to dioxygen (O_2), leading to an increased rate of ROS generation.

On the other hand, and in contrast to the conventional idea that reactive species mostly serve as a trigger for oxidative damage of biological structures, it is known that low physiologically

relevant concentrations of ROS can regulate a variety of key molecular mechanisms (Sen 2001). Since moderate and regular physical exercise causes *redox* changes in various cells and tissues, the molecular and functional implications of such changes, particularly on mitochondrial respiration, are not yet sufficiently characterized.

This article focuses on some possible mechanisms being described in the literature to be related to the hypothetical effect of acute and chronic exercise on cardiac mitochondrial respiratory function.

MITOCHONDRIAL GENERATION OF REACTIVE OXYGEN SPECIES

Up to 2% of the oxygen consumed by mitochondrial respiratory chain undergoes one electron reduction, mainly in steps corresponding to the NADH-coenzyme Q, succinate-coenzyme Q and coenzyme QH2-cytocrome c reductases (complexes I, II and III), to generate the superoxide radical ($O_2^{\cdot -}$), and subsequently other ROS such as hydrogen peroxide (H_2O_2) and the potentially harmful hydroxyl radical ($OH^{\cdot}$)(Halliwell and Gutteridge 1999). In mitochondria, electrons are transferred from NADH and $FADH_2$ to the oxidized form of coenzyme Q (UQ), yielding the reduced form of coenzyme Q (UQH_2), which transfers the electrons to cytocrome C oxidase, and is reconverted into UQ, passing first through the free radical semiquinone anion state ($UQ^{\cdot -}$) (see Fig. 1). In fact, the determination of mitochondrial ROS formation using different mitochondrial respiratory chain inhibitors has demonstrated that $O_2^{\cdot -}$ generation may occur preferably at the level of coenzyme Q, due to the accumulation of $UQ^{\cdot -}$ and consequent electron donation from $UQ^{\cdot -}$ to O_2 (Becker et al. 1999; Chen et al. 2003). However, although $O_2^{\cdot -}$ seems to be generated in most cases at the level of mitochondrial complex III, it is important to note that NADH dehydrogenase linked complex I can also be an important site of $O_2^{\cdot -}$ production (Ide et al. 1999).

Since $O_2^{\cdot -}$ formation is a continuous and physiological process, mitochondria are equipped with an efficient enzymatic and non-enzymatic antioxidants composed of superoxide dismutase (MnSOD), glutathione peroxidase (GPx), glutathione reductase (GR), and vitamins C and E. Superoxide anions generated by respiratory chain are readily dismutated by MnSOD, leading to the production of H_2O_2 which in turn is detoxified by GPx or by intra- and extra-mitochondrial catalase (Halliwell and Gutteridge 1999) (see Fig. 1). The reduced state of mitochondrial GPx is maintained by glutathione. Under conditions of increased mitochondrial $O_2^{\cdot -}$ generation , or when the antioxidant systems are unable to cope with the pro-oxidant *redox* state, H_2O_2 may accumulate and react with mitochondrial iron (Fe^{2+}), resulting in the formation of the highly reactive $OH^{\cdot}$ via Fenton reaction and consequently, leading to a condition of exacerbated oxidative stress. When this condition is aggravated, mitochondria and other cell components, namely lipid membranes, structural and enzymatic proteins, and DNA may suffer from deleterious effects of ROS attack, with consequent functional impairment (Kappus 1985; Lefer and Granger 2000; Tirosh and Reznick 2000).

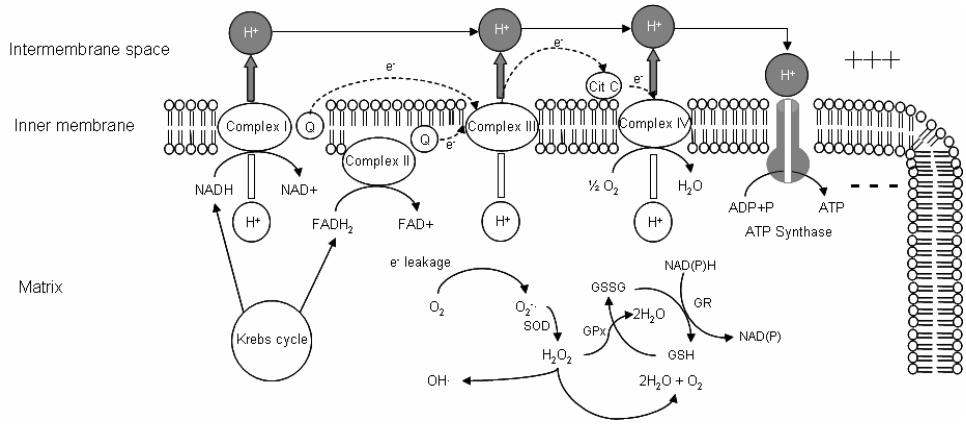

Figure 1. The scheme summarizes mitochondrial oxidative phosphorylation. Matrix reduced equivalents from Krebs cycle provide electrons to the mitochondria electron transport chain (ETC). The electrons flow through electron transport chain (ETC) carriers and the pumping of protons through the inner membrane create a transmembrane potential gradient. Protons return to matrix through several channels including ATP synthase, where ADP oxidative phosphorylation occurs with the production of ATP. The ETC, inserted in the inner mitochondrial membrane, constantly generates ROS by single electron leakage with monoelectron reduction of oxygen (superoxide radical, hydrogen peroxide and hydroxyl radical). These ROS are rapidly neutralized by mitochondria antioxidant defenses such as superoxide dismutase, catalase and glutathione peroxidase. The later enzyme uses reduced glutathione as electron donor resulting in the formation of oxidized glutathione, which in turn is reconverted in GSH by the action of GSSG reductase that uses NAD(P)H as cofactor.

ROS INTERACTION WITH MITOCHONDRIAL MEMBRANES

Being considered major sources of ROS and fundamental Ca^{2+} regulator organelles, mitochondria can become severely dysfunctional via permeability transition pores (PTP), which are formed under the synergistic effects of oxidative stress and deregulated cytosolic free Ca^{2+} (Crompton 2004). The functional changes exhibited by isolated mitochondria such as large amplitude swelling and non-specific increases in membrane permeability to small molecules with dissipation of transmembrane potential Δψ were originally considered as a sign of non-specific membrane damage and latter called PTP. This megachannel in the inner mitochondrial membrane may be opened in the presence of high concentrations of inorganic phosphate, fatty acids, uncouplers, or prooxidants under oxidative stress-related physiopathological conditions (Vercesi et al. 1997; Chakraborti et al. 1999). The PTP comprise a complex of a voltage-dependent anion channel, members of the pro- and anti-apoptotic Bax-Bcl2 protein family, adenine nucleotide translocases (ANT) and cyclophylin-D (Hirsch et al. 1997; Crompton 1999; Kokoszka et al. 2004). Under conditions of elevated intramitochondrial [Ca^{2+}] and oxidative stress, the ANT deforms from its native state, a gated channel that mediates ADP/ATP translocation, into a non-selective channel allowing free permeation of other molecules (small solutes with molecular weights of 1500kDa or less) rather

than ADP/ATP exchange (Hirsch et al. 1997; Vercesi et al. 1997). The ANT deformated state is catalysed by cyclophilin-D and this action is inhibited by cyclosporin A, preserving the native characteristic of ANT (Fig 2). When the ANT native form is destabilised into a PTP, it uncouples oxidative phosphorylation and collapses Δψ leading to a mitochondrial bioenergetic dysfunction. The susceptibility of ANT to lipid peroxides reflects its close relationship with cardiolipin. Cardiolipin, a phospholipid of unusual structure, is localized almost exclusively within the inner mitochondrial membrane where it is biosynthesized, and is particularly rich in unsaturated fatty acid. For this reason, cardiolipin is highly prone to ROS interaction, a condition that is favourable for the generation of reactive lipid fragments in the immediate surroundings of ANT. In fact, ANT has six or more strong bounds to cardiolipin and their break can cause an ANT protein structure modification and subsequent inactivation (Hoffmann et al. 1994). In addition, ANT contains three cystein residues, which are susceptible to oxidation if intramitochondrial concentrations of GSH and pyridine nucleotides like NADPH are not maintained under pro-oxidant *redox* conditions (Chernyak 1997). In fact, the formation of PTP occurs when thiol groups of the inner membrane proteins are oxidized, resulting in conformational changes that are dependent on the essential membrane thiol cross-linkage. The notion that *redox* changes in mitochondrial membranes are responsible for these conformational alterations is supported by the fact that PTP are regulated by thiol oxidants and reductants or by indirect changes in the *redox* state of pore thiols (for refs. see Kowaltowski et al. 2001). Nevertheless, a very recent study revealed that ANT are non-essential components of the PTP and are dispensable for at least some forms of PTP-related cell death, despite of their role in the regulation of permeability transition by modulating the sensitivity of the mitochondrial PTP to Ca^{2+} activation and ANT ligands (Kokoszka et al. 2004).

Because of the increased membrane permeability, mitochondria release apoptogenic factors and dissipate the electrochemical gradient of the inner membrane (Fig 2). The inhibition of PTP using pharmacological intervention countermeasures, such as the classic immunosuppressant specific inhibitor cyclosporine A or mitochondrial expression of the apoptosis-inhibitory Bcl-2, prevents cell death, suggesting that PTP is a rate-limiting event of the death process (see Hirsch et al. 1997; Hengartner 2000). Mitochondrial dysfunction is usually mediated by a transmembrane potential (Δψ) collapse, uncoupled respiration, overproduction of $O_2^{\cdot -}$, decrease in mitochondrial biogenesis, and diminished matrix Ca^{2+} outflow. These physiological phenomena entail a bioenergetic catastrophe culminating in the disruption of plasma membrane integrity (necrosis) and/or in the activation of specific **c**ysteine **asp**artate prote**ases** (caspases) by mitochondrial proteins that leak into the cytosol with secondary endonuclease activation (apoptosis) (reviewed in Kroemer et al. 1998; Hengartner 2000).

An important identified hallmark related to apoptotic cell death is mitochondrial Δψ. Indeed, the dissipation of Δψ is frequently associated with several physiological factors including ROS production and elevation of cytosolic Ca^{2+} concentration. Several lines of evidence suggest that a change in cellular Ca^{2+} handling and an increase in cytosolic Ca^{2+} concentration might be associated with apoptosis signalling (reviewed in Smaili et al. 2003). It is known that the opening of

PTP is triggered by increases in Ca^{2+} concentration and changes in mitochondrial voltage, pH and *redox* state (Kowaltowski et al. 2001). Consequently, because of the significant change in membrane permeability and the dramatic collapse in Δψ, oxidative phosphorylation uncouples and ATP synthesis is seriously compromised. In fact, these and other pathophysiological features may lead to the release of several pro-apoptotic compounds like cytocrome C, apoptosis-inducing factor, and smac/DIABLO from mitochondria with subsequent activation of caspases (see Skulachev 1998; Hengartner 2000).

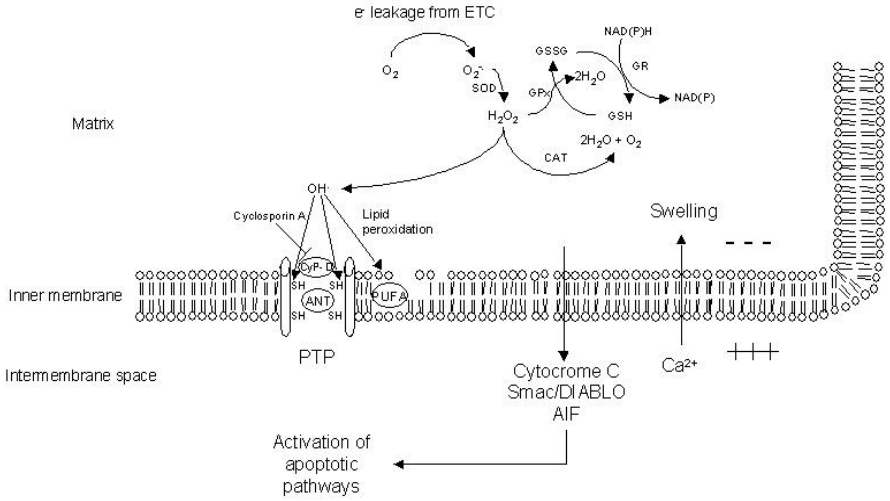

Figure 2. The scheme summarizes some proposed molecular processes related to the interaction of ROS with mitochondria membranes. When antioxidant defenses are insufficient to cope with pro-oxidant *redox* state, generated ROS interact with the phospholipids and with the proteins of mitochondria membranes opening permeability transition pores (PTP) that are responsible for loss of membrane electrical potential as well as for the release of pro-apoptotic compounds such as cytocrome C, Smac-DIABLO and apoptosis inducing factor (AIF). Concomitantly, mitochondria up-takes cellular Ca^{2+} and swell.

MEASUREMENT OF MITOCHONDRIAL O_2 CONSUMPTION

The classical method to study mitochondrial bioenergetics is based on measurements of mitochondrial oxygen consumption using an oxygen sensitive electrode. A common assessment of mitochondrial function provided by this equipment is the rate of oxygen consumption of energized mitochondria in the presence of ADP, the so-called state 3 respiration, and in the absence of the ADP, usually known as state 4 (Fig 3A). The referred equipment also allows to obtain information about other qualitative parameters of mitochondrial respiration, namely the respiratory control ratio (RCR), expressed through the ratio between state 3 and state 4, and the ADP/O (Fig 3A), which reflects the efficiency of mitochondria oxidative phosphorylation (Chance and Williams 1956). Furthermore, the technique provides some possibilities to study magnitudes and properties of

the non-coupled respiration (NCR), i.e. that is not coupled with ADP phosphorylation (see Fig 3B and 4). This NCR is due to the back leakage of protons into the mitochondrial matrix through other ion inner membrane channels rather than through Fo subunit of Fo F1 ATP synthase. It is assumed that this leakage is mediated by specific proteins such as uncoupling proteins (UCP) and ANT, and is involved in several cellular processes influencing basal metabolic rate (Diehl and Hoek 1999; Ricquier and Bouillaud 2000; Giacobino 2001).

According to the chemiosmotic theory, mitochondrial respiration and ATP synthesis are coupled by a proton electrochemical gradient across the mitochondrial inner membrane. Some stoechiometric rules sustain this coupling, including the ADP/O ratio and the low proton conductivity of the membrane that considers the H^+ leak negligible. As a consequence of this proton conductivity, *in vitro* experiments on mitochondrial O_2 consumption could be controlled by: (i) H^+ that re-enters into the matrix; (ii) the addition of protonophores - like carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) or carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (that permeabilizes the membrane to H^+) $^-$ into the reaction medium that destroys this control and leads to maximal respiratory rates, and (iii) the complete inhibition of ATPase by oligomycin that decreases O_2 consumption to a residual rate (the O_2 consumption after oligomycin addition may be due to H^+ leakage). This last feature corresponds to the conductivity of the phospholipid membrane for H^+. Thus, the determination of the respiratory rates using an O_2 sensitive electrode allows to identify and to localize the damage and dysfunction induced by both previous *in vivo* and *in vitro* stimuli (Fig 3B). For instance, comparing distinct experimental conditions, an increase in the rate of mitochondrial O_2 consumption with the addition of oligomycin indicates a H^+ leak that is considered an important sign of membrane proton conductivity and therefore, of phospholipids bilayer damage as well as of PTP opening. An increase in state 4 without change in state oligomycin usually means that intrinsic H^+ stoechiometry of ATP synthase is impaired and membranes are not damaged. In this case, an H^+ slip and intrinsic uncoupling of ATP synthase may occur. When state 3 and state CCCP or FCCP are similarly reduced, either the respiratory chain enzymes are damaged or the upstream oxidable substrates available in the matrix to provide electrons for ETC are limited. Nevertheless, when state 3 is reduced but not state CCCP or FCCP, no oxidative ETC enzyme impairment is suggested, but a decrease in the availability of ADP in the mitochondrial matrix may take place, probably due to ANT dysfunction. This increased proton leakage and intrinsic uncoupling of ETC redox pumps and/or ATPsynthase (proton slip) may cause a reduction in ADP/O and consequently, a decrease in oxidative phosphorylation efficiency.

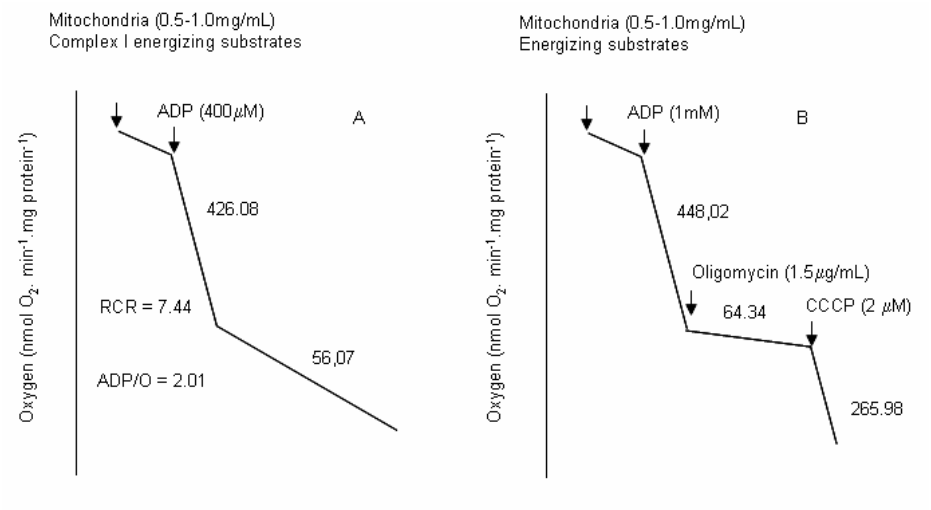

Figure 3. Examples of typical oxigraphic traces of mitochondria respiration in isolated heart mitochondria energized with respiratory substrates. A- State 3 respiration was initiated by adding ADP at a final concentration in the reaction medium of 0.4mM; B- saturating (1mM) ADP-linked state 3 respiration was inhibited by the addition of oligomycin (1.5μg/mL) and then, re-stimulated to a maximal rate through the addiction of CCCP (2μM), a protonophoric agent.

EXERCISE AND CARDIAC MITOCHONDRIAL RESPIRATION

Indisputably, mitochondrial function remains a central topic to understand cardiac bioenergetics. During exercise, energy turnover in cardiac muscle increases several fold when compared to resting condition, with a consequent rise in O_2 consumption and thus, in the rate of mitochondrial ATP production that matches the rate of ATP hydrolysis (Sen and Goldfarb 2000). In fact, cardiac tissue seems to demonstrate efficient cellular mechanisms in order to control mitochondrial ATP synthesis, providing sufficient energy for cellular demands during exercise.

In skeletal muscle, the increase in oxidative efficiency induced by endurance training is extensively associated with mitochondrial biogenesis and with biochemical changes at the level of single oxidative enzymes, up-regulating mitochondrial function (for refs see Tonkonogi and Sahlin 2002). However, much controversy has been noticed regarding those effects on myocardial mitochondria, particularly with training-induced increased mitochondria volume/density indirectly measured by cardiac citrate synthase activity (Leichtweis et al. 1997; Powers et al. 1998; Samelman 2000). In contrast to skeletal muscle, in which adaptive changes in the volume fraction of mitochondria can readily occur, increases beyond 35% are not so frequent in cardiac muscle, possibly because this would encroach on the volumes of contractile myofibrils and sarcoplasmatic reticulum within the cardiomyocyte and hence, limit heart performance (Hood et al. 1994).

An important biological process related to mitochondrial biogenesis that can be mediated by physical exercise is the production of ROS with the consequent alterations in the cell *redox*

environment. The assumption that ROS generation increases during exercise is not supported by some findings obtained from *in vitro* studies with isolated mitochondria in which ROS production was lower in state 3 (ADP stimulated respiration) than in state 4 (non ADP stimulated or basal respiration) (for refs. see Di Meo and Venditti 2001). The explanation for this *in vitro* phenomenon seems to be supported by the fact that mitochondrial ROS generation is related to the degree of reduction of electron chain carriers, and such a degree significantly falls during state 4 to state 3 transition. However, despite theoretical support and experimental controversial data tend to consider that ROS generation is lower in state 3 than in state 4, the hypothesis of exercise-induced enhanced oxidative stress should not be excluded. Indeed, other cellular disturbances induced by exercise, such as increased temperature and changes in Ca^{2+} homeostasis, could increase the capacity of mitochondria to produce ROS (Di Meo and Venditti 2001), interfering with some factors that affect mitochondrial respiration.

Until present, the limited number of studies that have accomplished the relationship between heart mitochondrial function and exercise used vigorous exercise regimens and thus, seems to be unconvincing to understand how regular and moderate training programs can regulate the expression of *in vitro* heart respiratory patterns. Ji and Mitchell (1994) analysed the effect of an acute bout of treadmill running exercise to exhaustion on heart mitochondrial function of doxorubicin treated and non-treated rats. The authors reported a significant increase in state 4 respiration with both complex I (malate-piruvate and 2-oxoglutarate) and complex II (succinate) substrates, which was attributed to an exercise-induced damage of the inner cardiac mitochondrial membrane that compromised the maintenance of the electrochemical proton gradient. These data seem to confirm that acute exhaustive exercise may alter mitochondrial inner membrane function, probably through the induction of PTP or other inner membrane channel disturbances that result, at least in part, from increased oxidative stress. Despite the apparently unexpected rise of state 3 respiration in exercised rat heart mitochondria, a significant reduction in RCR occurred, although only with site I electron donor energizer substrates. The enhanced state 3 after exhaustive running may be indicative either for increased energy utilization during exercise with consequent enhanced mitochondrial response to meet the additional energetic demands or, less probable, for hypothetical and non-confirmed factors modified by exercise including the activation of enzymes related to substrate dehydrogenation and membrane transport systems for the reducing equivalents NADH and $FADH_2$. Another not emphasized, although surprising finding of this study was that the function of cardiac mitochondria isolated from rats submitted to exhaustive exercise did not appear to be more impaired by free radicals ($O_2^{\cdot -}$ generated *in vitro* by the addition of hypoxanthine and xanthine oxidase - Hx+XO in the reaction medium) when compared to their non-exercised controls. Nevertheless, a tendency for an increase in state 4 respiration and for a reduction in RCR seems to occur in exercised mitochondria when exposed to such *in vitro* deleterious conditions.

Data from Leichtweis et al. (1997) revealed a significant decrease in state 4 respiration of heart mitochondria from rats submitted to a vigorous swim training program ($6h.day^{-1}$; $5\ days.week^{-1}$, 8-9 weeks) using malate-pyruvate and 2-oxoglutarate

as respiratory energizer substrates. This unexpected change induced by training occurred in heart mitochondria from both sham and ischemia-reperfused (I-R) rats. Although mitochondrial antioxidant defence mechanisms, namely glutathione contents and antioxidant enzymes, were not significantly modified with this overtraining-related stimulus, a decreased mitochondrial malondialdehyde concentration as a marker of lipid peroxidation, was observed in trained hearts; this contradicts a hypothetical condition of oxidative stress as suggested by the authors. Furthermore, the authors also considered that the depletion of cardiac vitamin E content previously reported after endurance training (Tiidus and Houston 1995; Asha Devi et al. 2003) may have contributed to aggravate damages in inner membrane. Vitamin E is a chain breaking antioxidant located in the lipid phase of the cell associated to membranes and acts as a powerful stabilizer, causing for example, inhibitory effects on the storage of heart lipofuscin-like particles (Asha Devi et al. 2003). Despite the absence of a close relationship between state 4 as an indirect sign of inner membrane permeability and oxidative stress, the supposed mitochondrial pro-oxidant *redox* state condition suggested in the study of Leichtweis (1997) was confirmed by the enhanced susceptibility of the vigorously trained and I-R heart mitochondria to different conditions of ROS attack. Accordingly, notorious functional reductions were found in mitochondria treated with Hx+XO, H_2O_2 and H_2O_2 + Fe^{2+}. Despite the reduction of state 4 in all experimental groups after ROS treatment, which does not seem to be in accordance with an increased mitochondrial inner membrane susceptibility, the evident perturbations induced by ROS exposure on ETC components and on ADP phosphorylation suggested by decreased state 3 and RCR confirm the enhanced susceptibility of mitochondria from overtrained rats. Taken together, these findings may have some clinical relevance when patients undergoing corrective cardiac surgery are subsequently exposed to considerable doses of free radicals produced by chemotherapeutic pro-oxidant drugs (Ji 2000; Wallace 2003).

Venditti and Di Meo (1996) did not find any change in RCR of heart rat mitochondria after both, acute exhaustive exercise and endurance training. Nevertheless, the RCR used by the authors was calculated as the ratio between uncoupling respiration (using FCCP) and basal respiration (using succinate plus rotenone), which is not the conventional method and could have, therefore, influenced the results. The stability and viscoelastic properties of heart mitochondrial membranes were analysed by Perez et al. (2003) after a variety of acute and chronic exercise. According to the data provided by fluorescence polarization methods, the acute bouts of exercise (exhaustive, maximal exhaustive and eccentric) affected mitochondrial membrane fluidity, at least in part due to the additional production of ROS, whereas 12 weeks of endurance treadmill-running training maintained membrane fluidity and stability. Furthermore, a sub-group of trained rats studied immediately after an exhaustive bout of exercise revealed no changes in the referred mitochondrial membrane properties. These data provides evidences that training induces heart mitochondria membrane protection against acute pro-oxidant deleterious stimuli.

MITOCHONDRIAL-LINKED SYSTEMS PREVENTING ROS FORMATION – THE HYPOTHETICAL ROLE OF EXERCISE

It has been argued that mitochondria possess various delicate respiration-linked mechanisms that interact independently or coordinated in an attempt to maintain sufficiently low intracellular O_2 levels and to decrease the life-time of one-electron O_2 reductants that ultimately lead to a decrease in ROS production. These mechanisms were reviewed by Skulachev (1997) and included (i) the so-called "mild uncoupling"; (ii) mitochondria "decoupling" that usually means a switching off or slipping of energy coupling respiration and occurs without any increase in membrane H^+ conductance; (iii) the short-lived PTP; and even (iv) the inhibition of respiratory substrates' oxidation due to superoxide-induced aconitase inactivation, an anti-ROS defense mechanism employed by the cell when ROS concentration is strongly increased. This author also suggests a mitochondria and cell selection phenomenon when the rate of ROS generation is high, i.e., the elimination of mitochondria or cells may take place with the involvement of ROS-dependent pore opening and ROS-dependent apoptosis mediating mitochondria and cell death.

Likewise, it has been mentioned that, during basal conditions, *in vitro* mitochondrial ROS generation seems to be controlled, at least in part, by a moderate losses of Δψ. In fact, it is known that cardiac mitochondrial respiratory chain form ROS in state 4, when the respiration rate is limited by the lack of ADP and the electrochemical protonic potential difference is high - above a certain critical threshold value (Korshunov et al. 1997; Miwa and Brand 2003). The group of Skulachev has demonstrated that any decrease in state 4 Δψ results in a strong inhibition of H_2O_2 formation and that mitochondria are equipped with a special mechanism of "mild uncoupling", which keeps the Δψ below the critical referred threshold for ROS production (Korshunov et al. 1997). The results obtained under those conditions were proposed to be related to the fact that some transients of ETC, capable of reducing O_2 to $O_2^{\cdot-}$, became long-lived. Another well-established ROS release regulator during non-stimulated mitochondrial respiration is the respiratory rate, whose increased flow possibly contributes to the shortening of live time of the ETC components. Indeed, the possible activation of mitochondrial pathways that decrease Δψ and increase respiration, such as UCP and ANT (Simonyan and Skulachev 1998), free fatty acids (FFA) (Korshunov et al. 1998), ATP-sensitive K^+ channels (Ozcan et al. 2002; Ferranti et al. 2003) all induced by exercise, should not be discharged with the purpose of preventing ROS release during *in vitro* rest respiration (state 4). Data obtained by Tonkonogi et al. (2000a) in isolated mitochondria from human skeletal muscle revealed that 6 weeks of endurance training increased the sensitivity of leak-dependent respiration in the presence of low concentrations of FFA. It was suggested that the referred adaptation might enhance the potential for metabolic regulation, increase the whole-body basal metabolic rate, and prevent the excessive formation of ROS by ETC, probably mediated by a mild-uncoupling phenomenon (Tonkonogi et al. 2000a).

Nevertheless, there is a lack of data regarding the effects of an endurance-training program on the regulation of heart respiratory function, particularly on the mechanisms related to the control of non-coupled oxygen consumption through other mitochondrial inner membrane ion carriers, rather

than UCP (Boss et al. 1998). Despite some controversy (Hesselink et al. 2003), data obtained from skeletal muscle showed that UCP1 and UCP3 expression as well as uncoupling respiration, when related to citrate synthase activity, seem to decrease after endurance training (Jones et al. 2003; Fernstrom et al. 2004), although a training-induced elevated sensitivity of non-coupled respiration to FFA had been reported (Tonkonogi et al. 2000a). Similarly, Boss et al (Boss et al. 1998) observed a decrease in heart UCP2, UCP3 expression and mRNA after 8wks of endurance training and suggested that these alterations result in a higher metabolic efficiency, diminishing the levels of energy dissipation. These and other hypothetical alterations should allow to speculate about the possible influence of endurance training in the regulation of electron flux through ETC and hence, about the shorter or longer living times of its complex components, with consequent regulation of ROS generation.

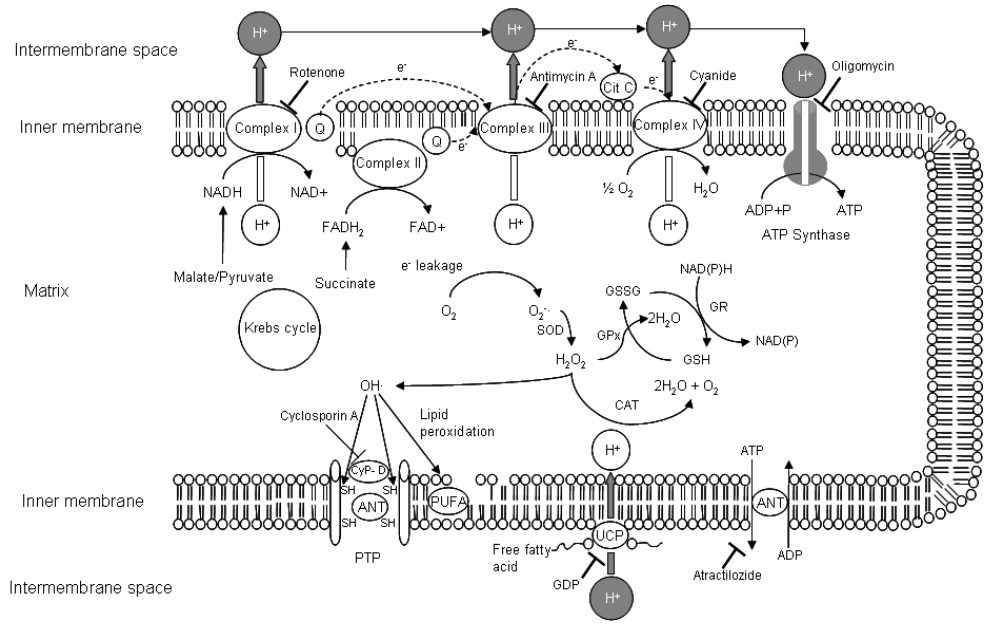

Figure 4. This scheme depicts some integrated systems related to mitochondrial respiratory function. The figure also shows some modulator compounds used during *in vitro* trials with isolated mitochondria such as ETC complex (rotenone, antimycin A and cyanide) ATPase (oligomycin), PTP (cyclosporine A) and UCP (GDP) inhibitors. Protons also return to matrix through other important membrane proteins that are responsible for uncoupling respiration, particularly at rest mitochondria state 4 respiration. These include UCP and ANT, which seem to be related to mild uncoupling.

Although acute high-intensity exercise resulting in an enhancement of oxidative stress can promote damage to heart mitochondria, culminating in its compromised function (Venditti and Di Meo 1996), chronic moderate exercise leads to mild free radical generation and may possibly be beneficial for mitochondrial respiratory function mediating the increased cell signalling for essential mitochondrial protein synthesis, with consequent organelle biogenesis and up-regulation of respiration. Though

this phenomenon appears to be less evident in cardiac than in skeletal muscle, those signals induced by cardiomyocyte contractions would activate protein kinases and phosphatases that could modify the activity of nuclear transcription factors and mRNA stability factors acting within the cytosol and increasing mRNA expression of nuclear-encoded mitochondrial proteins (NEMP) (Hood et al. 2002; Irrcher et al. 2003). Subsequently, these translated NEMP are then properly chaperoned to mitochondria and imported into the matrix space, inner or outer membranes (Voos and Rottgers 2002; Hood et al. 2003). Likewise, a higher contractile activity like in exercise is known to increase the rate of protein import and refolding, mediated by an enhanced expression of some machinery components, such as HSP60 and HSP70 (Powers et al. 1998; Samelman 2000; Hamilton et al. 2003). Moderate chronic exercise induces a cardiac HSP elevation; however, possible associations between a hypothetically enhanced mitochondrial energetic capacity and the expression of these proteins mediated by endurance training have not yet been characterized. In fact, this parallel linkage was successfully established in some studies using heat shock as protecting stimulus to enhance cardiac mitochondrial complex activity (Sammut et al. 2001; Suzuki et al. 2002; Sammut and Harrison 2003).

Furthermore, and as referred above in the text, mitochondria are considered the most important cellular Ca^{2+} buffers. Moreover, conditions of Ca^{2+} overload are frequently associated to several mitochondrial dysfunctions (Monteiro et al. 2003; Wallace 2003). In this sense and similarly to other heart protective models (Santos et al. 2002), it seems interesting to analyse the Ca^{2+} loading capacity of “trained” mitochondria as well as the susceptibility of mitochondria from trained hearts to Ca^{2+}-induced PTP.

In summary, the hypothetical role of exercise, particularly chronic exercise on the different systems known to prevent ROS formation linked with cardiac mitochondria deserves to be more profoundly investigated.

FUTURE DIRECTIONS

In order to establish a wide spectrum of preventive and therapeutic measures against cardiac pathological disorders, it seems important to clarify how physical training can regulate mitochondrial respiration. The effect of exercise training on the mechanisms related to mitochondrial membrane conductivity to protons as well as ETC enzymes’ functionality seems to be relevant research topics. Another problem that deserves to be experimentally tested is to know whether a moderate and well-established exercise training protocol contributes to elevate the tolerance of heart mitochondria to *in vitro* adverse conditions, mediating some uncoupling-based described mechanisms or others that possibly contribute to avoid rest (state 4) ROS production (Korshunov et al. 1997; Korshunov et al. 1998; Ferranti et al. 2003). Furthermore, the relationship between the hypothetical endurance training-induced enhanced mitochondrial function / tolerance to deleterious stimuli and the elevation of some important proteins, such as HSP, usually related to cardioprotection also needs to be experimentally addressed.

LIST OF ABBREVIATIONS

AIF - apoptosis inducing factor

ADP - adenosine triphosphate

ANT - adenine nucleotide transporters

ATP - adenosine diphosphate

Ca^{2+} - calcium ion

Caspases - cysteine aspartate proteases

CAT - catalase

cit C - cytocrome C

CyP-D - cyclophilin D

Q - coenzyme Q

e^- - electrons

ETC - electron transport chain

$FADH_2$ - flavine adenine dinucleotide

GDP - guanidine diphosphate

H^+ - hydrogen ion

H_2O_2 - hydrogen peroxide

HSP - heat shock proteins

$OH^·$ - hydroxyl radical

GPx - glutathione peroxidase

GR - GSSG reductase

GSH - reduced glutathione

GSSG - oxidized glutathione

NADPH - nicotinamide adenine dinucleotide phosphate

NCR - non-coupled respiration

NEMP - nuclear encoded mitochondrial proteins

PTP - permeability transition pore

PUFA - polyunsaturated fatty acids

RCR – respiratory control ratio

SH - sulfhydryl groups

$O_2^{-·}$ - superoxide radical

SOD - superoxide dismutase

UCP - uncoupling proteins

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EXPERIMENTAL WORK

Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice

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ABSTRACT

Background: There is a lack of studies reporting the influence of DOX treatment on chronically exercised animals. This study intended to determine the effect of endurance swimming training on cardiac muscle tolerance to *in vivo* DOX-induced damage, analyzing the levels of oxidative stress markers, the response of antioxidant system and the expression of 60 and 70 kDa heat shock proteins (HSP).

Methods: Forty-four Charles River CD1 male mice were randomly assigned to either non-trained placebo (NT+P) and non-trained DOX (NT+DOX) or trained placebo (T+P) and trained DOX (T+DOX). Twenty-four hours after completion of a 14-week training, cardiac ventricles were extracted for biochemical assays of oxidative stress and damage markers, antioxidant enzymes and HSPs.

Results: DOX treatment *per se* (single 20mg.kg^{-1} dose), administrated 24 hours after the last exercise bout, elevated ($p<0.05$) plasma cardiac troponin I (cTnI), HSP60, % oxidized glutathione, thiobarbituric acid reactive substances and carbonyl groups and reduced –SH groups. However, training induced a significant increase ($p<0.05$) on total and reduced glutathione (GSH), HSP60 expression, and decreased the rise of plasma cTnI as well as cardiac carbonyl groups contents in DOX hearts, when compared to NT+DOX mice. Although catalase activity of T+DOX was significantly higher than T+P, no changes were observed in the activities of superoxide dismutase, glutathione peroxidase and glutathione reductase. Neither DOX nor training induced significant variations in HSP70.

Conclusion: Training improved myocardial tolerance to DOX-induced damage. It is likely that the improvement in responses to DOX was related to training-induced increases in GSH and HSP60.

Key Words: HEART, ADRIAMYCIN, SWIMMING EXERCISE, ROS, ANTIOXIDANTS, HSP

INTRODUCTION

Previous research has established a number of different conditions that may precipitate various forms of cardiac muscle dysfunction (Colucci 1997). As the central organ of the cardiovascular system with elevated aerobic metabolism, the heart is continuously working and has one of the highest oxygen consumption rates among all body tissues, which seems to favor reactive oxygen species (ROS) production. In fact, the weakness of the heart to oxidative damage may be in part explained by the fact that heart demonstrates a slow turnover and relatively lower levels of antioxidant enzyme activity when compared to most other tissues (Ji 2000). Growing experimental evidence suggest that the increased production of ROS may play an important role in these dysfunctions (Lefer and Granger 2000). It seems probable that endurance exercise training provides myocardial protection against many cardiac insults. Although the exact mechanisms responsible for this protection continue to be debated, it has been argued that they are in part, associated with the decreased free radical production and with increased response of antioxidant defense systems (for a comprehensive review see Ji 1995).

Among other cell sources, heart mitochondria electron transport chain has been referred as one of the major sites of ROS production, through the so-called electron leakage. Whereas the activity of mammalian cytocrome C oxidase is O_2-saturated at very low O_2 tensions, the rate of electron leakage by mitochondria increases at high O_2 concentrations during certain conditions such as exercise (Di Meo and Venditti 2001), favoring enhanced ROS production. Nevertheless, when moderate and systematic, exercise could constitute an excellent tool either to prevent and/or to treat several diseases, providing enhanced parallel resistance to the cardiac muscle tissue (reviewed in Ji 2000; Ascensao et al. 2003). This phenomenon usually referred as cross-tolerance has been demonstrated by several studies in which endurance training seems to up-regulate heart antioxidant systems (Ramires and Ji 2001) and mitochondrial function (Venditti and Di Meo 1996), to reduce the formation of lipid peroxidation by-products (Venditti and Di Meo 1996) and to induce heat shock proteins (HSP) overexpression (Powers et al. 1998) after certain stress stimuli.

Due to its applicability and clinical usefulness, the majority of studies in the field of training-induced cross-tolerance have been widely supported on ischemia-reperfusion (I-R) as a model of cardiac oxidative damage and dysfunction (Powers et al. 1998; Ramires and Ji 2001). However, the study of cross-tolerance effect of endurance training to other stimuli rather than I-R should also be considered in order to increase the spectrum of training cross-tolerance applications. Accordingly, the *in vivo* treatment with doxorubicin (DOX), also known as adriamycin, can be considered an appropriate model to accomplish the above referred features and hence to test training-induced cardiac cross-tolerance, since DOX induces a dose-related and potentially lethal cardiotoxicity that may be in part due to increased oxidative stress (Horenstein et al. 2000; Hrdina et al. 2000).

To the best of our knowledge, there is only one study dealing with the effect of endurance training in DOX treated hearts (Kanter et al. 1985). The authors concluded that exercise ameliorated severe toxic damage caused by the drug, possibly

by increasing antioxidant enzymes activity. However, there is a clear diversity and variability in the effects of endurance training in modulating the various enzyme systems of cardiac antioxidant defense (for refs see Atalay and Sen 1999; Ascensao et al. 2003). Moreover, other systems rather than antioxidant enzymes could be involved in cardioprotection, such as glutathione and HSPs (Ji et al. 1994; Powers et al. 1998). Indeed, glutathione is also considered as an important system in cell protection during pro-oxidant *redox* conditions. In fact, a large amount of evidence has documented that multiple molecular mechanisms may contribute to the reduced glutathione (GSH) protecting action, including the scavenging of free radicals, thereby blocking the lipid chain reaction propagation in a manner similar to vitamin E (Halliwell and Gutteridge 1999). Likewise, HSPs as been proposed to provide cellular protection via their ability to orchestrate proper folding of nascent proteins, assist in protein repair, and facilitate degradation of irreparably damaged proteins (Mogk et al. 2002). They represent a family of highly conserved proteins, transiently expressed after acute or chronic exposure of the cardiac cells to sub-lethal environmental stimuli, such as hyperthermia or oxidative stress (for refs. see Starnes 2002). Therefore, it seems relevant to study the effect of endurance training, not only in the possible alterations on gene-modulated antioxidants enzymes, but also in other known protective systems hypothetically involved in cellular remodeling caused by prior endurance training in DOX-induced cardiomyopathy.

Therefore, the main purpose of this study was to analyze the effect of 14-wk swimming endurance training in cardiac muscle tolerance to *in vivo* DOX-induced oxidative stress and damage. We hypothesized that endurance training may induce some cardioprotection to DOX exposed myocardium with the contribution of enhanced responses of GSH, HSP and some gene-modulated antioxidant enzymes.

METHODS

Sample

44 Charles River CD1 male mice (aged six-eight weeks, weighing 30-35g at the beginning of the experiment) were used. During the experimental protocol, the animals were housed in collective cages (two mice per cage) and were maintained in a room at normal atmosphere (21-22º C; ~ 50-60% humidity) receiving commercial food for rodents and water *ad libitum* in an inverted 12-hour light/dark cycles. The animals were randomly divided into two groups: T (n=22, trained) and NT (n=22, non-trained). Body weights of the mice were monitored carefully throughout the experimental period. Only male animals were used because of the protective effect of estrogen on cardiac tissue in females (Voss et al. 2003). The Ethics Committee of the Scientific Board of the Faculty of Sport Sciences approved the experimental protocol, which followed the *Guidelines for Care and Use of Laboratory Animals* in research.

Endurance training protocol

The trained group was submitted to an endurance swimming training program, while not trained was not engaged in any exercise program. All the mice were adapted to water before the beginning of the experiment. The adaptation consisted of keeping the animals in shallow water at 31º C with the purpose of reducing the environment stress

without promoting any physical training adaptations.

The endurance-training program was performed in the morning (between 9 and 11 a.m.) and consisted of a swimming period 1h/day, 5 days/week for 14 weeks. Swimming was performed in a high filled and deep plastic container (100X100X100cm) with water maintained at a temperature between 31-35ºC. The animals were progressively familiarized with swimming during the first 3 weeks (Table 1), by increasing the swimming time for 20 min every seven days up to the final time of 1 h/day. Exercise sessions lasted 10 min on the first day of the training period and at the 7th day the animals swam continuously for 20 min. According to the protocol, at the end of the 14th day the animals swam 40 min/day and from the 21st day until the end of the training the period of swimming was 60 min/day (Gobatto et al. 2001). In order to optimize endurance-training adaptations, mice supported a 4% body weight load attached to the tail during the swimming periods (Evangelista et al. 2003). All mice were weighed once a week and when necessary the workload was adjusted to body weight changes. During training sessions, mice were allowed to swim at their own pace. Water burbling was produced sparingly to prevent mice floating.

Table 1. Exercise training protocol

	Weeks of training													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Exercise duration (min/day)	20	40	60	60	60	60	60	60	60	60	60	60	60	60
Load (% body weight)	0	0	0	1	2	3	4	4	4	4	4	4	4	4

Not trained mice were kept in shallow water at 31º C during the same periods of swimming animals, 5 days/week during the training period and were used as controls. After all the training and shallow immersion sessions, wet animals were carefully dried and placed in a warm environment to avoid additional cold physiological stress and health problems.

After the end of the endurance-training program, 22 trained and 22 sedentary animals were again randomly separated into four sub-groups. Thus, trained animals were distributed in trained plus placebo (T+P) and trained plus DOX (T+DOX); not trained animals were also distributed in non-trained plus placebo (NT+P) and non-trained plus DOX (NT+DOX). The placebo groups were injected i.p. with a saline solution (0.9% NaCl). The experimental groups were injected i.p. with a single dose of DOX (20mg.kg^{-1}) in solution according to Mohamed et al., (2000). Both treatments were carried 24 h after the last exercise bout and animals were sacrificed 24 h after DOX and placebo injections.

Tissue preparation

Animals were anaesthetized with diethyl ether and placed in supine position. After that, the opening of abdominal cavity exposed the inferior cava vein and a blood sample of approximately 1 ml was collected in a heparinized tube. The blood was immediately centrifuged (5 min at 5000*g*, 4ºC) and an aliquot of plasma was obtained and stored at –80ºC for

biochemical determination of cardiac troponin I (cTnI). After a quick opening chest, the whole mice hearts were then rapidly excised, rinsed with ice-cold saline, carefully dried and weighted. Then, the atria and the great vessels were removed and the ventricles were sliced and homogenized in a homogenization buffer (0.05M Tris, 0.03M L-serine, 0.06M boric acid, tissue:buffer ratio of 100mg/mL, pH 7.6) with a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4ºC 3-5 times for 5 seconds at speed low setting, with a final burst at a higher speed setting. Homogenates were centrifuged (2 min at 2000*g*, 4ºC, in order to eliminate cellular debris) and the resulting supernatant was stored at –80ªC for biochemical assays. An aliquot was separated immediately after centrifugation for measuring the antioxidant enzyme activity of superoxide dismutase (SOD), catalase (CAT), oxidized glutathione reductase (GR) and reduced glutathione peroxidase (GPx) on the day of animal's sacrifice to avoid lost of enzyme activity induced by freezing. For total (TGSH), reduced (GSH) and oxidized (GSSG) glutathione analysis a 500μl aliquot of heart homogenate was directly transferred into a 500μl of 10% perchloric acid. Oxidative capacity [citrate synthase (CS)], lipid peroxidation [thiobarbituric acid reactive substances (TBARS)], protein oxidation (carbonyl and protein SH groups), and HSP60 and HSP70 expression were also measured in the 2000*g* supernatant of heart homogenates. For the semi quantification of carbonyl groups and HSP60 and HSP70 using immunoassay methods (see Analysis of HSP 60, 70 and protein carbonylation sub-section below), eight (carbonyls) and five (HSPs) samples of muscle homogenate from each group were randomly chosen for independent Western Blots.

Both *soleus* muscles were excised and homogenized in tris buffer (200 mM, tissue:buffer ratio of 100mg/mL, pH 8.0) in a motor-driven Potter-glass homogenizer at 0–4°C at low speed. The homogenized samples were then centrifuged for 2 min at 2000*g*, the pellet was discharged and the supernatant was used for measuring muscle oxidative capacity through CS activity.

Biochemical assays

TGSH, GSH and GSSG measurements were determined as previously described by Tietze (1969) by spectrophotometric assay at 414nm. Lipid peroxidation on the whole cardiac muscle homogenate was assayed according to the method described by Bertholf et al. (1987) and measured by the formation of TBARS at 540 nm. Oxidative modification of protein SH groups was quantified by spectrophotometric measurement according to the method proposed by Hu (1990) at 414nm. Total SOD activity was measured according to the protocol of Beauchamp and Fridovich (Beauchamp and Fridovich 1971) using the RANSOD-kit (Randox Laboratories). GPx and GR activities were measured using RANSEL-kit and glutathione reductase-kit, respectively (Randox Laboratories). CAT activity was determined spectrophotometrically using the method proposed by Aebi (1984) at 240nm. Total antioxidant status (TAS) was determined spectrophotometrically at 600 nm with a commercial kit (Randox). cTnI concentration was quantitatively determined with an established immunoassay using Abbott kit. Cardiac and *soleus* CS activities were measured using the method proposed by Coore et al. (1971). The principle of assay was to initiate the reaction of acetyl-CoA with oxaloacetate and link the release of CoA-SH to 5,5-dithiobis (2-nitrobenzoate) at 412nm. Protein

contents from both cardiac and *soleus* muscles homogenates were assayed using bovine serum albumin as standard according to Lowry et al. (1951).

Analysis of HSP 60, 70 and protein carbonylation

To determine the levels of HSP60 and HSP70 in the heart, a certain volume of homogenate correspondent to 10μg protein was resolved by SDS-PAGE (12.5% acrylamide gels of 1mm thickness) as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes according to Locke et al. (1990). The immunoblotts were probed with 1:2000 dilution of monoclonal anti-HSP70 (Clone BRM-22, Sigma) and anti-HSP60 (Calbiochem) and with 1:2000 dilution of the secondary antibody (anti-mouse IgG peroxidase conjugate, Sigma, St. Louis, USA).

For protein carbonyl derivatives assay, a certain cardiac homogenate volume (V) containing 20μg of protein was derivatized with dinitrophenylhydrazine (DNPH). Briefly, the sample was mixed with 1 V of 12% SDS plus 2 V of 20mM DNPH 10% trifluoracetic acid (TFA), followed by a 30 min of dark incubation, after which 1,5 V of 2M Tris / 18.3% of β-mercaptoethanol were added. A negative control was simultaneously prepared for each sample. After diluting the derivatized proteins in TBS to obtain a final concentration of 0.001μg/μL, a 100μl volume was slot-blotted into a Hybond-PVDF membrane. Immunodetection of carbonyls was then performed using rabbit anti-DNP (DAKO) as the first antibody (1:2000 dilution), and anti-rabbit IgG-Peroxidase (Amersham Pharmacia) as the second antibody (1:2000 dilution).

For both referred methods, the bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (Bio Rad). Optical density results were expressed as percentage variation of control values.

Statistical analysis

Mean and mean standard errors were calculated for all variables in each of the experimental groups. One-way ANOVA followed by the Bonferroni post-hoc test was used to compare key variable between groups. The Pearson correlation coefficient was used to analyze the inter-correlations between variables. Statistical Package for the Social Sciences (SPSS Inc, version 10.0) was used for all the analysis. Statistical tests with a p-value < 0.05 were considered significant.

RESULTS

Mice body weights, absolute and relative heart weights are expressed in table 2. In accordance with the well-described body mass and cardiac adaptations induced by endurance training, the 14 weeks of swimming training decreased mice weight and increased the relative heart weight ($p<0.05$).

Training program resulted in a significant ($p<0.05$) improvement in skeletal muscle oxidative capacity as evidenced by CS activity in soleus muscle, whereas no changes were observed in cardiac CS activity among groups (see table 2). The improved enzymatic activity in soleus reflects that endurance swimming training was an efficient chronic stimulus to ameliorate muscle oxidative metabolism.

Table 2. Effect of endurance swimming training and doxorubicin treatment on mice weights, absolute and relative heart weights, skeletal muscle and cardiac CS activities for each group (N=11).

	NT+P	NT+DOX	T+P	T+DOX
Mice weight (g)	51.7± 5.5 *	49.3 ± 2.5	45.3 ± 4.0	44.7±4.3
Heart weight (mg)	214.2 ± 20.4 *	219.6±14.5	228.6 ± 17.8	234.6 ± 20.2
Heart weight/mice weight ($mg.g^{-1}$)	4.2 ± 0.45 *	4.5 ± 0.30 #	5.1 ± 0.41	5.28±0.62
Skeletal muscle CS ($\mu mol.mg^{-1}.min^{-1}$)	0.020±0.001 *	0.020±0.001 #	0.034±0.001	0.031±0.002
Cardiac CS ($\mu mol.mg^{-1}.min^{-1}$)	0.037±0.0006	0.037±0.003	0.034±0.002	0.038±0.001

Note: Not-trained plus placebo saline solution (NT+P); not-trained plus doxorubicin (NT+DOX); trained plus placebo saline solution (T+P); trained plus doxorubicin (T+DOX); citrate synthase (CS). All values are mean and SD. * NT+P vs. T+P and T+DOX; # NT+DOX vs. T+P and T+DOX. ($p<0.05$).

As can be depicted from table 3, DOX induced a significant increase in plasma levels of cTnI, which reflects enhanced cTnI release from heart and indicates loss of cardiomyocyte membrane integrity. However, endurance training resulted in a significant reversal ($p<0.05$) of DOX-induced increase in that leaked cardiac protein (NT+DOX vs. T+DOX).

Fig. 1 shows the effect of DOX and swimming training on the cardiac levels of TGSH, GSH, GSSG, %GSSG. Both DOX and training *per se* induced significant increases in TGSH and GSH ($p<0.05$). In addition, there was an increase in cardiac TGSH and GSH in T+DOX when compared to NT+DOX group. Animals receiving DOX underwent a substantial elevation in the cardiac GSSG content and %GSSG compared to the placebo control group. However, endurance training (T+DOX) significantly decreased the rise in heart GSSG and %GSSG induced by DOX administration.

A significant increase in cardiac levels of TBARS was observed in NT+DOX group compared to NT+P, whereas no significant decrease between NT+DOX (73.95±5.84 $nmol.g^{-1}$) and T+DOX (52.51±11.32 $nmol.g^{-1}$) had occurred. Concerning protein –SH groups, a significant ($p<0.05$) decrease was observed in NT+DOX when compared to all other groups. Conversely, as shown in fig. 2, protein carbonyl formation increased significantly from NT+P in relation to all other groups (46.29, 27.25 and 20.27% respectively in relation to NT+DOX, T+P and T+DOX). Endurance training reduced protein carbonyl formation in DOX treated groups (NT+DOX vs. T+DOX).

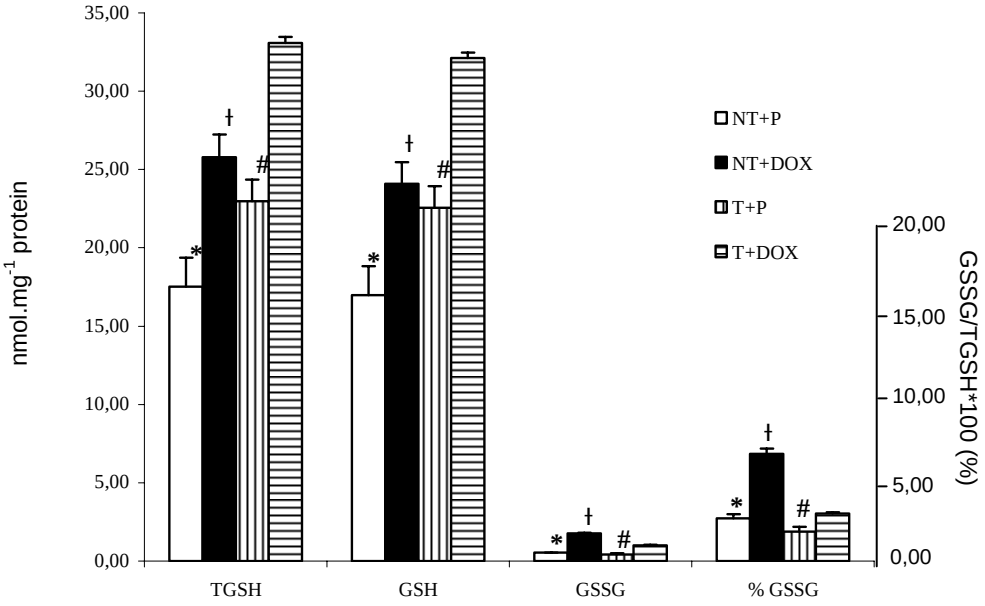

Figure 1. Effect of endurance swimming training and doxorubicin treatment on cardiac muscle TGSH, GSH, GSSG (nmol.mg^{-1}) and % GSSG for each group (N=11). Values represent mean and SEM. * p<0.05, NT+P vs. NT+DOX and T+P; ł p<0.05, NT+DOX vs. T+DOX; # p<0.05, T+P vs. T+DOX.

Table 3. Effect of endurance swimming training and doxorubicin treatment on plasma cTnI content and on cardiac TBARS and –SH content for each group (N=11).

	NT+P	NT+DOX	T+P	T+DOX
Plasma cTnI (ng.ml^{-1})	0.0±0,0	1.40±0.29 #	0.0±0.0 †	0.65±0.07
TBARS (nmol.g^{-1})	36.35±6.2 *	73.95±5.84	17.66±2.73 †	52.51±11.32
Protein -SH groups (mmol.g^{-1})	0.052±0.002	0.038±0.002 #	0.051±0.002	0.047±0.002

Note: Not-trained plus placebo saline solution (NT+P); not-trained plus doxorubicin (NT+DOX); trained plus placebo saline solution (T+P); trained plus doxorubicin (T+DOX); cardiac troponin I (cTnI); thiobarbituric acid reactive substances (TBARS). Values are mean and SEM. * NT+P vs. NT+DOX; # NT+DOX vs. all other groups; † T+P vs. T+DOX (p<0.05).

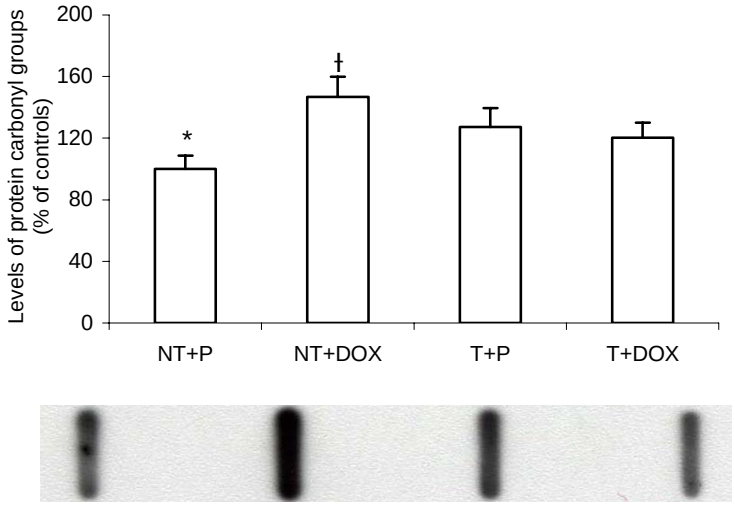

Figure 2. Effect of endurance swimming training and doxorubicin treatment on cardiac muscle protein carbonyl groups. Immediately below the histogram, the protein carbonyl formation panel shows a representative pattern of anti-denitrophenyl (DNP)-specific interaction with DNP for each group (N=8) as described in METHODS. Values are mean and SEM. * $p<0.05$, NT+P vs. all other groups; ł $p<0.05$, NT+DOX vs. T+DOX.

No changes were observed in the activity of any antioxidant enzyme and in total antioxidant status measured in cardiac homogenates, with the exception of CAT activity, which was higher in T+DOX than in T+P (table 4).

Table 4. Effect of endurance swimming training and doxorubicin treatment on cardiac muscle CAT, SOD, GPx, GR activities and TAS for each group (N=11).

	NT+P	NT+DOX	T+P	T+DOX
CAT (μmol.min.$^{-1}$mg.$^{-1}$)	112.92±4.31	103.25±2.90	100.34±3.18 *	122.00±6.95
SOD (U.mg^{-1})	95.8±3.1	81.3 ±9.3	93.0±10	81.2±7.5
GPx (U.mg^{-1})	9.5±0.6	9.9±0.9	8.9±0.4	9.4±0.5
GR (U.mg^{-1})	3.4±0.1	3.8±0.3	3.1±0.2	3.1±0.3
TAS (μmol.mg^{-1})	0.084±0.005	0.062±0.011	0.085±0.066	0.075±0.003

Note: Not-trained plus placebo saline solution (NT+P); not-trained plus doxorubicin (NT+DOX); trained plus placebo saline solution (T+P); trained plus doxorubicin (T+DOX); catalase (CAT); superoxide dismutase (SOD); glutathione peroxidase (GPx); glutathione reductase (GR); total antioxidant status (TAS). Values are mean and SEM. * T+P vs. T+DOX ($p<0.05$).

As can be seen in fig. 3, no changes were observed in HSP70 expression between groups, whereas significant differences were noted in HSP60. Indeed, all the studied experimental situations, i.e. DOX (NT+P vs. NT+DOX), training (NT+P vs. T+P) and training in the presence of DOX effects (NT+DOX vs. T+DOX) revealed significant ($p<0.05$) alterations in the expression of HSP60.

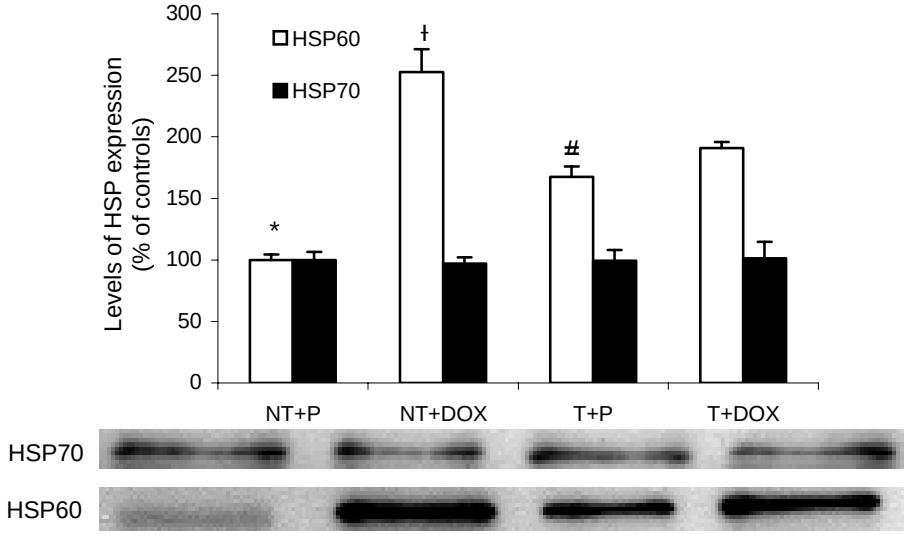

Figure 3. Effect of endurance swimming training and doxorubicin treatment on the percentages of cardiac muscle HSP60 and HSP70. Immediately below the histogram, the panel shows a representative western blotting of HSP60 and HSP70 for each group (N=5) as described in METHODS. Values are mean and SEM %. * $p<0.05$, NT+P vs. NT+DOX and T+P; † $p<0.05$, NT+DOX vs. T+DOX; # $p<0.05$, T+P vs. T+DOX.

Regarding the correlation coefficients between cardiac damage and oxidative stress variables, significant correlations were found between cTnI and GSSG ($r=0.821$, $p<0.01$), %GSSG ($r=0.682$, $p<0.01$), -SH ($r=-0.632$, $p<0.01$) and TBARS content ($r=0.518$, $p<0.01$). The response to cellular stress measured by HSP60 correlated significantly with tissue oxidative stress and damage markers, namely %GSSG ($r=0.629$, $p<0.05$), GSSG ($r=0.769$, $p<0.01$), –SH ($r=-0.608$, $p<0.05$) and carbonyl groups ($r=0.677$, $p<0.01$) as well as with the used specific systemic cardiac damage marker cTnI ($r=0.799$, $p<0.01$). Taken together, these correlations reinforce the oxidative etiology of cellular stress and of cardiac damage.

DISCUSSION

Overview of principal findings

The current study provided additional support to understand how regular physical exercise, particularly swimming training, could contribute to augmentation of cardiac muscle resistance against free radical-based cardiomyopathy induced by DOX administration. Two lines of evidence can be emphasized from the present study. First and considering cardiac stress markers, namely cTnI

and HSP60, endurance swimming training decreased the rise of cardiac disturbances induced by an acute single dose of DOX administration. Second, and according to changes observed in cardiac glutathione and HSP responses in both non-trained and trained mice hearts treated with DOX, it is likely that these systems might be considered as essential cellular defense against free radical-based cardiomyopathy caused by DOX, providing enhanced tolerance to trained myocardium at least in the first 48 hours after the end of training period.

Cardiac damage markers

DOX treatment caused a lower rise in myocardial cTnI release to the plasma in the T+DOX group compared to NT+DOX. In this study, since cTnI was used as an overall marker of myocardial damage and considering that plasma content of this protein correlates with loss of cardiomyocyte membrane integrity, the above results suggest that hearts from T+DOX animals suffered from less extent of membrane disturbances caused by DOX.

Given that DOX cardiotoxicity is clearly related to a ROS enhanced production-mediated deleterious action, several integrated *redox* mechanisms could be associated to the diminishing rise in the above-referred markers of cellular damage. In the present study, the pro-oxidative state induced by DOX can be suggested by the increase in %GSSG (discussed in the next section), which was accompanied by the significant accumulation of cardiac lipid peroxidation and protein oxidation by-products in the hearts from DOX treated animals. In fact, the increased oxidative stress caused by DOX seem to lead to peroxidative modification of membrane lipids measured as TBARS (NT+P vs. NT+DOX; T+P vs. T+DOX) probably altering normal cell function, since polyunsaturated fatty acids are usually considered highly susceptible to ROS attack. These results are in accordance with many other reports in which DOX induced increased heart lipid peroxidation by-products either throughout 24 hours after DOX administration (Luo et al. 1997), on the 3-4th day of DOX post-dosing injection (Wu and Kang 1998) or after cumulative treatment schedules (Luo et al. 1999). Although endurance swimming training did not significantly decrease the rise in cardiac TBARS content in DOX treated animals (NT+DOX vs. T+DOX), a declining trend was apparent in T+DOX group suggesting that training may have a protective effect against ROS-induced lipid peroxidation by DOX administration.

Regarding the level of oxidativelly modified proteins, DOX administration increased protein carbonyl content (NT+P vs. NT+DOX) (Figure 2), which probably contributed to disruption cellular function either by loss of catalytic and structural integrity or by interruption of some regulatory pathways (Stadtman and Levine 2000). DOX also induced a significant reduction in sulfhydryl residues (-SH), indicating increased disulfide linkages (-S-S-) from both proteins and GSH. In the present study, relatively elevated values of carbonyl groups in T+P were found, which could probably be explained by the effect of the last training sessions. However, our data importantly showed that these by products of oxidative protein damage were significantly reduced (18.01%) in T+DOX when compared to NT+DOX group, indicating that the cardiac *redox* adaptations induced by endurance swimming training offered some protection against DOX-induced cardiac protein oxidative damage.

Redox changes and antioxidant enzymes

It is sufficiently stated that endurance exercise results in many types of physiological (vascular, metabolic and functional) adaptations in the heart. Indeed, several studies have proposed that the increased cardiac function induced by regular exercise can be attributed to the enhanced cell defenses against oxidant production in the re-establishment of *redox* status (reviewed in Ji 2000; Ascensao et al. 2003). According to the fact that DOX-induced cardiomyopathy is in part, due to increased free radical generation and hence, to oxidative stress, it is possible that the cardiac *redox* adaptations induced by endurance training can contribute to the previously referred tolerance of myocardium to DOX.

The above-referred increase in %GSSG induced by DOX, both in NT+DOX and in T+DOX may be explained by GSH oxidation in order to cope with augmented levels of heart free radical production caused by DOX administration. In fact, a significant increase in cardiac GSSG content was observed in DOX treated animals. Although a reduction in GSH content could be expected, due to the amount of GSH oxidation (Sun and Kang 2002), a significant increase of heart tissue GSH was observed. One possible explanation is that, under tissue oxidative stress, GSH can be imported by cardiac muscle from plasma via the γ-glutamyl cycle to cope with DOX-induced increased oxidant production. The aforementioned changes may be supported by other studies as well. Indeed, as stated by Luo et al. (1997), DOX administration probably resulted in a net efflux of liver GSH and an increase in plasma GSH concentration, presumably under the influence of vasopressin and catecholamine stimulation, which is typical of a situation of pro-oxidant *redox* status. In addition, the authors interestingly observed increased plasma GSSG levels, which in their opinion could reflect an enhanced GSSG efflux from the myocardium under oxidative stress.

The notion that T+DOX hearts have a higher GSH importing capacity than those of NT+DOX needs to be importantly underlined. In fact, trained myocardium seems to have a greater ability to take-up GSH to cope with DOX toxic effects than those of non-trained mice, which may explain the diminished rise in cardiac %GSSG found in T+DOX group. Furthermore, a 14-wk of endurance training resulted in a significant increase in cardiac TGSH and GSH contents (NT+P vs. T+P), without altering GSSG concentrations. As described by others (Leichtweis et al. 1997), one possible explanation for that helpful adaptive response may be related to the independent increase in γ-glutamyl transpeptidase activity, which facilitates GSH breakdown and transmembrane transport, resulting in an increased GSH level in cardiomyocytes. Thus, the marked increase in GSH concentration and the consequent decrease in GSSG content and in %GSSG (NT+DOX vs. T+DOX) indicating a training effect on the re-establishment of physiological *redox* environment may help to maintain the cell membranes integrity and stability, promoting non-enzymatic detoxification of $OH^{\bullet}$ radicals and lipid peroxides (Horenstein et al. 2000). In addition, since one of known pathways of free radicals generation induced by DOX, particularly the $OH^{\bullet}$, is related to the formation of a DOX- Fe^{3+} complex (Horenstein et al. 2000), it is likely that the above described adaptations induced by training concerning glutathione system may be helpful in the detoxification of that potent radical. Thus, considering that DOX induced an increase in the

cardiac levels of GSH 24 hours after injection (NT+P vs. NT+DOX; T+P vs. T+DOX), GSH can be confirmed as one of the first lines of defense against DOX toxicity (Mohamed et al. 2000). In this sense, and taking into account that endurance training is an important chronic inductor (i) of cardiac GSH levels and (ii) of enhanced GSH importing capacity in the presence of DOX, it can be assumed that endurance training promotes some helpful effects in cardiac myocytes exposed to DOX toxicity through glutathione system, at least in the first post-administration hours.

This up-regulation of glutathione system was expected to be accompanied by an increased activity of antioxidant enzymes. However, in our study, endurance training did not induce any significant alteration in the activity of the selected enzymes. Although significant increases in cardiac GR and GPx had been reported elsewhere after endurance swimming training (Venditti and Di Meo 1996), other studies demonstrated that swimming training was *per se* ineffective in raising these and other gene-modulated antioxidant enzymes like SOD and CAT (Venditti and Di Meo 1997). Despite some studies reported that exercise-related to small but significant increases in myocardial SOD activity may be critical for protection against myocardial injury (Powers et al. 1998), no changes were observed in the present study. Regarding CAT, no variations were observed in CAT activity either induced by DOX treatment *per se* (NT+P vs. NT+DOX) or by training (NT+P vs. T+P), whereas the increase in T+DOX compared to T+P group reached significance. The biological rationale for this challenging adaptive response is unclear but could be associated with the fact that exercise-induced increases in myocardium ability to eliminate hydrogen peroxide and other organic peroxides resulting from DOX toxicity. In fact, the significant increased activity of CAT in DOX exposed hypertrophied hearts by endurance training (T+P vs. T+DOX) may be possibly attributed to DOX-induced activation of enzyme activity through protein synthesis, indicating that the adaptations induced by endurance training can be important for increasing the activity of this antioxidant enzyme in the presence of DOX. The absence of any significant variation on TAS, an indicator of the overall cardiac antioxidant capacity, seems to suggest that defense mechanisms that are more specific may be involved.

Heat shock proteins

Regardless of its role related to cardioprotection (Starnes 2002), HSPs overexpression can be undoubtedly interpreted as an acute sign of cellular stress. Despite the fact that cardiac HSP research has focused primarily on the abundant 70 KDa group, HSP60 in combination with HSP10 have similarly been shown to form an important mitochondria-located chaperonin complex expressed in doxorubicin-induced mitochondria apoptosis (Shan et al. 2003). The mechanistic link between doxorubicin-induced oxidative stress and cardiac muscle expression of HSP60 and HSP70 remains unclear. However, Kim et al., (Kim et al. 1999) suggest that the regulation of heat shock factor 1 (HSF1) transcriptional activity by anticancer drugs induces differential expression of cardiac HSPs, promoting elevation of cardiac mitochondrial HSP60 and reducing the relative expression of cytoplasmic HSP70. This absence of enhanced HSP70 overexpression after DOX treatment was also demonstrated by Ohtsuboa et al., (Ohtsuboa et al. 2000) in cell cultures. Therefore, and considering mitochondria as the site of DOX-

induced ROS production and consequently the main target of ROS deleterious and/or regulatory action, it was expected that an acute single dose of DOX resulted in an increase in HSP60, primarily considered a mitochondria abundant protein. In accordance, our data importantly showed a significant increase in HSP60 after DOX administration (NT+P vs. NT+DOX), whereas no change occurred regarding HSP70. Although DOX administration elevated the levels of cardiac HSP60 in hearts from NT+DOX and T+DOX mice, the rise in T+DOX was significantly lower than that of NT+DOX. Thus, and considering HSP as an acute and sensitive marker of cell stress, with consequent and important roles in the protein biochemical metabolism, the diminished increase in HSP60 in T+DOX compared to NT+DOX supports the idea that training augmented the tolerance of the myocardium against DOX toxicity.

It has been reported that endurance training induces elevation of these molecular chaperones. In addition to their feature of stress molecules discussed above, the mechanistic action of HSP has also been interpreted as tissue protector (Hutter et al. 1996; Sammut et al. 2001). Actually, numerous studies have suggested that HSPs contribute to elicit cellular defense against several stresses by aiding in the normal, unstressed transport and folding of nascent polypeptides and in the refolding of the denatured and/or aberrant proteins as may occur in cell stress (for refs. see Mogk et al. 2002). For instance, the data revealed by Sammut et al. (Sammut et al. 2001) demonstrated that post-ischemic mitochondrial integrity and function is preserved in myocardium previously expressing high levels of HSP60 and HSP72. In our study, since endurance training resulted in enhanced cardiac HSP60 and considering mitochondria as the main DOX-induced ROS generation site and target, possible mitochondrial mechanisms related to this training-induced cellular remodeling process may be involved. In fact, the observed increase in cardiac HSP60 expression may suggest important mitochondrial adaptations in order to cope with DOX toxicity (Fig 3). Although it is yet unknown which component of exercise is responsible for the up regulation of HSPs, a variety of stresses associated with endurance exercise has been suggested as contributors for HSP synthesis in cardiac muscle tissue. These include heat stress, metabolic overload, hypoxia and ROS production. In this sense, it is likely that endurance exercise results in the accumulation of redundant signals that probably act, independently or collectively, in the up-regulation of heart HSP levels (Powers et al. 1998). Unexpectedly, our data revealed no variations in HSP70 expression after training. We selected swimming as the endurance-training mode and there is evidence that acute and chronic hemodynamic responses to swimming, such as hypercapnemia and acidosis, are different from responses to other types of exercise such as treadmill running (Sturek et al. 1984). Moreover, swimming leads to different physical responses and mechanical stresses, compared to running, because of the effects of water pressure, utilization of different muscles and reduced effects of gravity. In addition, the differences in cardiac adaptations between swimming and running training were attributed to possible sympathoadrenal related mechanisms (Geenen et al. 1988). In the present study, these differences, in combination with distinct training intensities (Noble et al. 1999) and durations (Moran et al. 2004) may possibly explain

the inability of endurance swimming training to increase the levels of cardiac HSP70.

Conclusion

In summary, the data from the present study provides evidence that endurance swimming training improves myocardial tolerance to *in vivo* single dose of DOX-induced oxidative damage. It appears that aerobic training enhances cardiac GSH importing capacity and content, contributing to the observed decrease of DOX toxicity through the augmented response of glutathione system. Swimming training-induced HSP60 overexpression could also be considered as another possible strategy involved in the cardiomyocyte protection against DOX. In fact, the mechanisms by which endurance training seems to confer additional protection against DOX remain elusive and further studies need to be addressed in order to comprehend the role of the different systems, such as those related to mitochondria, in this process.

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Endurance exercise training attenuates morphological signs of cardiac muscle damage induced by doxorubicin in male mice

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ABSTRACT

The purpose of this study was to determine the effect of endurance swimming training (14 wks, 5day/wk, 1h/day) on cardiac muscle tolerance to *in vivo* doxorubicin (DOX)-induced toxicity, analyzing quantitative and qualitative histological signs of muscle damage and plasma cardiac troponin I (cTnI). Thirty-two Charles River CD1 male mice were randomly assigned to non-trained placebo (NT+P, n=8), non-trained DOX (NT+DOX, n=8), trained placebo (T+P, n=8) and trained DOX (T+DOX, n=8). DOX was administered i.p. 24 hours after the last exercise bout, in a single dose of 20mg.kg^{-1}. Twenty-four hours after DOX treatment, the animals were sacrificed and aliquots of plasma were obtained for measuring plasma concentrations of cTnI; cardiac ventricles were extracted for semi quantitative and qualitative morphological analysis of tissue damage using light and electron microscopy. DOX treatment *per se* elevated ($p<0.05$) the levels of cTnI, which were significantly attenuated in T+DOX group. Morphological examination revealed that the elevated extension and severity damage scores in sedentary DOX hearts were significantly ($p<0.05$) reduced in trained group treated with DOX. Moreover, the percentage the total abnormal mitochondria exhibiting extensive loss of cristea, intramitochondrial vacuoles and notorious myelin figures were 0%, 88.3%, 2.9% and 10.1% for NT+P, NT+DOX, T+P and T+DOX, respectively. According to our data, endurance training seems to attenuate the severe morphological and biochemical signs of cardiac muscle injury induced by DOX treatment. These improvements in trained hearts were accompanied by an enhanced mitochondrial protection against DOX side effects.

Key Words: ADRIAMYCIN, LIGHT AND ELECTRON MICROSCOPY, HEART, SWIMMING EXERCISE, MITOCHONDRIA, ULTRASTRUCTURE

INTRODUCTION

It is generally assumed that endurance exercise training provides myocardial protection against many cardiac insults. When moderately and systematically repeated, exercise could constitute an excellent tool either to prevent and/or to treat several diseases, providing enhanced parallel resistance to the cardiac muscle tissue (Moore and Palmer 1999). Although the exact mechanisms responsible for this protection continue to be debated, it has been argued that they are, at least in part, associated with the decreased ROS and with increased response of the several antioxidant defense systems (see Ji 1995; Ascensao et al. 2003). Accordingly, it has been demonstrated that endurance training up-regulates heart antioxidant enzymes and glutathione content (Ascensao et al. 2005b), improves mitochondrial respiratory function (Venditti and Di Meo 1996), reduces the formation of lipid peroxidation by-products (Venditti and Di Meo 1996) and induces heat shock proteins (HSP) overexpression (Powers et al. 1998; Ascensao et al. 2005b).

Most of the training-related cross-tolerance cardiac studies used ischemia-reperfusion (I-R) as a model to test cardiac susceptibility to oxidative damage and dysfunction (Powers et al. 2001). However, in addition to I-R, other stimuli associated with distinct pathways of cellular injury should also be considered in order to better understand all the mechanisms behind training cross-tolerance, which could enlarge its possible beneficial applications.

Doxorubicin (DOX) is a potent and broad-spectrum water-soluble anthracycline antibiotic prescribed for the treatment of a variety of malignancies including leukemia and solid tumors. The successful use of this antineoplastic therapeutic agent is limited by the development of a dose-dependent and irreversible cardiac toxicity (Wallace 2003). The referred toxicity is characterized by various electrocardiographic (Singal and Iliskovic 1998), echocardiographic (Bertinchant et al. 2003) and evident histomorphological changes (Bertinchant et al. 2003; Wallace 2003), seen as loss of myofibrils, distension of sarcoplasmatic reticulum, nuclear pyknosis, interstitial edema, myofibrilar vacuolization and hyalinization with loss of cross-striations (Bertinchant et al. 2003; Wallace 2003). Moreover, mitochondria have also been identified as primary DOX target organelles, and their involvement is evidenced by the results of many studies reporting functional and morphological alterations, such as extensive degeneration or even loss of cristea, intramitochondrial vacuoles and notorious myelin figures, mitochondria swelling and abnormal size and shape (Yen et al. 1996; Yen et al. 1999; Zhou et al. 2001; Oliveira et al. 2004).

To the best of our knowledge, there is only one study dealing with the effect of endurance training in DOX treated hearts analyzed through morphological alterations (Kanter et al. 1985). The authors concluded that exercise attenuated the severe toxicity caused by the drug, observed in the thin sections examined by light microscopy. However, qualitative and particularly, semi-quantitative information obtained by means of electron microscopy, may provide additional contribution to better understand the protective effect, at subcellular level, of previous endurance training on the toxicity caused by DOX. These new insights would consist in the recognition of the specific cardiomyocyte ultrastructures altered by the coordinated effects of endurance training against DOX treatment. In this sense, the main purpose of this study was to analyze the effect of 14-wk swimming endurance training in cardiac

muscle tolerance to *in vivo* DOX-induced damage throughout ultrastructural semi-quantitative and qualitative examination. Since mitochondria have been identified as important targets of DOX-induced subcellular damage in the heart, and that endurance training causes important biochemical, morphological and functional mitochondria adaptations (see Frenzel et al. 1988; Ascensao et al. 2005a), we hypothesized that these organelles could be a central target of endurance training-induced cardioprotection.

METHODS

Sample

32 Charles River CD1 male mice (aged six-eight weeks, weighting 30-35g at the beginning of the experiment) were used. During the experimental protocol, the animals were housed in collective cages (two mice per cage) and were maintained in a room at normal atmosphere (21-22º C; ~ 50-60% humidity) receiving commercial food for rodents and water *ad libitum* in a 12 h light/dark cycles. The animals were randomly divided into two groups: trained (n=16, trained) and non-trained (n=16, non-trained). Body weights of the mice were monitored carefully throughout the experimental period. Only male animals were used because of the protective effect of estrogen on cardiac tissue in females (Voss et al. 2003). The Ethics Committee of the Scientific Board of the Faculty of Sport Sciences approved the experimental protocol, which followed the *Guidelines for Care and Use of Laboratory Animals* in research.

Endurance training protocol

The trained group was submitted to an endurance swimming training program, while the non-trained was not engaged in any exercise program. All the mice were adapted to water before the beginning of the experiment. The adaptation consisted of keeping the animals in shallow water at 31º C with the purpose of reducing the environment stress without promoting any physical training adaptations. The endurance-training program was performed in the morning (between 9 and 11 a.m.) and consisted of a swimming period 1h/day, 5 days/week for 14 weeks (Ascensao et al. 2005b). Swimming was performed in a high filled and deep plastic container (100X100X100cm) with water maintained at a temperature between 31-35ºC. The animals were progressively familiarized with swimming during the first 3 weeks (Table 1), by increasing the swimming time for 20 min every seven days up to the final time of 1 h/day. Exercise sessions lasted 10 min on the first day of the training period and at the 7th day the animals swam continuously for 20 min. According to the protocol, at the end of the 14th day the animals swam 40 min/day and from the 21st day until the end of the training the period of swimming was 60 min/day. In order to optimize endurance-training adaptations, mice supported a 4% body weight load attached to the tail during the swimming periods (Evangelista et al. 2003). All mice were weighed once a week and when necessary the workload was adjusted to body weight changes. During training sessions, mice were allowed to swim at their own pace. Water burbling was produced sparingly to prevent mice floating.

Table 1. Exercise training protocol

	Weeks of training													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Exercise duration (min/day)	20	40	60	60	60	60	60	60	60	60	60	60	60	60
Load (% body weight)	0	0	0	0	0	1	2	3	4	4	5	5	5	5

DOX treatment

After the end of the endurance-training program, the 16 trained and 16 non-trained animals were again randomly separated into four sub-groups. Thus, trained animals were distributed in trained plus placebo (T+P, n=8) and trained plus DOX (T+DOX, n=8); non-trained animals were also distributed in non-trained plus placebo (NT+P, n=8) and non-trained plus DOX (NT+DOX, n=8). The placebo groups were injected i.p. with a 0.1ml of sterile saline solution (0.9% NaCl). The experimental groups were injected i.p. with a single dose of DOX (20mg.kg^{-1}) in 0.1ml solution according to others (Childs et al. 2002). Both treatments were carried 24 h after the last exercise bout and animals were sacrificed 24 h after DOX and placebo injections.

Plasma and muscle extraction

Animals were anaesthetized with diethyl ether and placed in supine position. After that, the opening of abdominal cavity exposed the inferior cava vein and a blood sample of approximately 1 ml was collected in a heparinized tube. The blood was immediately centrifuged (5 min at 5000*g*, 4ºC) and an aliquot of plasma was obtained and stored at –80ºC for biochemical determination of cardiac troponin I (cTnI). After a quick opening chest, the whole mice hearts were then rapidly excised, rinsed with ice-cold saline, carefully dried and weighted.

Both *soleus* muscles were excised and homogenized in tris buffer (200 mM, tissue:buffer ratio of 100mg/mL, pH 8.0) in a motor-driven Potter-glass homogenizer at 0–4°C at low speed. The homogenized samples were then centrifuged for two min at 2000*g*, the pellet was discharged and the supernatant was used for measuring skeletal muscle oxidative capacity through CS activity. A 25mg of left ventricle was immediately taken and homogenized for cardiac CS activity.

Tissue preparation for morphological analysis

After heart harvesting the atria and the great vessels were removed, and small pieces of left ventricle were cut into 1 mm cubic pieces and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer during two hours. The specimens were post-fixed with 2% osmiumtetroxide, dehydrated in graded alcohol, and embedded in Epon. Semithin sections for light microscopy (Zeiss Axioplan 2 Imaging System) were stained with toluidine blue and ultrathin sections for transmission electron microscopy (Zeiss EM 10A) were contrasted with 0.2% lead citrate and 0.5% uranylacetate. All used reagents were of analytical grade and purchased from acknowledged companies.

Procedures of morphological analysis

Quantitative analysis of cardiac muscle was performed using a final light microscopic magnification of ×400 on longitudinal and cross sections of heart ventricles. About 120 to 200 fibers

from each muscle were analyzed for quantification of the severity and the extension of lesions according to the criteria previously established (Sun et al. 2001) (Table 2).

Table 2. Criteria for morphological evaluation of cardiotoxicity

Degree	Severity
1	Sarcoplasmatic microvacuolizations and/or intersticial cellular edema
2	Same as 1 plus sarcoplasmatic macrovaluolizations or atrophia, necrosis, endocardial lesions, and thrombi
	Extension
0	No lesions
0.5	<10 single altered myocytes in the whole heart section
1	Scattered single altered myocytes
2	Scattered small groups of altered myocytes
3	Widely spread small groups of altered myocytes
4	Confluent groups of altered myocytes
5	Most cells damaged

The score obtained for each observed section was calculated as follows: severity * extension of damage based on Della Torre et al. (1999) with adaptations.

Ultra thin sections were examined using electron microscopy for a qualitative ultrastructural evaluation. The ultra thin sections of heart tissue were also semiquantitatively examined for histopathological evidence of cardiomyopathy, according to severity scores from 0-3, as previously described (Oliveira et al. 2004). Severity of damage was scored using electron microscopy grids: *grade 0*, no change from normal; *grade 1*, limited number of isolated cells (less than 5% of the total number of cells per block) exhibiting early myofibrillar loss and/or cytoplasmatic vacuolization; *grade 2*, groups of cells (5 to 30% of the total number) exhibiting early myofibrillar loss and/or cytoplasmatic vacuolization; and *grade 3*, diffuse cell damage (>30% of total number) with the majority of cells exhibiting marked loss of contractile elements, loss of organelles, and mitochondria and nuclear degeneration. All slides were scored independently by two examiners who were blinded to each tissue sample code.

The percentage of abnormal mitochondria (with extensive loss of cristae, intramitochondrial vacuoles and mitochondrial swelling) was evaluated in approximately one hundred random cells from each experimental group as previously described (Yen et al. 1996).

Biochemical assays

cTnI concentration was quantitatively determined with an established immunoassay using a commercial Abbott kit. Cardiac and *soleus* CS activities were measured using the method proposed by Coore et al., (Coore et al. 1971). The principle of assay was to initiate the reaction of acetyl-CoA with oxaloacetate and link the release of CoA-SH to 5,5-dithiobis (2-nitrobenzoate) at 412nm. Protein contents from both cardiac and *soleus* muscles homogenates were assayed using bovine serum albumin as standard according to Lowry et al. (1951).

Statistical analysis

Mean and mean standard errors were calculated for all variables in each of the experimental groups. One-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. Statistical Package for the Social Sciences (SPSS Inc, version 10.0) was used for all the analysis. The significance level was set at 5%.

RESULTS

Mice body weights, absolute and relative heart weights are expressed in Table 3. In accordance

with the well-described body mass and cardiac adaptations induced by endurance training, the 14 weeks of swimming training decreased mice weight and increased the relative heart weight ($p<0.05$).

Training program resulted in a significant ($p<0.05$) improvement in skeletal muscle oxidative capacity as evidenced by CS activity in soleus muscle, whereas no changes were observed in cardiac CS activity among groups (see Table 3). The improved enzymatic activity in soleus reflects that endurance swimming training was an efficient chronic stimulus to ameliorate muscle oxidative metabolism.

Table 3. Effect of endurance swimming training and DOX treatment on mice weights, absolute and relative heart weights. All values are mean and SD * NT+P vs. T+P and T+DOX; # NT+DOX vs. T+P and T+DOX. ($p<0.05$).

	NT+P	NT+DOX	T+P	T+DOX
Mice weight (g)	50.3 ± 3.5 *	52.1 ± 2.5	44.3 ± 4.1	43.7±3.3
Heart weight (mg)	212.2 ± 13.4 *	214.6±13.5	229.6 ± 15.8	224.8 ± 10.2
Heart weight/mice weight ($mg.g^{-1}$)	4.4 ± 0.35 *	4.3 ± 0.33 #	5.2 ± 0.21	5.36±0.48
Skeletal muscle CS ($\mu mol.mg^{-1}.min^{-1}$)	0.019±0.001 *	0.020±0.001 #	0.035±0.001	0.031±0.002
Cardiac CS ($\mu mol.mg^{-1}.min^{-1}$)	0.038±0.0004	0.036±0.003	0.035±0.002	0.037±0.001

As can be depicted from Fig 1, DOX induced a significant increase in plasma levels of cTnI. However, endurance training resulted in a significant reversal ($p<0.05$) of DOX-induced increase in that leaked cardiac protein (NT+DOX vs. T+DOX).

Morphological changes under light microscopy can be depicted from table 4. Briefly, in contrast with the normal appearance of NT+P group, the myocardium from NT+DOX was characterized by prominent and consistent vacuolization affecting a large number of cells with interstitial edema. In contrast, only vacuolar morphological changes were noted in a few small areas in the sections from T+DOX mouse hearts (Fig. 2). The most elevated damage score recorded in NT+DOX group were clearly attenuated in the T+DOX group (table 4).

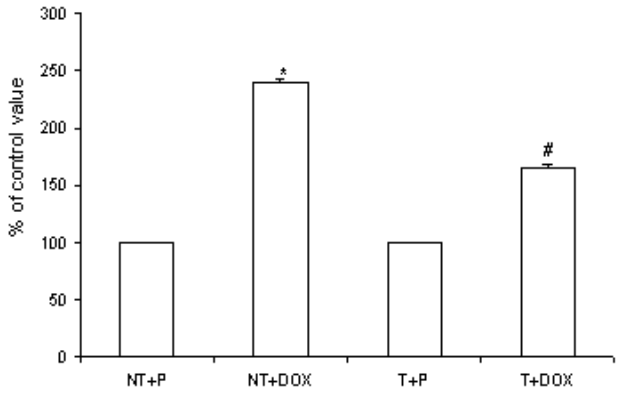

Figure 1. Effect of endurance swimming training and DOX treatment on plasma cTnI content. Values represent mean and SEM and are expressed as percentage of control (NT+P). * NT+DOX vs. all other groups; # T+DOX vs. all other groups ($p<0.05$).

Table 4. Cardiomyopathy scores recorded from hearts of all experimental groups seen under light microscopy.

Treatment	Animals	Cardiomyopathy score
Saline	NT+P	0
	T+P	0
DOX	NT+DOX	2,72*
	T+DOX	0,31**

* p<0.05 NT+DOX vs. all other groups; ** p<0.05 T+DOX vs. all other groups.

Ultrastructural analysis was performed in heart ventricles from animals of all experimental groups. As demonstrated in figure 3, non-trained DOX-treated mice (Figs. 3C and 3D) evidenced marked myocardial damage when compared to the normal appearance of their control (Fig. 3A) counterparts (NT+P vs. NT+DOX). These changes consisted of mitochondria damage with extensive degeneration or even loss of cristea, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysossomes, mitochondria swelling and abnormal size and shape. Moreover, the accumulation of intracytoplasmatic vacuoles, suggestive of sarcoplasmic reticulum dilatation, and myofilament disarray were also evident, compared to the normal histological appearance of non-trained control hearts. Endurance swimming training *per se* (NT+P vs. T+P) caused notable changes in myocardial structure seen as an apparent increased glycogen content, intercalated discs showing a notorious scalloped appearance and evident signs of mitochondria biogenesis with elevated number of encroached mitochondria per fiber area, probably resulting in an increased volume/density of mitochondria. It is important to note that mitochondria division, mild and focal loss of cristea density and organization within mitochondria and minimal degradation by-products, probably secondary lysossomes, were also present in non-treated trained hearts (Fig. 3B). Regarding hearts harvested from trained animals treated with DOX (T+DOX), although maintained ultrastructural alterations described for T+P group, the above-referred signs of morphologic damage seen in NT+DOX hearts were notoriously attenuated in T+DOX mouse hearts (Figs. 3E and 3F).

In accordance, the semiquantitative analysis of these histopathological changes confirmed that the severe ultrastructural abnormalities induced by DOX treatment in sedentary hearts were significantly attenuated in trained DOX treated group (Table 5).

Table 5. Effects of endurance running training and DOX treatment on ultrastructural histological cell damage scores

	Severity of cell damage	
Treatment	Non-trained	Trained
Saline	0.00±0.00	0.69±0.28
Doxorubicin	2.81±0.37*	1.49±0.27**

* p<0.05 NT+DOX vs. all other groups; ** p<0.05 T+DOX vs. NT+P; # T+P vs. T+DOX.

Semiquantitative analysis of the relative amount of abnormal mitochondria indicated that mitochondria from T+P group had a low frequency of mild alterations in mitochondrial morphology compared with NT+P, while most all mitochondria were markedly abnormal in hearts from NT+DOX. Most importantly, mitochondria from T+DOX hearts were distinctly protected from DOX-induced cardiac damage (Table 6).

Table 6. Semiquantitative analysis of mitochondrial damage in mouse hearts from all experimental groups.

Treatment	Percent abnormal mitochondria	
	Non-trained	Trained
Saline	0.0%	29.00% * 2.90% #
Doxorubicin	88.3% #	24.46%* 10.08% #

Note: Heart tissues of non-trained and trained mice with and without 20mg/kg of DOX treatment were examined by electron microscopy. About one hundred of random cells from each group were analysed for mitochondria morphology and the total number of normal and abnormal mitochondria were counted in each cell. Mitochondria were classified as abnormal as they exhibited extensive loss of cristea, intramitochondrial vacuoles and mitochondrial swelling.

The results are presented as percentage of abnormal mitochondria.

* Mitochondria were considered as abnormal only if presented mild focal loss of cristea density.

Mitochondria evidencing extensive degeneration or even loss of cristea, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysossomes and mitochondria swelling.

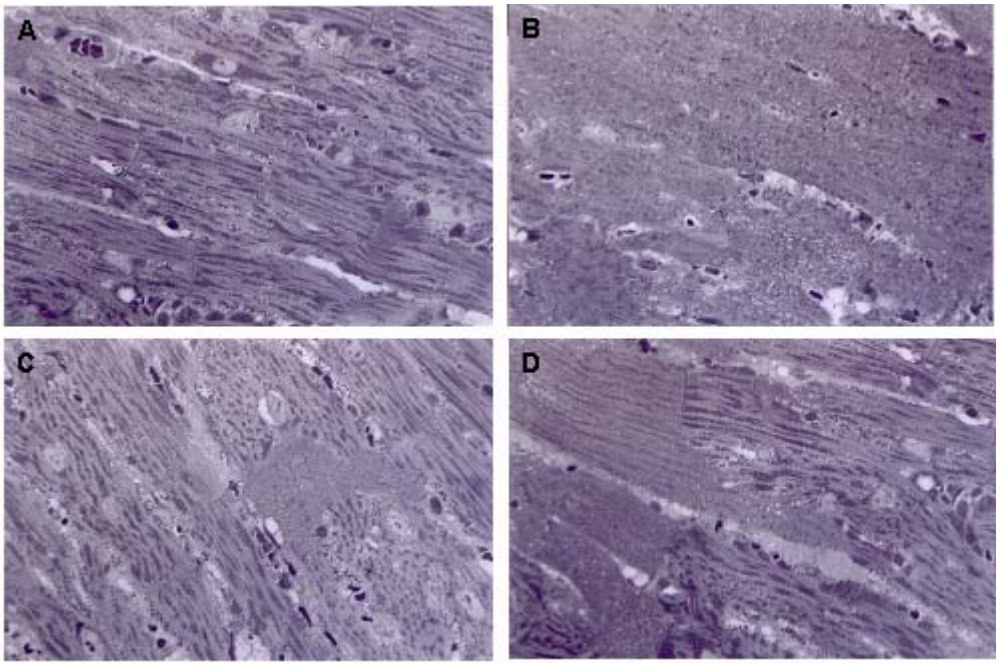

Figure 2. Representative photographs of the main histological features of cardiac tissue seen under light microscopy of the different groups. (A) NT+P group; (B) NT+DOX group; (C) T+P group; (D) T+DOX group (original magnifications: X870); In contrast with the normal appearance of NT+P note the area of sarcoplasmatic hyalinization and the general vacuolar degeneration affecting the majority of cells in NT+DOX. However, T+DOX group showed a slight degree of myocardial muscle fibers with vacuolization and slight interstitial edema. Cardiac muscle fibers from T+P hearts evidenced rare of vacuolization in a small number of cells.

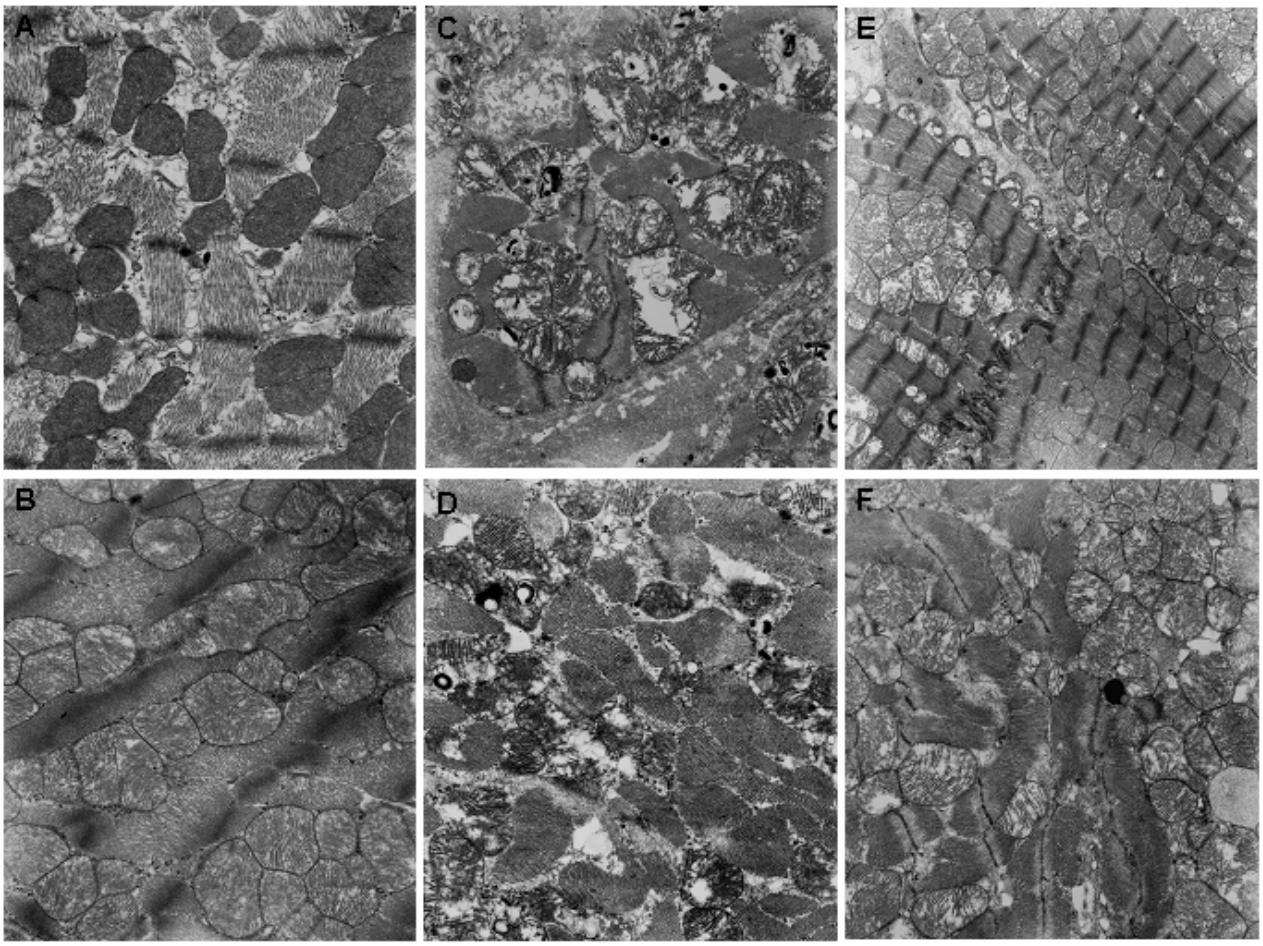

Figure 3. Representative electron micrographs of cardiac tissue from all the groups. (A) NT+P group (magnification: x16,000); (B) T+P group (magnification: x10,000); (C and D) NT+DOX group (magnifications: x10,000 and x12,500, respectively); (E and F) T+DOX group (magnifications: x6,500 and x10,000, respectively); Notice the cytoplasmatic vacuolation, myofibrillar disorganization, and the severe mitochondria damage with extensive degeneration or even loss of cristea, intramitochondrial vacuoles and notorious myelin figures in the NT+DOX group (C and D) that were partially attenuated in T+DOX group (E and F).

DISCUSSION

The present study provides biochemical and histological evidence that endurance training attenuates DOX acute cardiotoxicity. It was demonstrated that the deleterious and severe ultrastructural changes to the cardiac morphology induced by DOX treatment, particularly those affecting mitochondria, were largely attenuated by previous endurance swimming training.

An alternative approach for the detection of cardiac injury involves measurements of plasma concentrations of cardio-specific proteins that are released from damage myocytes. The plasma content of the highly sensitive cTnI, one of the components of the troponin complex of the muscle cells, has been widely recommended as a clinical parameter for the diagnosis of cardiac disease in various conditions (Bertinchant et al. 2000; Fredericks et al. 2001; Shave et al. 2002),

including DOX-induced cardiac damage (Bertinchant et al. 2003). The data from the present study showed that DOX administration induced a lower rise in cardiac cTnI release to the plasma in the T+DOX group compared to NT+DOX. Given that cTnI was used as a systemic marker of myocardial damage and considering that plasma content of this protein correlates with loss of cardiac cell membrane integrity, these results suggest that cardiomyocytes from T+DOX animals suffered from less extent of membrane disturbances caused by DOX treatment compared with their sedentary NT+DOX counterparts.

Under light microscopy, the obtained morphological lesions in NT+DOX hearts demonstrated that the cardiac myocytes exhibited an evident interstitial edema, suggestive of an inflammatory reaction. This is in agreement with other reports in which histopathological findings were consistent with increased mieloperoxidase (MPO) activation (Yagmurca et al. 2003; Fadillioglu et al. 2004) as well as with the attenuation of the cardiotoxic effects of DOX in mice treated with the anti-inflammatory agent ibuprofen (Inchiosa and Smith 1990). In fact, despite the importance of mitochondrial electron transport chain (Wallace 2003), other source of ROS that may contribute to cardiac injury include neutrophils activation (Arnhold et al. 2001), which migrate to the tissue during tissue injury and have a role in oxidative damage mechanisms through the action of either NADPH oxidase or MPO systems. In fact, stimulated neutrophils can increase production of large amounts of hypochlorous acid and superoxide radicals oxidizing other molecules, including proteins, lipids and nucleic acids, contributing to cause secondary damage by degrading the surrounding tissue and thus aggravating the injury (Halliwell and Gutteridge 1999). Nevertheless, the thin sections of hearts harvested from trained mice treated with DOX exhibited less extensive interstitial edema and cell vacuolization, with fewer, smaller and more sparsely distributed vacuoles when compared to their sedentary counterparts. Despite the indirect signs of inflammatory reaction induced by DOX administration, no evidence of infiltrative leukocytes were found in the analyzed sections of NT+DOX group.

The examination of ultrathin sections revealed that extensive sarcoplasmic vacuolization, mainly resulting from mitochondrial swelling/degeneration and sarcoplasmic reticulum distension, accompanied by other ultrastructural alterations, including myofilament disarray and fine-structure disruption predominates in the DOX-treated myocardium. However, all of these changes were dramatically attenuated in the hearts extracted from trained mice also treated with DOX, presenting less extensive swollen cardiac mitochondria and a lower intracellular edema evidenced by a less sarcoplasmic reticulum distension. Actually, the reduction of damage by endurance swimming training occurred at general cell level as demonstrated by the lower damage scores of T+DOX hearts compared with NT+DOX (Tables 4 and 5). This protective effect was accompanied by the diminished percentage of abnormal mitochondria exhibiting extensive degeneration or even loss of cristea, intramitochondrial vacuoles and notorious myelin figures and mitochondria swelling in T+DOX in opposition to NT+DOX group (Table 6).

The morphological data from the present study are in accordance with previous biochemical findings from our lab, comprising cardiac oxidative stress

and damage markers in DOX-treated mice (Ascensao et al. 2005b), evidencing cardiac protection induced by endurance training. Considering that DOX-induced cardiac toxicity has a marked oxidative etiology (Wallace 2003) and that chronic exercise ameliorates the cardiac capacity of antioxidant systems to counteract with deleterious ROS effects (Ji 2000), it can be suggested that the protection induced by exercise training against DOX could be mediated, at least in some extent to improvements in the cardiac antioxidant systems. Our results also support the concept that mitochondriopathy could be the primary event in DOX-induced cardiotoxicity (Wallace and Starkov 2000; Wallace 2003). It is known that DOX generates free radicals in cardiomyocytes by mitochondrial *redox* cycling between a semiquinone form and a quinone form (Davies and Doroshow 1986; Doroshow and Davies 1986). In fact, mitochondria have been identified as one of the targets of DOX-induced subcellular damage in the heart (Yen et al. 1996; Zhou et al. 2001; Oliveira et al. 2004). However, the ultrastructural semi quantitative evidence of training-induced mitochondrial protection in DOX-treated hearts has not been documented. As can be suspected from qualitative analysis of electron micrographs (see representative Fig 3) and from the analysis of table 6, the percentage of damaged mitochondria parallels the degree of other subcellular changes. Most relevant, the protection observed in mitochondria from trained hearts against DOX-induced cardiomyocyte damage was also evident regarding other ultrastructural alterations such as vacuolization and miofibrilar disarray largely observed in DOX sedentary hearts. In accordance, data from our group (unpublished) showed that heart mitochondria isolated from endurance trained rats had a higher respiratory function, a reduced susceptibility to calcium-induced uncoupled respiration as well as diminished signs of oxidative damage and apoptosis than their sedentary counterparts treated with DOX. Although biochemical and histological signs of DOX-induced apoptosis have been documented elsewhere (Arola et al. 2000; Childs et al. 2002; Liu et al. 2002), no evidence of apoptotic nucleus with peripheral condensed chromatin was observed in the present study.

Despite clear evidence of endurance training-induced cardioprotection against DOX, it was observed that training *per se* (NT+P vs. T+P) caused mild and focal degenerative alterations in mitochondrial structure that probably resulted in lysossome system activation, which could explain the appearance of secondary lysossomes surrounding mitochondria in T+P group. Probably due to the daily exercise stimuli-induced mild oxidative stress and damage, these changes may be responsible for enhanced mitochondrial turnover in endurance-trained hearts (Hood et al. 1994).

In summary, the data from the present study provides evidence that endurance swimming training improves myocardial tolerance to *in vivo* DOX-induced morphologic signs of damage. It is possible that these improvements can be related, at least partially, to training-induced enhanced mitochondrial protection against DOX side effects.

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Experimental Work

PAPER III

Moderate endurance training prevents doxorubicin-induced *in vivo* mitochondriopathy and reduces the development of cardiac apoptosis

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ABSTRACT

Endurance training is known to protect against myocardial dysfunction. Doxorubicin (DOX) is a potent anticancer drug that causes severe cardiomyopathy, probably mediated by the generation of highly reactive oxygen species derived from the mitochondrial electron transport chain. The objective of this work was to test the hypothesis that endurance training may be protective against *in vivo* DOX-induced cardiomyopathy through mitochondria-mediated mechanisms. Forty adult (6-8 weeks) Wistar male rats were randomly divided into the following four groups (n=10/group): not-trained, not-trained DOX ($20mg.kg^{-1}$), trained (14-wk endurance treadmill running, 60-90min/day) and trained DOX. Mitochondrial respiration, calcium tolerance, oxidative damage, heat shock proteins (HSP) and apoptosis markers were evaluated. DOX induces mitochondrial respiratory dysfunction, oxidative damage, histopathological lesions and trigger apoptosis ($P<0.05$, n=10). However, training limited the decrease in state 3, respiratory control ratio (RCR), uncoupled respiration, aconitase activity and protein SH content caused by DOX treatment as well as prevented the increased sensitivity to calcium observed in not-trained DOX-treated rats ($P<0.05$, n=10). Moreover, training inhibited the increase in mitochondrial protein carbonyl groups, malondialdehyde, Bax, Bax/Bcl2 ratio and tissue caspase 3 activity induced by DOX ($P<0.05$, n=10). Training *per se* also increased by approximately 2 fold the expression of mitochondrial HSP60 and tissue HSP70 ($P<0.05$, n=10).

We conclude that endurance training protects heart mitochondrial respiratory function from the toxic effects of DOX, probably by the improvement of mitochondria and cell defense systems and by a reduction in cell oxidative stress. In addition, endurance training limited the triggering of apoptosis induced by DOX treatment.

Key Words: HEART MITOCHONDRIA; EXERCISE TRAINING; DOXORUBICIN; RESPIRATION; OXIDATIVE PHOSPHORYLATION

INTRODUCTION

Alterations in heart physiology have been related to mitochondrial dysfunctions, including depressed mitochondrial respiration which is frequently associated with several cardiomyopathies particularly those linked with increased oxidative stress (Ferrari 1996; Wallace 2003). In fact, the heart is particularly sensitive to oxidative damage because it has relatively low levels of some antioxidant enzymes, it contains a large density/volume of mitochondria and it has an elevated rate of oxygen consumption (Halliwell and Gutteridge 1999). Moreover, increasing evidence indicate that under certain conditions, cardiac mitochondria are both major production sites and primary targets for reactive oxygen species (ROS) through the so-called electron leakage from the electron transport complexes (Kowaltowski and Vercesi 1999). For instance, an acute and severe bout of physical exercise increases oxygen consumption around six-fold, also enhancing mitochondria oxygen flux with subsequent additional ROS production (for refs. see Ascensao et al. 2003). Nevertheless, moderate and systematic exercise can generally constitute an excellent tool to provide enhanced parallel resistance to the cardiac muscle (Atalay and Sen 1999; Ascensao et al. 2003). It is possible that mitochondrial structural and biochemical adaptations induced by training, such as increased volume density, heat shock protein (HSP) expression and the up-regulation of antioxidant enzymes activity, could be related to training-induced enhanced cardiac resistance (Frenzel et al. 1988; Ji 2002; Powers et al. 2002). This phenomenon, usually referred to as cross-tolerance, has been demonstrated by several studies in which endurance training induced cardioprotection after acute stress stimuli (Demirel et al. 1998; Powers et al. 1998).

Most of the training-related cross-tolerance cardiac studies used ischemia-reperfusion (I-R) as a model to test cardiac susceptibility to oxidative damage and dysfunction. However, in addition to I-R, other stimuli associated with distinct known mechanism of cellular injury, such as the *in vivo* treatment with the antitumor antibiotic doxorubicin (DOX, or adriamycin), should also be considered in order to analyze the beneficial effects of training cross-tolerance. Actually, DOX induces a dose-related and potentially lethal cardiotoxicity that may be due, at least partially, to free radical production (Davies and Doroshow 1986; Doroshow and Davies 1986). Moreover, there is considerable evidence demonstrating that mitochondria are principal targets in the development of DOX-induced cardiomyopathy (Zhou et al. 2001) and that the onset and the severity of DOX toxicity correlates with disturbances in heart mitochondrial function and bioenergetics (Wallace 2003). In accordance, ultrastrutural data from rats exposed to acute and cumulative doses of DOX revealed important mitochondria morphological changes, namely significant swelling and loss of cristae, simultaneous with abnormal mitochondrial respiration and calcium loading capacity (Santos et al. 2002; Oliveira et al. 2004).

Recent data from our lab using heart tissue homogenate clearly demonstrated that endurance training induced cross-tolerance against DOX cardiotoxicity (Ascensao et al. 2005). However, studies dealing with the protective effect of endurance training on altered mitochondrial function of DOX-treated animals are missing. Taking into account the above-mentioned reasons, the main purpose and the novelty of this study was

the assessment of the effect of endurance training on DOX-induced mitochondriopathy (Yen et al. 1999; Santos et al. 2002), relating these findings with mitochondrial function and markers of oxidative stress. Given that gene transfection-mediated overexpression of cardiac HSPs have been extensively reported to result in enhanced myocardial mitochondrial tolerance (Suzuki et al. 2002), we also investigated if endurance training-induced increase in the levels of HSP could be related with protection of mitochondrial function. Furthermore, because it is known that mitochondrial dysfunction has an important role on DOX-induced apoptosis (Childs et al. 2002), another purpose of the present study was to analyze the effect of training on DOX-induced apoptosis.

METHODS

Sample

Forty Wistar male rats (aged 6-8 wks, weighting 200g at the beginning of the experiments) were used. During the experimental protocol, the animals were housed in collective cages (2 rats *per* cage) and were maintained in a room at normal atmosphere (21-22º C; ~ 50-60% humidity) receiving food and water *ad libitum* in a 12 hours light/dark cycles. The animals were randomly divided in two groups: Trained and not trained. Only male animals were used because of female estrogen protective effect on cardiac tissue reported elsewhere (Camper-Kirby et al. 2001). The Ethics Committee of the Scientific Board of Faculty of Sport Sciences approved the experimental protocol, which followed the *Guidelines for Care and Use of Laboratory Animals* in research.

Endurance training protocol

The trained animals were exercised 5 days/wk (Monday to Friday) for 14 wk on a motor driven treadmill. Both the treadmill speed and grade were gradually increased over the course of the 14-wk training period (Table 1), including 5 days of habituation to the treadmill with 10 min of running at 30m/min and 0% grade, with daily increases of 10 min until 50 min was achieved. Habituation was followed by one consecutive wk of continuous running (60 min/day) at 30m/min and 0% grade. This protocol proved to be efficient in rising cardiac dimensions (hypertrophy) and in antioxidant biochemical alterations protecting cardiac tissue during *in vivo* I/R (Powers et al. 1998). The not trained animals were not exercised but were placed on a nonmoving treadmill three times *per* wk (10-30 min/session) with the purpose of homogenizing the possible environment stress induced by treadmill without promoting any physical training adaptations.

Table 1 - Exercise training protocol

	Weeks of training													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Exercise duration (min/day)	30	60	90	90	90	90	90	90	90	90	90	90	90	90
Treadmill speed (m/min)	25	25	30	30	30	30	30	30	30	30	30	30	30	30
% Grade (treadmill inclination)	0	0	0	3	6	6	6	6	6	6	6	6	6	6

Note: Exercise-trained animals were exercised 5 days/wk on a motorized treadmill for 14wk.

DOX treatment

Twenty-four hours after the end of the endurance-training program, the animals from not trained and trained groups were again randomly separated into 2 sub groups, according to DOX or placebo treatment. Thus, not trained animals were distributed in non-trained plus placebo (NT+P, n=10) and non-trained plus DOX (NT+DOX, n=10); trained animals were also distributed in trained plus placebo (T+P, n=10) and trained plus DOX (T+DOX, n=10). The placebo groups were injected i.p. with a saline solution (0.9% NaCl) and DOX groups were injected i.p. with a single dose of DOX (20mg/Kg) in solution according to previous studies (Mohamed et al. 2000; Childs et al. 2002). Both treatments were carried 24 hours before the sacrifice.

Animals sacrifice and plasma, heart and soleus extractions

Animals were anaesthetized with diethyl ether and placed in supine position. After that, opening the abdominal cavity exposed the inferior cava vein and a blood sample of approximately 2 ml was collected in a heparinized tube. The blood was immediately centrifuged (5 min at 5000x*g*, 4ºC) and an aliquot of plasma was obtained and stored at –80ºC for biochemical determination of cardiac troponin T (cTnT). After a quick opening of the chest cavity, rat hearts were then rapidly excised, rinsed, carefully dried and weighted. A portion of approximately 20-25 mg of cardiac ventricle and one *soleus* muscle were separated, homogenized in homogenization buffer (0.05M Tris, 0.03M L-serine, 0.06M boric acid, tissue:buffer ratio of 100mg/mL, pH 7.6) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4ºC (3-5 times for 5 sec at speed low setting, with a final burst at a higher speed setting). Homogenates were centrifuged (2 min at 2000x*g*, 4ºC, in order to eliminate cellular debris) and the resulting supernatant was stored at –80ªC for later determination of HSP70 expression and caspase 3 activity (portion of cardiac ventricle) and citrate synthase activity (*soleus*). Protein content from cardiac muscle homogenate and from *soleus* was assayed using bovine serum albumin as standard according to Lowry et al. (1951). Additional small sample pieces from left ventricle papillary muscles were taken for morphological qualitative evaluation.

Isolation of rat heart mitochondria

Rat heart mitochondria were prepared using conventional methods of differential centrifugation as follows (Bhattacharya et al. 1991). Briefly, the animals were sacrificed as above stated and the hearts were immediately excised and finely minced in an ice-cold isolation medium containing 250mM sucrose, 0.5mM EGTA, 10mM Hepes-KOH (pH 7.4) and 0.1% defatted BSA (Sigma nº A-7030). The minced blood free tissue was then resuspended in 40mL of isolation medium containing 1mg protease subtilopeptidase A Type III (Sigma P-5380) *per* g of tissue and homogenized with a tightly fitted homogenizer (Teflon: glass pestle). The suspension was incubated for 1 min (4ºC) and then re-homogenized. The homogenate was then centrifuged at 14,500x*g* for 10 min. The supernatant fluid was decanted and the pellet, essentially devoided of protease, was gently re-suspended in its original volume (40mL) with a loose-fitting homogenizer. The suspension was centrifuged at 750x*g* for 10 min. and the resulting supernatant was centrifuged at 12,000x*g* for 10 min. The pellet was re-suspended using a paintbrush and re-pellet at 12,000x*g* for 10 min.

EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein content was determined by the Biuret method calibrated with BSA. All isolation procedures were performed at 0-4ºC.

An aliquot of mitochondria suspension was taken after isolation for immediate measuring of the enzyme activity of aconitase. Additional mitochondria aliquots were also separated and frozen at -80ºC for later determination of protein carbonyls, sulfhydryl groups (-SH), malondialdehyde (MDA) and HSP60, and pro- and anti-apoptotic Bax and Bcl2 proteins, respectively.

The remaining mitochondrial suspensions were used within 4 hours for respiratory assays and were maintained on ice (0-4ºC) throughout this period. Isolation procedures yielded well-coupled mitochondria: the respiratory control ratio (RCR) of isolated mitochondria varied from 7-10 (with glutamate-malate) or 3-4 (with succinate plus rotenone) for controls, as determined according to the method of Estabrook (1967).

Mitochondrial oxygen consumption assays

Mitochondrial respiratory function was measured polarographically, at 25ºC, using a Biological Oxygen Monitor System (Hansatech Instruments) and a Clark-type oxygen electrode (Hansatech DW 1, Norfolk, UK). Reactions were conducted in 0.75 ml closed thermostatted and magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein in a respiration buffer containing 65mM KCl, 125mM sucrose, 10mM Tris, 20μM EGTA, 2.5mM KH_2PO_4, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate and malate to a final concentration of 10 and 5 mM each, respectively, or succinate (10mM) plus rotenone (4μM). State 3 respiration was determined after adding 444 μM ADP; state 4 was measured as the rate of oxygen consumption in the absence of ADP. The RCR (state3/state 4) and the ADP/O ratios, the number of nmol ADP phosphorylated by nmol O_2 consumed, were calculated according to Estabrook (1967), using 474 ngatom O/ml as the value for oxygen solubility at 25ºC in doubly distilled water. State 4 respiration was also measured in the presence of glutamate (10mM) and malate (5mM), where ADP (1mM), oligomycin (1.5μg), carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (2μM) were added to induce, respectively, state 3 respiration, inhibition of state 3 respiration through inhibition of the ATP synthase and uncoupled respiration. In an independent trial, succinate energized mitochondria were subjected to 300μM of calcium chloride ($CaCl_2$) to analyze mitochondrial calcium loading capacity. The ratio between respiratory rates obtained before and one min after the addition of $CaCl_2$ was determined.

Plasma cardiac troponin I (cTnI) and skeletal muscle citrate sinthase (CS)

cTnI concentration was quantitatively determined with an established immunoassay using commercial Abbott kit. *Soleus* CS activities were measured using the method proposed by Coore et al. (1971). The principle of assay was to initiate the reaction of acetil-CoA with oxaloacetate and link the release of CoA-SH to 5,5-dithiobis (2-nitrobenzoate) at 412nm.

Aconitase activity

Immediately before measuring aconitase activity, mitochondrial fractions were resuspended in 0.5mL

buffer containing 50mM Tris-HCl (pH 7.4) containing 0.6mM $MnCl_2$ and sonicated for 2 sec. Aconitase activity was immediately measured spectrophotometrically by monitoring the formation of cis-aconitate from isocitrate at 240nm in 50mM Tris-HCl (pH 7.4), containing 0.6mM $MnCl_2$ and 20mM isocitrate at 25ºC according to Krebs and Holzach (1952). One unit was defined as the amount of enzyme necessary to produce 1μmol cis-aconitate *per* min (ε_{240}= 3.6mM^{-1}).

Sulfhydryl protein groups

The mitochondrial content of oxidative modified sulfhydryl protein groups was quantified by spectrophotometric measurement according to the method proposed by Hu (1990). Briefly, the colorimetric assay was performed after the reaction of 50μl aliquot of mitochondrial extract with 10μl of 5,5'-dithio-bis(2-nitrobenzoic acid) (10mM) in a medium containing 150μl of Tris (0,25M) and 790μl methanol, at 414nm against a blank test. SH content was expressed in nmol/g of mitochondrial protein (ε_{414} = 13,6 mM^{-1}cm^{-1}).

Malondialdehyde

Lipid peroxidation was measured by determining the levels of lipid peroxides as the amount of thiobarbituric acid reactive substances (TBARS) formed according to Rohn et al. (1993) with some modifications. Mitochondrial protein (0.5 mg) was incubated, at 25°C, in 500 μl of a medium consisting of 175 mM KCl, 10 mM Tris, pH 7.4. Samples of 50 μl were taken and mixed with 450 μl of a TBARS reagent (1% thiobarbituric acid, 0.6 N HCl, 0.0056% butylated hydroxytoluene). The mixture was heated at 80-90°C during 15 min, and recooled in ice for 10 min before centrifugation in Eppendorf centrifuge (1500*g*, 5 min). Lipid peroxidation was estimated by the appearance of TBARS spectrophotometrically quantified at 535 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nanomoles of MDA *per* milligram of protein (Buege and Aust 1978).

Analysis of protein carbonylation, HSP 60, HSP70, Bcl-2 and Bax

Equivalent amounts of proteins were electrophoresed on a 15% SDS-PAGE gel as described by Laemmli (1970), followed by blotting on a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech) according to Locke et al. (1990). After blotting, non-specific binding was blocked with 5% non fat dry milk in TTBS (Tris-buffered saline (TBS) with Tween 20) and the membrane was incubated with either anti-Bcl-2 (1:500; sc-7382 mouse monoclonal IgG; Santa Cruz Biotechnology) or anti-Bax (1:500; sc-493 rabbit polyclonal IgG; Santa Cruz Biotechnology) or anti-HSP60 (1:2000; 386028 mouse monoclonal IgG; Calbiochem) or anti-HSP70 (1:2000 dilution of monoclonal Clone BRM-22, Sigma) antibodies for 2 hours at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:1000; Amersham Pharmacia Biotech) for 2 hours.

Protein carbonyl derivatives were assayed according to Robinson et al., (1999) with some modifications. Briefly, a certain cardiac mitochondria volume (V) containing 20μg of protein was derivatized with dinitrophenylhydrazine (DNPH). The sample was then mixed with 1 V of 12% SDS plus 2 V of 20mM DNPH 10% TFA,

followed by a 30 min of dark incubation, after which 1,5 V of 2M Tris / 18.3% of β-mercaptoethanol were added. A negative control was simultaneously prepared for each sample. After diluting the derivatized proteins in TBS to obtain a final concentration of 0.001μg/μL, a 100μl volume was slot-blotted into a Hybond-PVDF membrane. Immunodetection of carbonyls was then performed using rabbit anti-DNP (DAKO) as the first antibody (1:2000 dilution), and anti-rabbit IgG-Peroxidase (Amersham Pharmacia) as the second antibody (1:2000 dilution).

For the referred methods, the bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (BioRad). Optical density results were expressed as percentage variation of control values.

Caspase-3 activation levels

100 μg of protein in muscle homogenate was used for analysis of caspase-3. After adding reaction buffer (10% sucrose; 0,1% CHAPS; 25 mM HEPES pH 7,4) and 40 μM DEVD-pNA (acetyl-Asp-Glu-Val-Asp-p-nitroaniline; catalogue number 235400; Calbiochem) substrate for caspase-3, the samples were incubated at 37ºC for 2 hours as previously described (Dirks and Leeuwenburgh 2002) with some adaptations. $A_{405\ nm}$ was read in a plate reader (Labsystem iEMS Reader MF). Percentage of increase in caspase activity was determined by comparing these results with the $A_{405\ nm}$ simultaneously incubated control sample.

Electron microscopy

Small sample pieces from left ventricle papillary muscles were cuted and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer during two hours. The specimens were post-fixed with 2% osmiumtetroxide, dehydrated in graded alcohol, and embedded in Epon. Ultrathin sections for transmission electron microscopy (TEM, Zeiss EM10A, Germany) were contrasted with 0.2% lead citrate and 0.5% uranylacetate for a qualitative ultrastructural analysis. The heart tissue was also semiquantitative examined for histopathological evidence of cardiomyopathy, according to severity scores from 0-3, as previously described (Santos et al. 2002; Oliveira et al. 2004). Severity of damage was scored using electron microscopy grids: *grade 0*, no change from normal; *grade 1*, limited number of isolated cells (less than 5% of the total number of cells per block) exhibiting early myofibrillar loss and/or cytoplasmatic vacuolization; *grade 2*, groups of cells (5 to 30% of the total number) exhibiting early myofibrillar loss and/or cytoplasmatic vacuolization; and *grade 3*, diffuse cell damage (>30% of total number) with the majority of cells exhibiting marked loss of contractile elements, loss of organelles, and mitochondria and nuclear degeneration. All slides were scored independently by an examiner who was blinded to each tissue sample code.

Statistical analysis

Mean and mean standard errors were calculated for all variables in each of the experimental groups. One-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. A *t*-test for independent measures was used to test the HSP70 and HSP60 differences between NT+P and T+P groups. Statistical Package for the Social Sciences

(SPSS Inc, version 10.0) was used for all analysis. The significance level was set at 5%.

RESULTS

Body weights, absolute and relative heart weights and *soleus* muscle citrate synthase activity are shown in Table 2. In accordance with the well-described body mass and cardiac adaptations induced by endurance training (Moore and Palmer 1999), the 14 wks of endurance running training decreased mice weight, and induced an increase in skeletal muscle citrate synthase activity, in heart weight and heart/body ratio ($p<0.05$). The improved enzymatic activity in *soleus* muscle reflects that endurance training was an efficient chronic stimulus to ameliorate muscle oxidative metabolism.

Table 2. Effects of endurance running training and DOX treatment on rat body and heart weights

Groups	Body weight (g)	Heart weight (g)	Heart/body ratio ($x10^{-4}$)	Skeletal muscle CS ($\mu mol.mg^{-1}.min^{-1}$)
NT+P	514.66 ± 16.3	0.96 ± 0.021	18.8 ± 0.44	0.017± 0.001
NT+DOX	496.0 ± 17.0	0.94 ± 0.046	18.9 ± 0.30	0.021 ± 0.004
T+P	425.0 ± 6.45 *	1.29 ± 0.054 *	30.4 ± 0.10 *	0.056 ± 0.004 *
T+DOX	417.75 ± 6.23 *	1.33 ± 0.037 *	31.9 ± 0.55 *	0.051 ± 0.002 *

Note: Data represent the means ± SEM for body and cardiac weights as well as *soleus* citrate synthase activity from ten individual rats corresponding to the four experimental groups, obtained from 10 independent experiments. * $p<0.05$ T+P and T+DOX compared to NT+P and NT+DOX.

To elucidate the effects of endurance training on heart mitochondria respiration of DOX treated rats, we studied respiratory parameters using both NADH and $FADH_2$-linked substrate oxidation through mitochondrial complex I and complex II, respectively. As it can be observed in Table 3, DOX treatment induced significant decrease in state 3 respiration (59%) as well as in the RCR (44%) of glutamate/malate energized heart mitochondria isolated from non-trained animals. However, no changes were noted concerning state 4 respiration and ADP/O when comparing the same groups (NT+P vs. NT+DOX). DOX-induced inhibition of respiration was completely prevented by endurance running training (NT+DOX vs. T+DOX), which means that DOX did not affect mitochondrial respiration in trained animals. Moreover, endurance training by itself significantly increased the RCR value in non-treated animals (42%), mostly due to a small although non-statistically significant decrease of state 4 respiration in trained animals.

Table 3. Effects of endurance running training and DOX treatment on rat heart mitochondria respiration using glutamate (10mM) and malate (5mM) as respiratory substrates

Groups	State 3	State 4	RCR	ADP/O
NT+P	462.71 ± 21.56	63.01 ± 3.13	7.34 ± 0.25	2.49 ± 0.16
NT+DOX	291.01 ± 10.04 *	56.47 ± 7.18	5.10 ± 0.37 *	2.18 ± 0.18
T+P	458.01 ± 69.62	48.24 ± 4.32	10.42 ± 0.76 **	2.27 ± 0.12
T+DOX	490.85 ± 54.17	71.44 ± 12.06	7.76 ± 0.47	2.47 ± 0.14

Note: Data represent the means ± SEM for rat heart mitochondria (0.5 mg/mL) isolated from all the experimental groups and obtained from 10 independent experiments. Oxidative phosphorylation was measured polarographically at 25ºC in a total volume of 0.75mL. Respiration medium and other experimental details are provided in methods. State 3 and state 4 respiration rates are expressed in nmol O_2.mL^{-1}.mg $protein^{-1}$. RCR – respiratory control ratio; ADP/O - number of nmol ADP phosphorylated by nmol O_2 consumed. * $p<0.05$ NT+DOX compared to all other groups; ** $p<0.05$ T+P compared to all other groups.

When the $FADH_2$-linked substrate succinate was used, state 3 respiration (47%) and RCR (31%) were significantly depressed by DOX administration, whereas no changes were detected in the rate state 4 respiration and on the phosphorylation efficiency expressed as the ADP/O ratio (NT+P vs. NT+DOX). As with complex I-related substrates, succinate-energized heart mitochondria isolated from trained rats treated with DOX showed values similar to control in the referred respiratory parameters. Mitochondria from trained animals did not show any significant difference in state 4, RCR and in ADP/O when compared to non-trained groups (Table 4).

Table 4. Effects of endurance running training and DOX treatment on rat heart mitochondria respiration using succinate (10mM) as respiratory substrate plus rotenone (4μM) to inhibit electron transport chain complex I

Groups	State 3	State 4	RCR	ADP/O
NT+P	548.66 ± 47.64	133.18 ± 10.96	4.12 ± 0.12	1.34 ± 0.08
NT+DOX	372.03 ± 30.92 *	121.52 ± 11.28	3.14 ± 0.11 *	1.25 ± 0.12
T+P	572.32 ± 18.87	168.95 ± 10.97	3.98 ± 0.32	1.23 ± 0.09
T+DOX	606.08 ± 61.78	156.76 ± 29.52	4.09 ± 0.12	1.39 ±0.09

Note: Data represent the means ± SEM for rat heart mitochondria (0.5 mg/mL) isolated from all the experimental groups and obtained from 10 independent experiments. Oxidative phosphorylation was measured polarographically at 25ºC in a total volume of 0.75mL. Respiration medium and other experimental details are provided in methods. State 3 and state 4 respiration rates are expressed in nmol O_2.mL^{-1}.mg $protein^{-1}$. RCR – respiratory control ratio; ADP/O - number of nmol ADP phosphorylated by nmol O_2 consumed. * $p<0.05$ NT+DOX compared to all other groups.

As it can be depicted from Fig. 1, neither DOX nor training altered state oligomycin whereas oxygen consumption during uncoupling respiration (state CCCP) was significantly depressed (22%) by DOX treatment (NT+P vs. NP+DOX) reflecting an impairment in the rate of electron transfer. However, training significantly attenuated the decrease in uncoupled respiration caused by DOX treatment.

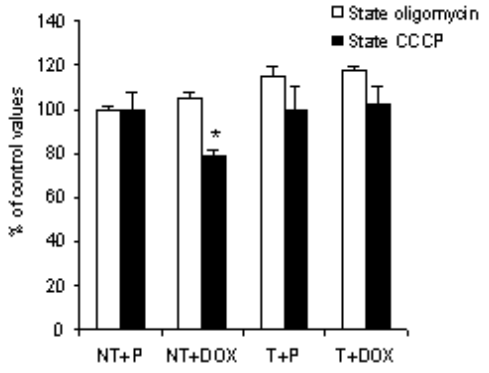

Figure 1. Effects of endurance running training and DOX treatment on olygomycin-inhibited state 3 respiration and CCCP-induced uncoupled respiration of rat heart mitochondria, obtained from 10 independent experiments. Respiration was induced with glutamate (10mM) - malate (5mM) as energizing substrates and saturated ADP concentration (1mM) to initiate state 3 respiration. State 3 was inhibited after the addition of oligomycin (1.5μg) and CCCP (2μM) was subsequently added to uncouple mitochondria respiration. Oxygen consumption by heart mitochondria was monitored polarographically with an oxygen electrode in a 0.75mL thermostated water-jacketed closed chamber with magnetic stirring, at 25ºC. Heart mitochondria (0.5mg/mL) were incubated in the standard reaction medium as described. Data represent means ± SEM and express the percentage of control values (NT+P). Open bars represent state oligomycin; closed bars represent state CCCP. * p<0.05 NT+DOX vs. all other groups.

We also analyzed the changes of mitochondria oxygen consumption after addition of calcium. Figure 2 shows that calcium-stimulated respiration largely failed to return to normal state 2 values in mitochondria isolated from DOX treated rats, and it demonstrated an accelerated and mostly uncoupled oxygen consumption (NT+P vs. NT+DOX), which was also reversed by cyclosporin A (1μM). In opposition, after calcium accumulation, heart mitochondria from trained animals were better able to recover from the increase in respiration rates, both with and without DOX treatment, which means that endurance training improved mitochondrial calcium control. These data can be confirmed by the increased (32% - NT+P vs. NT+DOX and 27% - NT+DOX vs. T+DOX) ratio between the respiratory rates of succinate-energized mitochondria after and before calcium addition. The ratio was significantly elevated in non-trained DOX treated rats compared to all other groups (Fig 2).

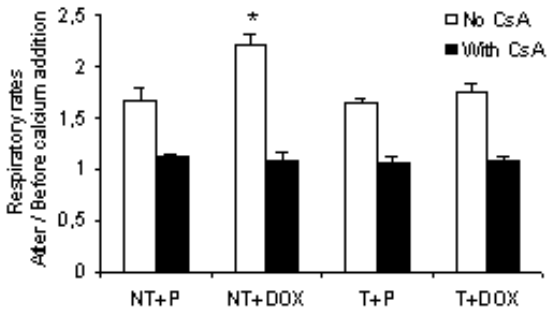

Figure 2. Effect of DOX and training on mitochondrial calcium tolerance, obtained from 10 independent experiments. Oxygen consumption by heart mitochondria was monitored polarographically with an oxygen electrode in a 0.75mL thermostated water-jacketed closed chamber with magnetic stirring, at 25ºC. Heart mitochondria (0.5mg/mL) were incubated in the standard reaction medium as described. A calcium pulse of 300μM was added to heart mitochondria energized with succinate (10mM) plus rotenone (4μM) to observe the effects of training and/or DOX in the recovery to state 4 respiration after calcium accumulation. As demonstrated by the ratios, DOX treated rats demonstrated an accelerated and mostly uncoupled oxygen consumption, which was limited by the incubation with 1μM cyclosporin A (CsA). Values are expressed as the ratio between respiratory rates assayed after and before the addition of calcium. * p<0.05 NT+DOX vs. all other groups.

As can be depicted from Table 5, DOX induced a significant increase in plasma levels of cTnI, which reflects enhanced cTnI release from heart and indicates loss of cardiomyocyte membrane integrity. However, endurance training resulted in a significant reversal ($p<0.05$) of DOX-induced cardiotoxicity (NT+DOX vs. T+DOX). We measured the activity levels of aconitase, a citric acid cycle enzyme containing Fe-S clusters, which are susceptible to ROS-induced damage and consequently to enzyme inactivation, as an indirect assessment of *in vivo* mitochondria superoxide radical production and associated damage (Melov et al. 1999).

As shown in Table 5, mitochondria aconitase activity from DOX group was significantly lower than those found in all other groups. The content of sulfhydryl groups measured in heart mitochondria isolated from DOX treated rats was also significantly lower than in heart mitochondria isolated from all other treatment groups (Table 5).

Concerning protein carbonyl groups and MDA from heart mitochondria extracts, a significant increase in the levels of both oxidative stress-related markers were found in the NT+DOX group (Fig. 3 and Table 5, respectively). However, mitochondria isolated from trained animals injected with DOX presented lower carbonyl and MDA levels when compared to their non-trained counterparts.

Table 5. Effects of endurance running training and DOX treatment on histological cell damage scores, plasma cTnI, mitochondria aconitase activity, MDA and –SH contents.

Groups	Plasma cTnI ($ng.ml^{-1}$)	Severity of cell damage	Aconitase ($U.mg\ protein^{-1}$)	MDA ($nmol.\ mg\ protein^{-1}$)	-SH ($mol.\ g\ protein^{-1}$)
NT+P	0.00±0.00	0.00±0.00	9.74 ± 0.78	1.899 ± 0.027	0.0124± 0.001
NT+DOX	1.53±0.11*	2.64±0.49*	4.88 ± 0.50*	2.576 ± 0.082*	0.0064 ± 0.0005*
T+P	0.00±0.00#	0.76±0.32	8.94 ± 0.83	1.754 ± 0.072	0.0124 ± 0.001
T+DOX	0.42±0.09**	1.52±0.38**	7.58 ± 0.24	1.758 ± 0.093	0.0121 ± 0.001

Note: Data represent the means ± SEM for all variables, obtained from 10 independent experiments. Cell damage scores, plasma cardiac troponin I (cTnI), malondialdehyde (MDA), sulfhydryl protein groups (SH); the severity of cell damage is expressed on a blinded score of 0-3. * $p<0.05$ NT+DOX vs. all other groups; ** $p<0.05$ T+DOX vs. NT+P; # T+P vs. T+DOX.

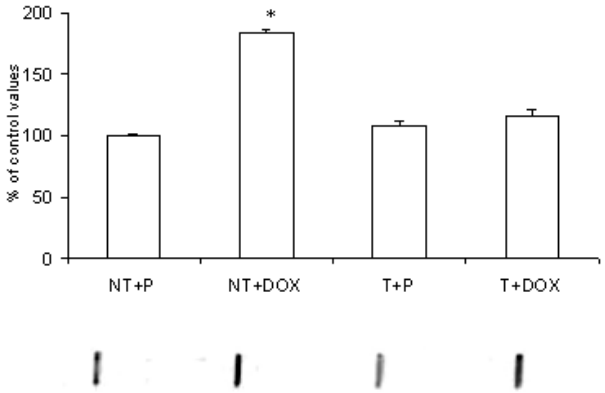

Figure 3. Effect of training and DOX treatment on the content of carbonyl derivatives of rat heart mitochondria, obtained from 10 independent experiments. Immediately below the histogram, the protein carbonyl formation panel shows a representative pattern of anti-denitrophenyl (DNP)-specific interaction with DNP for each group as described in METHODS. Values (mean and SEM) are expressed as percentage of control (NT+P). * p<0.05 NT+DOX vs. all other groups.

As illustrated in Fig. 4, endurance running training raised significantly the expression levels of both heart mitochondria HSP60 and whole cardiac muscle homogenate HSP70. In fact, when compared to trained controls, increases of 95% (HSP60) and 112% (HSP70) were found in trained hearts.

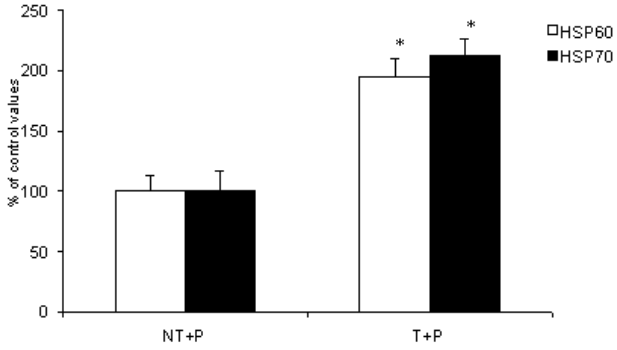

Figure 4. Effect of endurance running training on the expression of mitochondrial HSP60 (60 KDa) and tissue homogenate HSP70 (72 KDa), obtained from 10 independent experiments. Immediately below the histogram, the panel shows a representative western blotting of HSP60 and HSP70 for each group as described in METHODS. Values (mean and SEM) are expressed as percentage of control (NT+P). * p<0.05 T+P vs. NT+P.

We measured mitochondrial levels of Bax and Bcl2 to determine whether there were any DOX-induced changes in these pro- and anti- apoptotic and, if in presence of changes, to analyze the effect of training on those changes. As shown in Fig 5, DOX administration resulted in significant elevation of Bax and of Bax/Bcl2 ratio. However, training restored the increase in both Bax and Bax/Bcl2 ratio. No changes were observed in the expression of Bcl2 protein between groups.

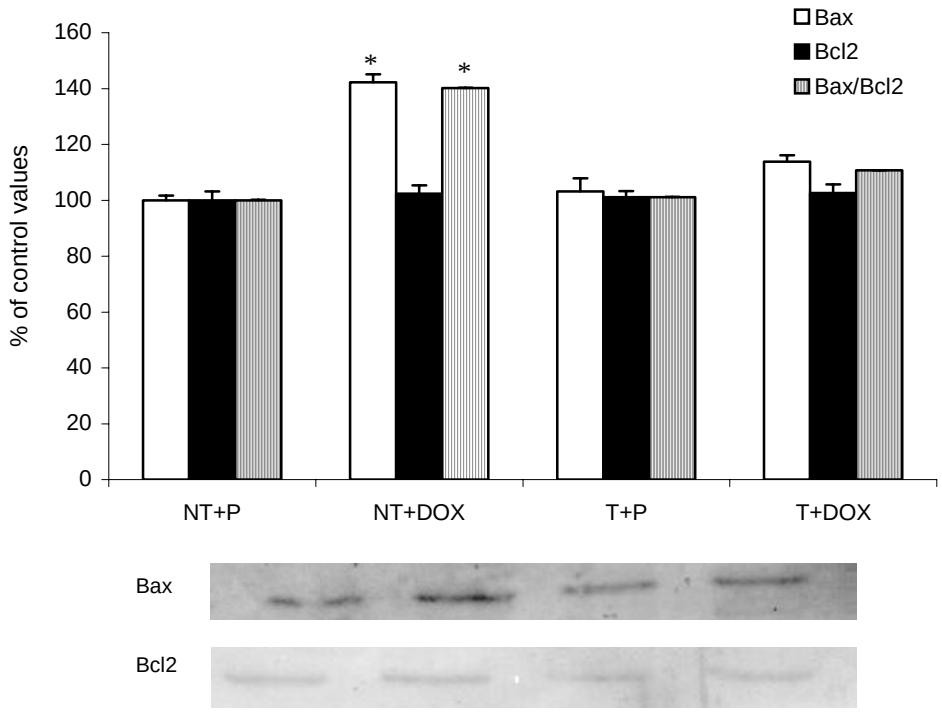

Figure 5. Effect of training and DOX treatment on the expression of Bax and Bcl2 proteins, as well as on Bax/Bcl2 ratio in rat heart mitochondria, obtained from 10 independent experiments. Immediately below the histogram, the panel shows a representative western blotting of Bax (21 KDa) and Bcl2 (25 KDa) for each group as described in METHODS. Values (mean and SEM) are expressed as percentage of control (NT+P). * $p<0.05$ NT+DOX vs. all other groups.

Fig. 6 shows that caspase-3 activity revealed a significant increase in cardiac muscle from non-trained DOX treated rats (NT+P vs. NT+DOX). However, training significantly attenuated the increase in caspase 3 activity induced by DOX (NT+DOX vs. T+DOX).

When qualitatively examined by electron microscopy, cardiac myocytes from DOX treated animals showed, without surprise, marked myocardial damage such as cytoplasmatic vacuolization, myofibrillar disorganization, mitochondria damage with extensive degeneration or even loss of cristae, intramitochondrial vacuoles and myelin figures, and mitochondria swelling, contrasting with the normal appearance of NT+P group (7A and 7B). Endurance training *per se* (NT+P vs. T+P) induced evident signs of mitochondria biogenesis with elevated number of encroached mitochondria. Mitochondria division, mild and focal loss of cristae density and loss of organization within mitochondria as well as scarce secondary lysosomes, were also present in non-treated trained hearts. Regarding hearts harvested from trained animals treated with DOX (T+DOX), although maintaining ultrastructural alterations described for T+P group, no major signs of DOX-induced cardiotoxicity were observed (Fig 7C and D). In accordance, the semiquantitative analysis of these histopathological changes confirmed that the severe ultrastructural abnormalities induced by DOX treatment in sedentary hearts were

significantly attenuated in trained DOX treated group (Table 5).

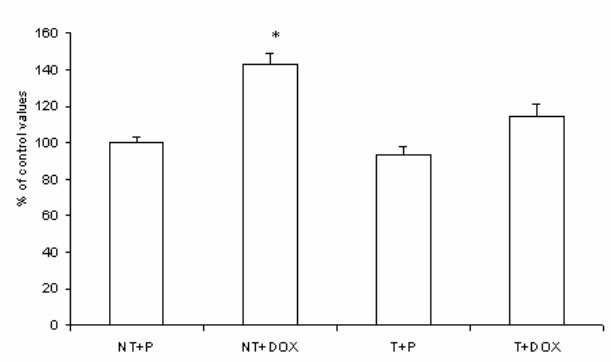

Figure 6. Effect of training and DOX treatment on the cardiac muscle homogenate caspase-3 activity, obtained from 10 independent experiments. Values (mean and SEM) are expressed as percentage of control (NT+P). * $p<0.05$ NT+DOX vs. all other groups.

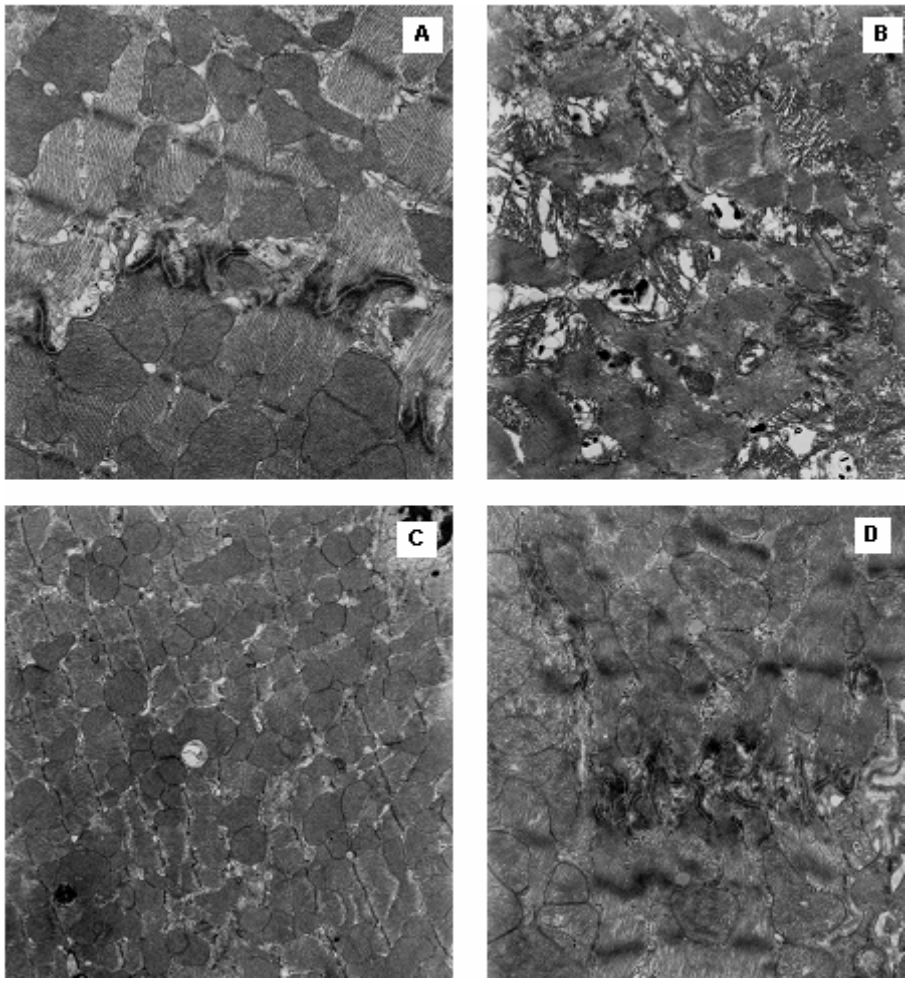

Figure 7. Representative electron micrograph of cardiac tissue from all the groups. (A) NT+P group (magnification: 12 500X); (B) NT+DOX group (magnification: 10 000X); (C) T+P group (magnification: 6 300 X); T+DOX group (magnification: 10 000X). Notice the cytoplasmatic vacuolation, myofibrillar disorganization, and the severe mitochondria damage with extensive degeneration or even loss of cristea, intramitochondrial vacuoles and notorious myelin figures in the NT+DOX group (B) that were partially attenuated in T+DOX group (D).

DISCUSSION

Overview of the principal findings

The results obtained in the present investigation provide new insights into the biochemical mechanisms by which endurance exercise training protects cardiac muscle tissue against the toxicity induced by DOX. Data confirmed that this drug induced mitochondrial superoxide production and oxidative damage, impaired mitochondrial respiratory function, and ultimately, triggered the mitochondrial pathway to apoptotic cell death. Moreover, in the presence of calcium, heart mitochondria from DOX treated animals have an increased sensitivity to the induction of permeability transition pore-mediated uncoupled respiration, which was limited in the T+DOX group. However, it was also noteworthy that 14-wk of endurance training limited the impact of DOX insult on heart mitochondrial superoxide production, oxidative damage, respiratory function and apoptotic cell fate. This protection was corroborated by the histological data as well as by the levels of the specific systemic marker of cardiac damage found in the different groups. Despite cardiovascular functional parameters were not determined in the present study, the elevated consistency between the variations in cTnI and those functional parameters reported in previous studies (Horton et al. 1995; Ikizler et al. 2002) might be indicative that the adaptations induced by endurance training translate into hemodynamic benefits in the T+DOX animals.

Evidence of training-induced protection on heart mitochondria respiration

Previous studies have shown that endurance training attenuates doxorubicin-induced cardiac damage, diminishing the increased biochemical and morphological signs of toxicity induced by *in vivo* DOX treatment (Kanter et al. 1985; Ascensao et al. 2005). However, no data is available concerning the cross-tolerance effect of moderate endurance training on DOX-induced mitochondria malfunction. Our present results demonstrate that 14-wk of moderate endurance running training prevented the inhibition of mitochondrial respiration caused by DOX. The depressed activity of mitochondrial enzyme components of complexes I and II caused by DOX (Yen et al. 1999; Santos et al. 2002) could partially justify the diminished electron transport through ETC in non-trained DOX-treated group. Thus, the unaltered state 3 observed in T+DOX suggests that, among other possible effects, training probably prevented the inactivation of complex I and II components in DOX-treated heart mitochondria.

The above-referred data concerning NADH-linked substrates were confirmed by mitochondrial oxygen consumption using oligomycin and CCCP (Fig. 1). Because both state 3 and state CCCP decreased after DOX insult and as endurance training restored these impairments, one can argue that exercise training prevented the damage of the respiratory chain enzymes and/or the upstream supply of electrons to the respiratory components that were limited after DOX treatment.

The Fe-S centers of aconitase can be reversely inactivated by superoxide ($O_2^{-\cdot}$) and related species, allowing the measurement of aconitase inactivity as an index of *in vivo* $O_2^{-\cdot}$ production in mitochondria (Melov et al. 1999). In the present study, enhanced mitochondrial $O_2^{-\cdot}$ generation in DOX group was indirectly demonstrated by significantly lower aconitase activity when compared to other groups (Table 5). This might lead to a decrease in the supply of reducing equivalents to the ETC, through the oxidative

damage inflicted to other citric acid cycle enzymes, namely -ketoglutarate dehydrogenase and succinate dehydrogenase (Nulton-Persson and Szweda 2001). Furthermore, given that mitochondria complexes include enzymes that consist of polypeptides that also comprise Fe-S clusters, namely those from complex I and II, they can also become prone to oxidative deactivation by $O_2^{-\cdot}$ or other ROS, resulting in accumulating products of protein oxidation such as carbonyl groups or oxidized thiol residues (Fig. 3). These alterations probably contributed to the decline of electron transfer through ETC, compromising state 3 respiratory rate and RCR (Lucas and Szweda 1999; Lucas et al. 2003). Considering that changes in markers of oxidative damage, measured by malondialdehyde, carbonyl and sulphydryl groups and aconitase activity, were in accordance with alterations in mitochondrial respiratory functionality of all groups, one could suggest that training counteract the changes in *redox* homeostasis induced by DOX. It is possible that training-induced up-regulation of mitochondria and cytosolic defenses might contribute to the referred mitochondrial tolerance against DOX effects. Indeed, data collected in studies using other models of cardiac and mitochondria dysfunction such as I-R, revealed that alterations in mitochondrial respiration are prevented in myocardium expressing high levels of HSPs (Sammut et al. 2001; Suzuki et al. 2002). In the present study, the finding that HSP60 and HSP70 were over-expressed in trained animals, together with the observed improvement of mitochondrial respiratory function strongly suggests that the up-regulation of these molecular chaperones possibly contributed to the preservation of mitochondria complexes' integrity and activity. This may be accomplished through facilitation of nuclear-encoded protein importation and assembly in the mitochondria matrix. Another possible linked explanation for the increased respiratory function of mitochondria isolated from trained rats compared to non-trained rats when exposed to DOX could be associated with the training-mediated effect in the up-regulation of mitochondrial antioxidant enzymes. As previously reported by others (Somani et al. 1995; Somani and Rybak 1996; Moran et al. 2004), endurance training improves the antioxidant capacity of mitochondria and thus, the ability to counteract DOX-induced free radical-mediated mitochondriopathy. Similar findings were obtained in rats overexpressing MnSOD and treated with DOX (Yen et al. 1999), in which impaired mitochondrial NADH- and $FADH_2$-linked state 3 and RCR were prevented. Moreover, given that the levels of mitochondrial HSP60 increased as well in trained groups, it is possible that the improved assisted folding of proteins within mitochondria also afforded some additional protection of mitochondria respiration of T+DOX group. Taken together, these findings suggest that protection of mitochondrial function exerted by training is important to counteract DOX cardiotoxicity.

Sensitivity of mitochondria to calcium-induced uncoupled respiration

In addition to interfering with mitochondrial respiration, there is growing evidence showing that DOX disrupts the ability of cardiac mitochondria to accumulate calcium, diminishing calcium-loading capacity (Zhou et al. 2001; Santos et al. 2002; Oliveira et al. 2004). Taking into account that mitochondria act as major sources of ROS and cytosolic calcium buffers, these organelles can become severely dysfunctional due to induction of

the permeability transition pore (PTP) under the synergistic effects of oxidative stress and increased matrix calcium (Crompton 1999). Under these conditions, induction of the PTP uncouples oxidative phosphorylation and collapses transmembrane potential leading to mitochondria bioenergetic dysfunction (Skulachev 1997; Crompton 1999). In the present study, the fact that this altered mitochondrial regulation of respiration was completely blocked by cyclosporin A, the specific inhibitor of the PTP (Broekemeier and Pfeiffer 1989), supports the notion that calcium-induced PTP can be responsible for the stimulation of mitochondrial respiration. After a 300 M pulse of calcium, the respiratory rate of succinate-energized mitochondria from DOX treated rats remained uncoupled. These results differed from the control assay (NT+P vs. NT+DOX), in which respiration was recovered following complete calcium accumulation by heart mitochondria. Moreover, this enhanced recovered respiratory rate after calcium addition was also accomplished by mitochondria from both T+P and T+DOX groups. The differences in oxygen consumption of heart mitochondria after calcium accumulation allow us to suggest that in T+DOX group, the subpopulation of mitochondria suffering from calcium-induced PTP was lower than in NT+DOX. From the results, we suggest that endurance training also protects heart mitochondria of DOX-treated rats from the enhanced susceptibility to calcium-induced PTP. Given that PTP has a marked oxidative etiology, one possible explanation for the enhanced tolerance of trained mitochondria to calcium could be related to the up-regulation of cardiomyocyte and mitochondria defenses caused by endurance training. In accordance, from the elevated content of SH-groups in T+DOX mitochondria compared with NT+DOX (Table 5) we wonder about the enhanced levels of sulfhydryl-donours in mitochondria from trained animals, such as GSH (Kowaltowski et al. 2001), which also contributes to the augmented capacity of these mitochondria to accumulate calcium, through inhibition of the PTP. Clearly, further studies need to be addressed to clarify the influence of training on this particular mechanism of mitochondria calcium regulation.

Impact of endurance training on DOX-induced mitochondria and cell apoptotic fate

Programmed cell death is a widely conserved general phenomenon that takes part in many processes involving the reconstruction of multicellular organisms, as well as in the elimination of old or damaged cells (Skulachev 1997, 2000). Given that mitochondria (dys)function during pro-oxidant *redox* changes is now increasingly considered as a key event in a variety of forms of cell death, including apoptosis, we analyzed the effects of *in vivo* DOX insult and training on the expression of the pro- and anti-apoptotic Bcl-2 family proteins (Bax and Bcl-2). The relative expression of these proteins in the mitochondrial outer membrane is thought to decide the fate of the cell by regulating membrane integrity (Hengartner 2000). DOX has recently been shown to cause apoptosis in rat heart (Arola et al. 2000; Heon et al. 2003) and this phenomenon was attributed, at least in part, to mitochondria-mediated pathways (Wang et al. 2001; Childs et al. 2002). In our study, mitochondria from non-trained DOX-treated rats seem to be more susceptible to apoptotic cell death when compared to control (NT+P), i.e., revealed a significant increase in Bax content and in Bax/Bcl2 ratio (Fig. 5). Our study also revealed that heart mitochondria isolated from trained hearts presented

lower Bax content and Bax/Bcl2 ratio suggesting that, similarly to mitochondrial respiration, a protective effect was observed in rats treated with DOX. It was however unexpected the lack of increase of Bcl2 in trained group (NT+P vs. T+P), considering its overexpression in trained hearts reported elsewhere (Lajoie et al. 2004; Siu et al. 2004).

Our data demonstrated that the activation of the caspase-3 apoptotic pathway by DOX was dramatically inhibited in trained hearts (Fig. 6) and followed the same trend of Bax content and Bax/Bcl2 ratio, suggesting a mitochondrial-mediated origin for caspase-3 activation. Training-induced increased HSP expression could be, among others, one of the protective mechanisms by which training limited apoptotic DOX side effects in heart mitochondria. The anti-apoptotic role of these important molecular chaperones was highlighted by the prevention of apoptotic cell death induced by I-R in HSP70 transfected hearts (Suzuki et al. 2002). Although other possible mechanisms for explaining this protection induced by endurance training cannot be excluded, our data suggest that it could be mediated by an improvement of mitochondria and cell defense systems.

In conclusion, the results highlight the advantage of endurance training against DOX cardiotoxicity and supplies evidence for a mitochondrial-mediated mechanism, which supports our initial hypothesis.

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Endurance training limits the functional alterations of heart rat mitochondria submitted to *in vitro* anoxia-reoxygenation

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ABSTRACT

Background: Studies analysing the effect of endurance training on heart mitochondrial function submitted to *in vitro* anoxia-reoxygenation (A-R) are missing. The present study aimed to investigate the protective effect of moderate endurance treadmill training (14-wk) against rat heart mitochondrial dysfunction induced by *in vitro* A-R. **Methods:** Respiratory parameters (state 3, state 4, ADP/O and respiratory control ratio- RCR) and oxidative damage markers (carbonyl groups and malondialdehyde) were determined in isolated mitochondria before and after 1 min anoxia followed by 4 min reoxygenation. Levels of heat shock protein 60 kDa (HSP60) and 70 kDa (HSP70) were measured before A-R in mitochondria and whole muscle homogenate, respectively. **Results:** State 3 was significantly decreased after A-R in both non-trained and trained group. However, state 3 was significantly higher in trained than in non-trained mitochondria both before and after A-R. A-R impaired RCR, ADP/O ratio and state 4 in non-trained group, which was significantly attenuated in endurance-trained group. The inhibition of state 4 induced by GDP was significantly higher in trained than in non-trained group. Oxidative modifications of mitochondrial proteins and phospholipids were found in non-trained group after A-R, although limited in trained group. Increased levels of mitochondrial HSP60 and tissue HSP70 accompanied the lower decrease in the respiratory function after A-R observed in trained group. **Conclusion:** We therefore concluded that endurance training protected rat heart mitochondria from the oxidant insult inflicted by *in vitro* A-R.

Key Words: CARDIAC MITOCHONDRIA; EXERCISE; RESPIRATION; OXIDATIVE PHOSPHORYLATION; OXIDATIVE DAMAGE; HEAT SHOCK PROTEINS

INTRODUCTION

It is well known that severe stimuli to the heart can cause structural and functional cardiac muscle impairments (Dhalla et al. 2000) and that, among other cell compartments, mitochondria are involved in the mechanisms related to some of these heart dysfunctions (Ferrari 1996; Jassem et al. 2002). Actually, mitochondria are the cells' powerhouses where the energy required to drive the endergonic and vital biochemical processes of cell life is produced through a well-coupled mechanism of oxidative phosphorylation (Cadenas 2004). Coupled with ATP synthesis, mitochondria are also critical organelles involved in the modulation of osmotic regulation, cell *redox* status and pH control, signal transduction, and in the establishment of Ca^{2+} homeostasis with consequent implications in the cell fate (Wallace et al. 1997).

It is often stated that regular exercise could constitute an excellent tool in the prevention of several heart dysfunctions as it increases the tolerance of cardiac tissue against stress conditions (Starnes and Bowles 1995; Powers et al. 2002). Since exercise represents an important heart mitochondrial stress-induced stimuli, it is possible that the structural and biochemical adaptations induced by training in these organelles, such as increased volume density, heat shock protein (HSP) expression and the up-regulation of antioxidant enzymes activity, could be related to the training-induced cardiac cross tolerance effect (Frenzel et al. 1988; Powers et al. 1998; Ji 2002). Therefore, it could be expected that these biochemical and structural adaptations parallel with improvements in several features of mitochondria functionality, including the respiratory function (Tonkonogi and Sahlin 2002). However, the influence of training on heart mitochondrial respiration has been scarcely addressed, namely alterations of basic parameters like state 4 and state 3 respiration, ADP/O and in the respiratory control ratio (RCR). Besides the study conducted by Leichtweis et al. (1997) reporting enhanced mitochondrial respiratory susceptibility after an atypical daily vigorous swimming training ($6h.day^{-1}$; $5\ days.wk^{-1}$, 8-9 wks), the limited number of studies using moderate training workloads did not find any positive changes in the ratio between uncoupled and basal respiration (Venditti and Di Meo 1996). Furthermore, it is also possible that the standard respiratory assays evaluated in isolated energized mitochondria are not sufficiently accurate to detect hypothetical improvements in respiratory function induced by chronic training in cardiac mitochondria and thus, an additional *in vitro* stimulus may be necessary to highlight possible differences in mitochondrial functional response.

I*n vitro* anoxia-reoxygenation (A-R) of isolated mitochondria is a standard and well-established deleterious stimulus, mediated by oxidative stress (Du et al. 1998; Du et al. 1999), that allows the exclusion of other cytosolic defense systems. Together with ADP-induced stimulation of mitochondria oxidative phosphorylation, constitutes a supplementary stress stimulus. Accordingly, it is possible that this model will be more sensitive to analyse the role of endurance training on respiratory rates, since in these conditions heart mitochondria from both sedentary and trained animals are submitted to an extra *in vitro* stimulus. Because the release of reactive oxygen species (ROS) is central to the mechanism of A-R-induced mitochondrial damage (Du et al. 1998; Du et al. 1999), it is logical to consider training-induced improved cardiac cell and mitochondria defences (Somani et al. 1995; Samelman 2000; Powers et al.

2002) as one of the possible candidates to counteract A-R-induced respiratory impairments. Therefore, the purpose of the present study was to analyse the effect of moderate endurance treadmill training on the heart mitochondrial respiratory susceptibility to *in vitro* A-R and relate the findings with the levels of mitochondrial oxidative damage. Moreover, since the gene transfection and hyperthermic stress-mediated overexpression of cardiac HSP60 and HSP70 have been extensively reported to result in enhanced myocardial mitochondrial tolerance (Sammut and Harrison 2003), we also investigated whether training-induced HSP overexpression would correspond to mitochondrial respiratory protection.

METHODS

Sample

Twenty Wistar male rats (aged 6-8 wks, weighting 200g at the beginning of the experiments) were used. During the experimental protocol, the animals were housed in collective cages (2 rats per cage) and were maintained in a room at normal atmosphere (21-22º C; ~ 50-60% humidity) receiving food and water *ad libitum* in 12 hours light/dark cycles. The animals were randomly divided in two groups: Trained (T) and Non Trained (NT). Only male animals were used because of female estrogen protective effect on cardiac tissue, as reported elsewhere (Camper-Kirby et al. 2001). The Ethics Committee of the Scientific Board of Faculty of Sport Sciences, University of Porto, Portugal, approved the experimental protocol, which followed the *Guidelines for Care and Use of Laboratory Animals* in research.

Endurance training protocol

The animals from T group were exercised 5 days/wk (Monday to Friday) for 14 wk on a motor driven treadmill. Both the treadmill speed and grade were gradually increased over the course of the 14-wk training period (Table 1), including 5 days of habituation to the treadmill with 10 min of running at 30m/min and 0% grade, with daily increases of 10 min until 50 min was achieved. Habituation was followed by one consecutive wk of continuous running (60 min/day) at 30m/min and 0% grade. This protocol proved to be efficient in rising cardiac dimensions (hypertrophy) and in antioxidant biochemical alterations protecting cardiac tissue during *in vivo* I/R (Powers et al. 1998). The animals from NT group were not exercised but were placed on a non-moving treadmill three times per wk (10-30 min/session) with the purpose of homogenizing the possible environment stress induced by treadmill without promoting any physical training adaptations.

Table 1 - Exercise training protocol

	Weeks of training													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Exercise duration (min/day)	30	60	90	90	90	90	90	90	90	90	90	90	90	90
Treadmill speed (m/min)	25	25	30	30	30	30	30	30	30	30	30	30	30	30
% Grade (treadmill inclination)	0	0	0	3	6	6	6	6	6	6	6	6	6	6

Note: Exercise-trained animals were exercised 5 days/wk on a motorized treadmill for 14wk.

Animals sacrifice heart and soleus extractions

Twenty-four hours after the last exercise bout, the animals were sacrificed by cervical dislocation. After a quick opening chest, rat hearts were then rapidly excised, rinsed, carefully dried and weighted. A portion of approximately 20-25mg of cardiac ventricles and one *soleus* muscle were separated, homogenized in homogenization buffer (0.05M Tris, 0.03M L-serine, 0.06M boric acid, tissue:buffer ratio of 100mg/mL, pH 7.6) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4ºC 3-5 times for 5 sec at speed low setting, with a final burst at a higher speed setting. Homogenates were centrifuged (2 min at 2000 x*g*, 4ºC, in order to eliminate cellular debris) and the resulting supernatant was stored at –80ºC for later determination of HSP70 expression (cardiac ventricle) and citrate synthase activity (*soleus*). Protein content from cardiac muscle and *soleus* homogenates were assayed using bovine serum albumin as standard according to Lowry et al. (1951).

Isolation of rat heart mitochondria

Rat heart mitochondria were prepared using conventional methods of differential centrifugation (Bhattacharya et al. 1991) as follows. Briefly, the animals were sacrificed as above stated and the hearts were immediately excised and finely minced in an ice-cold isolation medium containing 250mM sucrose, 0.5mM EGTA, 10mM Hepes-KOH (pH 7.4) and 0.1% defatted BSA (Sigma nº A-7030). The minced blood free tissue was then resuspended in 40mL of isolation medium containing 1mg protease subtilopeptidase A Type III (Sigma P-5380) per g of tissue and homogenized with a tightly fitted homogenizer (Teflon: glass pestle). The suspension was incubated for 1 min (4ºC) and then re-homogenized. The homogenate was then centrifuged at 14,500x*g* for 10 min. The supernatant fluid was decanted and the pellet, essentially devoided of protease, was gently re-suspended in its original volume (40mL) with a loose-fitting homogenizer. The suspension was centrifuged at 750x*g* for 10 min. and the resulting supernatant was centrifuged at 12,000x*g* for 10 min. The pellet was re-suspended using a paintbrush and re-pellet at 12,000x*g* for 10 min. EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein content was determined by the Biuret method calibrated with BSA. All isolation procedures were performed at 0-4ºC. Aliquots of mitochondrial suspension were separated and frozen at -80ºC for later determination of basal carbonyls, malondialdehyde (MDA) and HSP60 contents (see fig. 1A).

The remaining mitochondrial suspensions were used within 4 hours and were maintained on ice (0-4ºC) throughout this period. Isolation procedures yielded well-coupled mitochondria: RCR of isolated mitochondria varied from 7-10 (with glutamate-malate) or 3-4 (with succinate plus rotenone) for controls, as determined according to the method of Estabrook (1967). These mitochondrial suspensions were used to analyse the influence of uncoupling proteins (UCP) on basal mitochondrial respiration and also for testing the influence of *in vitro* A-R (see fig. 1A).

Mitochondrial oxygen consumption assays

Mitochondrial respiratory function was measured polarographically, at 25ºC, using a Clark-type oxygen electrode (Hansatech DW 1, Norfolk, UK). Reactions were conducted in 0.75 mL closed thermostatic and magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein

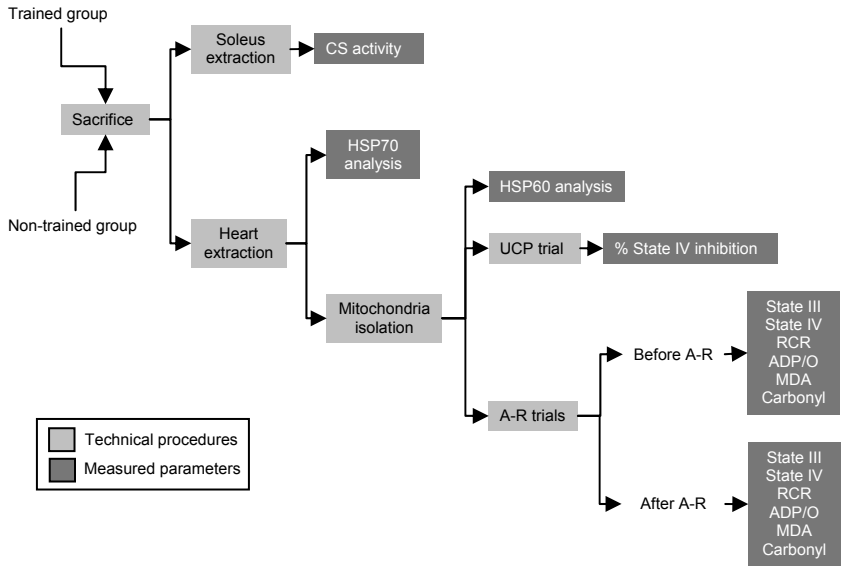
in a respiration buffer containing 65mM KCl, 125mM sucrose, 10mM Tris, 20μM EGTA, 2.5mM KH_2PO_4, pH 7.4.

After 1-minute equilibration period, mitochondrial respiration was initiated by adding glutamate and malate to a final concentration of 10 and 5 mM each, respectively. We measured some of the standard respiratory parameters that include state 3 respiration (oxygen consumption in the presence of externally added 444 μM ADP); state 4 (respiration rate observed upon complete ADP phosphorilation to ATP); RCR (a measure of the dependence of the respiratory rate on ATP synthesis and calculated as the ratio between the rate of oxygen consumption during state 3 and state 4) and ADP/O (a measure of mitochondrial oxidative phosphorylation efficiency that is defined as the ratio between the ADP added and oxygen consumed during ADP phosphorylation). The RCR and the ADP/O ratios were calculated according to Estabrook (1967), using 474 ngatom O/ml as the value of solubility of oxygen at 25ºC in doubly distilled water.

Anoxia and reoxygenation

Anoxia was performed by mitochondria (0.5mg/mL) consuming the oxygen content in the reaction medium after two ADP pulses into a close incubation chamber at 25ºC. Glutamate (10mM) and malate (5mM) were used as substrates. Energized cardiac mitochondria were stimulated with the first 444μM ADP pulse to obtain pre-anoxia respiratory rates. The anaerobic conditions were reached in state 4 through the addition of a second ADP pulse (1mM) and the period of anoxia was set to 1 minute (Fig. 1). Anoxia was followed by 4 min of *in vitro* reoxygenation by exposing the stirred medium containing the previously energized mitochondria to air (open chamber). Then, the polarographic oxygraph chamber was closed and the respiratory activities were measured again after the addition of another 444μM ADP pulse as shown in Figure 1.

A



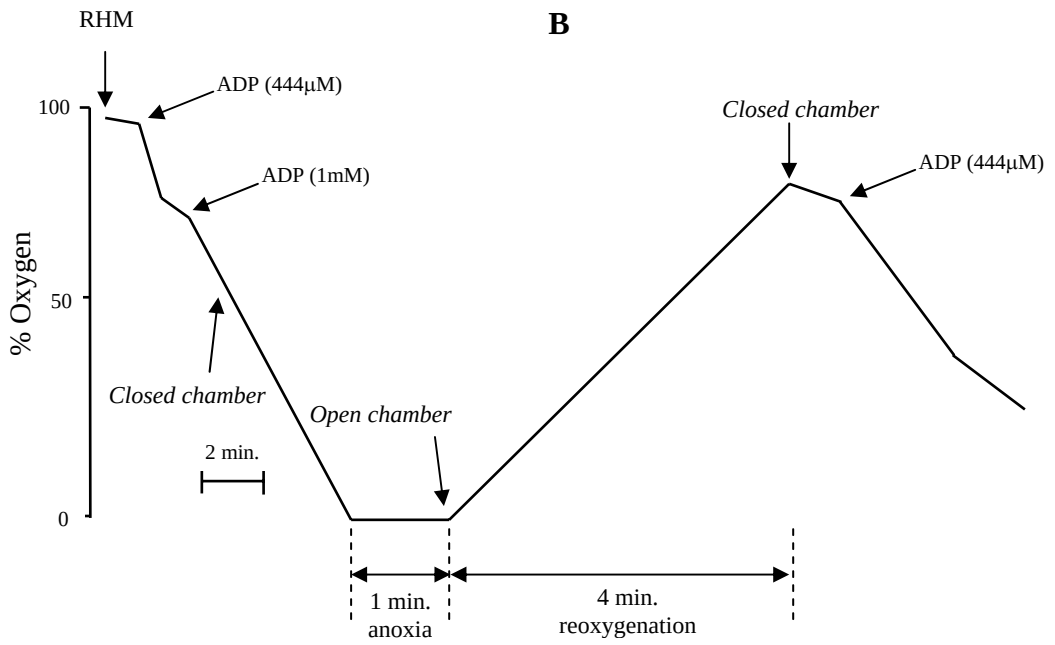


Figure 1. Experimental protocol. (A) Sequence of the technical procedures and the 10 independent measured parameters from each group or condition. (B) Typical polarographic oxygen electrode traces obtained in the A-R model. Oxygen consumption rates of energized (10mM glutamate+5mM malate) rat heart mitochondria (RHM) isolated from trained and non-trained rats were determined at 25ºC before and after stimulation through A-R. State 3 and state 4 respiration, respiratory control ratio (RCR) and ADP/O were calculated.

To analyse the susceptibility of heart mitochondria to A-R-mediated oxidative damage, mitochondria aliquots were separated and frozen at -80ºC for later determination of post A-R carbonyls and MDA contents (see fig. 1A).

With the purpose of ensuring whether the found changes were influenced by the *in vitro* mitochondria aging during respiratory assay, all the above referred respiratory parameters were also measured, in an independent trial (time-control trial), after 15-20 min incubation period in a stirred medium containing previously energized mitochondria exposed to air (Fig. 2). This time control was achieved in the presence of two pulses of ADP in order to mimic the pre-anoxic conditions reported in Fig. 1B.

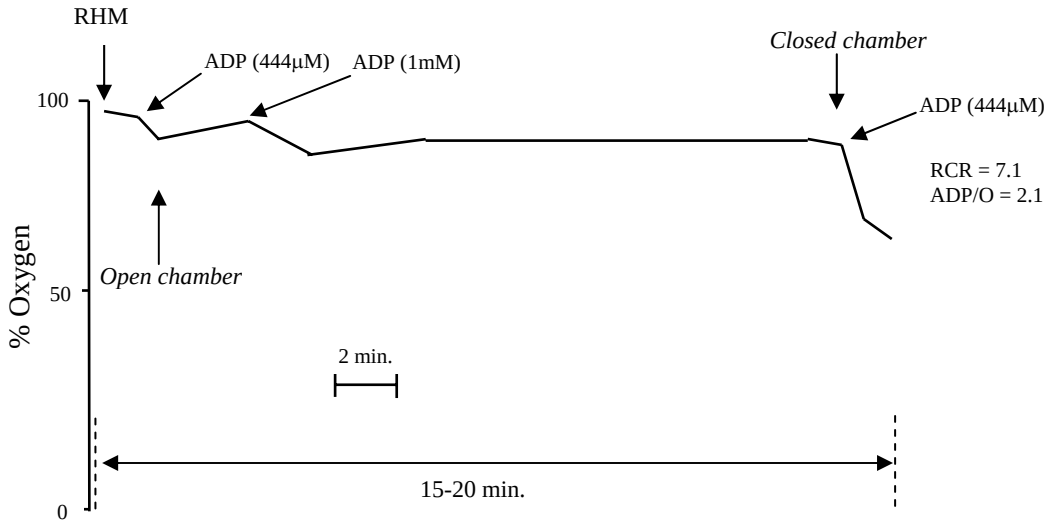


Figure 2. Typical polarographic oxygen electrode traces obtained in time control trials. Respiratory rates corresponding to oxygen consumption of energized (10mM glutamate+5mM malate) rat heart mitochondria (RHM) isolated from trained and non-trained rats were determined at 25ºC after 15-20 min of incubation in a stirred reaction medium exposed to air.

UCP-mediated basal respiration

In an independent trial performed with glutamate (10mM), malate (5mM) and ADP (444μM), state 4 respiration was determined and cardiac mitochondria were subsequently exposed to the addition of GDP (1mM) to inhibit uncoupling proteins (UCP). The addition of this inhibitor allowed testing the relative contribution of these membrane proteins to proton leak through the mitochondrial inner membrane, which may contribute to state 4 respiration, as well to a possible membrane-linked system related to the induction of the so-called mild uncoupling (Skulachev 1997).

Lipid peroxidation evaluation

Lipid peroxidation was measured by determining the levels of lipid peroxides as the amount of thiobarbituric acid reactive substances (TBARS) formed according to Rohn et al. (1993), with some modifications. Mitochondrial protein (0.5 mg) was incubated, at 25°C, in 500 μl of a medium consisting of 175 mM KCl, 10 mM Tris, pH 7.4. Samples of 50 μl were taken and mixed with 450 μl of a TBARS reagent (1% thiobarbituric acid, 0.6 N HCl, 0.0056% butylated hydroxytoluene). The mixture was heated at 80-90°C during 15 min, and recooled in ice for 10 min before centrifugation in Eppendorf centrifuge (1500*g*, 5 min). Lipid peroxidation was estimated by the appearance of TBARS spectrophotometrically quantified at 535 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nanomoles of MDA per milligram of protein (Buege and Aust 1978).

Analysis of protein carbonylation, HSP60 and HSP70

To determine the levels of HSP60 and HSP70 in the heart mitochondria and cardiac muscle, respectively, a certain volume of the suspension/homogenate correspondent to 10μg protein was resolved by SDS-PAGE (12.5% acrylamide gels of 1mm thickness) as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes according to Locke et al. (1990). The immunoblotts were probed with 1:2000 dilution of monoclonal anti-HSP 60 (Calbiochem) and monoclonal anti-HSP70 (Clone BRM-22, Sigma) and with 1:500 dilution of the secondary antibody (anti-mouse IgG peroxidase conjugate, Sigma, St. Louis, USA).

For protein carbonyl derivatives assay, a certain cardiac mitochondrial suspension volume (V) containing 20μg of protein was derivatised with dinitrophenylhydrazine (DNPH). Briefly, the sample was mixed with 1 V of 12% SDS plus 2 V of 20mM DNPH 10% TFA, followed by 30 min of dark incubation, after which 1,5 V of 2M Tris/18.3% of β-mercaptoethanol were added. A negative control was simultaneously prepared for each sample. After diluting the derivatised proteins in TBS to obtain a final concentration of 0.001μg/μL, a 100μL volume was slot-blotted into a Hybond-PVDF membrane. Immunodetection of carbonyls was then performed using rabbit anti-DNP (DAKO) as the first antibody, and anti-rabbit IgG-Peroxidase (Amersham Pharmacia) as the second antibody.

The bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (Bio Rad). Optical density results were expressed as percentage variation of control values (non-trained group).

Citrate synthase activity

Soleus citrate synthase activity was measured using the method proposed by Coore et al., (1971) by spectrophotometrically (412 nm) measuring the amount of 5,5-dithiobis (2-nitrobenzoate) that reacted with acetyl-Co upon release from the reaction of acetyl-CoA with oxaloacetate.

Statistical analysis

Mean and mean standard errors were calculated for all variables in each of the experimental groups (n=10) and paired Student *t*-test was performed to compare groups. A *t*-test for repeated measures was used to test the differences before and after A-R within groups. Statistical Package for the Social Sciences (SPSS Inc, version 10.0) was used for all analysis. The significance level was set at 5%.

RESULTS

Body weights, absolute and relative heart weights and *soleus* muscle citrate synthase activity are shown in table 2. In accordance with the well-described body mass and cardiac adaptations induced by endurance training, the 14 wks of endurance running training decreased rat weight, and induced an increased in heart weight and heart/body ratio ($p<0.05$). There was an increase in skeletal muscle citrate synthase activity in trained rats. This improved enzymatic activity in *soleus* reflects that endurance training was an efficient chronic stimulus to ameliorate muscle oxidative metabolism.

Table 2. Effects of endurance running training on rat body and heart weights as well as on the activity of skeletal muscle citrate synthase.

Groups	Body weight (g)	Heart weight (g)	Heart/body ratio ($x10^{-4}$)	Skeletal muscle CS ($\mu mol.mg^{-1}.min^{-1}$)
Non-trained	514.66 ± 16.3	0.96 ± 0.021	18.8 ± 0.44	0.017± 0.001
Trained	425.0 ± 6.45 *	1.29 ± 0.054 *	30.4 ± 0.10 *	0.056 ± 0.004 *

Note: Data represent the means ± SEM for body and cardiac weights as well as for skeletal muscle citrate synthase (CS) activity. * p<0.05 Trained vs. Non-Trained.

As illustrated in Fig. 3, endurance running training elevated significantly the expression of basal levels of both heart mitochondria HSP60 and whole cardiac muscle homogenate HSP70. In fact, when compared to non-trained controls, increases of 114% (HSP60) and 97% (HSP70) were verified in trained groups.

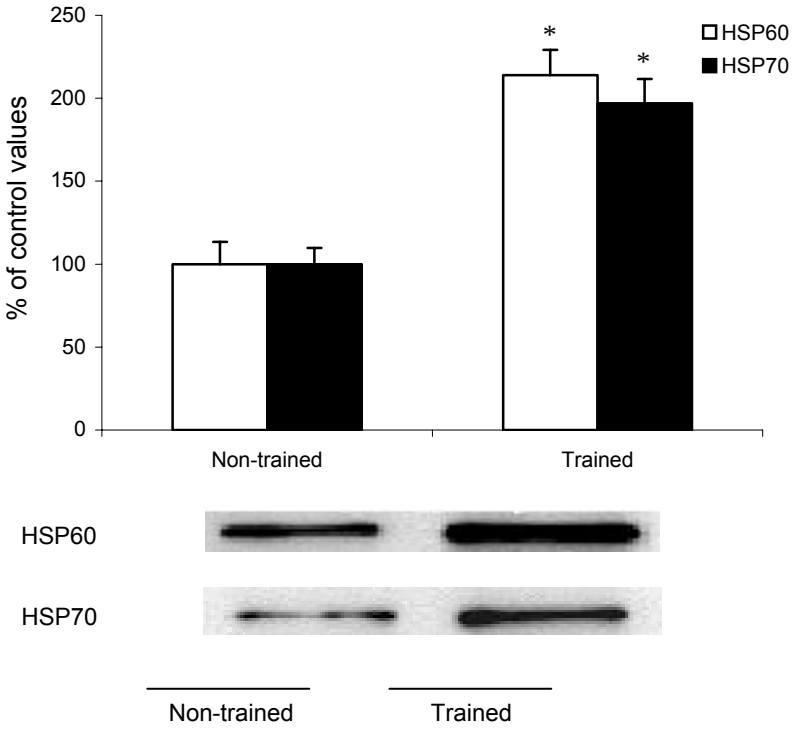


Figure 3. Effect of endurance running training on the expression of heart mitochondria HSP60 and tissue homogenate HSP70. Immediately below the histogram, the panel shows a representative western blotting of HSP60 and HSP70 for each group as described in METHODS. Values (mean and SEM) are expressed as percentage of control * p<0.05 Trained vs. non-trained.

The inhibition of state 4 respiration by 1mM GDP, a specific inhibitor of UCP, was significantly higher in heart mitochondria from trained animals than in heart mitochondria from non-trained animals. As illustrated in Fig. 4, respiratory rates in state 4 upon addition of GDP were inhibited at percentages of 13.44% and 24.33% in non-trained and trained groups, respectively.

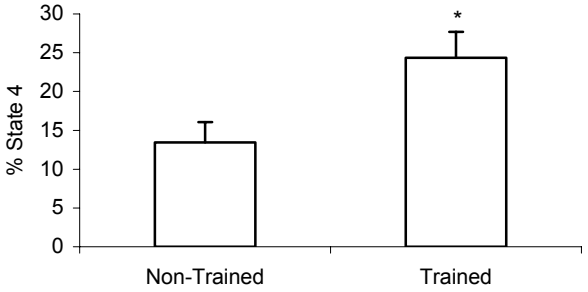
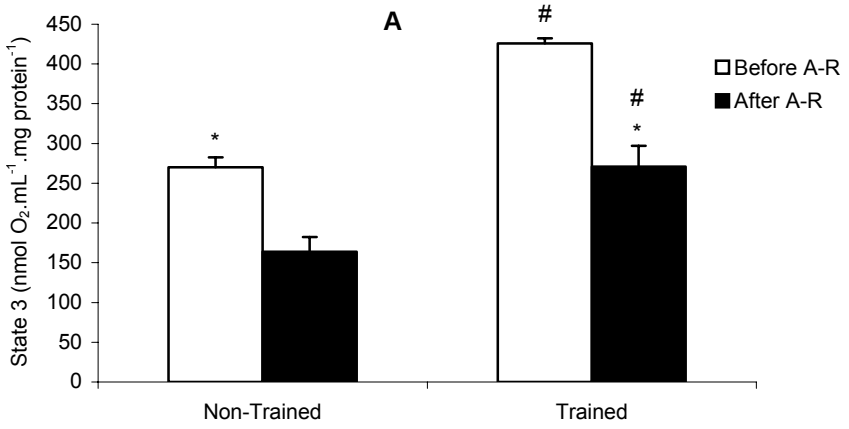


Figure 4. Effect of endurance training on the inhibition of state 4 respiration with the addition of GDP (1mM). Magnitude of respiratory inhibition induced by GDP on state 4 respiration rate, which was expressed as the percentage of inhibition of the rate in state 4. * $p<0.05$ compared with non-trained group.

Mitochondrial respiratory parameters after the "time-control trial" were not significantly different from those obtained before anoxia. Actually, RCR and ADP/O calculated after A-R were not different from those obtained before A-R in both non-trained and trained groups.

Concerning the anoxia-reoxygenation data, the exposure of mitochondria to this insult induced a significant decrease in state 3 respiration rate in non-trained group (from 270.0±0.5 to 164.0±18.3 nmol O_2.mL^{-1}.mg $protein^{-1}$) as well as in trained group (from 426.0±6.4 to 271.0±2.6 nmol O_2.mL^{-1}.mg $protein^{-1}$). However, it is noteworthy the significant difference between heart mitochondria absolute state 3 respiratory rates from trained animals when compared to their non-trained counterparts, both before and after A-R (Fig. 5A). State 4 respiration was only increased after A-R in non-training group (from 36.3±4.3 to 45.0±1.24 nmol O_2.mL^{-1}.mg $protein^{-1}$), whereas it further decreased slightly in mitochondria isolated from trained rats (from 44.2±3.12 to 40.0±2.81 nmol O_2.mL^{-1}.mg $protein^{-1}$). Endurance training remarkably increased the rate of oxygen consumption in state 4 respiration before A-R (Fig. 5B).



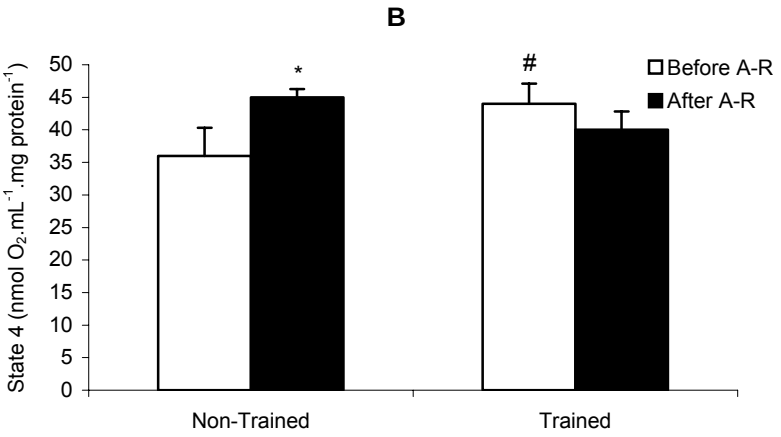
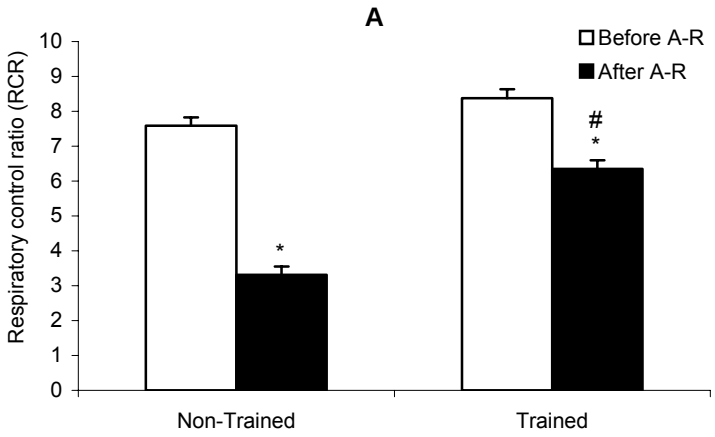


Figure 5. Influence of A-R on the respiratory parameters of rat heart mitochondria respiring with glutamate (10mM) and malate (5mM) from both non-trained and trained hypertrophied rat hearts. (A) Respiratory rates in state 3. (B) Respiratory rates in state 4. * $p<0.05$ compared with respiratory rates before A-R; # $p<0.05$ compared with respiratory rates of non-trained group.

The absolute RCR values were significantly depressed by A-R, both in trained (from 8.38±0.46 to 6.65±0.25) and in non-trained groups (from 7.59±0.5 to 3.31±0.24) (Fig. 6A). Nevertheless, a noticeable decreased fall was verified after A-R in trained when compared to non-trained group when expressed as the ratio of RCR after/before A-R (0.75±0.09 vs. 0.43±0.07, $p<0.05$). Concerning ADP/O ratio, and as can be seen from Fig. 6B, A-R induced significant decrease only in non-training group (from 2.54±0.19 to 1.91±0.57). Endurance running training limited the fall in ADP/O induced by A-R (from 2.2±0.06 to 2.07±0.05). Accordingly, the ratio of ADP/O after/before A-R was also closer to 1.0 in trained than in non-trained group (0.93±0.08 vs. 0.75±0.03).



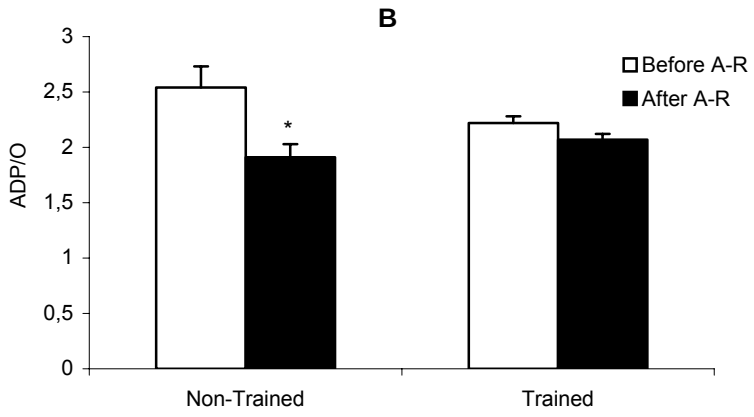


Figure 6. Influence of A-R on RCR (A) and on ADP/O (B) of rat heart mitochondria incubated with the complex I-linked substrates glutamate (10mM) and malate (5mM) from both non-trained and trained hypertrophied rat hearts. * $p<0.05$ compared with respiratory rates before A-R; # $p<0.05$ compared with respiratory rates of non-trained group.

Concerning oxidative damage markers assayed both before and after A-R insult, namely protein carbonyl groups and MDA from rat heart mitochondria extracts, significant increases were observed in non-trained, whereas no significant changes were noted in trained group (Fig. 7 and 8).

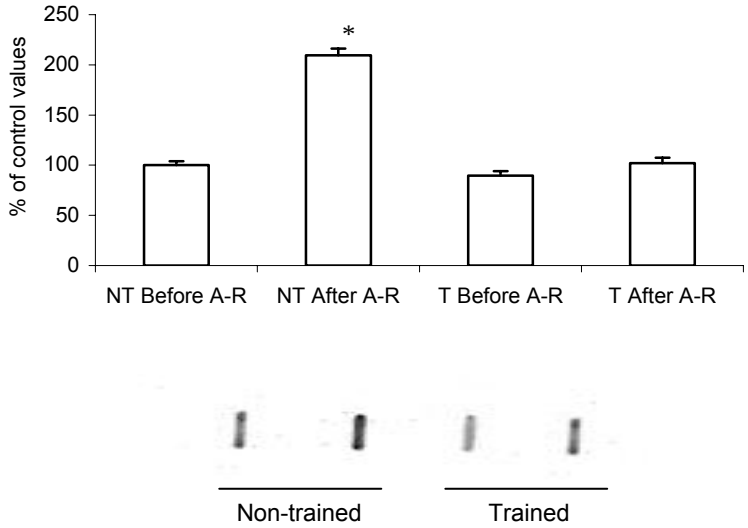


Figure 7. Effect of training and A-R on the content of carbonyl derivatives of heart mitochondria. Immediately below the histogram, the protein carbonyl formation panel shows a representative pattern of anti-denitrophenyl (DNP)-specific interaction with DNP for each group as described in METHODS. Values (mean and SEM) are expressed as percentage of control. * $p<0.05$ Before vs. after A-R.

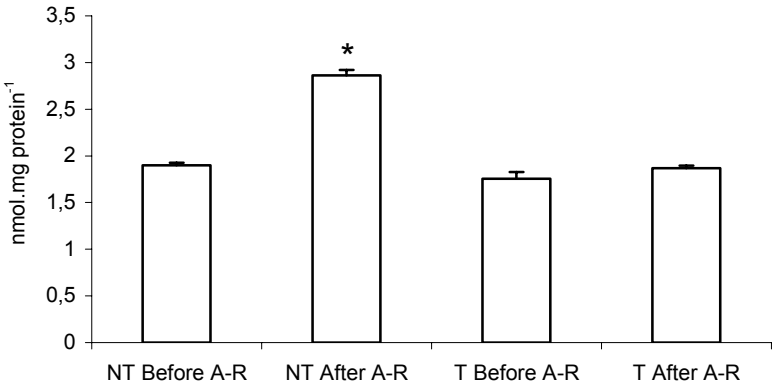


Figure 8. Effect of training and A-R on the content of malondialdehyde of heart mitochondria. Values represent mean and SEM. * $p<0.05$ Before vs. after A-R.

DISCUSSION

The use of A-R model of *in vitro* stimulation allows understanding the contribution of mitochondria to the trained hearts' tolerance against adverse stimuli. In fact, in studies that analyse the overall cardiac tissue response to deleterious stimuli, the complexity of the integrated cell defence systems limits the understanding and the precise description of the cell response mechanisms. Most of the times, differences in mitochondrial behaviour can only be uncovered after exposing the isolated mitochondrial fraction to stress conditions. One of the ways is by adding ADP, which mimics cellular need for extra energy production. Anoxia-reoxygenation of the isolated mitochondrial fraction is another inducer of stress that can be used to detect biochemical and functional changes present in mitochondrial fraction from different treated groups. In the present work, we proposed to study the protective role of endurance training on AR-induced mitochondrial dysfunction, a subjected only lightly explored in the literature. Our results showed that the A-R model is suitable to uncover differences between treatment groups.

As described in methods, we have verified in the "time-control trial" that incubation of mitochondria in the respiratory medium for 15-20 min did not alter respiratory features. This clears shows that aging of the mitochondrial fraction present in the reaction chamber was no cause or the differences observed upon A-R (Fig 2). These data were in accordance with other reports in lung (Willet et al. 2000b), brain (Morin et al. 2002) and liver (Du et al. 1998; Du et al. 1999) isolated mitochondria, where there was no significant *in vitro* aging during respiratory assays.

To the best of our knowledge, this is the first study that analyses the effect of moderate endurance treadmill training on rat heart mitochondrial respiratory function. In accordance with A-R experiments in mitochondria derived from other tissues like brain and liver (Du et al. 1998; Du et al. 1999; Morin et al. 2002), although in contrast to other similar experiments using isolated lung mitochondria (Willet et al. 2000b), the present study

revealed that A-R impaired all the measured respiratory parameters in non-trained heart mitochondria, namely state 3, state 4, RCR and ADP/O ratios (Figs. 5 and 6). The state 3 decrease is usually interpreted as a sign of respiratory chain dysfunction through intrinsic damage of its oxido-reductases and/or a limitation of reducing equivalents (Willet et al. 2000a; Willet et al. 2000b) with consequent decline of supplied electrons to mitochondria electron transport chain (ETC), or to specific damage to the mitochondrial phosphorylation system (ATP synthase, phosphate transporters or the adenine nucleotide translocator). Nevertheless, despite the fact that the inhibition of state 3 by A-R occurred in both non-trained and trained mitochondria, state 3 was higher in trained than in non-trained group before A-R. As the same glutamate-malate concentrations were provided to mitochondria in the respiratory medium, one can argue that enhanced activities or differential expression of substrate translocators, components of the ETC or other mitochondrial metabolic pathways, could contribute, at least partially, to explain these higher phosphorylation rates in mitochondria from trained animals. As seen by the enhanced basal state 4 respiratory rates, heart mitochondria inner membrane damage induced by A-R in non-trained group resulted in an increased permeability to protons most likely due to oxidative stress-induced damage to membrane lipids or proteins (Choksi et al. 2004) or by causing proton slippage through the ATP synthase or other proteins of the membrane respiratory complex (Willet et al. 2000a; Willet et al. 2000b). H^+ leak (membrane damage) and/or H^+ slip (modification of intrinsic H^+ stoichiometry of respiratory chain redox pumps and/or ATP synthase) led to a decrease in ADP/O ratio, i.e., compromised the efficiency of oxidative phosphorylation. However, our data importantly demonstrated that endurance training minimized the increased rate in state 4 as well as the decreased state 3, RCR and ADP/O caused by A-R, protecting rat heart mitochondria. This suggests an augmented resistance of the respiratory function in trained group against the referred stress stimuli.

Central to the considerations of this discussion is the property of mitochondria to be generators of oxygen-derived free radicals, such as superoxide anion, during A-R (Du et al. 1998; Du et al. 1999). The suggestion of an injury mechanism linked to oxidative protein modification and the protective effect of training are strongly supported by the observed significant increased formation of protein carbonyl derivatives in heart mitochondria from non-trained animals after A-R when compared with heart mitochondria from trained animals (Fig. 7). Moreover, a remarkable increase in the level of MDA produced during A-R occurred in the non-trained group (Fig. 8), as previously reported by others (Schild et al. 1997), which was also significantly attenuated in heart mitochondria from the trained group. In fact, as polyunsaturated fatty acids are considered highly susceptible to ROS attack, the increased oxidative stress caused by A-R in non-trained group seem to lead to peroxidative modification of membrane lipids, as assessed by MDA formation, altering normal mitochondria respiratory function (Halliwell and Gutteridge 1999).

Cardiac mitochondria ETC produce ROS during basal state 4, when the transmembrane electric potential ($\Delta\Psi$) is elevated above a determined critical threshold value. In fact, Korshunov et al. (1997) demonstrated that small decreases in $\Delta\Psi$ resulted in a strong inhibition of H_2O_2 formation in a mechanisms named by the authors as mild

uncoupling. This mechanism would keep the ΔΨ below the critical threshold for augmented ROS production. In our study, as can be depicted from Fig. 4, the inhibition of state 4 respiration by GDP was significantly higher in heart mitochondria from trained animals when compared with heart mitochondria from non-trained prior to A-R. Despite the observation of Boss et al. (1998) of a decrease in heart UCP2 mRNA after 8-wk endurance training, which does not favour our findings, a higher GDP-mediated decrease in state 4 in trained group compared with non-trained was observed. This suggests that possibly training induced a mild decrease of ΔΨ through an UCP-related mechanism, regulating the electron flux through ETC and reducing the amount of free radicals produced. The protective outcome of this uncoupling-mediated effect was previously demonstrated by the addition of small concentrations of fatty acids with concomitant decrease in ROS formation and by the increased stimulation of H_2O_2 production in the presence of GDP in mitochondria containing UCP (Negre-Salvayre et al. 1997; Korshunov et al. 1998; Kowaltowski et al. 1998).

Among other possible cell and mitochondria defence systems, the up-regulation of mitochondria HSP60 and cardiac HSP70 mediated by endurance training that was found in our study (Fig. 3) could be also involved in mitochondrial protection against oxidative-based damage, contributing to minimize the decrease in the respiratory function of this organelle when submitted to A-R. In fact, it has been clarified that HSP plays an essential role in keeping newly synthesized mitochondrial proteins (precursor proteins) in their correct unfolding conformation within the cytosol and transporting them into mitochondria (Deshaies et al. 1988). Therefore, one could suggest that cytoplasmic HSP70 overexpression could enhance translocation of precursor ETC and other proteins into mitochondria, which could contribute to the improved respiratory function of trained organelles after A-R when compared to non-trained. Moreover, given that the levels of mitochondrial HSP60 increased in trained groups as well, it is possible that the improved assisted folding of proteins within mitochondria also afforded additional protection to mitochondria respiration of trained group. This relationship is consistent with the data from other studies in which mitochondria abnormal respiration induced by I-R was reduced in myocardium expressing high levels of HSP 70, 60 and 32 (Sammut et al. 2001). In the present study, the relationship between HSP expression and mitochondrial respiratory function provides some evidence that the up-regulation of the referred molecular chaperones may be involved in the preservation of mitochondria complexes' integrity and activity.

Another possible explanation for the higher respiratory function of trained mitochondria than non-trained controls, when submitted to A-R deleterious effects, could be associated with the training-mediated effect on the up-regulation of mitochondrial antioxidant enzymes. Accordingly, this could have also been linked to HSP overexpression in trained group (Suzuki et al. 2002). As previously reported by others (Somani et al. 1995; Moran et al. 2004), endurance training ameliorated mitochondria antioxidant enzyme activity. This adaptation might also contribute to improve the capacity of mitochondria to counteract A-R-induced free radical-mediated malfunction. Similar findings were successfully obtained when isolated liver mitochondria demonstrated beneficial

effects on the impaired respiratory rates caused by *in vitro* hypoxia-reoxygenation in the presence of soluble antioxidants that were added during the incubation period (Schild et al. 1997).

In summary and regardless the hypothetical mechanisms, the results of the present study show that moderate endurance training improved heart mitochondrial respiratory function and increased their tolerance against A-R-induced dysfunction. Moreover, these findings suggest that mitochondrial adaptations might be involved in the well-described training-induced protection against cardiac deleterious insults.

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OVERALL DISCUSSION

OVERALL DISCUSSION

The results of the studies comprised in this dissertation support the notion that, both swimming and running endurance training have cardioprotective effects against oxidative-based insults, namely *in vivo* DOX treatment and *in vitro* A-R, at distinct levels of cellular organization. These are in accordance with the current knowledge of many other experimental reports (Kanter et al. 1985; Ji et al. 1994; Venditti and Di Meo 1996; Demirel et al. 1998; Powers et al. 1998; Taylor et al. 1999; Demirel et al. 2001; Hamilton et al. 2001; Ramires and Ji 2001; Demirel et al. 2003; Hamilton et al. 2003; Lennon et al. 2004a) that used endurance training as a strategy to prevent or attenuate the morphological, biochemical and functional signs of cardiac damage caused by several harmful stimuli (reviewed in Starnes and Bowles 1995; Powers et al. 2001; Powers et al. 2002; Starnes 2002; Ascensao et al. 2003; Taylor and Starnes 2003; Powers et al. 2004). Data from our studies contribute to enlarge the spectrum of cross-tolerance covered by the chronic adaptations induced by exercise, highlighting the involvement of mitochondrial mechanisms behind cardioprotection (studies III and IV). In fact, despite the wide range of data attesting the benefits of prior regular exercise in the protection of heart against I-R injury, the most frequent physiopathological manifestation associated with myocardial damage, other *in vivo* and *in vitro* deleterious insults, namely doxorubicin (studies I, II and III) and mitochondria anoxia-reoxygenation (A-R) (study IV) were analyzed.

Both cardiotoxic models used in the studies included in this work are associated with a clear and well-defined tissue (studies I, II and III) and mitochondrial (studies III and IV) pro-oxidative imbalance resulting from excessive ROS production and/or from the inability of the several endogenous antioxidant systems to coupe with enhanced ROS generation. These models resulted from either DOX administration (Davies and Doroshow 1986; Doroshow and Davies 1986; Singal and Iliskovic 1998; Horenstein et al. 2000; Hrdina et al. 2000; Wallace 2003) or mitochondrial A-R (Schild et al. 1997; Du et al. 1998; Du et al. 1999; Morin et al. 2002; Morin et al. 2003a; Morin et al. 2003b). As a result, several biochemical, histological and functional alterations occured at different levels of cellular organization, as increased accumulation of oxidative stress and damage, apoptosis markers, stress proteins and serum levels of specific cardiac proteins, alteration in the normal morphological appearance of both whole cardiac cells and mitochondria, and diminished functional respiratory capacity.

Most of the training-related cross-tolerance studies that investigate cardiac overall tissue used I-R as a model to test cardiac susceptibility to oxidative damage. However, in addition to I-R, other stimuli associated with distinct known mechanism of cellular damage, such as the *in vivo* treatment with the antitumor antibiotic DOX, should also be considered in order to analyze the beneficial effects and increase the spectrum of training cross-tolerance applications. The potent oxidant species resulting from DOX treatment are considered important aggressive agents, due to the high affinity of these compounds with some cellular components, including polyunsaturated membrane fatty acids, some protein groups and both nuclear and mitochondrial DNA. The consequent harmful effects on mitochondria and on other cell structures lead to cellular injury and consequently to heart dysfunction. The primary specific systemic evidence of heart damage induced by DOX administration and the protective effect of regular endurance training was evidenced by the levels of plasma cardiac troponin I (cTnI) (studies I, II and III), a method to detect cardiac injury. In fact, plasma content of this highly sensitive component of the troponin complex of cardiomyocytes has been extensively suggested as a specific clinical marker for the diagnosis of cardiac disease in various conditions (Bertinchant et al. 2000; Fredericks et al. 2001; Shave et al. 2002), including DOX-induced cardiac damage (Bertinchant et al. 2003). The studies comprised in this dissertation lack the evaluation of cardiac functional parameters. However, variations in left ventricular developed pressure (LVDP), maximum rate of left ventricular pressure development (+dP/dt), and maximum rate of left ventricular pressure decline (-dP/dt) demonstrated to be significantly consistent with the levels of cTnI (Horton et al. 1995; Ikizler et al. 2002; Margreiter et al. 2002; Angheloiu et al. 2004; Scheule et al. 2004) and even with other non specific intracellular proteins such as lactate dehydrogenase (Ramires and Ji 2001; Lennon et al. 2004b) or creatine phosphate kinase (Liu et al. 2002). In our study, plasma content of cTnI was consistent with tissue (study I) and mitochondrial (study III) concentrations of oxidative damage markers. In fact, lipid peroxidation assessed through the variations in the levels of cardiac thiobarbituric acid reactive substances (TBARS) and mitochondrial malondialdehyde (MDA), followed the expected tendency after *in vivo* acute and severe DOX treatment (I and III) and after *in vitro* mitochondrial A-R (study IV). Their rise was attenuated with both endurance swimming (study I) and treadmill training (studies III and IV). Furthermore, protein oxidation estimated by increased contents of protein carbonyl groups (CGs) and by decreased levels sulfhydryl groups (-SH) were found in cardiac tissue (study I) and mitochondria (studies III and IV) pro-oxidant environments

caused by DOX or A-R, being significantly and inversely altered in trained groups submitted to the same *in vivo* or *in vitro* stimuli.

In addition to other explanations discussed below, the mitochondrial superoxide production estimated by the levels of mitochondrial aconitase inactivity (study III) supports that endurance training also attenuated ROS generation in heart mitochondria isolated from DOX-treated animals. Indeed, the validation of the lower aconitase activity as a marker of mitochondrial superoxide formation arises from the demonstration that aconitase activity, but not fumarase, was drastically diminished (by ~70-80 %) in mitochondrial fractions of brain regions from SOD2 knockout mice (Melov et al. 1999). The selective loss of mitochondrial aconitase activity in aged SOD2 knockout mice and the specific loss of mitochondrial, but not cytosolic aconitase, were demonstrated by Williams et al. (1998) and confirm that aconitase inactivation is a selective measure of mitochondrial superoxide, highlighting the compartment-specific inactivation of aconitase following mitochondrial ROS-mediated oxidative stress and damage. These data are in accordance with other studies revealing increased signs of oxidative damage in heart homogenates and in heart mitochondria under the effect of acute deleterious stimuli (Venditti and Di Meo 1996; Schild et al. 1997; Demirel et al. 1998; Powers et al. 1998; Abreu et al. 2000; Santos and Moreno 2001), increasing the spectrum of cross tolerance applications of endurance training and confirming cardioprotection found in other cytotoxic models (Venditti and Di Meo 1996; Demirel et al. 1998; Powers et al. 1998). Actually, data from our work provide evidence that endurance training improved myocardial and mitochondrial tolerance to *in vivo* DOX-induced oxidative stress (studies I and III) and damage (studies I, II and III). Concerning the results revealed by study I, this was probably and at least partially, due to the improved importing capacity and content of cardiac glutathione of trained animals. Despite myocardium contains only approximately 1-2 mM of reduced glutathione (GSH), compared with the higher levels found in other tissues like skeletal muscle and liver (Halliwell and Gutteridge 1999), this compound is regarded as the most important endogenous nonenzymatic antioxidant in the heart, because of the limited antioxidant enzyme activity (Ferrari et al. 1991). The relevance in cardioprotection during conditions of enhanced oxidative stress was evident by the increased levels observed 24h after DOX administration (study I). This was probably due to GSH importation from the circulation via γ-glutamyl cycle, since most of the *de novo* synthesis of GSH occurs in the liver (Ji and Leeuwenburgh 1996). In fact, and in accordance with current knowledge, in which rat heart GSH has been reported to be elevated after swimming

training program (Kihlstrom 1990; Kihlstrom 1992), our data also demonstrated an important GSH increase in trained heart muscles (study I). Moreover, the lower percentage of oxidized glutathione, an important index of tissue oxidative stress, observed in trained hearts treated with DOX compared with their sedentary counterparts, were consistent with the cardioprotection against distinct pro-oxidant cell environments found in other studies (Ramires and Ji 2001). Thus, it seems likely that the protective effect of endurance training against harmful DOX effects seems to be associated with an up-regulation of cardiomyocyte antioxidant defense capacity to coupe with DOX-related enhanced ROS generation. The results provided by study I and III, concerning oxidative stress and damage markers, seem to be in agreement with data of other investigations, where the morphological, biochemical and functional deleterious effects induced either by DOX or by other oxidant insults like I-R were attenuated or completely abolished by the administration of antioxidants (Janero 1991; Kozluca et al. 1996; DeAtley et al. 1999; Suzuki et al. 1999; Venditti et al. 1999; Coombes et al. 2000a; Coombes et al. 2000b; Li and Singal 2000; Mohamed et al. 2000; Agapito et al. 2001; Breitbart et al. 2001; Quiles et al. 2002; Santos et al. 2002; Fadillioglu et al. 2003; Yamanaka et al. 2003; Zieba and Wagrowska-Danilewicz 2003; Oliveira et al. 2004). It is also important to highlight the consistency of the biochemical data (studies I, II and III) with the histological findings (studies II and III). In these later studies, in clear contrast with the normal appearance of cardiac tissue from sedentary control animals, hearts extracted from sedentary DOX treated animals evidenced marked structural alterations such as the accumulation of intracytoplasmatic vacuoles and myofilament disarray, mitochondria damage with extensive degeneration or even loss of cristea, intramitochondrial vacuoles and evident myelin figures. Confirming the cardioprotective effect of endurance training against deleterious effects induced by DOX, these morphological disarrangements were significantly inhibited in trained hearts. Since damaged mitochondria corresponded to the degree of other non-mitochondrial subcellular changes and, most importantly, the protection observed in mitochondria from trained hearts against DOX-induced damage was evident in other fine ultrastructural alterations, we may suspect of the important role of this particular non-pharmacological countermeasure (exercise training) in the modulation of mitochondria regarding cardioprotection. In fact, several pharmacological models of mitochondria modulation have been largely studied regarding the potential involvement of these organelles in cardioprotection (Santos et al. 2002; Szewczyk and Wojtczak 2002; Monteiro et al. 2003a; Monteiro et al. 2003b; Halestrap et al. 2004; Marin-Garcia and Goldenthal 2004;

McLeod et al. 2004a; McLeod et al. 2004b; Oliveira et al. 2004). However, the involvement of endurance training in cardiac mitochondria remodelling during physiopathological conditions has been scarcely analysed considering biochemical (studies III and IV), morphological (study II) and respiratory functional parameters (studies III and IV).

DOX interferes with a number of different mitochondrial functions, when both isolated mitochondria are exposed to the drug *in vitro* and when mitochondria are isolated from *in vivo* treated animals. However, it is important to distinguish between these two types of observations. In *in vitro* experiments, DOX is present in the reaction chamber and available as an alternate electron acceptor to directly interfere with mitochondria electron transport and respiration. On the other hand, studies of mitochondria isolated from *in vivo* exposures reveal the effects on mitochondrial bioenergetics that persist after the drug has been washed from the purified membranes (Solem and Wallace 1993; Solem et al. 1994). The *in vitro* addition of DOX to isolated mitochondria stimulates state 4 (basal, non-phosphorylative) respiration and inhibits state 3 (phosphorylative), resulting in an impairement of the respiratory control ratio, although with no changes in the efficiency of oxidative phosphorylation seen as ADP/O ratio (for refs. see Wallace 2003). However, the results of study III considering respiratory parameters obtained in mitochondria isolated from animals treated *in vivo* with DOX were in accordance with others reporting significant impairments in state 3 and respiratory control ratio (RCR), but unchanged state 4 and ADP/O ratio (Yen et al. 1999; Santos et al. 2002). These later studies using overexpressed MnSOD transgenic model and rats treated with carvedilol (a β-adrenergic antagonist with potent antioxidant activity), respectively, were well succeeded in providing significant protection against both cardiac mitochondria respiratory dysfunction and abnormal morphological appearance as consequence of the harmful effects of DOX. The protection induced by training to DOX dysfunctional mitochondria observed in study III confirmed moderate chronic exercise as an extremely useful and efficient non pharmacological tool to counteract the harmful effects associated with DOX cardiotoxicity and highlight the influence of endurance training on the mitochondrial pathways related to cardioprotection. This protective effect was also evident when using an *in vitro* oxidative-based deleterious insult like A-R, which demonstrated to be suitable to uncover differences between treatment groups after endurance training (study IV).

From the results obtained in study III regarding the effect of training on mitochondrial sensitivity to externally added calcium-induced uncoupled respiration, as well as on apoptosis-related markers, it is

reasonable to suggest that exercise training may exert some modulation effects on one of the major mitochondrial targets related to cardioprotection, the permeability transition pore (PTP) (Halestrap et al. 2004), through the attenuation of the favorable conditions for its opening. Under the synergistic effects of oxidative stress and deregulated cytosolic free Ca^{2+}, PTP formation has been recognized as one of the most relevant mitochondrial mechanism that may precede apoptotic cell death (Hirsch et al. 1997; Crompton 1999). In fact, there is a clear dependence of PTP on mitochondria *redox* state regulation, since it can be independently induced by mitochondrial pyridine nucleotide and/or thiol oxidation (Costantini et al. 1996) and inhibited by antioxidants (reviewed in Chernyak 1997; Kowaltowski et al. 2001). The protective effect of training on calcium-induced uncoupling respiration in heart mitochondria isolated from DOX treated rats observed in study III was probably linked to oxi-reductive mitochondrial and cell adaptations that attenuated PTP induction. This hypothesis can be supported by the higher content of –SH groups in DOX trained mitochondria compared to their sedentary DOX counterparts, which is probably indicative of diminished thiol pool damage in trained mitochondria treated with the drug. Despite the inner membrane proteins that must suffer from oxidative damage to result in PTP have not yet been determined, the involvement of ADP/ATP translocator is considered a serious candidate, given promptness of the three critical cysteine residue of ADP/ATP translocator for being oxidized (Costantini et al. 2000) and its anchored state on cardiolipin (Hoffmann et al. 1994). Cardiolipin is an inner membrane phospholipid particularly rich in unsaturated fatty acids and thus, highly prone to ROS interaction in the immediate surroundings of ADP/ATP translocator. Besides the influence of PTP on the decrease of mitochondrial membrane potential, PTP is also referred as a necessary step for the release of several pro-apoptotic compounds including apoptosis-inducing factor, smac/DIABLO and cytocrome *c* to the cytosol stimulating a cascade of subsequent events leading to activation of caspases, with consequent compromise of cell viability (for comprehensive review see Skulachev 1998; Hengartner 2000; Lawen 2003).

According with present understanding, our data (study III) confirmed DOX as a stressful agent capable of inducing increased signs of cardiac apoptosis (Arola et al. 2000; Wang et al. 2001; Childs et al. 2002; Green and Leeuwenburgh 2002; Yamanaka et al. 2003). However, also consistent with the other biochemical, morphological and functional features of analysis used in the present work, there was a clear attenuation of DOX-induced apoptosis in trained hearts and mitochondria suggested by the decreased levels

of mitochondria Bax/Bcl2 ratio and by the lower caspase 3 activity (study III). Since it was suggested that, in the present study, endurance training exerted its anti-apoptotic effects through improved mitochondria and cell oxi-reductive equilibrium, our data seems to be in accordance with the results of many studies, in which the ameliorated *redox* state of DOX-treated hearts followed by antioxidant supplementation reduced the signs of apoptosis (Wang et al. 2001; Liu et al. 2002; Tokudome et al. 2002; Yamanaka et al. 2003). Furthermore, these results seem to reinforce the evidence for a mitochondrial-mediated mechanism by which endurance training-induced cardioprotection against DOX.

Among the several possible explanations that have been suggested for the evident training-mediated attenuation of cardiac damage (studies I and II) and respiratory dysfunction caused either by *in vivo* DOX (study III) or by *in vitr*o A-R (study IV), the overexpression of cytosolic (HSP70) and mitochondrial (HSP60) chaperones were successfully tested. This beneficial role of exercise by means of increasing this proteins is supported by several reports (Salo et al. 1991; Locke et al. 1995a; Locke et al. 1995b; Demirel et al. 1998; Powers et al. 1998; Demirel et al. 2001; Locke 2002; Noble 2002a, b; Starnes 2002; Thomason and Menon 2002; Demirel et al. 2003; Hamilton et al. 2003; Taylor and Starnes 2003; Lennon et al. 2004a). Using enzymatic kinetic analysis on purified mitochondrial samples derived from ischemic-reperfused hearts, several authors demonstrated that hyperthermic stress-mediated overexpression of cardiac HSP60 and HSP70 resulted in enhanced cardiac mitochondrial tolerance through improved individual mitochondrial respiratory chain enzyme activity (Sammut et al. 2001; Sammut and Harrison 2003). In fact, hyperthermic stress-induced enhanced HSP expression independently increased the activities of mitochondrial complexes I, IV and V and suggested a trend to increase in complex I and III (Sammut et al. 2001). Moreover, a study from the same group revealed that mitochondria isolated from hearts transfected with HSP70 gene exhibited enhanced MnSOD activity and this improved antioxidant capacity was associated with increased mechanical cardiac function, improved mitochondria respiratory function and with decreased levels of myocardial damage and apoptosis caused by I-R. This is in accordance with our data (studies III and IV) and confirms the importance of these molecular chaperones in cardioprotection, suggesting that training-induced beneficial effects on mitochondrial function are exerted, at least partially, through an HSP-mediated action. For example, McLeod et al. (2004b) shown that delayed preconditioned mitochondria displayed increased tolerance against A-R. Bearing in mind that preconditioning is thought to induce HSP in rat hearts (Sun et al.

1995), one can therefore hypothesize that these and other adaptations may occur during endurance training process, including those associated to the variety of HSP cellular roles, contributing to increase the tolerance of heart mitochondria to *in vitro* stimuli like A-R (study IV) and to other *in vivo* stimuli that cause respiratory dysfunction like DOX (study III) or I-R.

Actually, it is known that exercise stimulates several biochemical cascades of signalling in the heart at distinct levels of cellular organization that ultimately lead to the synthesis of fundamental proteins responsible for a series of beneficial adaptations (Roth et al. 1998; Iemitsu et al. 2002; Coven et al. 2003; Iemitsu et al. 2004), including physiological cardiac hypertrophy (Wakatsuki et al. 2004) and improved endogenous antioxidant response with known consequences on redox-sensitive cell signaling processes (Ji 2002). Although not yet experimentally tested in cardiac muscle, the enhanced gene expression of skeletal muscle MnSOD after an acute bout of exercise was preceded by an elevated level of NF-*k*B and AP1 binding (Hollander et al. 2001). Furthermore, a large amount of evidence has documented that exercise training not only increases heart antioxidant enzyme activity trough increasing its mRNA levels, but also by post-transcriptional modifications improving mRNA stability (Somani and Rybak 1996; Wilson and Johnson 2000).

It has been postulated that mitochondria have several accurate membrane-linked systems that interact independently or coordinated to keep sufficiently low intracellular O_2 concentrations and to diminish the lifetime of one-electron O_2 reductants that ultimately lead to a decrease in ROS production. These mechanisms were reviewed by Skulachev (1997) and included, among others, the so-called mild uncoupling that was postulated to be a protein-mediated process controlled by several factors (Starkov 1997). According to the recent data, uncouplers of oxidative phosphorylation operate as protonophores traversing the hydrophobic membrane region thereby increasing the proton permeability of coupled membrane and wasting energy by means of a futile proton cycle. Several protein complexes from the mitochondrial inner membrane have suggested participating in this phenomenon such as uncoupling proteins (UCP) and adenine nucleotide translocators (Korshunov et al. 1998; Simonyan and Skulachev 1998), ATP-sensitive K^+ channels (Ozcan et al. 2002; Ferranti et al. 2003). Despite Boss et al (1998) observed a decrease in heart UCP2, UCP3 expression and mRNA after 8wks of endurance training, an increased inhibition of state 4 respiration through GDP addition on trained mitochondria was observed in the study IV. Moreover, this was consistent with the higher tolerance of trained mitochondria against A-R harmfulness, which preliminary suggested that

endurance training might also exert some modulation effect on mitochondria involving UCP, with hypothetical influence on basal mitochondrial ROS generation.

DIRECTIONS FOR FUTURE RESEARCH

1. In addition to interfere with mitochondrial respiration, there is growing evidence that the primary mechanism by which DOX disrupts mitochondrial function is by inducing permeability transition pore (Solem and Wallace 1993; Solem et al. 1994; Solem et al. 1996; Zhou et al. 2001). Since permeability transition pore has a recognized etiology, based on excessive levels of intramitochondrial calcium and/or oxidative stress (Vercesi et al. 1997; Kowaltowski et al. 2001), and that endurance training demonstrated cardioprotection against DOX, consistent with tissue improved defense systems and diminished damage (study I), mitochondria and cell histopathological findings (studies II and III), mitochondrial signs of oxidative damage and apoptosis (study III) and respiratory function (study III), the effects of moderate endurance training on DOX-induced increased mitochondrial susceptibility to calcium overload, higher swelling amplitudes and permeability transition pore formation would be interesting topics to be addressed in future researches.

2. It seems clinical relevant to provide more accurate information regarding the precise mitochondrial inner membrane locations altered by exercise training. Unfortunately, due to the limited mitochondrial yield and the time spent on each experiment of our study, we measured mitochondrial respiratory parameters only in the presence of complex I and II-linked substrates, namely glutamate-malate and succinate, respectively (study III) or only substrates for complex I (study IV). Additionally and due to the same reasons, the use of specific mitochondrial complex inhibitors as well as uncouplers with protonophoric actions were limited. It was therefore difficult to provide more insights into particular location of impaired or improved sites throughout mitochondrial ETC and/or on the inner membrane components caused by endurance training, which should be further addressed in future functional and biochemical investigations.

3. As referred above in the discussion section, the inhibition of state 4 with GDP in the trained mitochondria (study IV) suggests that endurance training may exert some modulator effect on mitochondrial UCP, possibly influencing rest (state 4) mitochondrial ROS production. The further analysis of whether or not

exercise has some influence on these and other inner membrane protein complexes, such as the ATP-sensitive K^+ channels or ANT, involved in the regulation of ROS production by mitochondria through a phenomenon known as mild uncoupling, seems to be pertinently important. It would therefore contribute to better comprehend the role of exercise training on the hypothetical control of mitochondrially generated ROS and consequently, on the mechanisms related to cardioprotection.

4. The effects of different exercise durations (Powers et al. 1998; Demirel et al. 2001; Moran et al. 2004) and intensities (Lennon et al. 2004b) in the protection of cardiac function, as well as the time for loss of cardioprotective effects of exercise (Lennon et al. 2004a) were effectively tested by others, providing additional information related to exercise-induced cardioprotection. These and other studies were based on the analysis of enzymatic antioxidant defense systems and heat shock proteins-related mechanisms in cardiac homogenates, and parameters of cardiac function. In order to understand the potential of exercise in the alteration of mitochondrial-related mechanisms involved in cardioprotection, those would be interesting research topics for future research using isolated heart mitochondria affected either by the harmful DOX effects or by other cardiotoxic interventions such as ischemia-reperfusion.

CONCLUSIONS

CONCLUSIONS

Based on the findings of the different studies comprised in this dissertation, it seems reasonable to emphasize the following conclusions:

(i) As described, *in vivo* DOX treatment was confirmed as a proficient model of cardiotoxicity, resulting in an increased oxidative stress and damage, in the triggering of apoptosis and mitochondrial respiratory dysfunction with important biochemical, morphological and functional outcomes both in whole cardiac muscle and in isolated mitochondria.

(ii) The several above-mentioned impairments caused by DOX treatment are attenuated by previous endurance exercise training being evident at distinct levels of cellular organization.

(iii) The protective effect of endurance training against DOX is visible on mitochondrial functionality, namely on standard respiratory parameters.

(iv) Highlighting the advantage of endurance training against DOX cardiotoxicity, the biochemical, histological and functional findings supply evidence for a mitochondrial-mediated mechanism behind this cardioprotective effect

(v) Endurance training improves heart mitochondrial respiratory function and increases their tolerance against A-R-induced oxidative-mediated dysfunction.

(vi) Confirming the important role in cardioprotection against other deleterious stimuli, exercise-induced HSP overexpression is also determinant in protecting both cardiac muscle homogenate and isolated mitochondria against *in vivo* DOX and/or *in vitro* A-R

In summary, the findings of the studies presented in this work may contribute to enlarge the spectrum of cross-tolerance covered by the adaptations induced by chronic exercise, suggesting that mitochondrial adaptations might be involved in the well-described training-induced protection against cardiac deleterious insults.

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