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Effects of hyperglycaemia and endurance training on rat skeletal and cardiac muscle mitochondrial function and apoptotic signaling

Special reference to the permeability transition

A presente dissertação foi escrita para a obtenção do título de Doutor no âmbito do curso de Doutoramento em Actividade Física e Saúde organizado pelo Centro de Investigação em Actividade Física, Saúde e Lazer (CIAFEL), da Faculdade de Desporto da Universidade do Porto, nos termos do artigo 81º do Decreto de Lei 74/2006

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KEY-WORDS: *ENDURANCE EXERCISE, DIABETES, HEART*, *SKELETAL MUSCLE, MITOCHONDRIAL PERMEABILITY TRANSITION PORE, CELL DEATH.*

“The best way to prepare for life is to begin to live.”

Elbert Hubbard

To my parents and grandparents who gave me life and taught me life.

“On the hopes of reaching the moon men fail to see the flowers that blossom at their feet.”

Albert Schweitzer

To Di for making me see the flowers while accompanying me in the journey to the moon and for been forever my love.

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TABLE OF CONTENTS

FIGURES

TABLES

ABBREVIATIONS

$\Delta\psi_{H+}$ electrochemical proton gradient

$\Delta\Psi$ transmembrane potential

ADP adenine dinucleotide

ANT adenine nucleotide translocator

BKA bongkrekic acid

Ca^{2+}calcium

CCCP carbonyl cyanide m-chlorophenylhydrazone

CsA cyclosporin A

Cyp D cyclophilin D

DM diabetes mellitus

ETC electron transport chain

HbA1C glycated hemoglobin

HK hexokinase

HSP heat shock proteins

MPTP mitochondrial permeability transition pore

NEM N-ethylmaleimide

OPR oxidative phosphorylation rate

OS oxidative stress

OXPHOS oxidative phosphorylation

PBR peripheral benzodiazepine receptor

Pi phosphate

PiC phosphate carrier

PGC1-α protein peroxisome proliferator activated receptor γ coactivator 1 α

PTP permeability transition pore

RCR respiratory control ratio

ROS reactive oxygen species (ROS).

SPSS Statistical Package for the Social Sciences

STZ streptozotocin

T1DM type 1 *diabetes mellitus*

T2DM type 2 *diabetes mellitus*

TCA tricarboxylic acid

Tfam; mitochondrial transcription factor A

UCP uncoupling proteins

VDAC voltage-dependent anions channel

RESUMO

A *diabetes mellitus* (DM) tem sido associada à disfunção mitocondrial observada no músculo-esquelético e coração diabético. O exercício físico não só produz adaptações benéficas a nível mitocondrial como é habitualmente utilizado na prevenção e no tratamento da DM. O poro de transição de permeabilidade mitocondrial (PTPM) é uma entidade importante que tem sido associada a disfunções mitocondriais e à morte celular por apoptose. Esta dissertação, compreendida por um artigo de revisão e três experimentais, pretendeu analisar o efeito de uma exposição prolongada a um estado severo da hiperglicemia, resultado da administração de *estreptozotocina* (STZ) (estudo I) e do treino de resistência (estudo II) na bioenergética mitocondrial avaliada através do consumo de oxigénio e potencial transmembranar ($\Delta\Psi$), marcadores de apoptose e susceptibilidade à indução do PTPM em mitocôndrias isoladas de músculo-esquelético. No estudo III, foram analisados os efeitos de tolerância cruzada do treino de resistência contra disfunção resultante da hiperglicemia na disfunção mitocondrial cardíaca, susceptibilidade e modulação do PTPM e sinalização de apoptose. Adicionalmente, foram quantificados o canal iónico dependente de voltagem (VDAC), translocador nucleotídeo de adenina (ANT) e ciclofilina D (Cyp-D) para avaliar os mecanismos de modulação do PTPM (estudo III). A sinalização para apoptose foi avaliada através da actividade da caspase 3 (estudo III) e 9 (estudos I-III) bem como da quantificação dos conteúdos mitocondriais de Bax e Bcl-2 (estudo III). A DM resultou na disfunção da respiração mitocondrial tanto no músculo-esquelético como no coração que, no entanto, foram atenuados pelo treino. A hiperglicemia e o treino de resistência tiveram efeitos opostos na susceptibilidade do PTPM. Enquanto que a hiperglicemia aumentou a resistência, o treino aumentou a susceptibilidade à abertura do PTPM. Nas mitocôndrias diabéticas cardíacas o treino de resistência produziu uma atenuação ou reversão dos efeitos deletérios resultantes da hiperglicemia. Os resultados deste trabalho sugerem que os efeitos mitocondriais do treino e da hiperglicemia sejam variáveis com o tipo de tecido em estudo.

PALAVRAS-CHAVE: *TREINO DE RESISTÊNCIA,* DIABETES, CORAÇÃO*, MÚSCULO-ESQUELÉTICO, PORO DE TRANSIÇÃO DE PERMEABILIDADE MITOCONDRIAL, MORTE CELULAR.*

ABSTRACT

Diabetes mellitus (DM) has been closely associated with mitochondrial dysfunction of diabetic skeletal and cardiac muscle. Physical exercise not only induces beneficial mitochondrial adaptation but also is used in the prevention and treatment of DM. The mitochondrial permeability transition pore (MPTP) is an important entity that has been associated with mitochondrial dysfunction and apoptotic cell death. This dissertation, comprising one review and three experimental studies, intended to analyze the effect of long-term exposure to a severe state of hyperglycaemia induced by streptozotocin (STZ) administration (study I) and of endurance treadmill training (study II) on mitochondrial bioenergetics evaluated through oxygen consumption, transmembrane potential endpoints ($\Delta\Psi$), apoptosis markers, and susceptibility to calcium-induced MPTP in skeletal muscle and isolated mitochondria. In study III, we analyzed the cross tolerance effect of endurance training against hyperglycaemia-induced cardiac mitochondria dysfunction, MPTP susceptibility and modulation, and apoptosis signaling. Addicionally, voltage-dependent anions channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin D (Cyp D) were quantified to access MPTP modulation mechanisms (studies III). Apoptotic signaling was followed by caspase 3 (study III) and 9 (studies I-III) activity as well as mitochondrial Bax and Bcl-2 contents (study III). DM resulted in impairments on mitochondrial respiration on both skeletal and cardiac muscle, which were attenuated by endurance training. Hyperglycaemia and endurance training had opposite effects on MPTP susceptibility in skeletal muscle. Long-term severe hyperglycaemia increased resistance while training increased susceptibility to MPTP-opening. In the diabetic cardiac mitochondria the deleterious endpoints as a result of hyperglycaemia, were attenuated or reverted by endurance training. Results from this work also suggest that the mitochondrial effects of training and hyperglycaemia are tissue specific.

KEY-WORDS: *ENDURANCE EXERCISE, DIABETES, HEART*, *SKELETAL MUSCLE, MITOCHONDRIAL PERMEABILITY TRANSITION PORE, CELL DEATH.*

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Diabetes mellitus are a group of metabolic diseases characterized by defects in the secretion and/or action of insulin as a result of genetic and/or environmental factors. There are basically three basic types of diabetes: type 1 *diabetes mellitus* (T1DM), an autoimmune disease which most often strikes children and young adults, characterized by the destruction of pancreatic cells which causes insulin deficiency; type 2 *diabetes mellitus* (T2DM), the most common form (accounts for 85-95 percent of all cases), usually associated with insulin resistance, as a result of a inability of the body to use insulin properly; and gestational *diabetes mellitus*, which appears only during pregnancy and whose hallmark is insulin resistance. A number of other types of diabetes exist and a person may even exhibit characteristics of more than one type, showing signs of both T1DM and T2DM (Inzucchi, 2005).

DM is currently one of the most common diseases in the world representing the fourth or fifth leading cause of death in most developed countries. Moreover, there are substantial evidence that has become epidemic in many developing and newly industrialized nations. Currently, about 194 million people over 20 years suffers from diabetes and it is estimated that it would affect by the year 2025, 333 million adults worldwide (International Diabetes Federation, 2003).

Chronic elevation of blood glucose, even when no symptoms are present represents the most well known clinical manifestation of the disease and usually alerts the individual to the presence of DM. In addition to increased blood glucose levels, metabolic dysfunctions associated with a variety of pathophysiological complications are also a hallmark of all types and forms of DM (Rahimi, Nikfar, Larijani, & Abdollahi, 2005). These complications are responsible for significant morbidity and mortality of millions of persons all over the world, leading to tissue damage and often to serious diseases. They are multidimensional and include stroke, peripheral vascular disease, blindness, kidney disease, and lower-extremity amputation, coronary heart disease, heart tissue dysfunction and abnormalities in peripheral tissues as skeletal muscle (Dumas, Simard, Flamment, Ducluzeau, & Ritz, 2009).

Both the skeletal and cardiac muscle are extremely energy demanding tissues, highly reliant on mitochondrial oxidative metabolism and in both tissues mitochondrial impairments have been associated with diabetic dysfunction (for review see Boudina & Abel, 2007; Sun, Liu, Liu, & Liu, 2008).

The mitochondrion represents one of the most important steps in the evolution of higher organisms as 98% of the oxygen that we breathe is consumed in mitochondria. Moreover, these are organelles that although varying in both shape and size are critical in the regulation of several cellular physiological processes, like ATP production, osmotic regulation, modulation of redox status, pH control and calcium homeostasis (Brookes, Yoon, Robotham, Anders, & Sheu, 2004; Cadenas, 2004).

Mitochondria have long been recognized as the cell's powerhouses as they are the centres of energy production within the cell. The enzyme system of the tricarboxylic acid (TCA) cycle breaks acetyl CoA derived to generate carbon dioxide and in the process to reduce NAD^+ to NADH and succinate into fumarate, generating $FADH_2$ in the process (Skulachev, 1999b). These intermediates provide reducing equivalents to the electron transport chain (ETC) moving progressively from a reduced to an oxidized state in which the final step is the reduction of oxygen to water (Mitchell, 1976). The energy yield from the electrons transferred from the reduced equivalents along the ETC is used, not only to drive electrons across the ETC but also to transfer protons across the mitochondrial inner membrane from the matrix into the inter-membrane space, establishing an electrochemical proton gradient ($\Delta\psi_{H+}$), which the major consequence is the formation of a mitochondrial transmembrane potential ($\Delta\psi$), usually estimated at -150 to -180 mV (Yaguzhinsky, Yurkov, & Krasinskaya, 2006). In the presence of ADP, as protons return to the matrix through the proton channel of the F1F0-ATP synthase the energy released by the proton flow is used to depolarise the mitochondrial inner membrane driving the enzyme like a motor to phosphorylate ADP and to release ATP (Mitchell, 1966).

The respiration-coupled energy conservation in form of ATP is one of the most important mitochondrial functions. In aerobic cells, 90-95% of the total amount of ATP produced is a result of oxidative phosphorylation (OXPHOS) and the remain is synthesized during glycolysis (Scheffler, 2007). However, none of this oxidative-derived energy would be possible without the generation of $\Delta\psi$ which is usually sustained by cellular respiration. Mitochondrial $\Delta\psi$ lies at the center of all the major bioenergetic functions of the mitochondrion. From the manufacture of ATP to the accumulation of calcium, $\Delta\psi$ provides a force that drives the influx of protons or of calcium ions that simply move into mitochondria down their electrochemical potential gradients. The loss of $\Delta\psi$ may result in the inhibition of respiration, or be a consequence of a failure on the provision of substrate or some kind of uncoupling

mechanism that shunts the proton circuit and so dissipates the potential (Duchen, 2004).

Comprehensively, oxygen consumption and Δψ have often been used for quantitative studies of the regulation of OXPHOS system of mitochondria from several tissues including skeletal and cardiac muscle.

Several studies have consistently demonstrated a defective OXPHOS system of diabetic heart mitochondria, usually accompanied by inactivation and/or diminishing gene expression of mitochondrial dehydrogenases (Bugger et al., 2009; Lashin, Szweda, Szweda, & Romani, 2006; Savabi & Kirsch, 1991), and/or activity of ETC complexes (Bugger et al., 2009; Dabkowski et al., 2009; Lashin, Szweda, Szweda, & Romani, 2006; Lin, Brownsey, & MacLeod, 2009) and/or depression of ATP synthase (Bugger et al., 2009; Tomita et al., 1996). Moreover, there is an increase in fatty acid oxidation and an up-regulation of fatty acid oxidation genes in diabetic models (Buchanan et al., 2005; Herrero et al., 2006), which is also associated with increased production of reactive oxygen species (ROS) (Ye, Metreveli, Ren, & Epstein, 2003) and uncoupled respiration. Under these conditions, the energy of the proton gradient is not used to phosphorylate ADP to ATP. The increase in content and/or the activation of the uncoupling proteins (UCPs), a class of mitochondrial inner membrane proteins that can dissipate the proton gradient before it can be used to provide the energy for OXPHOS, represents one of the possible mechanisms by which protons can re-enter the mitochondrial matrix bypassing ATP synthesis. While UCP2, the most common form in the heart mitochondria, seems to be unchanged or decreased (Murray, Panagia, Hauton, Gibbons, & Clarke, 2005; Van Der Lee, Willemsen, Van Der Vusse, & Van Bilsen, 2000), another isoform, UCP3, common in the skeletal muscle, seems to be increased in some diabetic models (Hidaka et al., 1999; Young et al., 2001). In addition to UCPs, the adenine nucleotide translocase (ANT) is another important candidate for the modulation of mitochondrial energy efficiency. This protein was shown to mediate uncoupling of mitochondrial respiration by free fatty acids and to lower mitochondrial Δψ in both skeletal and cardiac muscle (Roussel, Chainier, Rouanet, & Barre, 2000; Skulachev, 1999a). Moreover, myocardial overexpression of ANT1 isoform, the most common in the heart, ameliorates diabetic cardiomyopathy in mice (Wang et al., 2009).

When we look at diabetic skeletal muscle mitochondria one study reports a decrease in protein oxidative damage in 8 wks STZ-treated rats (Kayali et al., 2004). This however, is not in accordance with most studies which suggest that hyperglycaemia leads to pro-

oxidative and damaging conditions for both skeletal and cardiac muscle mitochondria. Early studies clearly refer to an altered metabolism and decreased oxidative phosphorylation capacity of skeletal muscle in both insulin-resistant and diabetic; human and animal subjects with changes in some critical enzymes and/or proteins or protein complexes closely related with mitochondria (el Midaoui, Tancrede, & Nadeau, 1996; Gross, Harris, & Beyer, 1972; Noble & Ianuzzo, 1985; Petersen, Dufour, Befroy, Garcia, & Shulman, 2004; Simoneau & Kelley, 1997) some of which seem inherit (Befroy et al., 2007). This includes decreases in mitochondrial DNA copy number (Antonetti, Reynet, & Kahn, 1995); downregulation of oxidative genes (Mootha et al., 2003; Patti et al., 2003; Sreekumar, Halvatsiotis, Schimke, & Nair, 2002); transcriptional coactivators like *PGC-1 α and* content of the ATP synthase β-subunits (Hojlund et al., 2003). A decreased capacity of the ETC activity and changes in mitochondrial morphology and density (Kelley, He, Menshikova, & Ritov, 2002; Morino et al., 2005; Ritov et al., 2005) have also been described.

One of the critical biological phenomenons for the regulation of cellular homeostasis is the redox equilibrium, intrinsically associated with mechanisms of production and removal of ROS. Among other cellular and systemic sources of ROS, mitochondria are often referred as one of the most relevant contributors, being at the same time targets of its own ROS generation (Brookes, 2005; Turrens, 2003). During the electron flow along the ETC, some electrons leak and react with oxygen in the matrix to produce ROS. These molecules are important intermediaries of cellular signal transduction, vascular tone, erythropoietin synthesis, regulation of oxygen tension, mitochondria function and cell cycle. Thus, mitochondria are also considered vital for signalling and regulation of cellular homeostasis and death pathways. Nevertheless, an increased ROS production above the capacity of the antioxidant systems to counteract them, ultimately affects cellular function (Gakh et al., 2006) and has been implicated in several known diseases as well as in aging process (Droge, 2002). In fact, when ROS are overproduced above the coping capacities of the antioxidant system, an increase of oxidative stress (OS) and oxidative damage may occur (Cadenas, 2004; Droge, 2002) which can lead to cellular dysfunction and ultimately to cell death (Orrenius, Gogvadze, & Zhivotovsky, 2006). Although the clinical nature of the disease is well known, the precise mechanisms underlying the pathophysiology of DM remains yet unclear. All diabetic states ultimately lead to persistent hyperglycaemia (Inzucchi, 2005). It is generally accepted that hyperglycaemia-induced OS is a key process which upsets cellular homeostasis (Brownlee, 2005) and promotes cell damage in diabetic tissues

(Brownlee, 2001; Green, Brand, & Murphy, 2004) inducing several abnormalities in mitochondria isolated from skeletal muscle (Bonnard et al., 2008; Chen & Ianuzzo, 1982; Gross, Harris, & Beyer, 1972; Rouyer et al., 2007; Schrauwen & Hesselink, 2004) and heart (for review see Boudina & Abel, 2007).

Another part of the puzzle comes from an obscure but very interestingly phenomenon, the mitochondrial permeability transition pore (MPTP).

Mitochondrial outer membrane contains embedded or attached enzymes that act as communicating gateways with the rest of the cellular network. The inner membrane encloses the matrix, where mitochondrial DNA and various enzymes such as the tricarboxylic acid cycle and the β-oxidation enzymes. However, this membrane is not freely permeable and forms the major barrier between the cytosol and the mitochondrial matrix, containing special membrane proteins that transport selected metabolites across the membrane (Scheffler, 2007). Mitochondrial function is so fundamental to cell life that any disturbances will lead to disruption of cellular function, disease or even death (Duchen, 2004).

High levels of mitochondrial membrane permeabilization can be considered as the “point-of-no-return” in both the intrinsic and extrinsic route of apoptosis. By disrupting mitochondrial ion and volume homeostasis and dissipating the mitochondrial transmembrane $\Delta\Psi$, the release of proteins that normally are confined in the mitochondrial intermembrane space occurs (Kroemer, Galluzzi, & Brenner, 2007). However, independently of the end-stage cell death, mitochondrial membrane permeability is frequently the decisive event between life and death, culminating in the release of certain apoptotic factors by mitochondria. This event, is thought to be partially dependent of the increase in mitochondrial permeability as the result of the formation of a large-conductance multiprotein specific channel complex within the inner mitochondrial membrane known as the MPTP (Kroemer, Galluzzi, & Brenner, 2007). Although the mechanistic regulation and composition of the MPTP is yet unclear and remains somewhat controversy, as virtually every constituent of the MPTP have been challenged (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007; Basso et al., 2005; Kokoszka et al., 2004), the most widely and classic model considers the opening of a large-conductance multiprotein channel involving the outer membrane voltage-dependent anion channel (VDAC), the inner membrane ANT and cyclophilin-D (Cyp-D), either as constituents or sensitizers of the MPTP (Crompton, 1999).

When we analysed the role of Cyp-D, initially Crompton et al. (1999) reported that cyclosporin A (CsA) acts as a potent inhibitor of MPTP opening. Later, it was demonstrated that inhibition of pore opening by CsA and its analogues was mediated by Cyp-D (Halestrap, 1991; Tanveer et al., 1996). More recently Cyp D knockout mice were found to be highly resistant to calcium-induced MPTP opening and behave like mitochondria from wild-type mice treated with CsA (Basso et al., 2005; Nakagawa et al., 2005). The inhibitory effect of CsA was associated with a reduction in the calcium sensitivity of pore opening rather than a total blockade. Even in the presence of CsA, pore opening can still be induced at high calcium concentrations implying that MPTP opening involves a conformational change in a membrane protein that is triggered by calcium and facilitated by (rather than totally dependent on) Cyp-D. Furthermore, variations in the expression of the MPTP component/regulatory proteins Cyp-D and ANT were reported to correlate with the susceptibility of isolated mitochondria to undergo MPTP opening (Csukly et al., 2006; Matas et al., 2009; Oliveira & Wallace, 2006).

Regarding ANT, several studies have demonstrated that while opening of the MPTP can be inhibited by ATP or ANT inhibitors like bongkrekic acid (BKA) or carboxyatractyloside, pore activation is achieved by adenine nucleotide depletion which induces different ANT conformations (Halestrap, 1991; Hunter & Haworth, 1979; Novgorodov, Gudz, Jung, & Brierley, 1991). As the specificity for inhibition of the MPTP by nucleotides matches their ability to be transported by ANT (Halestrap, Woodfield, & Connern, 1997) it has been proposed that triggered by calcium, Cyp-D binds to ANT and causes it to undergo a conformational change to induce pore formation which is increased by OS (Halestrap, 2009a). Although ANT seems to be relevant to MPTP formation, an essential role has been ruled out since mouse liver mitochondria lacking ANT1 and ANT2 can still exhibit a CsA-sensitive permeability transition (Brustovetsky, Tropschug, Heimpel, Heidkamper, & Klingenberg, 2002).

When we look at the results regarding the involvement of VDAC in the formation of MPTP, although there were initial evidence that VDAC1 binding to ubiquinone analogues could inhibit the MPTP (Fontaine, Ichas, & Bernardi, 1998; Walter et al., 2000), later studies demonstrated that mitochondria lacking VDAC1 were still inhibited by these compounds (Krauskopf, Eriksson, Craigen, Forte, & Bernardi, 2006). Moreover, mitochondria lacking all three isoforms of VDAC could still undergo MPTP opening (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007). These data eliminate

VDAC as an essential component of the MPTP. However, a regulatory role cannot be completely dismissed.

It has been recognized for many years that MPTP opening is activated by phosphate (Pi). The effect of Pi may partly reflect its ability to enhance calcium uptake, but there may be additional effects. Through its effect on pH, matrix Pi contributes to the maintenance of optimal pH values for PTP opening. Besides its effect on matrix pH, Pi facilitates PTP opening by decreasing divalent cations like Mg^{2+} (which decreases the probability of PTP opening) and by generating polyphosphate which acts as a PTP inducer (Di Lisa & Bernardi, 2009; Halestrap, 2009a).

The specificity of Pi over other inorganic ions suggests that a specific carrier like the mitochondrial phosphate carrier (PiC) might be involved. Moreover, as N-ethylmaleimide (NEM), ubiquinone 0 and Ro 68-3400, which all inhibit PTP opening are also PiC inhibitors (Di Lisa & Bernardi, 2009), the mitochondrial PiC represents at the moment, one of the most preeminent constituents of the MPTP.

Several other proteins have been proposed to be either structural or regulatory components of the MPTP. These include: the mitochondrial phosphate carrier (PiC), hexokinase (HK), mitochondrial creatine kinase and the peripheral benzodiazepine receptor (PBR) (this last one with only circumstantial evidences in its favour) (for review see Halestrap, 2009b). Recent studies have reported that phosphate is required for inhibition of MPTP opening by CsA of Cyp-D (Basso, Petronilli, Forte, & Bernardi, 2008). Moreover, decreasing HK binding to VDAC decreases susceptibility to MPTP opening in heart mitochondria which could render the heart more resistant to ischemia-reperfusion injury (Halestrap & Pasdois, 2009). Despite these studies, the involvement of PiC or HK in MPTP formation remains unproven. Additional regulatory pathways seem to involve the pro and anti-apoptotic proteins of the Bcl-2 family. These proteins are thought to directly or indirectly control MPTP formation at contact sites between the mitochondrial outer and inner membranes (Orrenius, Gogvadze, & Zhivotovsky, 2006).

Under MPTP-opening mitochondria show a sudden increase in the permeability of the inner mitochondrial membrane which results in the dissipation of the mitochondrial $\Delta\Psi$ and mitochondria calcium-loading capability, mitochondrial swelling, and rupture of the outer mitochondrial membrane (Tsujimoto & Shimizu, 2007). The MPTP can be induced under various conditions, such as exposure of mitochondria to calcium in the presence of phosphate, ROS, fatty acids, and reduction in mitochondrial membrane

potential, while adenine nucleotides, and low pH are known to inhibit MPTP opening (Murphy & Steenbergen, 2006).

When cytosolic calcium concentrations rise due to disturbances of cellular calcium homeostasis, mitochondria act as calcium buffers through its uptake and accumulation (Gunter, Buntinas, Sparagna, Eliseev, & Gunter, 2000; Gunter & Gunter, 2001; Gunter, Yule, Gunter, Eliseev, & Salter, 2004). However, moderate changes in mitochondrial calcium concentrations play a critical role in regulating oxidative phosphorylation. By activating mitochondrial dehydrogenases, calcium indirectly increases the supply of NADH to fuel the respiratory chain, enabling the supply of ATP to match ATP demand in response to an increased work load of the translocase (Gunter, Buntinas, Sparagna, Eliseev, & Gunter, 2000; Gunter & Gunter, 2001; Gunter, Yule, Gunter, Eliseev, & Salter, 2004). Additional calcium-dependent mechanisms include stimulation of the proton translocating ATPase and activation of the respiratory chain by increases in matrix volume (Harris, 1993). However, there are pathological conditions in which calcium levels become excessively high and can lead to mitochondrial calcium overload. This can result in an increased risk of damage to the mitochondria through MPTP opening and in a decreased mitochondrial function. Flarsheim el al. (1996) showed a reduced calcium uptake in isolated heart mitochondria from STZ-injected rats. More recently, Oliveira and colleagues (2003) proposed that the decrease in calcium uptake in STZ-diabetic heart dysfunction was not due to dysfunctional calcium uptake machinery and could, be the result of enhanced mitochondrial permeability transition. It seems that the major functional significance of mitochondrial calcium uptake is in the regulation of mitochondrial metabolism. However, little is known about the precise physiological role of this channel, whether it is affected or what roles play in a chronic disease like diabetes. An increased susceptibility to MPTP opening has been associated with cellular dysfunction in brain (Moreira et al., 2004), heart (Halestrap & Pasdois, 2009; Oliveira et al., 2003) and kidney (Oliveira, Esteves, Seica, Moreno, & Santos, 2004) of diabetic animal models, which suggests a connection between an altered MPTP susceptibility and DM. However, until the present, no data exists regarding the diabetic skeletal muscle, considering that the mitochondrial phenotype that characterizes diabetic skeletal muscle may be associated with alterations in the susceptibility of the MPTP to assume an opened conformation.

Together with administration of antioxidants, thiazolidinediones and metformin, exercise is one of the preventive and therapeutic strategies used to tackle mitochondrial dysfunction associated with diabetes. In fact, it is well known that

physical inactivity is a risk factor for DM that can not only trigger, accelerate or enhance the pathogenesis of DM, but can also increase the associated morbidity and mortality (LaMonte, Blair, & Church, 2005).

Exercise is considered a simple and inexpensive intervention that can improve insulin action and insulin-independent glucose transport, attenuating diabetic-associated risk factors (Short et al., 2003) and its complications (Roberts, Won, Pruthi, Lin, & Barnard, 2006).

Moreover, exercise is proposed to strengthen the antioxidant defence mechanisms, reducing acute and chronic effects of diabetic-related OS and increasing mitochondrial biogenesis and coupling (for review see Lumini, Magalhaes, Oliveira, & Ascensao, 2008).

Being extremely adaptive organelles, mitochondria are able to respond and adjust to physiological demands imposed to the cells. Since earlier work by Holloszy's (Holloszy, 1967), an extensive body of literature has demonstrated that endurance exercise effectively stimulates muscle mitochondrial oxidative capacity and biogenesis. Endurance exercise training not only enhances the activity of mitochondrial enzymes and respiratory chain complexes, but it also increases OXPHOS, overall improving mitochondrial function (Ferstrom et al, 2004) as reflected by increased mitochondrial enzyme activities, maximal rate of ATP synthesis, increased transcript levels of nuclear and mitochondrial genes encoding mitochondrial proteins, mitochondrial DNA abundance, and expression of peroxisome proliferator activated receptor γ coactivator 1 α (PGC-1 α) and mitochondrial transcription factor A (TFAM; mRNA) (Chow et al., 2007).

The analysis of the impact of endurance training on the oxidative phosphorylation rate (OPR) in mitochondria isolated from *gastrocnemius* and red *vastus lateralis* muscles of rats with or without chronic STZ-induced DM revealed that although OPR was significantly depressed by DM in mitochondria from both muscles, it was significantly increased by training in both muscles of diabetic and nondiabetic rats (el Midaoui, Tancrede, & Nadeau, 1996). Similar results were found in the analysis of cardiac mitochondrial respiratory parameters of STZ-rats as both state 3 respiration and OPR rates were significantly depressed in mitochondria of diabetic rats (Mokhtar, Lavoie, Rousseau-Migneron, & Nadeau, 1993). However, in contrast with what was observed in the skeletal muscle, these alterations were reverted by endurance physical training,

without any significant changes in plasma glucose or insulin levels, suggesting mechanisms independent of blood glucose control.

Mitochondrial dysfunction caused by hyperglycaemia induced OS, or subsequent to nuclear or mitochondrial DNA mutations, can lead to a diminished mitochondrial content, compromising energy production as well as other functions dependent of mitochondrial-associated signalling cascades. When submitted to some physiological stimulus, such as endurance training, mitochondria are able to increase in size, number and/or volume in a well establish adaptation phenomenon termed mitochondrial biogenesis (Hood, 2001). Preferentially acting upon subsarcolemal mitochondria (Adhihetty, Ljubicic, & Hood, 2006), biogenesis increases the ratio of mitochondria to myofribrilar volume density, potentially providing an additional tolerance of these tissues to potential harmful stimuli. The phenotypic changes undergone by mitochondria in human and animal skeletal and cardiac muscles in response to training interventions or pathophysiological states are well documented and occur at both structural and functional levels. Endurance training is not only associated with an increase in mitochondrial volume density and enzyme activity in oxidative metabolism, but also with an improvement of coupling and regulatory properties of mitochondrial respiration in human and animal skeletal and cardiac muscles (Hood, 2001; Hood et al., 1994).

Notwithstanding, the overall response of mitochondria of some tissues, like the liver (Ferreira, Seica et al., 2003; Palmeira et al., 1999) and testis (Palmeira, Santos, Seica, Moreno, & Santos, 2001) in some diabetic models that exhibit an increase in oxidative, phosphorylative and antioxidant capabilities, others, like the heart (Oliveira et al., 2003), brain (Santos et al., 2001) and kidney (Oliveira, Esteves, Seica, Moreno, & Santos, 2004) fail to cope with the imposed demands, with consequences to the mitochondrial integrity and function.

Although transitory enhancement in RONS production beyond the capacity of the antioxidant system results in an altered redox status (Alessio, 1993; Davies, Quintanilha, Brooks, & Packer, 1982; Sen, 1995), organisms engaged in long-term heavy exercise are seemingly more resistant to OS (Ji, Gomez-Cabrera, & Vina, 2006). In fact, several modalities and different levels of physical activity, mainly endurance exercise, have proven to protect heart cells against pro-oxidant insults like doxorubicin-induced mitochondrionopathy (Ascensao et al., 2005a; Ascensao et al., 2005b) and mitochondrial anoxia-reoxygenation (Ascensao et al., 2006b).

Venditti and colleagues (1999) were the first group to report that chronic endurance training could reduce mitochondrial ROS production in mitochondria isolated from skeletal muscle. Judge et al. (2005) provided evidence that lifelong voluntary wheel activity decreased H_2O_2 production by 10% in isolated heart mitochondria from 24-month-old male Fischer compared with their sedentary counterparts. This demonstrates a multi-tissue effect of exercise that, according to both authors, was the result of decreased superoxide generation particularly at complex I (Starnes, Barnes, & Olsen, 2007). Furthermore, endurance-trained animals seem to exhibit a higher tolerance to increasing levels of calcium as isolated heart mitochondria from female Sprague–Dawley rats that underwent an exercise program of 10 wks of endurance treadmill running increased the amounts of calcium necessary to induce MPTP particularly when using complex II-linked substrates (Marcil, Bourduas, Ascah, & Burelle, 2006).

Considering the above mentioning reasoning, it is clear from the literature that hyperglycaemia causes perturbations in both skeletal and cardiac muscles mitochondria at the level of respiratory system. What is still unclear and was never a subject of research is the effect of this condition on the resistance to MPTP opening and apoptosis in skeletal muscle. Despite these issues were addressed in mitochondria derived from other tissues such as liver, heart, kidney and brain, it is a matter of interest to know how skeletal muscle mitochondria in hyperglycaemia respond to calcium-induced MPTP opening.

In addition, and as mentioned above, physical exercise is particularly advised against systemic and tissue abnormalities resulting from hyperglycaemic conditions. Although the improvements of chronic exercise stimulation on functional endpoints of skeletal muscle mitochondrial respiratory system, namely oxygen consumption and membrane potential are already described, no definitive clues exists regarding biochemical and functional markers/parameters of mitochondrial-related apoptosis. Despite the reports from Siu and colleagues (Siu, Bryner, Martyn, & Alway, 2004), suggesting increased resistance of muscle mitochondria to apoptosis (increased Bcl-2 to Bax ratio), it is still not clear whether whole body chronic exercise results in a protective phenotype against apoptosis induction. In fact, results from O'Learry and colleagues (O'Leary & Hood, 2008) and also from Adhihetty and colleagues (Adhihetty, O'Leary, Chabi, Wicks, & Hood, 2007) using chronic low-frequency electrical stimulation of nerve-induced contractile activity are not conclusive regarding the mitochondrial resistance to apoptotic stimulus. Moreover, despite this model of chronic stimulation has provided

positive outcomes, for instance on the induction of mitochondrial biogenesis, it may differ from whole body exercise models such as treadmill or free wheel running. In an attempt to contribute to better understand this issue, we therefore proposed to analyze the effect of a long-term endurance treadmill running training program on skeletal muscle resistance to MPTP induction and apoptotic signaling.

With respect to the effects of both hyperglycaemia and exercise training on cardiac mitochondrial phenotypes, particularly respiratory response as well as the tolerance of mitochondria to apoptotic stimuli and susceptibility to MPTP gating, much agreement cames from the literature. In fact, heart mitochondria of with severe hyperglycaemia is known to be impaired regarding the refered endpoints, including MPTP gating (Oliveira et al., 2002) and endurance training has been proved to decrease ROS production (Starnes, Barnes, & Olsen, 2007) and to provide mitochondrial protection against several *in vivo* and *in vitro* conditions causing heart mitochondrial dysfunction such as ischemia-reperfusion, doxorubicin treatment, anoxia-reoxigenation (Ascensao et al., 2005a; Ascensao et al., 2005b, 2006b; Ciminelli, Ascah, Bourduas, & Burelle, 2006). Moreover, endurance training *per se* exerted positive modulation of heart MPTP gating in a substrate specific manner (Marcil, Bourduas, Ascah, & Burelle, 2006). With these previous findings in mind, it is tempting to hypothesize that the heart diabetic mitochondrionopathy and the associated increased apoptotic signaling may benefit from endurance training. Furthermore, given that MPTP may be regulated by some proteins known to be part and/or sensitize the pore such as ANT, VDAC and Cyp D, an additional insight into the combined effect of these two stimuli (hyperglycaemia and exercise) on the expression of these proteins and its relation with the susceptibility to MPTP induction may be of interest.

CHAPTER I

AIMS

AIMS

Despite being a long known and studied condition, the exact underlying mechanisms associated with DM complications-induced mitochondrinopathy and how exercise modulates the function of diabetic mitochondria are still unclear. Consequently, the general objective of the present thesis was to analyze the effects of moderate endurance treadmill training on skeletal and cardiac muscles mitochondrial bioenergetics in long-term and severe hyperglycaemia, particularly focusing on the susceptibility to MPTP-opening and on the apoptotic signaling

To achieve this general objective, specific purposes were design for each of the original articles corresponding to the chapters of the experimental work of this thesis:

Paper I

To analyze the effect of long-term severe hyperglycaemia on *gastrocnemius*:

(i) mitochondrial oxygen consumption and transmembrane potential endpoints;
(ii) susceptibility to mitochondrial permeability transition pore induction by calcium;
(iii) mitochondrial calcium handling;
(iv) apoptotic levels.

Paper II

To study the effects of 14wk of whole body endurance treadmill training on *gastrocnemius*:

(i) mitochondrial oxygen consumption and transmembrane potential parameters;
(ii) susceptibility to mitochondrial permeability transition pore;
(iii) calcium handling;
(iv) susceptibility to intrinsic apoptosis.

Paper III

To determine the cross-tolerance the effect of endurance training against cardiac mitochondrial abnormalities and apoptotic signaling that characterizes severe hyperglycaemia, following:

(i) Classic mitochondrial bioenergetics endpoints, namely oxygen consumption and transmembrane potential;

The susceptibility to MPTP gating through different methodologies;

(ii) Mitochondrial ability for calcium handling;

(iii) The semi-quantitative expression of some important proteins, known to be constituents and sensitizers of the MPTP;

(iv) Apoptotic signalling as expressed by the content of Bcl-2, Bax and Bax/Bcl-2 ratio as well as by the activities of caspases 3 and 9;

(v) The content of Tfam as a marker of mitochondrial biogenesis.

This thesis is based on the following review and original articles, referred in the text by Arabic and Roman numbers, respectively:

THEORETICAL BACKGROUND

1.Lumini, J.A. Magalhães, J. Oliveira, PJ., Ascensão, A. (2008). Beneficial effects of exercise on muscle mitochondrial function in diabetes. *Sports Med*. 38(9):735-50.

EXPERIMENTAL WORK

I. Lumini-Oliveira, J. Ascensão, A. Pereira, C.V. Magalhães, S. Marques, F. Oliveira, PJ. Magalhães, J. (2009). Long-term hyperglycaemia decreases *gastrocnemius* susceptibility to permeability transition. *Eur J Clin Invest* 40 (4): 319–329,

II. Lumini-Oliveira, J. Magalhães, J. Pereira, C.V. Aleixo, I. Oliveira, PJ. Ascensão, A. (2009). Endurance training improves *gastrocnemius* mitochondrial function despite increased susceptibility to permeability transition. *Mitochondrion* 9(6): 454-462

III. Lumini-Oliveira, J. Magalhães, J. Pereira, C.V. Marques, F. Oliveira, PJ. Ascensão, A. (2009). Effect of moderate endurance training against *in vitro* heart mitochondrial dysfunction in long-term severe hyperglycaemia – special relevance on the susceptibility to permeability transition pore. *Mitochondrion* (under review)

CHAPTER II

THEORETICAL BACKGROUND

PAPER 1

Review Article

Beneficial effects of exercise on muscle mitochondrial function in diabetes

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Running title: Exercise and diabetic mitochondria

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CONTENTS

ABSTRACT

The physiopathology of diabetes mellitus has been closely associated to a variety of alterations in mitochondrial histology, biochemistry and function. Generally, the alterations comprise increased mitochondrial reactive oxygen and nitrogen species (RONS) generation resulting in oxidative stress and damage, decreased capacity to metabolize lipids leading to intramyocyte lipid accumulation, diminished mitochondrial density and reduced levels of uncoupling proteins (UCP) with consequent impairment in mitochondrial function. Chronic physical exercise is a physiological stimulus able to induce mitochondrial adaptations that can counteract the adverse effects of diabetes on muscle mitochondria. However, the mechanisms responsible for mitochondrial adaptations in muscles from diabetic patients are still unclear. The main mechanisms by which exercise may be considered an important non-pharmacological strategy for preventing and/or attenuating diabetes-induced mitochondrial impairments may involve (i) increased mitochondrial biogenesis, which is dependent on the increased expression of some important proteins such as the “master switch” peroxisome proliferator-activated receptor (PPAR)-γcoactivator-1α (PGC-1α) and heat shock proteins (HSP), both severely down-regulated in muscles from diabetic patients, and (ii) the restoration or attenuation of the low UCP3 expression in skeletal muscle mitochondria from diabetic patients, suggested to play a pivotal role in mitochondrial dysfunction.

There is evidence that chronic exercise and lifestyle interventions reverse impairments in mitochondrial density and size, in the activity of respiratory chain complexes and in cardiolipin content; however, the mechanisms by which chronic exercise alters mitochondrial respiratory parameters, mitochondrial antioxidant systems and other specific proteins involved in mitochondrial metabolism in muscle from diabetic patients remain to be elucidated.

1. INTRODUCTION

Diabetes mellitus is an heterogeneous group of diseases characterized by increased blood glucose and abnormalities in carbohydrate, fat and protein metabolism that are associated with a variety of pathophysiological complications (Rahimi, Nikfar, Larijani, & Abdollahi, 2005). These complications are often responsible for the decreased quality of life of millions of persons all over the world. Diabetes was estimated to be, directly or indirectly, responsible for 5.2% of the total number of deaths worldwide in 2000 (Roglic et al., 2005). Currently, about 194 million people over a period of 20 years have suffered from diabetes. The disease is estimated to affect 333 million adults by 2025 (International Diabetes Federation, 2003).

It is well established that mitochondria have been associated to the physiopathology of a variety of diseases (Duchen, 2004; Fosslien, 2001; Luft, 1994). In addition of being primarily identified as the cause of disease, mitochondrial DNA mutations and impaired substrate oxidation have now been found to occur as secondary phenomena in aging as well as in age-related degenerative diseases such as Parkinson, Alzheimer, and Huntington diseases, amyotrophic lateral sclerosis, cardiomyopathies, atherosclerosis and also diabetes mellitus (Luft, 1994). Coupled with ATP synthesis, mitochondria are also critical organelles involved in cell osmotic regulation, in the modulation of redox status and pH control, in signal transduction and in the establishment of calcium homeostasis (Brookes, 2005; Brookes, Yoon, Robotham, Anders, & Sheu, 2004; Cadenas, 2004). Mitochondria also provide important contributions for the regulation of cell fate through its role in the regulation of cellular death pathways (Brown & Borutaite, 2001, 2002; Skulachev, 1999b).

In diabetic patients, skeletal muscle mitochondria have a reduced size and a decreased activity of the electron transport chain when compared with control counterparts (Kelley, He, Menshikova, & Ritov, 2002; Toledo, Watkins, & Kelley, 2006). Moreover, in skeletal muscle of nondiabetic individuals with a family history of type 2 diabetes, a decreased expression of nuclear genes encoding mitochondrial proteins involved in oxidative phosphorylation has been reported (Mootha et al., 2003; Patti et al., 2003), along with reduced in vivo oxidative phosphorylation (Morino et al., 2005; Petersen, Dufour, Befroy, Garcia, & Shulman, 2004). However, it is also well established that mitochondria are rather adaptable organelles and that muscle tissue can manifest considerable plasticity regarding mitochondrial content, namely

in response to exercise-induced contractile activity (Adhihetty, Irrcher, Joseph, Ljubicic, & Hood, 2003; Irrcher, Adhihetty, Joseph, Ljubicic, & Hood, 2003).

In fact, the increased understanding of the mechanisms underlying mitochondrial dysfunction associated with diabetes has lead to the use of preventing and/or therapeutic parallel strategies, based mostly on the administration of antioxidants, thiazolidinediones, metformin and exercise.

Presently, exercise is considered a simple and inexpensive intervention that can improve insulin action and insulin-independent glucose transport (Henriksen, 2002). It also stimulates enzymatic-dependent substrate oxidation pathways, mitochondrial biogenesis, as well as attenuates diabetic-associated risk factors and complications (Roberts, Won, Pruthi, Lin, & Barnard, 2006; Short et al., 2003). Given that several types of exercise can induce considerable alterations on mitochondrial metabolism, morphology and function(see Ascensao, Ferreira, & Magalhaes, 2007), a wide variety of exercise interventions has been reported, showing beneficial mitochondrial alterations (Baar, 2004; el Midaoui, Tancrede, & Nadeau, 1996; Fritz et al., 2006; Toledo et al., 2007; Toledo, Watkins, & Kelley, 2006; van Loon & Goodpaster, 2006) and therefore improving the muscle metabolism in diabetic patients (McCarty, 2005; Short et al., 2003; Sriwijitkamol et al., 2006; Toledo et al., 2007; Toledo, Watkins, & Kelley, 2006; van Loon & Goodpaster, 2006).

The present review will focus on some important cellular mechanisms by which exercise can be considered an important strategy against diabetes-induced mitochondrial dysfunction. The alterations include two major referred redox-related mechanisms: exercise-induced mitochondrial biogenesis and the modulator effect of exercise on mitochondrial uncoupled respiration through uncoupling protein (UCP) in diabetic mitochondria.

2. DIABETES-INDUCED MUSCLE MITOCHONDRIAL IMPAIRMENT

Data analysis of major measurements of respiratory indexes of oxygen consumption, transmembrane electric potential, calcium loading capacity and swelling amplitude as well as biochemical markers and histological indexes suggests that mitochondria from tissues obtained from patients or animal models develop several adaptive responses. Only some of these biological responses may be interpreted as improvements, suggestive of increased resistance against deleterious conditions, such as

that verified in cardiac mitochondria from GK rats against calcium-induced permeability transition,(Oliveira et al., 2001; Oliveira, Seica et al., 2004) the decreased protein oxidative damage observed in pancreas, liver, kidney and skeletal muscle mitochondria from STZ-treated rats (Kayali et al., 2004) and the increased markers of cardiac mitochondrial remodelling (enhanced mitochondrial area despite mitochondria being damaged and dysfunctional and a surprisingly high proportion of altered proteins with mitochondrial origin) observed in OVE 26 mice, a model of type 1 diabetes (Shen et al., 2004). The observations would suggest that in less severe diabetic states, tissues are likely more prone to adaptation. However, the majority of studies reveal a clear trend to the conclusion that diabetes is harmful for muscle mitochondria.

In the search for casual factors that explain the decreased oxidative capacity of skeletal muscle in insulin-resistant and diabetic subjects, most investigators have focused on alterations in some critical enzymes and/or proteins or protein complexes closely related with mitochondrial metabolism. Antonetti et al. (1995) described a 2.5-fold increased expression of mitochondrial-encoded genes when expressed relative to mitochondrial DNA copy number (which decreased ~ 50%) in hyperglycaemic muscle. However, other studies using DNA microarrays have shown evidence of a coordinated downregulation of oxidative genes in skeletal muscle from human type 2 diabetic subjects (Mootha et al., 2003; Patti et al., 2003; Sreekumar, Halvatsiotis, Schimke, & Nair, 2002). The studies reported a cluster of oxidative genes under the control of peroxisome proliferator-activated receptor (PPAR)-γ–coactivator-1α (PGC-1α), a “master switch” involved in mitochondrial biogenesis, was reduced, pointing toward a phenotype of general mitochondrial dysfunction in hyperglycaemic skeletal muscle.

It is well recognized that mitochondria from type 2 diabetic patients present morphological and biochemical signs suggestive of a dysfunctional condition. Proteomic analysis demonstrated a decreased content of the ATP synthase β-subunits in skeletal muscle mitochondria from type 2 diabetic patients (Hojlund et al., 2003). Additionally, several studies described a decreased capacity of the electron transport chain (ETC) as measured by decreased rotenone-sensitive NADH oxireductase activity, and these data were accompanied by significant alterations in mitochondrial morphology and density (Kelley, He, Menshikova, & Ritov, 2002; Morino et al., 2005; Ritov et al., 2005). For instance, a 38% reduction in mitochondrial content

was found in insulin-resistance subjects by Morino et al., (Morino et al., 2005), which might be the major factor responsible for the reduced mitochondrial function. Furthermore, Petersen et al. (2004) observed a marked decrease in oxidative phosphorylation in skeletal muscle mitochondria from patients with type 2 diabetes. Further work from the same group using magnetic resonance spectroscopy found that the rates of muscle mitochondrial substrate oxidation were decreased by 30% in lean insulin-resistant offspring subjects compared with insulin-sensitive controls, supporting the hypothesis that insulin resistance in skeletal muscle may be associated with deregulation of intramyocellular fatty acid metabolism, possibly because of an inherited defect in the activity of mitochondrial oxidative phosphorylation (Befroy et al., 2007). In fact, some authors suggested that the intramyocellular lipid accumulation typical from diabetic patients may have been responsible for mitochondrial changes and, in turn, mitochondrial dysfunction may predispose these patients to intramyocellular lipid accumulation (He, Goodpaster, & Kelley, 2004; Morino et al., 2005; Petersen et al., 2003). The above referred biochemical and ultrastructural data observed in diabetic mitochondria were also observed in skeletal muscle-derived mitochondria from obese patients (Ritov et al., 2005; Toledo, Watkins, & Kelley, 2006), despite the observation by Mogensen et al., (Mogensen et al., 2007) of functional impairments in mitochondrial respiration in obese type 2 diabetic skeletal muscle compared with obese nondiabetic counterparts. In fact, this study showed a significant decrease in ADP-stimulated respiration with pyruvate plus malate as substrates in mitochondria isolated from diabetic obese muscle compared to those from nondiabetic obese group. Despite some controversy, the significant correlation found between some of the alterations and insulin sensitivity supports the hypothesis that insulin resistance in skeletal muscle of type 2 diabetic patients is associated with deregulation of intramuscular fatty acid metabolism, possibly due to defects in mitochondrial function.

The most highlighted strategy for preventing and reversing some of mitochondrial defects included in the diabetic phenotype, is the up-regulation of PGC-1α (Franks & Loos, 2006) which is normally down-regulated in skeletal muscle from diabetic patients and may largely account for the diminished oxidative capacity of these patients (McCarty, 2005; Tanaka et al., 2003). In this regard, feasible strategies which up-regulate PGC-1α may be useful for preventing and treating diabetes. The strategies may include exercise,

thiazolidinediones, metformin and other agents capable to stimulate AMP kinase or serve as PPARγ agonists (see McCarty, 2005). The following sections will therefore focus on some described potential targets by which exercise can be considered an important therapeutic or preventive agent against the "mitochondrionopathy" that characterize the diabetic phenotype. Mitochondrial dysfunction markers include the diminished mitochondrial content/density and the down-regulated expression of skeletal muscle UCP3.

3. POTENTIAL MECHANISMS AND TARGETS BY WHICH EXERCISE CAN COUNTERACT DIABETES-INDUCED MITOCHONDRIAL IMPAIRMENTS

3.1. EXERCISE-INDUCED MITOCHONDRIAL BIOGENESIS

It is now established that mitochondrial dysfunction, caused by either nuclear or DNA mutations, can lead to a wide variety of pathophysiological conditions, including diabetes. Fortunately, muscle tissue has a remarkable capacity to alter the gene expression profile and phenotype in response to changes in functional demands imposed by several stimuli. Regularly performed exercise in the form of endurance training induces, among others, a well-established muscle adaptation termed mitochondrial biogenesis. The phenomenon can be visualised by electron microscopy as an increase in mitochondrial content in both subsarcolemal and intermyofibrilar regions of skeletal or cardiac myocytes. It is known that mitochondrial biogenesis acts preferentially in subsarcolemal mitochondria, with consequent increase in the ratio of mitochondrial to myofibrillar volume density (Frenzel et al., 1988; Hoppeler & Fluck, 2003).

Presently, the sedentary and/or diseased population possesses increased tolerance against deleterious effects of pathologies, with consequent enhanced quality of life and with greater functional independency. The adaptability of mitochondria allows us to be optimistic about the role of exercise in preventing the appearance of markers of mitochondrial dysfunction, which is typical of several pathologies (*viz,* diabetes). In this regard, understanding the cellular mechanisms that govern the increase in mitochondrial volume with repeated bouts of exercise can provide new insights into possible preventive and therapeutic interventions.

3.1.1. Transcriptional regulatory mechanisms

The biogenesis and function of mitochondria rely upon the regulated expression of a large number of genes, most of which reside in the nuclear genome. The protein-coding capacity of

mitochondrial DNA (mtDNA) is limited to 13 subunits of the mitochondrial respiratory chain. Nuclear regulation plays thus an important role in governing nuclear-mitochondrial interactions. Signalling events related with muscle contraction and involved in mitochondrial biogenesis are known to activate protein kinases and phosphatases. Such proteins can modify the activity of some DNA-binding transcription factors acting in the nucleus, as well as mRNA stability factors acting within the cytosol (Figure 2) resulting in increased mRNA expression of nuclear-encoded mitochondrial proteins (NEMP) (for references see Adhihetty, Irrcher, Joseph, Ljubicic, & Hood, 2003; Hood, 2001; Irrcher, Adhihetty, Joseph, Ljubicic, & Hood, 2003). New mitochondrial proteins are targeted to mitochondria and imported into different organelle locations, such as the matrix or the inner and outer membranes (Figure 3) (Hood, 2001; Hood et al., 2003). A small number of NEMP include transcription factors that act directly on mtDNA, increasing mRNA expression of mitochondrial gene products, such as some subunits of the ETC complexes and other mitochondrial enzymes.
Some transcription factors and/or co-factors have been identified as implicated in the phenomenon, including the peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α), the nuclear respiratory factor 1 and 2 (NRF1 and NRF2), specificity protein 1 (Sp1), and the products of the immediate genes, *c-jun* and *c-fos*(Scarpulla, 2002a, 2002b); in fact, some of the proteins described are down-regulated in skeletal muscle from diabetic patients. Contractile activity has been shown to increase mRNA and/or protein contents of several of transcription factors, which is consistent with their roles in mediating phenotypic changes due to exercise (Baar, 2004; Baar et al., 2002; Bergeron et al., 2001; Garnier et al., 2005; Lehman et al., 2000; Murakami, Shimomura, Yoshimura, Sokabe, & Fujitsuka, 1998; Xia, Buja, & McMillin, 1998; Xia, Buja, Scarpulla, & McMillin, 1997). In fact, Patti et al.,(2003) observed that the decreased expression of mitochondrial-related genes was well correlated with whole-body aerobic capacity. Moreover, experimental evidence suggested that the expression of PGC-1α mimic mitochondrial adaptations that follow exercise training, resulting in a decrease of lipid-induced mitochondrial dysfunction (Koves et al., 2005).

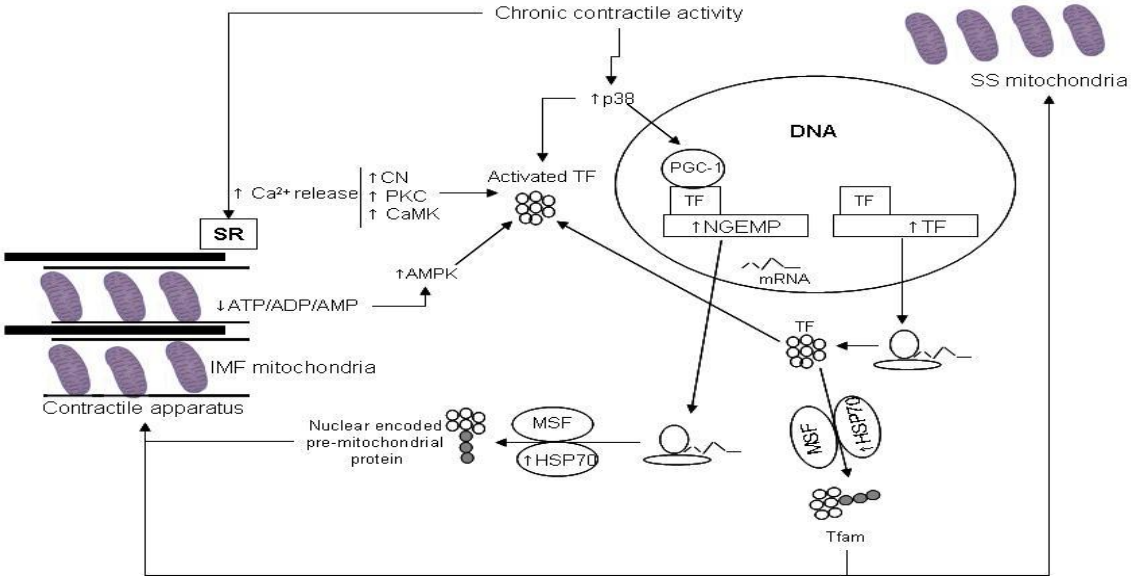

Figure 1 Summary of signalling pathways induced by contractile activity that lead to the expression of transcription factors. The activation of transcription factors is closely involved in the synthesis of mitochondrial proteins and mitochondrial biogenesis. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine monophosphate kinase (AMPK), calcineurin (CN), calcium (Ca^{2+}), Ca^{2+}-calmodulin-activated protein kinase (CaMK), cytosolic heat shock protein 70 (HSP70), intermyofibrillar (IMF) and subsarcolemal (SS) mitochondria, mitochondrial import stimulation factor (MSF), nuclear genes encoding mitochondrial proteins (NGEMP), sarcoplasmatic reticulum (SR), peroxisome proliferators-activator receptor-γ-coactivator (PGC), protein kinase C (PKC), transcription factor (TF), mitochondrial transcription factor A (Tfam).

Even though the quantitative role of mtDNA is relatively small when comparing to the nuclear contribution, it is nevertheless crucial for the function and assembly of a full respiratory chain. One transcription factor involved in the coordination and regulation of mitochondrial proteins and in mtDNA replication is the mitochondrial transcription factor A (Tfam). In fact, Tfam has been shown to be up-regulated (protein, mRNA levels and import into mitochondria) after contractile activity (Bengtsson, Gustafsson, Widegren, Jansson, & Sundberg, 2001; Garnier et al., 2005; Gordon, Rungi, Inagaki, & Hood, 2001). Tfam, the crucial messenger in the crosstalk between nucleus and mitochondria, is a nuclear-encoded DNA-binding protein able to translocate into mitochondria, assuming relevant importance in the efficient transcription of genes of mitochondrial-encoded proteins such as complex IV cytochrome oxidase subunits I-III (Bengtsson, Gustafsson, Widegren, Jansson, & Sundberg, 2001). Although mechanical factors have been reported to increase the expression of signalling molecules potentially involved in mitochondrial plasticity (such as c-Jun

NH_2-terminal kinase-induced activator protein-1, AP-1), high-intensity/low repetitive strength training seems to leave mitochondrial compartment relatively unaffected (Chilibeck, Syrotuik, & Bell, 1999), although this kind of exercise has been proved to be effective in the improvement of insulin sensitivity (Holten et al., 2004).

A literature summary of the main proteins involved in exercise-induced increased mitochondrial biogenesis is provided in table 1.

Table 1. Effect of exercise on some mitochondrial biogenesis-associated markers

Study	Model	Exercise Type	Outcomes
(Goto et al., 2000)	Sprague-Dawley rats	3-7d, 2 times/d, 2h swimming bouts	↑ PGC-1α Mrna
(Bengtsson, Gustafsson, Widegren, Jansson, & Sundberg, 2001)	Healthy humans	4 wk, 4d/wk, 45 min/d, one-legged training	↑ Tfam
(Gordon, Rungi, Inagaki, & Hood, 2001)	Sprague-Dawley rats	14d electric stimulation (10 Hz, 3 h/day,)	↑ Tfam mRNA
(Baar et al., 2002)	Wistar rats	1-5d, 2 times/d, 3h swimming bouts	↑ PGC-1α mRNA and protein, NRF-1 and NRF-2 protein
(Pilegaard, Saltin, & Neufer, 2003)	Healthy humans	4 wk, 5 times/wk, 1h one-legged training	↑ PGC-1α protein and mRNA
(Akimoto et al., 2005)	C57BL/6J mice	4 wk voluntary wheel running	= NRF-1 mRNA ↑ PGC-1α
(Cartoni et al., 2005)	Healthy trained cyclists	10 km cycling time trial	↑ PGC-1α, ERRα mRNA, Mfn1, Mfn2, NRF-2
(Garnier et al., 2005)	Healthy humans with different fitness levels	Not applicable	Correlation of VO_{2max} with PGC-1α, NRF-1, Tfam
(Mahoney, Parise, Melov, Safdar, & Tarnopolsky, 2005)	Healthy sedentary humans	High-intensity cycling until exhaustion	↑ PPAR-γ, NRBF-2, PGC-1α, PPAR-δ
(Russell, Hesselink, Lo, & Schrauwen, 2005)	Healthy humans	2h endurance exercise	↑ PGC-1α and PPAR β/δmRNA
(Terada, Kawanaka, Goto, Shimokawa, & Tabata, 2005)	Sprague-Dawley rats	Intermittent swimming bout	↑ PGC-1α

(Adhihetty, Taivassalo, Haller, Walkinshaw, & Hood, 2007)	Patients with mitochondrial myopathy	Endurance training	= PGC-1α and Tfam
(Wright et al., 2007)	Wistar rats	2 times/d, 3h running bouts or 2h swimming	↑ binding of NRF-1,NRF-2 ↑ PGC-1α

ERRα - oestrogen related receptor α; **Mfn 1** - Mitofusin 1; **Mfn 2** -Mitofusin 2; **NRF 1** - nuclear respiratory binding factor 1; **NRF 2** - nuclear respiratory factor 2; **PGC-1α** peroxisome proliferator-activated receptor (**PPAR**)-γ-coactivator-1α; **Tfam** - mitochondrial transcription factor A; **VO_{2max}** - maximal oxygen uptake; ↑ indicates improvement; **=** indicates unchanged

3.1.2. Protein import machinery

During mitochondrial biogenesis, the majority of mitochondrial proteins derived from the nuclear genome depend on the incorporation of nuclear-encoded matrix and membrane proteins into the organelle through protein import machinery (Figure 2). The targeting of proteins from cytosol to specific mitochondrial compartments can represent a rate-limiting step in the entire process. Most proteins are made-up as “precursor” proteins, having a signal sequence that initially interacts with cytosolic molecular chaperones. Important molecules include cytosolic heat shock proteins (HSP) and the mitochondrial import stimulating factor (MSF) (for a detailed review see Adhihetty, Irrcher, Joseph, Ljubicic, & Hood, 2003; Hood, 2001; Hood et al., 2003; Hood & Joseph, 2004; Irrcher, Adhihetty, Joseph, Ljubicic, & Hood, 2003).

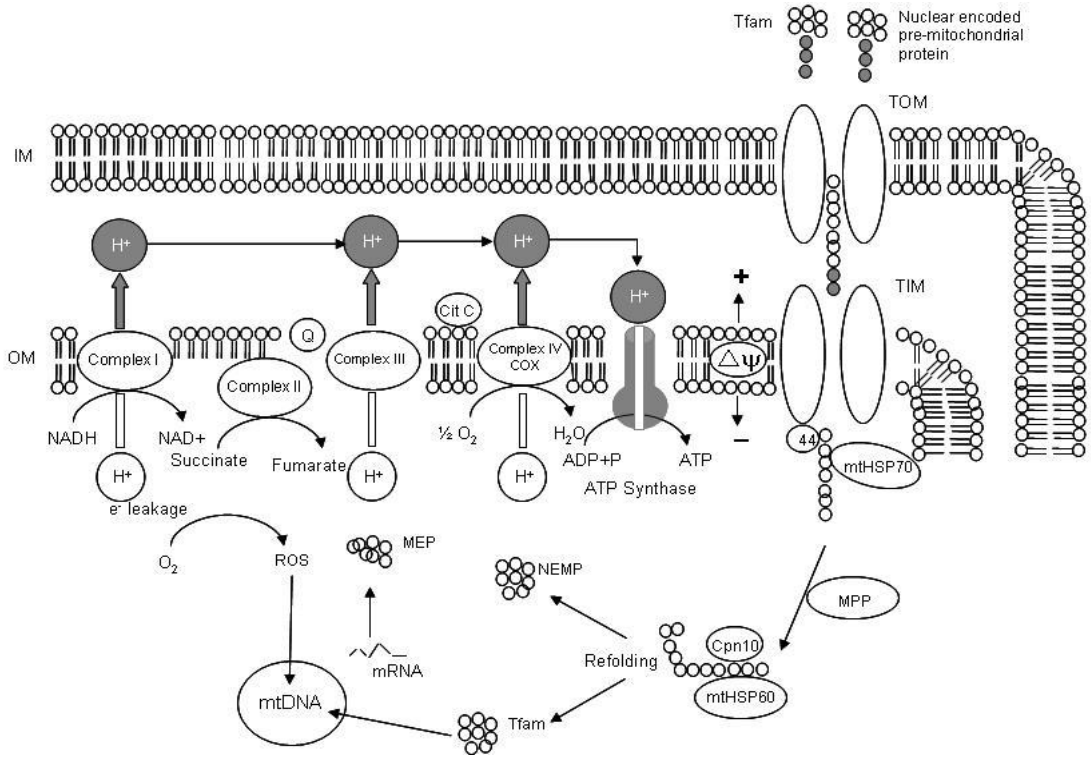

Figure 2 Overview of mitochondrial protein import pathway and the targeting of a matrix protein into mitochondria. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), chaperonin (Cpn), cytochrome C (Cyt C), cytochrome C oxidase (COX), inner membrane (IM), mitochondrial encoded protein (MPP), mitochondrial heat shock protein (mtHSP), mitochondrial processing peptidase (MPP), oxidized nicotine adenine dinucleotide (NAD^+), reduced nicotine adenine dinucleotide (NADH), nuclear-encoded mitochondrial protein (NEMP), outer membrane (OM), reactive oxygen an nitrogen species (RONS), mitochondrial transcription factor A (Tfam), translocase of inner membrane (TIM), Tim 44 (44), translocase of outer membrane (TOM), transmembrane potential (Δψ), ubiquinone (CoQ).

Contractile activity has been shown to increase the expression of some components of the protein import machinery, including MSF, cHSP70, mtHSP70, HSP60, Cpn10 and Tom20 (Hood, Rungi, Colavecchia, Gordon, & Schneider, 2002; Ornatsky, Connor, & Hood, 1995; Takahashi, Chesley, Freyssenet, & Hood, 1998). The effect was also observed in cardiac mitochondria both after thyroid hormone treatment and endurance training-induced mitochondrial biogenesis (Ascensao, Ferreira, Oliveira, & Magalhaes, 2006; Ascensao et al., 2005a; Ascensao et al., 2005b, 2006a; Craig, Chesley, & Hood, 1998; Hamilton et al., 2003; Lennon et al., 2004; Powers et al., 1998). Moreover, exercise-induced regulation of inner membrane cardiolipin content is also a

determinant event related to the assembly of the mitochondrial reticulum by interfering in the rate of protein import capacity (Takahashi & Hood, 1993).
The cross tolerance effect of endurance training against the reduced expression of skeletal and cardiac muscles HSP70 and HSF-1 caused by STZ-induced diabetes was analysed by Atalay et al. (2004). The authors reported that endurance treadmill training may offset some of the adverse effects of diabetes such as the increased oxidative damage and tissue inflammation by upregulating tissue HSP expression. Collectively, the results provide evidence of how chronic exercise and experimentally-induced contractile activity result in an accelerated protein import capacity into muscle mitochondria. It appears logical to assume that the adaptations are advantageous against mitochondrial dysfunction in diabetes.

3.1.3. Mitochondrial turnover and network organization

The increase in mitochondrial mass also depends on the balance between production and degradation of several mitochondrial components. The degradation process is known to be mediated, at least partially, by specific pathways involving intramitochondrial proteins such as the ATP-dependent Lon protease (*Lon P*) (for a detailed review see Bota & Davies, 2001). *Lon P*, which is also expressed in skeletal and cardiac muscle, can control mtDNA replication and gene expression, being involved in mitochondrial biogenesis through a possible role in the proper assembly of mitochondrial proteins by eliminating non-assembled subunits (Luciakova, Sokolikova, Chloupkova, & Nelson, 1999). The significant correlation found by Garnier et al. (2005) between gene expression of *Lon P* and maximal mitochondrial respiratory capacity as well as with PCG-1α mRNA in subjects with different levels of aerobic fitness suggests that degradation process of non-functional mitochondrial protein might participate in mitochondrial adaptation induced by endurance training.
Morphological data have demonstrated that mitochondria are organized into dynamic tubular structures or networks; the balance between continuous fission and fusion regulates the dynamic nature of mitochondria in mammals (Santel et al., 2003; Smirnova, Griparic, Shurland, & van der Bliek, 2001). Among the most cited proteins involved in the fast dynamic, there are two mitochondrial GTPases related to fusion and fission, mitofusin (*Mfn*) (Rojo, Legros, Chateau, & Lombes, 2002; Santel et al., 2003) and dynamin-related protein 1 (*Drp1*), (Legros, Lombes, Frachon, & Rojo, 2002; Smirnova, Griparic, Shurland, & van der Bliek, 2001) respectively. Both proteins

are highly expressed in both skeletal and cardiac muscles.

Recent data reported that alterations in the regulatory pathways of *Mfn* may participate in the pathophysiology of insulin-resistant condition and type 2 diabetes (Bach et al., 2005; Soriano et al., 2006). Bach et al., (Bach et al., 2005) found that muscle *Mfn2* protein expression was reduced in lean and in obese type 2 diabetic patients. A positive correlation between the *Mfn2* expression and the insulin sensitivity was also detected in nondiabetic and type 2 diabetic subjects before and after a weight loss intervention, which increased threefold the muscle *Mfn2* mRNA. The observation suggests that *in vivo* modulation of muscle *Mfn2* levels may be considered another molecular mechanism explaining alterations in mitochondrial function and in insulin sensitivity in obesity or type 2 diabetes.

It was observed that exercise can represent an important stimulus for the up-regulation of muscle *Mfn* expression. The transcript levels of proteins that act to oppose fusion and fission pathways in order to maintain the dynamics of tubular mitochondrial networks were followed in skeletal muscle from subjects with distinct endurance levels (Garnier et al., 2005). The authors observed a coordinated increase in maximal mitochondrial respiratory capacity and mRNA levels of *Mfn2* and *Drp1* was in accordance with the training status of the subjects. The result suggests that mitochondrial dynamics may also be an important process behind the functional adaptations to endurance training. Similar findings were also obtained by Cartoni et al., (2005) regarding the skeletal muscle content in *Mfn1* and *Mfn2* mRNA 24 hours after acute exercise. In this regard, it appears reasonable to presume that the refereed adaptations in mitochondrial turnover are advantageous against mitochondrial impairments observed in diabetes.

3.2. MITOCHONDRIAL UNCOUPLED RESPIRATION – THE ROLE OF UCP3

The coupling between oxygen consumption and ATP synthesis is vital to muscle energetics. However, the coupling is not fully efficient and part of the oxygen consumed is attributed to mitochondrial uncoupling (Brand et al., 1999). Uncoupled respiration (UCR) is associated with the dissipation of the mitochondrial proton gradient as heat, instead of being used as a mediator in ATP synthesis. The mechanisms behind UCR and its physiological importance are not completely understood. One hypothesis is that some mitochondrial proteins, such as uncoupling proteins (UCPs) and the adenine nucleotide translocase (ANT) mediate back-leakage

of protons serving as uncouplers of oxidative phosphorylation (Ricquier & Bouillaud, 2000).

3.2.1. Relevance of UCP for mitochondrial physiology

3.2.1.1. UCP3 as fatty acid peroxides carriers through inner mitochondrial membrane

Concerning its physiological relevance, some research groups (Hesselink et al., 2003; Himms-Hagen & Harper, 2001; Schrauwen, Saris, & Hesselink, 2001) suggested that UCP3 can act as an exporter of fatty acid anions from the mitochondrial matrix when fatty acids delivery to the mitochondria exceeds its capacity to oxidize them, a condition typical of type 2 diabetes pathophysiology in skeletal muscle. In fact, it is suggested by Schrauwen et al., (2001) that although the majority of the non-esterified fatty acids delivery to mitochondria is generally oxidized, in several conditions, such as fasting, high-fat diet, diabetes, obesity, and acute exercise, some may actually accumulate in the mitochondrial matrix. Since non-esterified fatty acids are not able to cross back the inner membrane, their deleterious effects when accumulated in the matrix can be a major reason for the up-regulation of UCP3 described, for example, during acute exercise and diabetes. On the other hand, when fat oxidation capacity is improved, UCP3 expression could be hypothetically down-regulated, which agrees with findings regarding UCP3 modulation through endurance training. The high expression of UCP3 in type IIb muscle fibers (Hesselink et al., 2001) is also consistent with this hypothesis, since these muscle fibers, with a reduced rate of fatty acid oxidation, need to be protected against mitochondrial non-esterified fatty acids accumulation. In contrast, type I fibers have a high capacity to oxidize fatty acids and therefore are less prone to its accumulation; under this reasoning, UCP3 expression was described to be lower (Schrauwen, Saris, & Hesselink, 2001).

However, it is noteworthy that the export of excess fatty acid anions from the matrix to the mitochondrial intermembrane space will also reduce the proton gradient and the transmembrane potential resulting in mild uncoupling as a secondary consequence (Hesselink et al., 2003). Thus, it cannot be excluded at the present that a reduction in UCP3 content would also result in improved energetic efficiency as a side effect.

Additionally, some studies (Goglia & Skulachev, 2003; Schrauwen, Saris, & Hesselink, 2001) have also suggested that UCPs, including UCP3, act to translocate fatty acid peroxides from the inner to the outer membrane leaflet

through a complex flip-flop mechanism (Goglia & Skulachev, 2003). By using a protective mechanism, the inner leaflet is liberated of fatty acid peroxides that otherwise would form very aggressive oxidants damaging mitochondrial DNA and other mitochondrial matrix-localized components of vital importance.

3.2.1.2. UCP3-mediated mild uncoupling decreases mitochondrial RONS production

The modulation of UCP3 content by physical activity, particularly in diabetic patients where basal levels of UCP3 are dramatically diminished, might also be interpreted as an antioxidant defence mechanism. It has been previously suggested that UCP3 reduces the formation of RONS in skeletal muscle (Vidal-Puig et al., 2000). In fact, considering that the mitochondrial membrane potential regulates the production of RONS, the mild uncoupling mediated by UCPs and other proteins (Goglia & Skulachev, 2003) may markedly prevent the hyperpolarization of mitochondrial membranes. In this mechanism, the transmembrane electrochemical gradient would not rise above a critical threshold for RONS formation, decreasing superoxide and hydrogen peroxide production (Goglia & Skulachev, 2003; Korshunov, Skulachev, & Starkov, 1997). Vidal-Puig et al.,(2000) provide evidence that UCP3 KO mice are apparently healthy animals and have increased coupling of mitochondrial respiration in skeletal muscle. Nevertheless, the increased RONS generation found in those mice clearly suggests that one of the functions of UCP3 in skeletal muscle could be the reduction of oxidative stress. Such a defence mechanism may represent a feedback system to limit further generation of RONS under conditions in which mitochondrial membrane potential is higher than required for ATP production, (Brand et al., 2002) as occurs in the post-exercise period. Since oxygen consumption by skeletal muscle remains elevated for several hours after the end of an exercise bout, an attractive antioxidant defence mechanism proposed for UCPs can be delineated (Hildebrandt, Pilegaard, & Neufer, 2003).

3.2.2. Effects of exercise on UCP3 expression

3.2.2.1. Acute exercise

A substantial amount of data has been published from studies conducted with animals and humans on the effect of acute and chronic exercise-induced modulation of muscle UCP3 (Table 2). In rats submitted to 200-min-long intermittent bout of treadmill running or swimming, a 7-fold increased UCP3

mRNA and 5.6-fold UCP3 protein content was observed in skeletal muscle, showing that the acute regulation of UCP3 gene expression by exercise has immediate and functionally important consequences (Zhou, Lin, Coughlin, Vallega, & Pilch, 2000). Kasaoka et al.,(1998) reported that a single 1h bout of treadmill running increased 7-fold the levels of rat skeletal muscle UCP3 mRNA in *gastrocnemius* after 3h. The levels returned to normal within 24h after exercise. Moreover, 2h treadmill running increased rat UCP3 gene expression levels 252% and 63% above the values found for sedentary animals in white and red *gastrocnemius* muscles, respectively (Cortright et al., 1999). Taken together, the studies strongly suggest that the remarkable sensitivity of the UCP3 to acute exercise should serve an important function in skeletal muscle during and after exercise (Table 2).

It is also important to note that in such exercise conditions, plasma levels of free fatty acids (FFA) are increased. Actually, it was previously demonstrated that fasting-induced increase in FFA levels leads *per se* to a rapid and specific regulation of UCP3 mRNA (Tunstall, Mehan, Hargreaves, Spriet, & Cameron-Smith, 2002). In fact, the data suggests that instead of acute exercise *per se*, increased levels of FFA levels and/or fatty acid oxidation seem to be responsible for the up-regulation of UCP3 after acute exercise (Pilegaard, Ordway, Saltin, & Neufer, 2000; 2002). Nevertheless, in contrast with the above reasoning, Busquets et al., (2005) observed in a manipulated model of tumor-induced muscle wasting in mice that the activation of UCP3 gene expression in skeletal muscle can be independent on the circulating levels of FFA.

3.2.2.2. Chronic exercise

Concerning the effect of endurance training on the modulation of UCP3 expression, cross-sectional studies (Russell et al., 2002; Schrauwen et al., 2002; Schrauwen, Troost, Xia, Ravussin, & Saris, 1999) showed that UCP3 mRNA content is significantly lower in endurance-trained athletes than in lean untrained human subjects. The data have also been recently confirmed by evaluating UCP3 protein levels (Russell, Somm et al., 2003; Russell, Wadley et al., 2003). Russell et al., (2003) showed that UCP3 protein expression measured in skeletal muscle biopsies was 46% lower in the trained compared to the untrained group. Also, the authors demonstrated that UCP3 protein expression in the different muscle fibers was expressed as IIx>IIa>I in the fibres for both groups but lower in all fibre types of the endurance trained subjects when compared to sedentary subjects. The result suggests

that training status did not change the skeletal muscle fibre hierarchical UCP3 protein expression. These and other studies revealing the tendency for unchanged or decreased mitochondrial UCP3 upon chronic stimuli such as endurance training or voluntary wheel running are summarized in table 2 (Boss et al., 1998; Cortright et al., 1999; Fernstrom, Tonkonogi, & Sahlin, 2004; Jones, Baar, Ojuka, Chen, & Holloszy, 2003; Schrauwen, Russell, Moonen-Kornips, Boon, & Hesselink, 2005; Tonkonogi, Krook, Walsh, & Sahlin, 2000).

Table 2. Effect of acute and chronic exercise on muscle UCP3

Study	Model	Exercise Type	Major outcomes
		Acute	
(Schrauwen, Troost, Xia, Ravussin, & Saris, 1999)	Healthy trained vs. untrained humans	Cycling exercise until exhaustion	↓ mRNA levels of UCP3 (trained)
(Russell et al., 2002)	Endurance-trained vs. active humans	Cycling VO_2max test	↓ UCP2 and UCP3 mRNA (trained)
(Russell, Wadley et al., 2003)	Trained cyclists vs. Untrained	Cycling VO_2max test	↓ UCP3 mRNA in (trained)
(Zhou, Lin, Coughlin, Vallega, & Pilch, 2000)	Spragley-Dawley rats	Swimming bout of 10-200 min or single bout of running (24m/min).	↑ UCP3 mRNA
(Pilegaard et al., 2002)	Healthy humans	One-legged cycling intermittent and continue exercise (55-90% VO2max) 2h exercise	↑ UCP3 mRNA (glycogen depleted leg)
(Schrauwen et al., 2002)	Healthy humans	(50%VO_2max), 4 h rest with glucose ingestion (Gi) and fasting conditions (F)	↑ UCP3 mRNA (F) = UCP3 mRNA (Gi) = UCP3 protein (GI,F)
		Chronic	
(Boss et al., 1998)	OFA rats	4 wks running, 5 d/wk	↓ UCP2 and UCP3 mRNA
(Hjeltnes, Fernstrom, Zierath, & Krook, 1999)	Tetraplegic humans	8 wks, electric stimulated leg cycle	↓ UCP3 and UCP2 mRNA
(Tonkonogi, Krook, Walsh, & Sahlin, 2000)	Healthy humans	6 wks endurance training	= UCP2 and UCP3 mRNA
(Jones, Baar, Ojuka, Chen, & Holloszy, 2003)	Wistar rats	1,3 and 10d swimming, 2 times/d, 3h bouts	↑ UCP3 mRNA ↑ UCP3
(Russell, Somm et al., 2003)	Healthy active humans	6 wks, endurance (70-80 % VO_2max) or sprint (90-100% maximal speed) training	↓ UCP3 mRNA and protein
(Fernstrom, Tonkonogi, & Sahlin, 2004)	Healthy humans	6 wks intermittent endurance exercise (70-80% VO_2 peak)	= UCP3 and ANT ↓ UCP3 (relative to cytochrome c and citrate synthase)

(Schrauwen, Russell, Moonen-Kornips, Boon, & Hesselink, 2005)	Healthy untrained humans	12d, 2h endurance exercise or 45 min of intermittent exercise.	↓ UCP3

UCP2 – uncoupling protein 2; **UCP3** – uncoupling protein 3; **ANT** – adenine nucleotide translocator; **VO_{2max}** - maximal oxygen uptake; ↑ indicates improvement; ↓ indicates decrement; **=** indicates unchanged

4. CROSS TOLERANCE EFFECT OF ENDURANCE TRAINING AGAINST MITOCHONDRIAL DYSFUNCTION IN DIABETIC MUSCLE

As mentioned above, skeletal muscle from type 2 diabetic patients is characterized by a larger accumulation of intramyocyte triglycerides, reduced mitochondrial ETC capacity and oxidative phosphorylation, increased mitochondrial RONS production and oxidative damage, decreased mitochondrial biogenesis and a diminished UCP3 content (Schrauwen & Hesselink, 2004). Isolated or in combination, the possibly interconnected data may suggest that in these type 2 diabetic patients, as well as in the elderly and in the obesity, the low levels of UCP3 fail to sufficiently counteract mitochondrial RONS generation and to export fatty acid anions and/or peroxides, ultimately leading to lipid peroxide-induced mitochondrial damage and dysfunction. Fortunately, the low UCP3 levels in diabetic patients increased significantly in response to low intensity exercise program; interestingly, the up-regulation paralleled with the improvement of several clinical parameters (Fritz et al., 2006). In the study, the increase in UCP3 protein and mRNA contents was also accompanied by the up-regulation of PPAR γ protein and mRNA levels as well as NRF1 mRNA. Whether the augmented UCP3 protein and mRNA levels is a result of the increased mitochondrial mass is still a matter of discussion (Hesselink, Schrauwen, Holloszy, & Jones, 2003; Jones, Baar, Ojuka, Chen, & Holloszy, 2003).

As AMPK has been identified as a potential target for the treatment of insulin resistance and type 2 diabetes (Winder & Hardie, 1999) and since the stimulation of the AMPK pathway leads to increased mitochondrial biogenesis and function (Bergeron et al., 2001). Sriwijitkamol et al, (2006) determined whether sedentary obese insulin-resistant Zucker rats have abnormalities in the *gastrocnemius* AMPK signaling pathway (LKB1-AMPK-PGC-1). The authors also examined whether 7 wk of exercise training on a treadmill reversed the possible abnormalities in the AMPK pathway. The results showed that the decreased AMPK phosphorylation, AMPK kinase LKB1 expression and PGC-1α protein decrease in sedentary obese

insulin-resistant Zucker rats were restored by endurance training. However, in a recent effort to analyse the effect of exercise intensity (50 vs. 70% VO_2max) on AMPK stimulation, at different signalling intermediate steps, spanning from the upstream kinase, LKB1, to the putative AMPK target, PGC-1α, a study from the same research group provided evidence that low intensity exercise increased the expression of PGC-1α in type 2 diabetes, and this effect does not appear to require significant AMPK stimulation (Sriwijitkamol et al., 2007). The finding highlights the utility of exercise to ameliorate molecular defects in type 2 diabetes.

The impact of 16 weeks of moderate-intensity aerobic exercise training on age-related changes in skeletal muscle oxidative capacity from insulin-resistant patients was analyzed by Short et al. (2003). The training program increased peak oxygen uptake, the activity of some muscle mitochondrial enzymes (citrate synthase and cytochrome c oxidase) as well as mRNA levels of selected genes involved in mitochondrial biogenesis (PGC-1α, NRF-1 and Tfam), which are usually down-regulated in diseased conditions. Moreover, a combined intervention of weight loss and physical activity in sedentary obese adults with insulin resistance was associated with enlargement of intermyofibrillar mitochondria from *vastus lateralis*, resulting in a significant increase in myofiber volume occupied by mitochondria after intervention (Toledo, Watkins, & Kelley, 2006). Recent data from the same research group showed that a similar intervention of chronic exercise and weight loss resulted in significant increases in mitochondrial density and size, cardiolipin content as well as in the activity of citrate synthase and NADH oxidase (Toledo et al., 2007). The results indicate that intensive short-term lifestyle modifications improve mitochondrial metabolism and oxidative functional capacity by restoring mitochondrial content and paralleled with enhanced insulin sensitivity in these patients (Short et al., 2003; Toledo et al., 2007; Toledo, Watkins, & Kelley, 2006). Respiratory data from *gastrocnemius* and red *vastus lateralis*-derived mitochondria of STZ-treated rats demonstrated that the overall capacity to oxidize substrates and produce energy also appeared to be greatly increased by endurance treadmill training in a experimental diabetic group (el Midaoui, Tancrede, & Nadeau, 1996). An histological-based study conducted by Searls et al., (2004) indicated that a program of 9 weeks of endurance treadmill training attenuated the left ventricle ultrastructural changes observed in STZ-induced diabetes, including signs of mitochondrial damage such as

mitochondrial swelling and disrupted inner and outer membranes. The morphological improvements induced by endurance training in heart mitochondria from STZ-treated rats are in agreement with an enhanced response of heart mitochondrial respiratory parameters reported by Mokhtar et al., (1993). Adaptative responses, suggestive of increased mitochondrial function in diabetic muscle, would also benefit lipid metabolism, avoiding the intramitochondrial accumulation of fatty acid anions and/or peroxides as well as RONS-induced mitochondrial DNA, mRNA and enzyme damage, certainly contributing to attenuate diabetic phenotype.

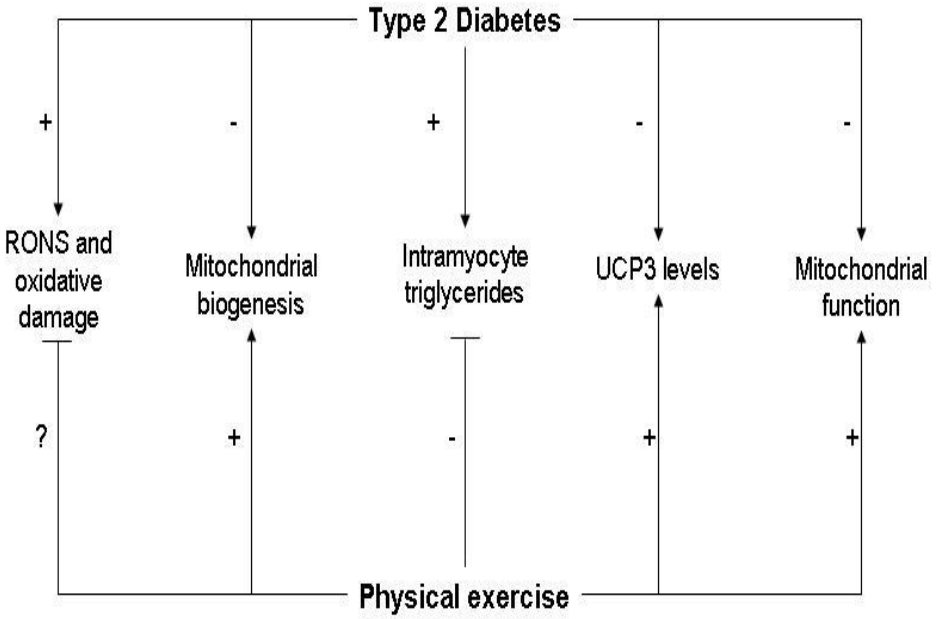

Figure 3 Summary of the proposed mechanisms by which exercise modulates mitochondrial function in subjects with diabetes. We propose that alterations of morphological, biochemical and functional characteristics of muscle mitochondria found in diabetic subjects might be attenuated or even prevented by chronic exercise, particularly endurance training. The alterations may include production of increased reactive oxygen and nitrogen species (RONS) with consequent oxidative damage, diminished mitochondrial density, elevated intramyocyte content of triglycerides and reduced uncoupling proteins (UCP) levels, resulting in diabetic "mitochondrionopathy".

5. CONCLUDING REMARKS

As can be depicted from figure 4, diabetes is generally characterized by diminished density, accumulation of intramyocyte triglycerides, enhanced mitochondrial RONS generation, leading to increased oxidative damage of mitochondrial proteins and

phospholipids, reduced levels of skeletal muscle UCP and reduced mitochondrial turnover. Biochemical end-points studied, associated with marked mitochondrial morphological disturbances, are suggestive of mitochondrial dysfunction. Physical exercise is a physiological stimulus able to induce some mitochondrial adaptations that are favourable for counteracting the referred impairments in diabetic muscle mitochondria. Some critical targets of the modulator role of exercise as an important intervention against diabetes-induced mitochondrionopathy involve the increased expression of PGC-1α and HSP, both severely down-regulated in skeletal muscle of diabetic patients and increase in UCP3 content. How exercise alters mitochondrial respiratory parameters, mitochondrial antioxidant systems as well as other specific important proteins involved in mitochondrial metabolism should be the subject of further research.

REFERENCES

Adhihetty, P. J., Irrcher, I., Joseph, A. M., Ljubicic, V., & Hood, D. A. (2003). Plasticity of skeletal muscle mitochondria in response to contractile activity. *Exp Physiol, 88*(1), 99-107.

Adhihetty, P. J., Taivassalo, T., Haller, R. G., Walkinshaw, D. R., & Hood, D. A. (2007). The effect of training on the expression of mitochondrial biogenesis- and apoptosis-related proteins in skeletal muscle of patients with mtDNA defects. *Am J Physiol Endocrinol Metab, 293*(3), E672-680.

Akimoto, T., Pohnert, S. C., Li, P., Zhang, M., Gumbs, C., Rosenberg, P. B., et al. (2005). Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J Biol Chem, 280*(20), 19587-19593.

Antonetti, D. A., Reynet, C., & Kahn, C. R. (1995). Increased expression of mitochondrial-encoded genes in skeletal muscle of humans with diabetes mellitus. *J Clin Invest, 95*(3), 1383-1388.

Ascensao, A., Ferreira, R., & Magalhaes, J. (2007). Exercise-induced cardioprotection - biochemical, morphological and functional evidence in whole tissue and isolated mitochondria. *Int J Cardiol, 117*, 16-30.

Ascensao, A., Ferreira, R., Oliveira, P. J., & Magalhaes, J. (2006). Effects of endurance training and acute Doxorubicin treatment on rat heart mitochondrial alterations induced by in vitro anoxia-reoxygenation. *Cardiovasc Toxicol, 6*(3-4), 159-172.

Ascensao, A., Magalhaes, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., et al. (2005a). Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. *Int J Cardiol, 100*(3), 451-460.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., et al. (2005b). Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *Am J Physiol Heart Circ Physiol, 289*(2), H722-731.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., et al. (2006). Endurance training limits the functional alterations of heart rat mitochondria submitted to in vitro anoxia-reoxygenation. *Int J Cardiol, 109*(2), 169-178.

Atalay, M., Oksala, N. K., Laaksonen, D. E., Khanna, S., Nakao, C., Lappalainen, J., et al. (2004). Exercise training modulates heat shock protein response in diabetic rats. *J Appl Physiol, 97*(2), 605-611.

Baar, K. (2004). Involvement of PPAR gamma co-activator-1, nuclear respiratory factors 1 and 2, and PPAR alpha in the adaptive response to endurance exercise. *Proc Nutr Soc, 63*(2), 269-273.

Baar, K., Wende, A. R., Jones, T. E., Marison, M., Nolte, L. A., Chen, M., et al. (2002). Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *Faseb J, 16*(14), 1879-1886.

Bach, D., Naon, D., Pich, S., Soriano, F. X., Vega, N., Rieusset, J., et al. (2005). Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6. *Diabetes, 54*(9), 2685-2693.

Befroy, D. E., Petersen, K. F., Dufour, S., Mason, G. F., de Graaf, R. A., Rothman, D. L., et al. (2007). Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. *Diabetes, 56*(5), 1376-1381.

Bengtsson, J., Gustafsson, T., Widegren, U., Jansson, E., & Sundberg, C. J. (2001). Mitochondrial transcription factor A and respiratory complex IV increase in response to exercise training in humans. *Pflugers Arch, 443*(1), 61-66.

Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., et al. (2001). Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am J Physiol Endocrinol Metab, 281*(6), E1340-1346.

Boss, O., Samec, S., Desplanches, D., Mayet, M. H., Seydoux, J., Muzzin, P., et al. (1998). Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 in the rat. *Faseb J, 12*(3), 335-339.

Bota, D. A., & Davies, K. J. (2001). Protein degradation in mitochondria: implications for oxidative stress, aging and disease: a novel etiological classification of mitochondrial proteolytic disorders. *Mitochondrion, 1*(1), 33-49.

Brand, M. D., Brindle, K. M., Buckingham, J. A., Harper, J. A., Rolfe, D. F., & Stuart, J. A. (1999). The significance and mechanism of mitochondrial proton conductance. *Int J Obes Relat Metab Disord, 23 Suppl 6*, S4-11.

Brand, M. D., Pamplona, R., Portero-Otin, M., Requena, J. R., Roebuck, S. J., Buckingham, J. A., et al. (2002). Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3. *Biochem J, 368*(Pt 2), 597-603.

Brookes, P. S. (2005). Mitochondrial H(+) leak and ROS generation: an odd couple. *Free Radic Biol Med, 38*(1), 12-23.

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol, 287*(4), C817-833.

Brown, G. C., & Borutaite, V. (2001). Nitric oxide, mitochondria, and cell death. *IUBMB Life, 52*(3-5), 189-195.

Brown, G. C., & Borutaite, V. (2002). Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radic Biol Med, 33*(11), 1440-1450.

Busquets, S., Almendro, V., Barreiro, E., Figueras, M., Argiles, J. M., & Lopez-Soriano, F. J. (2005). Activation of UCPs gene expression in skeletal muscle can be independent on both circulating fatty acids and food intake. Involvement of ROS in a model of mouse cancer cachexia. *FEBS Lett, 579*(3), 717-722.

Cadenas, E. (2004). Mitochondrial free radical production and cell signaling. *Mol Aspects Med, 25*(1-2), 17-26.

Cartoni, R., Leger, B., Hock, M. B., Praz, M., Crettenand, A., Pich, S., et al. (2005). Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. *J Physiol, 567*(Pt 1), 349-358.

Chilibeck, P. D., Syrotuik, D. G., & Bell, G. J. (1999). The effect of strength training on estimates of mitochondrial density and distribution throughout muscle fibres. *Eur J Appl Physiol Occup Physiol, 80*(6), 604-609.

Cortright, R. N., Zheng, D., Jones, J. P., Fluckey, J. D., DiCarlo, S. E., Grujic, D., et al. (1999). Regulation of skeletal muscle UCP-2 and UCP-3 gene expression by exercise and denervation. *Am J Physiol, 276*(1 Pt 1), E217-221.

Craig, E. E., Chesley, A., & Hood, D. A. (1998). Thyroid hormone modifies mitochondrial phenotype by increasing protein import without altering degradation. *Am J Physiol, 275*(6 Pt 1), C1508-1515.

Duchen, M. R. (2004). Roles of mitochondria in health and disease. *Diabetes, 53 Suppl 1*, S96-102.

el Midaoui, A., Tancrede, G., & Nadeau, A. (1996). Effect of physical training on mitochondrial function in skeletal muscle of normal and diabetic rats. *Metabolism, 45*(7), 810-816.

Fernstrom, M., Tonkonogi, M., & Sahlin, K. (2004). Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle. *J Physiol, 554*(Pt 3), 755-763.

Fosslien, E. (2001). Mitochondrial medicine--molecular pathology of defective oxidative phosphorylation. *Ann Clin Lab Sci, 31*(1), 25-67.

Franks, P. W., & Loos, R. J. (2006). PGC-1alpha gene and physical activity in type 2 diabetes mellitus. *Exerc Sport Sci Rev, 34*(4), 171-175.

Frenzel, H., Schwartzkopff, B., Holtermann, W., Schnurch, H. G., Novi, A., & Hort, W. (1988). Regression of cardiac hypertrophy: morphometric and biochemical studies in rat heart after swimming training. *J Mol Cell Cardiol, 20*(8), 737-751.

Fritz, T., Kramer, D. K., Karlsson, H. K., Galuska, D., Engfeldt, P., Zierath, J. R., et al. (2006). Low-intensity exercise increases skeletal muscle protein expression of PPARdelta and UCP3 in type 2 diabetic patients. *Diabetes Metab Res Rev*.

Garnier, A., Fortin, D., Zoll, J., N'Guessan, B., Mettauer, B., Lampert, E., et al. (2005). Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. *Faseb J, 19*(1), 43-52.

Goglia, F., & Skulachev, V. P. (2003). A function for novel uncoupling proteins: antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer membrane leaflet. *Faseb J, 17*(12), 1585-1591.

Gordon, J. W., Rungi, A. A., Inagaki, H., & Hood, D. A. (2001). Effects of contractile activity on mitochondrial transcription factor A expression in skeletal muscle. *J Appl Physiol, 90*(1), 389-396.

Goto, M., Terada, S., Kato, M., Katoh, M., Yokozeki, T., Tabata, I., et al. (2000). cDNA Cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. *Biochem Biophys Res Commun, 274*(2), 350-354.

Hamilton, K. L., Staib, J. L., Phillips, T., Hess, A., Lennon, S. L., & Powers, S. K. (2003). Exercise, antioxidants, and HSP72: protection against myocardial ischemia/reperfusion. *Free Radic Biol Med, 34*(7), 800-809.

He, J., Goodpaster, B. H., & Kelley, D. E. (2004). Effects of weight loss and physical activity on muscle lipid content and droplet size. *Obes Res, 12*(5), 761-769.

Henriksen, E. J. (2002). Invited review: Effects of acute exercise and exercise training on insulin resistance. *J Appl Physiol, 93*(2), 788-796.

Hesselink, M. K., Greenhaff, P. L., Constantin-Teodosiu, D., Hultman, E., Saris, W. H., Nieuwlaat, R., et al. (2003). Increased uncoupling protein 3 content does not affect mitochondrial function in human skeletal muscle in vivo. *J Clin Invest, 111*(4), 479-486.

Hesselink, M. K., Keizer, H. A., Borghouts, L. B., Schaart, G., Kornips, C. F., Slieker, L. J., et al. (2001). Protein expression of

UCP3 differs between human type 1, type 2a, and type 2b fibers. *Faseb J, 15*(6), 1071-1073.

Hesselink, M. K., Schrauwen, P., Holloszy, J. O., & Jones, T. E. (2003). Divergent effects of acute exercise and endurance training on UCP3 expression. *Am J Physiol Endocrinol Metab, 284*(2), E449-450; author reply 450-441.

Hildebrandt, A. L., Pilegaard, H., & Neufer, P. D. (2003). Differential transcriptional activation of select metabolic genes in response to variations in exercise intensity and duration. *Am J Physiol Endocrinol Metab, 285*(5), E1021-1027.

Himms-Hagen, J., & Harper, M. E. (2001). Physiological role of UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis. *Exp Biol Med (Maywood), 226*(2), 78-84.

Hjeltnes, N., Fernstrom, M., Zierath, J. R., & Krook, A. (1999). Regulation of UCP2 and UCP3 by muscle disuse and physical activity in tetraplegic subjects. *Diabetologia, 42*(7), 826-830.

Hojlund, K., Wrzesinski, K., Larsen, P. M., Fey, S. J., Roepstorff, P., Handberg, A., et al. (2003). Proteome analysis reveals phosphorylation of ATP synthase beta - subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. *J Biol Chem, 278*(12), 10436-10442.

Holten, M. K., Zacho, M., Gaster, M., Juel, C., Wojtaszewski, J. F., & Dela, F. (2004). Strength training increases insulin-mediated glucose uptake, GLUT4 content, and insulin signaling in skeletal muscle in patients with type 2 diabetes. *Diabetes, 53*(2), 294-305.

Hood, D., Rungi, A., Colavecchia, M., Gordon, J., & Schneider, J. (2002). Stress proteins and mitochondria. In M. Locke & E. Noble (Eds.), *Exercise and stress response - The role of stress proteins* (pp. 151-162). Boca Raton - Florida: CRC Press.

Hood, D. A. (2001). Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J Appl Physiol, 90*(3), 1137-1157.

Hood, D. A., Adhihetty, P. J., Colavecchia, M., Gordon, J. W., Irrcher, I., Joseph, A. M., et al. (2003). Mitochondrial biogenesis and the role of the protein import pathway. *Med Sci Sports Exerc, 35*(1), 86-94.

Hood, D. A., & Joseph, A. M. (2004). Mitochondrial assembly: protein import. *Proc Nutr Soc, 63*(2), 293-300.

Hoppeler, H., & Fluck, M. (2003). Plasticity of skeletal muscle mitochondria: structure and function. *Med Sci Sports Exerc, 35*(1), 95-104.

International Diabetes Federation. (2003). *Diabetes Atlas 2003* (2nd ed.). Brussels: International Diabetes Federation.

Irrcher, I., Adhihetty, P. J., Joseph, A. M., Ljubicic, V., & Hood, D. A. (2003). Regulation of mitochondrial biogenesis in muscle by endurance exercise. *Sports Med, 33*(11), 783-793.

Jones, T. E., Baar, K., Ojuka, E., Chen, M., & Holloszy, J. O. (2003). Exercise induces an increase in muscle UCP3 as a component of the increase in mitochondrial biogenesis. *Am J Physiol Endocrinol Metab, 284*(1), E96-101.

Kayali, R., Cakatay, U., Telci, A., Akcay, T., Sivas, A., & Altug, T. (2004). Decrease in mitochondrial oxidative protein damage parameters in the streptozotocin-diabetic rat. *Diabetes Metab Res Rev, 20*(4), 315-321.

Kelley, D. E., He, J., Menshikova, E. V., & Ritov, V. B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes, 51*(10), 2944-2950.

Korshunov, S. S., Skulachev, V. P., & Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett, 416*(1), 15-18.

Koves, T. R., Li, P., An, J., Akimoto, T., Slentz, D., Ilkayeva, O., et al. (2005). Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-

induced mitochondrial inefficiency. *J Biol Chem, 280*(39), 33588-33598.

Legros, F., Lombes, A., Frachon, P., & Rojo, M. (2002). Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. *Mol Biol Cell, 13*(12), 4343-4354.

Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M., & Kelly, D. P. (2000). Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest, 106*(7), 847-856.

Lennon, S. L., Quindry, J. C., French, J. P., Kim, S., Mehta, J. L., & Powers, S. K. (2004). Exercise and myocardial tolerance to ischaemia-reperfusion. *Acta Physiol Scand, 182*(2), 161-169.

Luciakova, K., Sokolikova, B., Chloupkova, M., & Nelson, B. D. (1999). Enhanced mitochondrial biogenesis is associated with increased expression of the mitochondrial ATP-dependent Lon protease. *FEBS Lett, 444*(2-3), 186-188.

Luft, R. (1994). The development of mitochondrial medicine. *Proc Natl Acad Sci U S A, 91*(19), 8731-8738.

Mahoney, D. J., Parise, G., Melov, S., Safdar, A., & Tarnopolsky, M. A. (2005). Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *Faseb J, 19*(11), 1498-1500.

McCarty, M. F. (2005). Up-regulation of PPARgamma coactivator-1alpha as a strategy for preventing and reversing insulin resistance and obesity. *Med Hypotheses, 64*(2), 399-407.

Mogensen, M., Sahlin, K., Fernstrom, M., Glintborg, D., Vind, B. F., Beck-Nielsen, H., et al. (2007). Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. *Diabetes, 56*(6), 1592-1599.

Mokhtar, N., Lavoie, J. P., Rousseau-Migneron, S., & Nadeau, A. (1993). Physical training reverses defect in mitochondrial energy production in heart of chronically diabetic rats. *Diabetes, 42*(5), 682-687.

Mootha, V. K., Lindgren, C. M., Eriksson, K. F., Subramanian, A., Sihag, S., Lehar, J., et al. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet, 34*(3), 267-273.

Morino, K., Petersen, K. F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., et al. (2005). Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest, 115*(12), 3587-3593.

Murakami, T., Shimomura, Y., Yoshimura, A., Sokabe, M., & Fujitsuka, N. (1998). Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. *Biochim Biophys Acta, 1381*(1), 113-122.

Oliveira, P. J., Rolo, A. P., Seica, R., Palmeira, C. M., Santos, M. S., & Moreno, A. J. (2001). Decreased susceptibility of heart mitochondria from diabetic GK rats to mitochondrial permeability transition induced by calcium phosphate. *Biosci Rep, 21*(1), 45-53.

Oliveira, P. J., Seica, R., Santos, D. L., Rolo, A. P., Sardao, V. A., Ferreira, F. M., et al. (2004). Vitamin E or coenzyme Q10 administration is not fully advantageous for heart mitochondrial function in diabetic goto kakizaki rats. *Mitochondrion, 3*(6), 337-345.

Ornatsky, O. I., Connor, M. K., & Hood, D. A. (1995). Expression of stress proteins and mitochondrial chaperonins in chronically stimulated skeletal muscle. *Biochem J, 311 (Pt 1)*, 119-123.

Patti, M. E., Butte, A. J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., et al. (2003). Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A, 100*(14), 8466-8471.

Petersen, K. F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D. L., et al. (2003). Mitochondrial dysfunction in the

elderly: possible role in insulin resistance. *Science, 300*(5622), 1140-1142.

Petersen, K. F., Dufour, S., Befroy, D., Garcia, R., & Shulman, G. I. (2004). Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med, 350*(7), 664-671.

Pilegaard, H., Keller, C., Steensberg, A., Helge, J. W., Pedersen, B. K., Saltin, B., et al. (2002). Influence of pre-exercise muscle glycogen content on exercise-induced transcriptional regulation of metabolic genes. *J Physiol, 541*(Pt 1), 261-271.

Pilegaard, H., Ordway, G. A., Saltin, B., & Neufer, P. D. (2000). Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab, 279*(4), E806-814.

Pilegaard, H., Saltin, B., & Neufer, P. D. (2003). Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol, 546*(Pt 3), 851-858.

Powers, S. K., Demirel, H. A., Vincent, H. K., Coombes, J. S., Naito, H., Hamilton, K. L., et al. (1998). Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. *Am J Physiol, 275*(5 Pt 2), R1468-1477.

Rahimi, R., Nikfar, S., Larijani, B., & Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomed Pharmacother, 59*(7), 365-373.

Ricquier, D., & Bouillaud, F. (2000). Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *J Physiol, 529 Pt 1*, 3-10.

Ritov, V. B., Menshikova, E. V., He, J., Ferrell, R. E., Goodpaster, B. H., & Kelley, D. E. (2005). Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes, 54*(1), 8-14.

Roberts, C. K., Won, D., Pruthi, S., Lin, S. S., & Barnard, R. J. (2006). Effect of a diet and exercise intervention on oxidative stress, inflammation and monocyte adhesion in diabetic men. *Diabetes Res Clin Pract*.

Roglic, G., Unwin, N., Bennett, P. H., Mathers, C., Tuomilehto, J., Nag, S., et al. (2005). The burden of mortality attributable to diabetes: realistic estimates for the year 2000. *Diabetes Care, 28*(9), 2130-2135.

Rojo, M., Legros, F., Chateau, D., & Lombes, A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J Cell Sci, 115*(Pt 8), 1663-1674.

Russell, A., Wadley, G., Snow, R., Giacobino, J. P., Muzzin, P., Garnham, A., et al. (2002). Slow component of [V]O(2) kinetics: the effect of training status, fibre type, UCP3 mRNA and citrate synthase activity. *Int J Obes Relat Metab Disord, 26*(2), 157-164.

Russell, A. P., Hesselink, M. K., Lo, S. K., & Schrauwen, P. (2005). Regulation of metabolic transcriptional co-activators and transcription factors with acute exercise. *Faseb J, 19*(8), 986-988.

Russell, A. P., Somm, E., Praz, M., Crettenand, A., Hartley, O., Melotti, A., et al. (2003). UCP3 protein regulation in human skeletal muscle fibre types I, IIa and IIx is dependent on exercise intensity. *J Physiol, 550*(Pt 3), 855-861.

Russell, A. P., Wadley, G., Hesselink, M. K., Schaart, G., Lo, S., Leger, B., et al. (2003). UCP3 protein expression is lower in type I, IIa and IIx muscle fiber types of endurance-trained compared to untrained subjects. *Pflugers Arch, 445*(5), 563-569.

Santel, A., Frank, S., Gaume, B., Herrler, M., Youle, R. J., & Fuller, M. T. (2003). Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. *J Cell Sci, 116*(Pt 13), 2763-2774.

Scarpulla, R. C. (2002a). Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim Biophys Acta, 1576*(1-2), 1-14.

Scarpulla, R. C. (2002b). Transcriptional activators and coactivators in the nuclear

control of mitochondrial function in mammalian cells. *Gene, 286*(1), 81-89.

Schrauwen, P., & Hesselink, M. K. (2004). Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. *Diabetes, 53*(6), 1412-1417.

Schrauwen, P., Hesselink, M. K., Vaartjes, I., Kornips, E., Saris, W. H., Giacobino, J. P., et al. (2002). Effect of acute exercise on uncoupling protein 3 is a fat metabolism-mediated effect. *Am J Physiol Endocrinol Metab, 282*(1), E11-17.

Schrauwen, P., Russell, A. P., Moonen-Kornips, E., Boon, N., & Hesselink, M. K. (2005). Effect of 2 weeks of endurance training on uncoupling protein 3 content in untrained human subjects. *Acta Physiol Scand, 183*(3), 273-280.

Schrauwen, P., Saris, W. H., & Hesselink, M. K. (2001). An alternative function for human uncoupling protein 3: protection of mitochondria against accumulation of nonesterified fatty acids inside the mitochondrial matrix. *Faseb J, 15*(13), 2497-2502.

Schrauwen, P., Troost, F. J., Xia, J., Ravussin, E., & Saris, W. H. (1999). Skeletal muscle UCP2 and UCP3 expression in trained and untrained male subjects. *Int J Obes Relat Metab Disord, 23*(9), 966-972.

Searls, Y. M., Smirnova, I. V., Fegley, B. R., & Stehno-Bittel, L. (2004). Exercise attenuates diabetes-induced ultrastructural changes in rat cardiac tissue. *Med Sci Sports Exerc, 36*(11), 1863-1870.

Shen, X., Zheng, S., Thongboonkerd, V., Xu, M., Pierce, W. M., Jr., Klein, J. B., et al. (2004). Cardiac mitochondrial damage and biogenesis in a chronic model of type 1 diabetes. *Am J Physiol Endocrinol Metab, 287*(5), E896-905.

Short, K. R., Vittone, J. L., Bigelow, M. L., Proctor, D. N., Rizza, R. A., Coenen-Schimke, J. M., et al. (2003). Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes, 52*(8), 1888-1896.

Skulachev, V. P. (1999). Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. *Mol Aspects Med, 20*(3), 139-184.

Smirnova, E., Griparic, L., Shurland, D. L., & van der Bliek, A. M. (2001). Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell, 12*(8), 2245-2256.

Soriano, F. X., Liesa, M., Bach, D., Chan, D. C., Palacin, M., & Zorzano, A. (2006). Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2. *Diabetes, 55*(6), 1783-1791.

Sreekumar, R., Halvatsiotis, P., Schimke, J. C., & Nair, K. S. (2002). Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes, 51*(6), 1913-1920.

Sriwijitkamol, A., Coletta, D. K., Wajcberg, E., Balbontin, G. B., Reyna, S. M., Barrientes, J., et al. (2007). Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. *Diabetes, 56*(3), 836-848.

Sriwijitkamol, A., Ivy, J. L., Christ-Roberts, C., DeFronzo, R. A., Mandarino, L. J., & Musi, N. (2006). LKB1-AMPK signaling in muscle from obese insulin-resistant Zucker rats and effects of training. *Am J Physiol Endocrinol Metab, 290*(5), E925-932.

Takahashi, M., Chesley, A., Freyssenet, D., & Hood, D. A. (1998). Contractile activity-induced adaptations in the mitochondrial protein import system. *Am J Physiol, 274*(5 Pt 1), C1380-1387.

Takahashi, M., & Hood, D. A. (1993). Chronic stimulation-induced changes in mitochondria and performance in rat skeletal muscle. *J Appl Physiol, 74*(2), 934-941.

Tanaka, T., Yamamoto, J., Iwasaki, S., Asaba, H., Hamura, H., Ikeda, Y., et al. (2003). Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle

and attenuates metabolic syndrome. *Proc Natl Acad Sci U S A, 100*(26), 15924-15929.

Terada, S., Kawanaka, K., Goto, M., Shimokawa, T., & Tabata, I. (2005). Effects of high-intensity intermittent swimming on PGC-1alpha protein expression in rat skeletal muscle. *Acta Physiol Scand, 184*(1), 59-65.

Toledo, F. G., Menshikova, E. V., Ritov, V. B., Azuma, K., Radikova, Z., DeLany, J., et al. (2007). Effects of physical activity and weight loss on skeletal muscle mitochondria and relationship with glucose control in type 2 diabetes. *Diabetes, 56*(8), 2142-2147.

Toledo, F. G., Watkins, S., & Kelley, D. E. (2006). Changes induced by physical activity and weight loss in the morphology of intermyofibrillar mitochondria in obese men and women. *J Clin Endocrinol Metab, 91*(8), 3224-3227.

Tonkonogi, M., Krook, A., Walsh, B., & Sahlin, K. (2000). Endurance training increases stimulation of uncoupling of skeletal muscle mitochondria in humans by non-esterified fatty acids: an uncoupling-protein-mediated effect? *Biochem J, 351 Pt 3*, 805-810.

Tsuboyama-Kasaoka, N., Tsunoda, N., Maruyama, K., Takahashi, M., Kim, H., Ikemoto, S., et al. (1998). Up-regulation of uncoupling protein 3 (UCP3) mRNA by exercise training and down-regulation of UCP3 by denervation in skeletal muscles. *Biochem Biophys Res Commun, 247*(2), 498-503.

Tunstall, R. J., Mehan, K. A., Hargreaves, M., Spriet, L. L., & Cameron-Smith, D. (2002). Fasting activates the gene expression of UCP3 independent of genes necessary for lipid transport and oxidation in skeletal muscle. *Biochem Biophys Res Commun, 294*(2), 301-308.

van Loon, L. J., & Goodpaster, B. H. (2006). Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state. *Pflugers Arch, 451*(5), 606-616.

Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ido, Y., et al. (2000). Energy metabolism in uncoupling protein 3 gene knockout mice. *J Biol Chem, 275*(21), 16258-16266.

Winder, W. W., & Hardie, D. G. (1999). AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol, 277*(1 Pt 1), E1-10.

Wright, D. C., Han, D. H., Garcia-Roves, P. M., Geiger, P. C., Jones, T. E., & Holloszy, J. O. (2007). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. *J Biol Chem, 282*(1), 194-199.

Xia, Y., Buja, L. M., & McMillin, J. B. (1998). Activation of the cytochrome c gene by electrical stimulation in neonatal rat cardiac myocytes. Role of NRF-1 and c-Jun. *J Biol Chem, 273*(20), 12593-12598.

Xia, Y., Buja, L. M., Scarpulla, R. C., & McMillin, J. B. (1997). Electrical stimulation of neonatal cardiomyocytes results in the sequential activation of nuclear genes governing mitochondrial proliferation and differentiation. *Proc Natl Acad Sci U S A, 94*(21), 11399-11404.

Zhou, M., Lin, B. Z., Coughlin, S., Vallega, G., & Pilch, P. F. (2000). UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase. *Am J Physiol Endocrinol Metab, 279*(3), E622-629.

CHAPTER III

EXPERIMENTAL WORK

Study I

Experimental article

Long-term hyperglycaemia decreases *gastrocnemius* susceptibility to permeability transition

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Running head: Diabetes and muscle mitochondria

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Abstract

Background: Hyperglycaemia-resulting in mitochondrial bioenergetics' complications is associated with skeletal muscle dysfunction. The aim of the present work was to analyse the effect of long-term severe hyperglycaemia on *gastrocnemius* mitochondrial bioenergetics, with special relevance on the susceptibility to mitochondrial permeability transition pore (MPTP) opening.

Methods: Sixteen adult (6–8 wk old) male Wistar rats were randomly divided into two groups (n=8/group): control and diabetic. A single dose (50mg.kg^{-1}) of streptozotocin (STZ) was administrated i.p. to induce hyperglycaemia. *In vitro* mitochondrial oxygen consumption rates, membrane potential (Δψ) fluctuations, MPTP induction as followed by osmotic swelling and extramitochondrial calcium movements and caspase 9-like activity were evaluated 18 wks after STZ treatment.

Results: STZ treatment induced an increase in state 4 and a decrease in the respiratory control ratio with complex I substrates ($p<0.05$), while no differences were observed using complex II substrates. In both conditions, no significant differences were observed when measuring maximal Δψ, although STZ treatment increased Δψ during ADP-induced depolarization when succinate was used. The most critical result was that muscle mitochondria isolated from STZ-treated rats showed a decrease susceptibility to MPTP induction by calcium, as followed by two different experimental protocols. Interestingly, the protection was accompanied by a decrease in muscle caspase 9-like activity.

Conclusions: These data demonstrate that 18 wks of STZ treatment lead to a decrease in *gastrocnemius* mitochondrial respiratory control ratio and to decreased calcium-dependent mitochondrial MPTP. Results from this and other works suggest that mitochondrial effects of hyperglycaemia are time and organ specific.

1. Introduction

Diabetes mellitus is a group of common metabolic and degenerative diseases clinically characterized by a phenotype of increased blood glucose, decreased insulin secretion and/or resistance and impairments in carbohydrate, fat and protein metabolism, associated with a variety of pathophysiological complications (Rahimi, Nikfar, Larijani, & Abdollahi, 2005), responsible for the decreased quality of life of millions of people all over the world and for 5.2% of the total number of deaths worldwide in 2000 (Roglic et al., 2005). Type I diabetes is characterized by an absolute insulin deficiency, requiring daily insulin replacement. To understand the physiological and pathological changes of the disease, animal models of diabetes of hyperglycaemia are important research tools, allowing a tight control over experimental conditions, which is difficult with humans (Rees & Alcolado, 2005). In the present study, we used streptozotocin (STZ)-induced rats as a model for human type I diabetes mellitus. STZ-induced hyperglycaemia is obtained after selective destruction of β-cell by STZ, a broad spectrum antibiotic with diabetogenic effects. STZ-treated rats present many characteristics seen in insulin-dependent diabetic human patients such as hypoinsulinemia, hyperglycaemia, ketonuria, and hyperlipidaemia. Therefore, this model is of great use to evaluate the alterations promoted by uncontrolled type I diabetes. Recent and early studies have reported morphological, biochemical and functional data suggestive of mitochondrial dysfunction in experimental diabetes induced by streptozotocin (STZ) administration (Bonnard et al., 2008; Chen & Ianuzzo, 1982; el Midaoui, Tancrede, & Nadeau, 1996; Fahim, el-Sabban, & Davidson, 1998; Noble & Ianuzzo, 1985; Rouyer et al., 2007). Besides the energy producing process, mitochondria are also critical organelles in cell osmotic regulation, modulation of redox status and pH control, signal transduction and in the maintenance of calcium (Ca^{2+}) homeostasis (Brookes, Yoon, Robotham, Anders, & Sheu, 2004). Mitochondria also provide important contributions for the regulation of cell fate through its role in several cell death signalling pathways (Skulachev, 1999b). It has been described that induction of cell death is often associated with disrupted mitochondrial membrane structure. In fact, the low permeability of inner mitochondrial membrane to ions and solutes is critical to the mechanism of oxidative phosphorylation (Brookes, Yoon, Robotham, Anders, & Sheu, 2004). When mitochondrial Ca^{2+} accumulates over a threshold, mitochondria can undergo increased membrane

permeability to molecules with masses up to 1,500 Da, in an event called mitochondrial permeability transition (MPT). The MPT is caused by the opening of a multiprotein pore (MPTP) composed by several proteins of the outer and inner mitochondrial membranes (Crompton, 1999). It is well established that the susceptibility to MPTP opening occurs under conditions of increased oxidative stress and Ca^{2+} overload (Crompton, 1999; Kowaltowski, Castilho, & Vercesi, 2001). Despite some debate on the role of MPTP in apoptosis, the majority of the literature published to date supports a role for the MPTP in the release of proapoptotic proteins during apoptosis (Crompton, 1999; Hengartner, 2000).

Skeletal muscle, which makes up 40% of the body mass of humans and other mammalian species, is the primary tissue responsible for the peripheral disposal of glucose in response to a glucose or insulin challenge (Henriksen, 2002). By nature of its metabolic processes, the skeletal muscle has a high reliance on mitochondrial oxidative phosphorylation. It is described that diverse diabetic tissues have distinct capacities to respond to stress stimuli. For instance, microarray-based studies revealed tissue-specific transcriptome response to STZ-induced diabetes, suggesting that tissue-specific mechanisms are involved in the adaptation to diabetes complications (Knoll, Pietrusz, & Liang, 2005). In addition, Najemnikova et al., (2007) recently reported that heat shock factor 1 response to heat stress was increased in liver, heart and kidney from STZ treated rats but not in *gastrocnemius* and *plantaris* skeletal muscles. Moreover, despite absence of differences in the constitutive levels of heat shock proteins (HSP) 25 and 72 between diabetic and non-diabetic tissues, all with the exception of the skeletal muscles overexpressed HSP25 after heat stress. The data published so far clearly indicates that differences exist regarding organ response to hyperglycaemia, which are further modulated by the time and type of treatment used to produce hyperglycaemia.

There is an absence of literature regarding the effects of hyperglycaemia, namely induced by STZ, on skeletal muscle mitochondrial bioenergetics and MPTP regulation. The hypothesis behind the present study is that STZ-induced 18 weeks hyperglycaemia causes alterations in mitochondrial bioenergetics in *gastrocnemius* muscle, as well as an increase of susceptibility to MPTP induction, as previously described for the heart (Oliveira et al., 2003). Decreased mitochondrial function and increased susceptibility to MPTP opening during uncontrolled type I diabetes may explain

some of the skeletal muscle phenotype observed in patients harboring that condition (Lumini, Magalhaes, Oliveira, & Ascensao, 2008).

2. Methods

2.1 Materials

Streptozotocin [N-(Methylnitrosocarbamoyl)-a-D-glucosamine] was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and prepared prior to use in 100 mM citrate, pH 4.5. Calcium Green 5-N was obtained from Molecular Probes (Eugene, OR, USA). All other reagents and chemicals used were of the highest grade of purity commercially available.

2.2 Animals

Sixteen Wistar male rats (aged 6-8 wks, weighing 200g at the beginning of the experiments) were used. During the experimental protocol, 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. Male Wistar rats weighing about 200 g were randomly divided into two groups (n=8 *per* group): control (C) and diabetic (STZ). 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.

2.3 Induction and characterization of STZ-induced hyperglycaemia

In order to induce hyperglycaemia, one group was injected intraperitoneally with a single injection of STZ (50 mg/kg), after a 16-h fasting period. The volume used was always 0.5 ml/200 g body weight. Control animals were injected with the same volume of citrate solution. In the following 24 h, animals were orally feed with glycosilated serum in order to avoid hypoglycaemia resulting from massive destruction of ß-cells and consequent release of intracellular insulin. During this period, weight was measured and glycaemia was determined from the tail vein. Values were taken in fasting conditions just before STZ administration and in non-fasting conditions in the weeks after. If feeding blood glucose in the tail vein exceeded 250 mg/dl, animals were considered hyperglycaemic.

2.4 Glycaemia and HbA1C evaluation

Blood glucose concentration was determined immediately after animal sacrifice (Glucometer-Elite, Bayer, Berlin, Germany). The glycated hemoglobin (HbA1C) values were determined in blood collected at the time of animal sacrifice

through ionic exchange chromatography (Abbott Imx Glicohemoglobin, Abbott Laboratories, Amadora, Portugal).

2.5 Tissue preparation and skeletal muscle mitochondria isolation

After exposed to isoflurane inhalation in a glass container to perform anesthesia, the animals were sacrificed by cervical dislocation and *gastrocnemius* muscles were extracted for the preparation of isolated mitochondria. Before mitochondrial isolation, a portion of approximately 20-25 mg of *gastrocnemius* muscle was separated, homogenized in homogenization buffer (20mM Tris, 137mM NaCl, 0.2mM EDTA, 0.5mM EGTA, 1% Triton X-100, 10% Glycerol, pH 7.6) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C 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*, 4°C, in order to eliminate cellular debris) and the resulting supernatant was stored at –80°C for later determination of caspase 9 activity. Protein content from muscle homogenates were assayed using bovine serum albumin as standard according to Lowry et al. (1951).

Skeletal muscle mitochondria were prepared by conventional methods involving differential centrifugation, as previously described (Magalhaes et al., 2007a). Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing 100mM sucrose, 0.1mM EGTA, 50mM Tris-HCl, 100mM KCl, 1mM KH_2PO_4 and 0.2% BSA, pH 7.4. Minced blood-free tissue was rinsed and suspended in 10 ml of fresh medium containing 0.2 mg/ml bacterial proteinase (Nagarse E.C.3.4.21.62, Type XXVII; Sigma) and stirred for 2 minutes. The sample was then carefully homogenized with a tightly fitted Potter-Elvehjen homogenizer and a Teflon pestle. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then fractionated by centrifugation at 700x*g* for 10 minutes. The resulting pellet was removed and the supernatant suspension centrifuged at 10,000x*g* during 10 minutes. The supernatant was decanted and the pellet was gently resuspended in isolation medium (1.3ml per 100mg of the initial tissue) and centrifuged at 7,000x*g* for 3 minutes. The supernatant was discarded and the final pellet, containing the mitochondrial fraction, was gently resuspended ($0.4\mu l.mg^{-1}$ initial tissue) in a medium containing 225mM mannitol, 75mM sucrose, 10mM Tris and 0.1mM EDTA, pH 7.4. All mitochondrial isolation procedures were performed at 0-4ºC. Mitochondrial protein concentration was spectrophotometrically estimated

according to Lowry et al. (1951) using bovine serum albumin as standard. The mitochondrial suspensions were used within 4 hours after the excision of the muscles and were maintained on ice (0-4°C) throughout this period. There was no significant alteration of the mitochondrial RCR between the first and the last measurements in the same animal.

2.6 Measurement of mitochondrial respiratory activity

Mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Spring, OH, USA) connected to a recorder (Kipp & Zonen, BD 112, Delft, Netherlands) in a thermostated water-jacketed sealed glass chamber. Reactions were conducted in a 1ml closed thermostated (25 ºC) and magnetically stirred glass chamber containing 0.5mg of mitochondrial protein in a reaction buffer containing 225mM mannitol 75mM sucrose, 10mM Tris, 10mM KCl, 10mM K_2HPO_4 and 0.1mM EDTA, pH 7.5 in accordance to Tonkonogi *et al* (2000). After 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5mM) plus malate (2mM) or succinate (10mM) plus rotenone (4µM). State 3 respiration was determined after adding 800 nmol ADP; state 4 respiration was measured as the rate of oxygen consumption which follows total ADP phosphorylation. The respiratory control ratio (RCR, the ratio between state 3 and state 4) and the ratio between the amount of ADP phosphorylated per oxygen consumed (ADP/O) were calculated according to Estabrook (1967) and by using 235 $nmolO_2$/ml as the value for the solubility of oxygen at 25ºC. In an independent experiment, oligomycin (final concentration 1.5µg.ml^{-1}) and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 2µM) were added during state 3 respiration promoted by adding saturated (2mmol) amount of ADP. An estimate of the level of inner membrane integrity was made by measuring the ratio of uncoupled respiration induced by CCCP addition versus the rate in state oligomycin (CCCP/oligomycin) using succinate as substrate, according to Benamar et al. (2003) with adaptations. It has been described that the ratio increases as the inner membrane impermeability to protons increases (Benamar, Tallon, & Macherel, 2003).

2.7 Mitochondrial Membrane Potential

Mitochondrial transmembrane potential (Δψ) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+ selective electrode prepared in our laboratory as described by Kamo et al. (Kamo, Muratsugu, Hongoh, & Kobatake,

1979) using a AgCL reference electrode (Tacussel, Model MI 402) and as previously described (Lumini-Oliveira et al., 2009). No correction factor was used to correct the passive binding contribution of TPP^+ to membrane potential as the purpose of this study was to show the relative changes in the potential, rather than absolute values. As a consequence, a slight overestimation of the Δψ values is anticipated.

A mitochondrial matrix volume of 1.1 µl/mg of protein was assumed. Reactions were carried out in 1 ml of reaction buffer containing 225mM mannitol 75mM sucrose, 10mM Tris, 10mM KCl, 10mM K_2HPO_4 and 0.1mM EDTA, pH 7.5, supplemented with 3 µM TPP^+ and 0.5 mg/ ml of protein with the temperature maintained at 25ºC. For measurements of Δψ with complex I-linked substrates, energization was carry out with 2 mM of malate and 5 mM of pyruvate and ADP-induced phosphorylation was achieved by adding 800nmol ADP. For measurements of Δψ with complex II-linked substrates, 10mM succinate supplemented with 4 µM rotenone were added to the medium containing 3µM TPP^+ and mitochondria. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured for both substrates.

2.8 Determination of Mitochondrial Swelling

Mitochondrial osmotic volume changes were followed by monitoring the classic decrease of absorbance at 540 nm with a Jasco V-560 spectrophotometer. The reaction was continuously stirred and the temperature was maintained at 25ºC. The assays were performed in 2 ml of reaction medium containing 200 mM sucrose, 10 mM Tris, 10 µM EGTA, 5 mM KH_2PO_4, pH 7.4, supplemented with 4 µM rotenone, 10 mM succinate and 240 µM of calcium with 0.5 mg/ml protein. Control trials were made by using 1 µM of cyclosporin-A, the MPT inhibitor (Broekemeier, Dempsey, & Pfeiffer, 1989).

2.9 Mitochondrial Calcium Loading Capacity

Extramitochondrial free calcium was measured with the hexapotassium salt of the fluorescent probe Calcium Green 5-N. Muscle mitochondria (0.1 mg/ml) were resuspended in 2 ml reaction buffer containing 200 mM Sucrose, 10 mM Tris, 10 µM EGTA, 5 mM KH_2PO_4 (pH 7.4) supplemented with 4 µM rotenone. Prior calibration was made using subsequent calcium pulses to a final concentration of 300 nmoles of calcium/mg prot. Calcium uptake was initiated by adding 10 mM succinate to the reaction chamber. Free calcium was monitored with 100 nM Calcium Green 5-N. Fluorescence was

continuously recorded in a water-jacketed cuvet holder at 25°C with a Perkin-Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. From the fluorescence decrease (i.e. calcium entry to mitochondria) and with proper calibration (as previously described), the rate of calcium uptake was determined. After calcium accumulation, fluorescence increased (calcium release from mitochondria). At the end of each experiment, a calibration was performed for calculating the maximum and minimum fluorescence signal, with an excess of calcium and EGTA, respectively. Control trial was made using 1 μM cyclosporin-A. The used dissociation constant for the complex calcium probe was 4 μM (Rajdev & Reynolds, 1993).

2.10 Caspase-like activity assay

To measure caspase 9-like activity, aliquots of *gastrocnemius* tissue homogenate containing 50 μg (for caspase 9) were incubated in a reaction buffer containing 25 mM Hepes (pH 7.4), 10% sucrose; 10 mM DTT, 0.1% CHAPS and 100 μM caspase substrate Ac-LEHD-*p*NA (235400, Calbiochem, Darmstadt, Germany) for 2 h at 37°C. Caspase-like activity was determined by following the detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate Ac-LEHD-*p*-nitroanilide. The method was calibrated with known concentrations of *p*-nitroanilide (Calbiochem, Darmstadt, Germany).

2.11 Statistical analysis

Mean and standard error of the mean were calculated for all variables in each group. Students' *t*-test for independent measures was used to compare differences between groups. Statistical Package for the Social Sciences (SPSS Inc, version 12.0, Chicago, IL, USA) was used for all analyses. The significance level was set at 5%.

3. Results

3.1 Characterization of the animals

Body and *gastrocnemius* muscle weights, heart to body weight ratio, blood glucose and glycated hemoglobin are shown in Table 1. After 18 wks of STZ treatment, whole animal and *gastrocnemius* muscle weights decreased significantly ($p<0.05$). However, as muscle/body weight was not affected by hyperglicaemic conditions, *gastrocnemius* weight decrease was not tissue specific but rather a result of the whole animal body weight decrease. Blood glucose concentration, assessed immediately after the sacrifice of the animals, was significantly higher in STZ-diabetic rats when compared to controls.

To estimate the severity of diabetes, glycated hemoglobin (HbA1c) was also evaluated since HbA1c is a very helpful marker to determine the severity of diabetes, indicating the average glycaemia presented 2–3 months prior to the analysis. The measured content of HbA1c confirmed that blood glucose levels were significantly increased in STZ-treated rats (table 1).

Table 1 Characterization of the animals used in the present study

Groups	*Body weight (g)*	*Muscle weight (g)*	*Muscle/body ratio ($mg.g^{-1}$)*	*Glycaemia ($mg.dL^{-1}$)*	*HbA1c (% total Hb)*
Control	452.0 ± 11.4	5.5 ± 0.3	11.2 ± 0.2	105.5 ± 1.8	4.2 ± 0.5
STZ	255.5 ± 3.3*	3.2 ± 0.6*	12.4 ± 0.4	580.5 ± 12.8*	12.6 ± 0.5*

Data are means±SEM; * vs. control ($p<0.05$). Blood glucose and glycated hemoglobin (HbA1c) were determined as described in methods.

3.2 Mitochondrial oxygen consumption

To elucidate the effects of STZ treatment on skeletal muscle mitochondrial respiration, we determined oxygen consumption using both NADH and succinate-linked substrate oxidation through mitochondrial complex I and complex II, respectively (table 2). As it can be observed in Table 2, STZ treatment decreased the RCR (49%) and an increased state 4 respiration (72%) of malate-pyruvate-energized muscle mitochondria. No changes were noted regarding state 3 and ADP/O when comparing control and STZ groups. When succinate was used, no significant changes were detected in the rate of state 3 and state 4 respiration and on the RCR or on the ADP/O ratio (table 2).

Table 2 Respiratory parameters (state 3 and state 4 rates, respiratory control rate - RCR and ADP/O ratios) of malate (2mM)/pyruvate (5mM) and succinate (10mM) energized *gastrocnemius* muscle mitochondria isolated from control and STZ rats (18 wks after treatment).

Malate-Pyruvate	*State 3 ($natomO.min^{-1}.mg\ prot^{-1}$)*	*State 4 ($natomO.min^{-1}.mg\ prot^{-1}$)*	*RCR*	*ADP/O*
Control	167.2±5.7	15.9±0.5	9.6±0.6	2.5±0.1
STZ	136.7±5.7	27.3±2.5*	4.9±0.*	2.4±0.2
Succinate				
Control	173.3±6.4	53.7±2.7	3.3±0.3	1.7±0.1
STZ	152.1±3.0	44.7±4.6	3.6±0.1	1.6±0.2

Note: Data are means±SEM for skeletal muscle mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group. Oxidative phosphorylation was polarographically measured at 25ºC in a total volume of 1 ml. Respiration medium and other experimental details are provided in the methods section. RCR – respiratory control ratio (state 3/state 4); ADP/O - number of nmol ADP phosphorylated by natom O consumed. * vs. control ($p<0.05$).

By using succinate as a substrate, oligomycin-inhibited state 3 respiration, uncoupled respiration (state CCCP) and the ratio CCCP/oligomycin (which is a measure of mitochondrial integrity) were unaffected by treatment, which is in accordance to the results obtained for the different respiratory rates (Table 2) using the same substrate.

3.3 Mitochondrial transmembrane electric potential

To attain further insight into the effects of STZ-induced severe hyperglycaemia on mitochondria capacity to perform oxidative metabolism, Δψ fluctuations associated with mitochondrial bioenergetics were also investigated.

Both trials with complex I and complex II-related substrates, mitochondria isolated from control and STZ treated animals did not show significant differences regarding energization, ADP-induced depolarization and repolarization Δψ fluctuations, with the exception of depolarization when using succinate as substrate (Table 3). Upon energization with malate plus pyruvate, the two groups of muscle mitochondria (isolated from control and STZ animals) developed a Δψ around -198 mV and -185mV, respectively, although the difference was not statistically significant.

No significant changes were observed regarding the phosphorylative lag phase with both substrates (Table 3).

Table 3 Effect of STZ treatment on *gastrocnemius* muscle mitochondrial transmembrane potential (Δψ) fluctuations with malate plus pyruvate or succinate as substrates.

	Δψ (-mV)			Lag phase (s)
Malate-pyruvate	*Maximal energization*	*ADP Depolarization*	*Repolarization*	
Control	198.1±2.5	179.4±2.5	199.1±3.9	102.0±9.2
STZ	184.9±2.9	169.1±4.9	196.1±4.1	118.0±3.5
Succinate				
Control	209.7±4.6	170.1±1.8	205.8±3.7	122.8±6.8
STZ	215.3±1.0	183.7±2.2*	209.7±2.1	132.0±11.4

Note: Data are means±SEM for skeletal muscle mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group. Table shows the average response of mitochondrial membrane potential developed with malate (2mM) plus pyruvate (5mM) or succinate (10mM) (energization), the decrease in membrane potential after ADP addition (depolarization), the repolarization value after ADP phosphorylation. Mitochondrial transmembrane potential was measured using a TPP^+-selective electrode at 25ºC in a total volume of 1 ml. Reaction medium and other experimental details are provided in the methods section. * vs. control ($p<0.05$)

3.4 Mitochondrial swelling caused by MPT induction

We hypothesize that the persistent and severe hyperglycaemic condition may favour the modulation of skeletal muscle mitochondrial function by altering the susceptibility to MPTPopening. Following this idea, the possible effects of 18 wks STZ treatment on *in vitro* induction of the calcium-induced MPTP was also investigated. Figure 1A represents a typical recording from swelling experiments in mitochondria from control and STZ-treated rats. As pictured, the addition of calcium results into a decrease in the mitochondrial suspension absorbance with three distinct phases. Initially, an increase in absorbance is observed, which most likely results from the formation of opaque calcium crystals inside mitochondria (Andreyev, Fahy, & Fiskum, 1998). Upon MPTP opening, a decrease of absorbance with a slow followed by a fast kinetic rate was observed. Incubation of mitochondrial suspension with cyclosporin-A, a specific MPTP inhibitor (Broekemeier, Dempsey, & Pfeiffer, 1989), completely inhibits the absorbance decrease after calcium addition, which demonstrate the association with MPTP opening. Figure 1 (panels B-E) shows several end-points measured from the recordings obtained, namely swelling amplitude (the difference between the initial suspension absorbance and the final value upon absorbance stabilization), the average swelling rate, maximal swelling rate and time elapsed until the faster swelling kinetics starts. The results of swelling experiments demonstrate that *gastrocnemius* mitochondria isolated from STZ-treated rats are less susceptible to calcium-induced PTP opening when compared with controls.

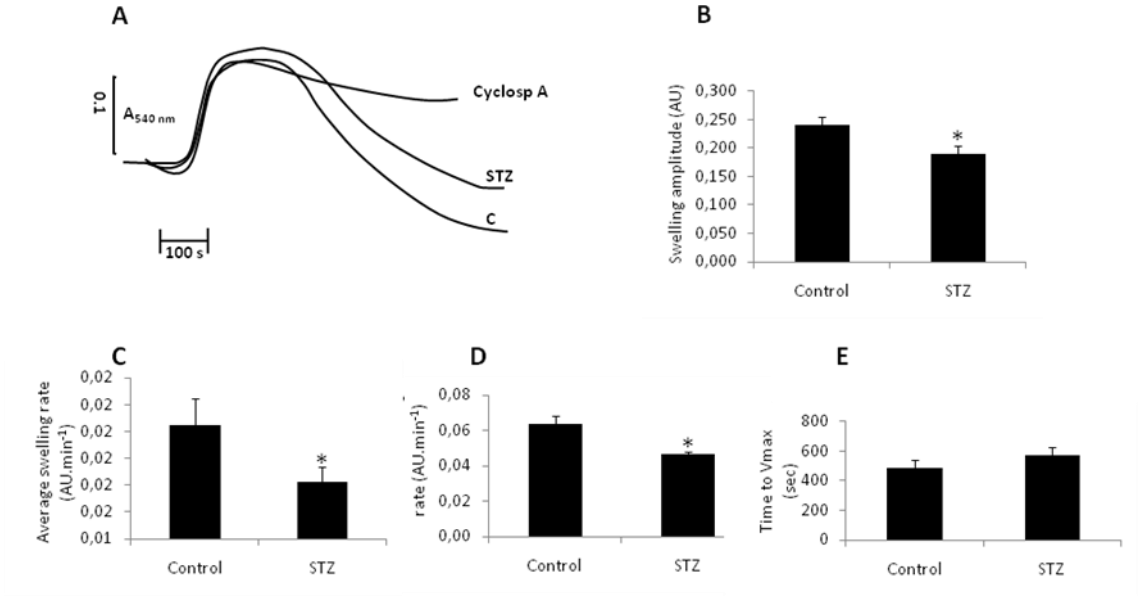

Figure 1 Effect of 18 wks STZ treatment on mitochondrial MPTP induction in succinate-energized *gastrocnemius* muscle mitochondria. The absorbance of mitochondrial suspension was followed at 540nm. Mitochondria were incubated as described in the methods section. A 240 μM calcium pulse, corresponding to 960nmol.mg protein^{-1} was added to 0.5 mg of mitochondrial protein in order to attain the cyclosporin A-sensitive swelling, due to MPTP opening. Cyclosporin-A curve corresponds to both the control and STZ groups. Panel A - Typical recording of mitochondrial swelling (decrease of optical density) of different mitochondrial preparations for each experimental group is shown; Panel B - swelling amplitude; Panel C - average swelling rate; Panel D - maximal swelling rate and Panel E – Time to Vmax, i.e., the lag phase elapsed until large scale swelling starts. Data are means±SEM; * vs. control ($p<0.05$).

3.5 Calcium loading capacity

The susceptibility of *gastrocnemius* mitochondria to MPTP opening was further analysed by following calcium fluxes through the mitochondrial membranes. Figure 2A represents a typical recording of the fluorimetric monitoring of extramitochondrial calcium movements through the fluorescent probe Calcium Green 5-N of both groups.

Surprisingly, *gastrocnemius* mitochondria isolated from STZ rats were not only able to accumulate a higher amount of calcium (figure 2B) when compared to their control counterparts, but also released a significantly lower amount of calcium than controls (figure 2C). Cyclosporin-A was able to prevent calcium release to the external buffer (figure 2A).

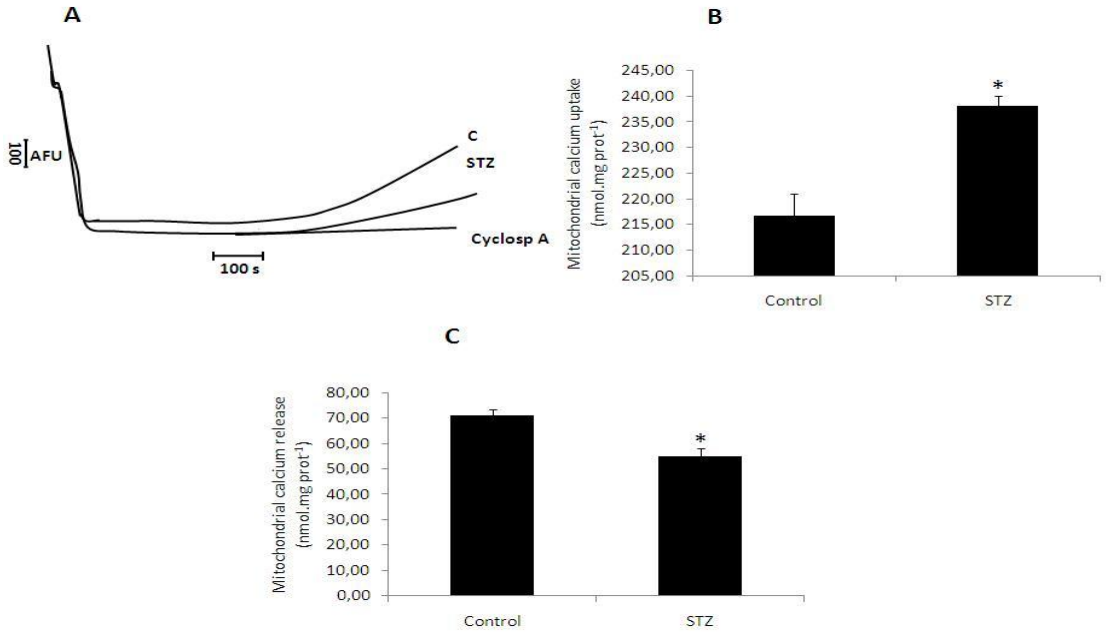

Figure 2 Effect of 18 wks of STZ treatment on extramitochondrial calcium fluxes in succinate-energized *gastrocnemius* muscle mitochondria. Panel A - Typical measurements of calcium movements using the fluorescent calcium sensitive probe Calcium Green 5-N as described in the method section. The previous calibration curve is not shown. The reactions were carried out in 2ml of reaction medium supplemented with 0.1 mg protein, 4µM rotenone, 100nM Calcium Green 5-N. Calcium entry into mitochondria (indicated by fluorescence decay) was initiated by 10mM succinate addition. Fluorescence was monitored continuously and the increase in signal indicates calcium release. The release of calcium from mitochondria of both experimental groups was inhibited by 1µM cyclosporin-A, indicating that the observed release was due to MPTP opening. Calcium fluxes are representatively expressed as arbitrary units of fluorescence (AFU); Panel B – mitochondrial calcium uptake; and Panel C – mitochondrial calcium release. Data are means±SEM; * vs. control ($p<0.05$).

3.6 Caspase 9 activity

One indication of intrinsic mitochondrial-driven activation of apoptotic cell death is the activity of caspase 9. The analysis revealed that STZ-induced severe and prolonged hyperglycaemia decreased the basal activity of caspase 9 in *gastrocnemius* muscle.

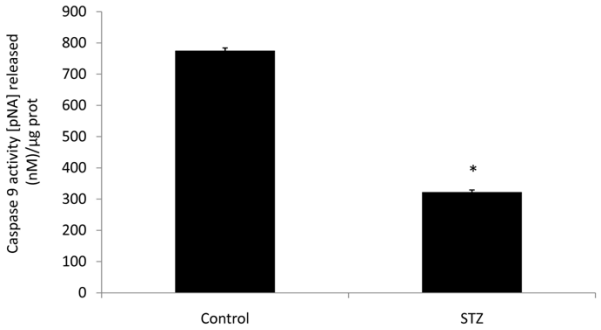

Figure 3 Effect of 18 wks of STZ treatment on caspase 9 activity in *gastrocnemius* muscle. Caspase-9-like activity was measured by following the cleavage of the colorimetric substrates Ac-LEHD-pNA. The caspase-like activity was expressed by [pNA] released nM/µg prot. Data is expressed as means±SEM; * vs. STZ ($p<0.05$).

4. Discussion

4.1 Overview of principal findings

The present investigation provides new insights into the possible mechanisms by which persistent (18 wks) and severe hyperglycaemia induced by STZ treatment alters mitochondrial bioenergetics in rat *gastrocnemius* muscle.

Data from this study suggest that the respiratory control ratio (RCR) of mitochondria isolated from the *gastrocnemius* muscle of STZ-treated rats was affected when complex I substrates were used. However, the present data showed for the first time that *gastrocnemius* mitochondria isolated from STZ-treated animals had an enhanced tolerance to permeability transition pore opening in a calcium/phosphate medium. Somewhat unexpectedly, *gastrocnemius* mitochondria isolated from STZ rats were able to accumulate higher amounts of calcium and were demonstrated to be less susceptible to PTP opening than the control group, as mitochondrial swelling amplitude and rates as well as calcium release were lower in the STZ group.

4.2 Mitochondrial oxygen consumption and transmembrane potential

The data from Tables 2 and 3 suggest that long-term experimentally induced hyperglycaemia resulted in mitochondrial respiratory dysfunction as seen by a decreased respiratory control ratio, a measure of the dependence of the

respiratory rate on ATP synthesis, and alterations on state 4 respiratory rate. The alterations were more visible when complex I-linked substrates were used. Regardless the increase in Δψ observed on repolarization after ADP phosphorylation, no significant changes were observed in the remaining Δψ endpoints between groups (Table 3). The apparent contradiction between respiratory and Δψ data can be explained by two distinct reasons. Although the two methods are different, we are inclined to think that the unchanged maximal membrane potential using Complex I substrates and the equivalent increased state 4 respiration agrees with a possibility of decoupling at the Complex I level (see below). As for the differences observed in ADP-induced depolarization for complex II substrates, the simpler explanation is that this end-point cannot be easily accessed from respiratory data values and thus is not comparable. Thus, our data show that long-term experimental hyperglycaemia causes altered coupling between respiration and phosphorylation in *gastrocnemius* muscle. The lack of coupling may be due to several causes, although the lack of differences in uncoupled/state 3 respiration and at the ADP/O ratio suggests that no damage to the respiratory chain or to the phosphorylation system occurred. The increase in state 4 respiration would suggest an increased inner membrane permeability, leading to higher proton influx (i.e. uncoupling) (Brand, Chien, Ainscow, Rolfe, & Porter, 1994). Nevertheless, the increase in state 4 respiration with malate+pyruvate, but not succinate, occur without alteration in Δψ (Table III). A new explanation may involve an intrinsic slippage of protons (decoupling) through complex I due to modifications of the intrinsic H^+/e^- stoichiometry. This would lead to a decreased number of protons pumped per electron transferred, which would increase the rate of electron transfer to compensate for the decreased proton pumping (Papa, Lorusso, & Di Paola, 2006). It must be stressed that as intrinsic decoupling at mitochondrial complexes with exception of complex IV is still a matter of debate (Kadenbach, 2003).
As stated, several studies reported decreased muscle mitochondrial metabolism and morphological disturbances in STZ-treated rats, including decreased mitochondrial mass, increased number of disarrayed cristae and mitochondrial swelling, reduced matrix electron density and compromised activity of several Krebs Cycle enzymes (Bonnard et al., 2008; Chen & Ianuzzo, 1982; Fahim, el-Sabban, & Davidson, 1998; Noble & Ianuzzo, 1985; Rouyer et al., 2007). Mitochondrial alterations associated with decreased function seem

to be related with increased oxidative stress and damage within the muscle from hyperglycaemic subjects (Bonnard et al., 2008). Bonnard and co-workers (2008) showed that both type I and II diabetic mice skeletal muscle presented mitochondrial morphological disturbances accompanied by increased levels of protein carbonylation, cytochrome c release, decreased mtDNA/nDNA ratio that were reverted by antioxidants and insulin. It is thus likely that sustained hyperglycaemia-induced enhanced muscle free radical generation can contribute to alterations in mitochondrial respiration.

However, it must be noted that enhanced muscle oxidative stress may also lead to up-regulated antioxidant defences, including mitochondrial chaperons and low molecular weight antioxidants, which may contribute to an apparent increased resistance to MPTP induction in the STZ group.

4.3 Modulation of the permeability transition pore

Mitochondrial dysfunction can also be triggered by stress-induced opening of the MPTP, which has been involved in calcium dynamics and cell death (Crompton, 1999; Hengartner, 2000). A hallmark of MPTP is the cyclosporin A-inhibited large-scale swelling induced by calcium in the presence of phosphate, which occurs through increase in oxidative stress (Kowaltowski, Castilho, Grijalba, Bechara, & Vercesi, 1996). The main consequences of MPTP opening are loss or collapse of mitochondrial potential, depending on the reversibility of the phenomenon, and an efflux of calcium into the extramitochondrial space. The irreversible opening of a high conductance MPTP in cells inevitably cause mitochondrial swelling, cytochome c release, caspase activation and apoptosis, or the fall of the mitochondrial potential, ATP depletion and energetic collapse followed by necrotic cell death (Tsujimoto & Shimizu, 2007). However, it is not at all clear whether the pore plays any other physiological role for mitochondria, or indeed whether it even exists except under extremely pathological conditions (see Duchen, 2004). Some studies have also shown that an increased vulnerability to skeletal muscle MPTP opening develops under various physiological and pathological conditions including aging (Chabi et al., 2008), denervation-induced atrophy (Adhihetty, O'Leary, Chabi, Wicks, & Hood, 2007; Csukly et al., 2006), bupivacaine administration (Irwin et al., 2002), and muscle dystrophies (Millay et al., 2008). Moreover, other studies in hyperglycaemic animals showed an increased susceptibility to MPTP induction in the heart (Oliveira et al.,

2003), brain (Moreira, Santos, Moreno, Seica, & Oliveira, 2003) and kidney (Oliveira, Esteves, Seica, Moreno, & Santos, 2004). However, the present work is the first to demonstrate the effect of severe and persistent hyperglycaemia on the susceptibility to MPTP opening in *gastrocnemius* skeletal muscle.

As mentioned, as MPTP opening can be induced by increasing amounts of calcium and by oxidative stress, and given that diabetes leads to increased free radical generation, due to hyperglycaemia (Simmons, 2006), our hypothesis was that hyperglycaemic animals would have an increase susceptibility of *gastrocnemius* mitochondria to undergo MPTP induction. However, our results showed that *gastrocnemius* muscle mitochondria isolated from rats after 18 wks of severe hyperglycaemia had a higher calcium loading capacity before MPTP induction (Figs 1 and 2). The data obtained is not completely unexpected if compared with data obtained in other hyperglycaemic animal models, including previous results obtained in heart and liver mitochondria isolated from hyperglycaemic animals, showing a decreased susceptibility to MPTP induction (Ferreira, Seica et al., 2003; Oliveira et al., 2001). Ferreira et al. (2003) tested the susceptibility of liver mitochondria to MPTP induction in both type I (STZ) and type II (lean Goto-Kakizaki) experimental diabetes models and suggested that there is an apparent positive correlation between the severity and the duration of the disease, and the susceptibility to MPTP induction, with also a clear organ specific difference present. In this regard, it is important to note that in our study, muscle mitochondria were tested 18 wks after STZ treatment, and this long-term experimental diabetes model may contribute to some tissue and specifically mitochondrial metabolic adaptations to oxidative stress conditions caused by severe and persistent hyperglycaemia. These may include increases in mitochondria coenzyme Q and cardiolipin contents (Ferreira, Seica et al., 2003), up-regulation of antioxidant defences including vitamin E content, reduced (GSH) to oxidised (GSSG) glutathione ratio and GSH peroxidase and GSSG reductase activities (Ferreira, Palmeira, Matos, Seica, & Santos, 1999). Despite the recent reports showing that short-term STZ treatment does not induce improvements in some extramitochondrial defence systems such as muscle HSF-1, HSP72, HSP25 (Najemnikova, Rodgers, & Locke, 2007), it is likely that long-term severe hyperglycaemic condition would target other cellular protecting mechanisms, i.e. heme oxygenase 1 and catalase (Shen et al., 2008), possibly contributing to the observed additional tolerance to stress-induced MPTP.

Another hypothesis which seems

attractive involves a possible reduction in the levels of muscle hexokinase bound to mitochondria that may contribute to these results, as hexokinase expression is both known to be regulated by insulin (Kruszynska, Mulford, Baloga, Yu, & Olefsky, 1998; Pendergrass et al., 1998) and to be involved in an inhibitory regulation of the MPTP complex (Crompton, 1999). Further studies are needed to determine how long-term hyperglycaemia results in decreased susceptibility to the MPTP in *gastrocnemius* skeletal muscle.

Regardless of the mechanism involved, the decreased susceptibility of the MPTP in hyperglycaemic animals may be a countermeasure to prevent mitochondrial dysfunction, which limits damage during the alterations in ionic and redox status in muscle cells under chronic hyperglycaemic conditions.

Interestingly, no STZ effects were observed on succinate-sustained respiration despite the fact that differences exist between the two groups regarding calcium-induced MPTP in the presence of succinate. There are several possibilities for that apparent discrepancy, including STZ-induced alterations in proteins that do not participate directly in oxidative phosphorylation but that are part of the MPTP complex (e.g. cyclophilin D) or even the existence of a reserve of mitochondrial proteins that can be recruited to form the MPTP upon the presence the calcium but whose recruitment have no effect in the yield and fluxes of oxidative phosphorylation. Also, it must be stressed that the MPTP experiments are conducted in the mandatory presence of calcium, which is a MPTP agonist. When measuring oxidative phosphorylation, no calcium is present; which may mask alterations in oxidative phosphorylation that are secondary to MPTP opening.

We further determined the activity of caspase 9-like in the whole tissue homogenate as a marker of mitochondrial apoptotic signaling. The results of the present study show that long-term severe hyperglycaemia decreased caspase 9-like activity suggesting that this condition may predispose *gastrocnemius* to a lower induction of the intrinsic, mitochondrial, pathway for apoptosis.

In summary, the data from the present study indicates that in an animal model for prolonged severe hyperglycaemia, mimicking uncontrolled type I diabetes in humans, the mitochondrial respiratory control ratio is affected and a decreased susceptibility to MPTP induction exists in *gastrocnemius* skeletal muscle. Specific and differential adaptations within muscle mitochondria to the 18wks of high blood glucose levels were likely translated into protection against MPTP induction and impairments in the respiratory system.

A more accurate and organ-specific understanding of mitochondrial impairments would possibly contribute to better design preventive and/or therapeutic countermeasures against the disease.

References

Adhihetty, P. J., O'Leary, M. F., Chabi, B., Wicks, K. L., & Hood, D. A. (2007). Effect of denervation on mitochondrially mediated apoptosis in skeletal muscle. *J Appl Physiol, 102*(3), 1143-1151.

Andreyev, A. Y., Fahy, B., & Fiskum, G. (1998). Cytochrome c release from brain mitochondria is independent of the mitochondrial permeability transition. *FEBS Lett, 439*(3), 373-376.

Benamar, A., Tallon, C., & Macherel, D. (2003). Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing. *Seed Sci Res, 13*, 35-45.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M. A., Morio, B., et al. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest, 118*(2), 789-800.

Brand, M. D., Chien, L. F., Ainscow, E. K., Rolfe, D. F., & Porter, R. K. (1994). The causes and functions of mitochondrial proton leak. *Biochim Biophys Acta, 1187*(2), 132-139.

Broekemeier, K. M., Dempsey, M. E., & Pfeiffer, D. R. (1989). Cyclosporin A is a potent inhibitor of the inner membrane

permeability transition in liver mitochondria. *J Biol Chem, 264*(14), 7826-7830.

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol, 287*(4), C817-833.

Chabi, B., Ljubicic, V., Menzies, K. J., Huang, J. H., Saleem, A., & Hood, D. A. (2008). Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. *Aging Cell, 7*(1), 2-12.

Chen, V., & Ianuzzo, C. D. (1982). Metabolic alterations in skeletal muscle of chronically streptozotocin-diabetic rats. *Arch Biochem Biophys, 217*(1), 131-138.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *Biochem J, 341 (Pt 2)*, 233-249.

Csukly, K., Ascah, A., Matas, J., Gardiner, P. F., Fontaine, E., & Burelle, Y. (2006). Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D. *J Physiol, 574*(Pt 1), 319-327.

Duchen, M. R. (2004). Roles of mitochondria in health and disease. *Diabetes, 53 Suppl 1*, S96-102.

el Midaoui, A., Tancrede, G., & Nadeau, A. (1996). Effect of physical training on mitochondrial function in skeletal muscle of normal and diabetic rats. *Metabolism, 45*(7), 810-816.

Estabrook, R. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods Enzymol, 10*, 41-47.

Fahim, M. A., el-Sabban, F., & Davidson, N. (1998). Muscle contractility decrement and correlated morphology during the pathogenesis of streptozotocin-diabetic mice. *Anat Rec, 251*(2), 240-244.

Ferreira, F. M., Palmeira, C. M., Matos, M. J., Seica, R., & Santos, M. S. (1999). Decreased susceptibility to lipid peroxidation of Goto-Kakizaki rats: relationship to mitochondrial antioxidant capacity. *Life Sci, 65*(10), 1013-1025.

Ferreira, F. M., Seica, R., Oliveira, P. J., Coxito, P. M., Moreno, A. J., Palmeira, C. M., et al. (2003). Diabetes induces metabolic adaptations in rat liver mitochondria: role of coenzyme Q and cardiolipin contents. *Biochim Biophys Acta, 1639*(2), 113-120.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Henriksen, E. J. (2002). Invited review: Effects of acute exercise and exercise training on insulin resistance. *J Appl Physiol, 93*(2), 788-796.

Irwin, W., Fontaine, E., Agnolucci, L., Penzo, D., Betto, R., Bortolotto, S., et al. (2002). Bupivacaine myotoxicity is mediated by mitochondria. *J Biol Chem, 277*(14), 12221-12227.

Kadenbach, B. (2003). Intrinsic and extrinsic uncoupling of oxidative phosphorylation. *Biochim Biophys Acta, 1604*(2), 77-94.

Kamo, N., Muratsugu, M., Hongoh, R., & Kobatake, Y. (1979). Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol, 49*(2), 105-121.

Knoll, K. E., Pietrusz, J. L., & Liang, M. (2005). Tissue-specific transcriptome responses in rats with early streptozotocin-induced diabetes. *Physiol Genomics, 21*(2), 222-229.

Kowaltowski, A. J., Castilho, R. F., Grijalba, M. T., Bechara, E. J., & Vercesi, A. E. (1996). Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca2+ ions. A proposed model for phosphate-stimulated lipid peroxidation. *J Biol Chem, 271*(6), 2929-2934.

Kowaltowski, A. J., Castilho, R. F., & Vercesi, A. E. (2001). Mitochondrial permeability transition and oxidative stress. *FEBS Lett, 495*(1-2), 12-15.

Kruszynska, Y. T., Mulford, M. I., Baloga, J., Yu, J. G., & Olefsky, J. M. (1998). Regulation of skeletal muscle hexokinase II by insulin in nondiabetic and NIDDM subjects. *Diabetes, 47*(7), 1107-1113.

Lowry, O. H., Rosenbrough, N., Farr, A. L., & Radall, R. J. (1951). Protein measurement with the folin phenol reagent. *J Biol Chem*(193), 265-275.

Lumini-Oliveira, J., Magalhaes, J., Pereira, C. V., Aleixo, I., Oliveira, P. J., & Ascensao, A. (2009). Endurance training improves gastrocnemius mitochondrial function despite increased susceptibility to permeability transition. *Mitochondrion, 9*, 454-462.

Lumini, J. A., Magalhaes, J., Oliveira, P. J., & Ascensao, A. (2008). Beneficial effects of exercise on muscle mitochondrial function in diabetes mellitus. *Sports Med, 38*(9), 735-750.

Magalhaes, J., Ferreira, R., Neuparth, M. J., Oliveira, P. J., Marques, F., & Ascensao, A. (2007). Vitamin E prevents hypobaric hypoxia-induced mitochondrial dysfunction in skeletal muscle. *Clin Sci (Lond), 113*(12), 459-466.

Millay, D. P., Sargent, M. A., Osinska, H., Baines, C. P., Barton, E. R., Vuagniaux, G., et al. (2008). Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. *Nat Med, 14*(4), 442-447.

Moreira, P. I., Santos, M. S., Moreno, A. M., Seica, R., & Oliveira, C. R. (2003). Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid-beta exposure. *Diabetes, 52*(6), 1449-1456.

Najemnikova, E., Rodgers, C. D., & Locke, M. (2007). Altered heat stress response following streptozotocin-induced diabetes. *Cell Stress Chaperones, 12*(4), 342-352.

Noble, E. G., & Ianuzzo, C. D. (1985). Influence of training on skeletal muscle enzymatic adaptations in normal and diabetic rats. *Am J Physiol, 249*(4 Pt 1), E360-365.

Oliveira, P. J., Esteves, T. C., Seica, R., Moreno, A. J., & Santos, M. S. (2004). Calcium-dependent mitochondrial permeability transition is augmented in the kidney of Goto-Kakizaki diabetic rat. *Diabetes Metab Res Rev, 20*(2), 131-136.

Oliveira, P. J., Rolo, A. P., Seica, R., Palmeira, C. M., Santos, M. S., & Moreno, A. J. (2001). Decreased susceptibility of heart mitochondria from diabetic GK rats to mitochondrial permeability transition induced by calcium phosphate. *Biosci Rep, 21*(1), 45-53.

Oliveira, P. J., Seica, R., Coxito, P. M., Rolo, A. P., Palmeira, C. M., Santos, M. S., et al.

(2003). Enhanced permeability transition explains the reduced calcium uptake in cardiac mitochondria from streptozotocin-induced diabetic rats. *FEBS Lett, 554*(3), 511-514.

Papa, S., Lorusso, M., & Di Paola, M. (2006). Cooperativity and flexibility of the protonmotive activity of mitochondrial respiratory chain. *Biochim Biophys Acta, 1757*(5-6), 428-436.

Pendergrass, M., Koval, J., Vogt, C., Yki-Jarvinen, H., Iozzo, P., Pipek, R., et al. (1998). Insulin-induced hexokinase II expression is reduced in obesity and NIDDM. *Diabetes, 47*(3), 387-394.

Rahimi, R., Nikfar, S., Larijani, B., & Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomed Pharmacother, 59*(7), 365-373.

Rajdev, S., & Reynolds, I. J. (1993). Calcium green-5N, a novel fluorescent probe for monitoring high intracellular free Ca2+ concentrations associated with glutamate excitotoxicity in cultured rat brain neurons. *Neurosci Lett, 162*(1-2), 149-152.

Rees, D. A., & Alcolado, J. C. (2005). Animal models of diabetes mellitus. *Diabet Med, 22*(4), 359-370.

Roglic, G., Unwin, N., Bennett, P. H., Mathers, C., Tuomilehto, J., Nag, S., et al. (2005). The burden of mortality attributable to diabetes: realistic estimates for the year 2000. *Diabetes Care, 28*(9), 2130-2135.

Rouyer, O., Zoll, J., Daussin, F., Damge, C., Helms, P., Talha, S., et al. (2007). Effect of angiotensin-converting enzyme inhibition on skeletal muscle oxidative function and exercise capacity in streptozotocin-induced diabetic rats. *Exp Physiol, 92*(6), 1047-1056.

Shen, W. L., Zhong, M. F., Ding, W. L., Wang, J., Zheng, L., Zhu, P., et al. (2008). Elevated catalase and heme oxygenase-1 may contribute to improved postischaemic cardiac function in long-term type 1 diabetes. *Clin Exp Pharmacol Physiol, 35*(7), 820-826.

Simmons, R. A. (2006). Developmental origins of diabetes: the role of oxidative stress. *Free Radic Biol Med, 40*(6), 917-922.

Skulachev, V. P. (1999). Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. *Mol Aspects Med, 20*(3), 139-184.

Tonkonogi, M., Walsh, B., Svensson, M., & Sahlin, K. (2000). Mitochondrial function and antioxidative defence in human muscle: effects of endurance training and oxidative stress. *J Physiol, 528 Pt 2*, 379-388.

Tsujimoto, Y., & Shimizu, S. (2007). Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis, 12*(5), 835-840.

Experimental article

Endurance Training Improves *Gastrocnemius* Mitochondrial Function Despite Increased Susceptibility to Permeability Transition

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Abstract

The aim of the present work was to test the hypothesis that moderate endurance treadmill training ameliorates *gastrocnemius* mitochondrial bioenergetics and increases the tolerance to the calcium-induced mitochondrial permeability transition pore (MPTP) opening. Twelve adult (6–8 wk old) male Wistar rats were randomly divided into two groups (n=6\ per group): sedentary and trained (14 wk of endurance treadmill running, 60min/day). Several end-points for *in vitro gastrocnemius* mitochondrial function including oxygen consumption, transmembrane electric potential and susceptibility to calcium-induced MPTP opening were evaluated. Caspase 9 activity was measured in the intact tissue. Endurance training induced significant increases in state 3 and in respiratory control ratio both with complex I and II-linked substrates (malate+pyruvate and succinate, respectively). Increased CCCP-induced uncoupled respiration with succinate as substrate was also observed ($p<0.05$). No differences were found regarding state 4 and ADP/O ratio with both substrates. In addition, training significantly decreased the phosphorylative lag phase, whereas no changes were observed on maximal transmembrane electric potential, ADP-induced depolarization and repolarization potential ($p<0.05$). Interestingly and as opposed to our hypothesis, muscle mitochondria isolated from trained rats presented an increased susceptibility to MPTP induction by calcium, although in an initial phase muscle mitochondria isolated from trained rats had an increased calcium uptake. Interestingly, we also verified that endurance training increased the activity of caspase 9. The data obtained confirms that endurance training results in a general improvement in the *gastrocnemius* mitochondrial respiratory function, although mitochondrial and cellular alterations during training also result in increased calcium-induced MPTP opening.

1. Introduction

Skeletal muscle has a high reliance on oxidative metabolism, with mitochondria being critical organelles involved in ATP synthesis, cell osmotic regulation, modulation of redox status and pH control, signal transduction and in the establishment of calcium homeostasis (Brookes, 2005; Brookes, Yoon, Robotham, Anders, & Sheu, 2004; Cadenas, 2004). Mitochondria also provide an important contribution for the regulation of cell fate through its role on the regulation of cellular death, either by directly triggering apoptosis or acting by amplifying the apoptotic signals (Skulachev, 1999b). The release of pro-apoptotic proteins contained in mitochondria has been referred to be dependent, at least partially, on the formation and opening of pores that span the inner and outer mitochondrial membranes leading to the loss of mitochondrial membrane potential ($\Delta\Psi$), to mitochondrial osmotic swelling and rupture of the outer mitochondrial membrane, in a phenomenon denominated the mitochondrial permeability transition. The pores that originate the mitochondrial permeability transition are termed mitochondrial permeability transition pores (MPTP). It is well established that the susceptibility to MPTP opening occurs under conditions of increased oxidative stress and Ca^{2+} overload (Crompton, 1999; Kowaltowski, Castilho, & Vercesi, 2001). In fact, despite the recognized buffering role of mitochondria in the regulation of cellular calcium homeostasis both under basal and stressful conditions, the synergetic effect of increased free radical production and deregulated calcium levels lead to a physiopathological condition favoring MPTP opening. Although some debate exists on the role of the MPTP in apoptosis, most of the literature published so far supports the role of the MPTP in the facilitation of the release of proapoptotic proteins triggering apoptosis (Crompton, 1999; Hengartner, 2000). Physical exercise, particularly endurance training, has been proven to provide tissue protection against several deleterious conditions. It is well known that a program of regular endurance exercise, undertaken over a number of weeks, produces significant adaptations in skeletal muscle, causing noticeable improvements in the mitochondrial oxidative capacity (Fluck & Hoppeler, 2003). The improvements allow the duration of activity for a longer period before the signs of exercise-induced fatigue become limiting and can have therapeutic beneficial effects for muscle tissue under pathophysiological conditions such as myopathies diminishing exercise intolerance (Lumini,

Magalhaes, Oliveira, & Ascensao, 2008; Tarnopolsky & Raha, 2005).

Some beneficial adaptations in skeletal muscle induced by endurance exercise training include among others, increased mitochondrial volume density (Fluck & Hoppeler, 2003), improved oxidative enzyme activities (Holloszy, 1967) and the up-regulation of antioxidant and/or antiapoptotic-related proteins such as superoxide dismutase (Gore et al., 1998; Hollander et al., 1999), glutathione peroxidase (Gore et al., 1998) or heat shock proteins (HSP) (Atalay et al., 2004; Desplanches, Ecochard, Sempore, Mayet-Sornay, & Favier, 2004; Naito, Powers, Demirel, & Aoki, 2001). Moreover, endurance training results in the improvement of skeletal muscle mitochondrial function and in the decreased susceptibility to *in vitro* mitochondrial oxidative stress (for refs see Ascensao & Magalhaes, 2006; Tonkonogi & Sahlin, 2002). A recent study from Adhihetty et al. (2007) showed that 7 days of low-intensity *in situ* electrical stimulation of rat hindlimb (*tibialis anterior*) muscles, an artificial model of exercise produced by electrical stimulation of the motor nerve, induced predominantly antiapoptotic adaptations in skeletal muscle mitochondrial.

The hypothesis for the present work is that endurance training not only improves skeletal muscle mitochondrial function but also decreases the susceptibility to the MPT, which would explain the previously described positive adaptations on skeletal muscle antioxidant and antiapoptotic networks. On the other hand, as the whole body endurance treadmill training induction of mitochondrial biogenesis can produce an obligatory elevation in a number of proapoptotic mitochondrial proteins, the effect could potentially result into an exacerbated apoptotic response following a triggering stimulus. To confirm our working hypothesis, we used an animal model for mitochondrial alterations following endurance training. The analysis of the effects of a whole body exercise training model, such as treadmill running, on the susceptibility of *gastrocnemius* mitochondria to Ca^{2+}-induced MPTP has not been studied so far, being pertinent to the study of exercise-induced mitochondrial adaptations and advantages.

2. Methods

2.1. Animals

Twelve Wistar male rats (aged 6-8 wks, weighting 200g at the beginning of the experiments) were used. During the experimental protocol, 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 into two groups (n=6 *per* group): Sedentary (Sed) and endurance trained (T). 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.

2.2. 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). The protocol included 5 days of habituation to the treadmill with 10 min of running at 15m/min and 0% grade, with daily increases of 5-10 min until 30 min was achieved. Habituation was followed by one consecutive wk of continuous running (30 min/day) at 15m/min and 0% grade and was gradually increased until 60min/day (20m/min) on the second wk. The animals from Sed group were not exercised but were placed on a non-moving treadmill five times per wk (10-30 min/session) with the purpose of habituate animals to 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	60	60	60	60	60	60	60	60	60	60	60	60
Treadmill speed (m/min)	15	20	25	25	25	25	25	25	25	25	25	25	25	25
% Grade (treadmill inclination)	0	0	0	0	0	3	3	6	6	6	6	6	6	6

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

2.3. Tissue preparation and skeletal muscle mitochondria isolation

Twenty-four hours after the last training session, the animals were sacrificed by cervical dislocation and *gastrocnemius* muscles were extracted for the preparation of isolated mitochondria. Before mitochondrial isolation, a representative portion of approximately 100 mg of *gastrocnemius* muscle was separated, homogenized in homogenization buffer (20mM Tris, 137mM NaCl, 0.2mM EDTA, 0.5mM EGTA, 1% Triton X-100, 10% Glycerol, 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 assays of caspase 9 activity. Protein content from muscle homogenates were assayed using bovine serum albumin as standard according to Lowry et al. (1951).

Skeletal muscle mitochondria were prepared by conventional methods involving differential centrifugation, as previously described (Magalhaes et al., 2005; Magalhaes et al., 2007b). Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing 100mM sucrose, 0.1mM EGTA, 50mM Tris-HCl, 100mM KCl, 1mM KH_2PO_4 and 0.2% BSA, pH 7.4. Minced blood-free tissue was rinsed and suspended in 10 ml of fresh medium containing 0.2 mg/ml bacterial proteinase (Nagarse E.C.3.4.21.62, Type XXVII; Sigma) and stirred for 2 minutes. The sample was then carefully homogenized with a tightly fitted Potter-Elvehjem homogenizer and a Teflon pestle. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then fractionated by centrifugation at 700x*g* for 10 minutes. The resulting pellet was removed and the supernatant suspension centrifuged at 10,000x*g* during 10 minutes. The supernatant was decanted and the pellet was gently resuspended in isolation medium (1.3ml per 100mg of the initial tissue) and centrifuged at 7,000x*g* for 3 minutes. The supernatant was discarded and the final pellet, containing the mitochondrial fraction, was gently resuspended ($0.4\mu l.mg^{-1}$ initial tissue) in a medium containing 225mM mannitol, 75mM sucrose, 10mM Tris and 0.1mM EDTA, pH 7.4. All mitochondrial isolation procedures were performed at 0-4ºC. Mitochondrial protein concentration was spectrophotometrically estimated with the biuret method using bovine serum albumin as standard (Gornall, Bardawill, & David, 1949). The mitochondrial suspensions were used within 4 hours after the excision of the muscles and were maintained on ice (0-4ºC) throughout this period. There was no significant alteration of the mitochondrial RCR between the first and the last measurements of the same animal. The isolation protocol yields a mixed population of both intermyofibrillar (IMF) and subsarcolemmal (SS) mitochondrial subfractions.

2.4. Measurement of mitochondrial respiratory activity

Mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode (Yellow Springs Instruments, OH) connected to a recorder (Kipp & Zonen, BD 112) in a thermostated water-jacketed sealed glass chamber. Reactions were conducted in a 1ml closed thermostated (25 ºC) and magnetically stirred glass chamber containing 0.5mg of mitochondrial protein in a reaction buffer containing 225mM mannitol 75mM sucrose, 10mM Tris, 10mM KCl, 10mM K_2HPO_4 and 0.1mM EDTA, pH 7.5 in accordance to

Tonkonogi *et al* (2000) and in order to maintain mitochondrial integrity for a longer time period. After 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5mM) plus malate (2mM) or succinate (10mM) plus rotenone (4µM). State 3 respiration was determined after adding ADP to a final concentration of 400 µM; state 4 respiration was measured as the rate of oxygen consumption which follows total ADP phosphorylation. The respiratory control ratio (RCR, the ratio between state 3 and state 4) and the ratio between the amount of ADP phosphorylated per oxygen consumed (ADP/O) were calculated according to Estabrook (1967) and by using 235 nmolO_2/ml as the value for the solubility of oxygen at 25ºC.

Oligomycin (final concentration 1.5µg.ml^{-1}) and carbonyl cyanide m-chlorophenylhydrazone (CCCP, 2µM) were added in sequence during state 3 respiration sustained by the addition of a saturating amount of ADP (final concentration 1mM).

An estimate of the level of inner membrane integrity was made by measuring the ratio between uncoupled respiration induced by CCCP addition and the respiratory rate in the presence of oligomycin (CCCP/oligomycin) using succinate as substrate, according to Benamar et al. (2003) with adaptations (Magalhaes et al., 2007b). It was described that the ratio increases as the inner membrane impermeability to protons increases (Benamar, Tallon, & Macherel, 2003).

2.5. Mitochondrial Membrane Potential

Mitochondrial transmembrane potential (Δψ) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+ selective electrode prepared in our laboratory as described by Kamo et al. (Kamo, Muratsugu, Hongoh, & Kobatake, 1979) using a AgCL reference electrode (Tacussel, Model MI 402). Both the TPP^+ electrode and the reference electrode were inserted into an open vessel with magnetic stirring and connected to a pH meter (Jenway, Model 3305). The signals were fed to a potentiometric recorder (Kipp & Zonen, Model BD 121). No correction factor was used to correct the passive binding contribution of TPP^+ to membrane potential as the purpose of this study was to show the relative changes in the potential, rather than absolute values. As a consequence, a slight overestimation of the Δψ values is anticipated. The Δψ was estimated from the following equation (at 25ºC): $\Delta\psi = 59 \times \log(v/V) - 59 \times \log(10^{\Delta E/59} - 1)$, where v, V, and ΔE stand for mitochondrial volume, volume of the incubation

medium, and deflection of the electrode potential from the baseline, respectively. A mitochondrial matricial volume of 1.1 µl/mg of protein was assumed. Reactions were carried out in 1 ml of reaction buffer containing 225mM mannitol 75mM sucrose, 10mM Tris, 10mM KCl, 10mM K_2HPO_4 and 0.1mM EDTA, pH 7.5, supplemented with 3 µM TPP^+ and 0.5 mg/ ml of protein with the temperature maintained at 25ºC. For measurements of Δψ with complex I-linked substrates, energization was performed with 2 mM of malate and 5 mM of pyruvate and depolarisation potential was achieved by adding 400 µM of ADP. For measurements of Δψ with complex II-linked substrates, 10mM succinate supplemented with 4 µM rotenone were added to the medium containing 3 µM TPP^+ and mitochondria. Recordings of Δψ without any exogenous substrate addition were also obtained in the absence and presence of rotenone in the reaction chamber.

The lag phase, determined as the time in seconds reflecting the capacity of mitochondria to depolarize and repolarize after ADP addition, was also measured for both malate+pyruvate and succinate as substrates.

2.6. Determination of Mitochondrial Swelling

Mitochondrial osmotic volume changes due to MPTP induction were followed by monitoring the classic decrease of absorbance at 540 nm (Oliveira, Bjork et al., 2004) with a Jasco V-560 spectrophotometer. The reaction was stirred continuously and the temperature was maintained at 25ºC. The assays were performed in 2 ml of reaction medium containing 200 mM sucrose, 10 mM Tris, 10 µM EGTA, 5 mM KH_2PO_4, pH 7.4, supplemented with 4 µM rotenone, 10 mM succinate and 240 µM of calcium with 0.5 mg/ml protein. A negative control was performed by using 1 µM of cyclosporin A.

2.7. Mitochondria Calcium Fluxes

Extramitochondrial free calcium was measured with the hexapotassium salt of the fluorescent probe Calcium Green 5-N. Muscle mitochondria (0.1 mg/ml) were resuspended in 2 ml reaction buffer containing 200 mM Sucrose, 10 mM Tris, 10 µM EGTA, 5 mM KH_2PO_4 (pH 7.4) supplemented with 4 µM rotenone. In the presence of non-energized mitochondria, calibration was made using sequential calcium pulses to a final concentration of 300 nmoles of calcium/mg prot. Calcium uptake was initiated by adding 10 mM succinate to the reaction chamber. Free calcium was monitored with 100 nM

Calcium Green 5-N. Fluorescence was continuously recorded in a water-jacketed cuvet holder at 25°C with a Perkin-Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. From the fluorescent decrease (calcium entry in mitochondria) and with proper calibration (as described previously), we determined the rate of calcium uptake. After calcium accumulation, the florescence signal increased (meaning calcium release from mitochondria). At the end of each experiment, a calibration was performed for calculating the maximum and minimum fluorescence signal, with an excess of calcium and EGTA, respectively.

A negative control was performed by using 1 μM of cyclosporin-A. The used dissociation constant for the complex calcium probe was 4 μM (Rajdev & Reynolds, 1993).

2.8. Caspase-like activity assay

To measure caspase 9-like activity, aliquots of *gastrocnemius* tissue homogenate containing 50 μg (for caspase 9) were incubated in a reaction buffer containing 25 mM Hepes (pH 7.4), 10% sucrose; 10 mM DTT, 0.1% CHAPS and 100 μM caspase substrate Ac-LEHD-*p*NA (235400, Calbiochem) for 2 h at 37°C. Caspase-like activity was determined by following the detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate Ac-LEHD-*p*-nitroanilide. The method was calibrated with known concentrations of *p*-nitroanilide (Calbiochem).

2.9. Statistical analysis

Mean and standard error of the mean were calculated for all variables in each group. Students' *t*-test for independent measures was used to compare differences between groups. Statistical Package for the Social Sciences (SPSS Inc, version 12.0) was used for all analyses. The significance level was set at 5%.

3. Results

3.1. Characterization of the animals

Body, *gastrocnemius* muscle absolute weights, heart and muscle to body weight ratio as well as the mitochondrial protein yielding in the two groups are shown in Table 2. Mitochondrial protein yield was higher in trained than in the sedentary group ($p<0.05$) reflecting the widely reported increase in mitochondrial content induced by endurance training (Adhihetty, Ljubicic, & Hood, 2007). In accordance with the well-described body mass and cardiac adaptations induced by endurance training (Moore & Palmer, 1999), the 14 wks of endurance running

training decreased animal weight, and induced an increase heart weight and heart/body ratio ($p<0.05$).

Table 2 Effects of endurance running training on rat body, *gastrocnemius* muscle weight and muscle or heart/body weight ratio.

Groups	*Body weight (g)*	*Muscle weight (g)*	*Muscle/body ratio ($mg.g^{-1}$)*	*Heart/body ratio ($mg.g^{-1}$)*	*Mitochondrial protein yield ($mg.g^{-1}$)*
Sedentary	459.0 ± 12.3	5.4 ± 0.1	11.8 ± 0.3	2.6 ± 0.1	3.1 ± 0.2
Trained	373.3 ± 7.2 *	4.6 ± 0.2 *	12.3 ± 0.5	3.0 ± 0.1 *	5.7 ± 0.3 *

Data are means±SEM; * vs. sedentary ($p<0.05$).

3.2. Mitochondrial oxygen consumption

To elucidate the effects of endurance training on skeletal muscle mitochondrial respiration, we determined oxygen consumption using both complex I and complex II-linked substrates (table 3). As it can be observed in Table 3, endurance treadmill training increased the state 3 (12%) and the RCR (42%) of malate-pyruvate-energized muscle mitochondria. No changes were noted regarding state 4 respiration and on the ADP/O when comparing sedentary and trained groups. When the complex II-linked substrate succinate was used, state 3 respiration (25%) and the RCR (44%) were also significantly increased after 14wks of endurance treadmill training, whereas no changes were detected in the rate of state 4 respiration and on the phosphorylation efficiency expressed as the ADP/O ratio (table 3).

As it can be depicted from Figure 1, endurance training did not alter state 3 respiration in the presence of oligomycin whereas oxygen consumption during uncoupling respiration (state CCCP) was significantly increased (25%) reflecting an improvement in the rate of electron transfer.

Table 3 Effect of endurance treadmill training on respiratory parameters of malate (2mM) plus pyruvate (5mM) and succinate (10mM) energized *gastrocnemius* muscle mitochondria isolated from sedentary control and trained rats.

Pyruvate-malate	*State 3 (natomO.min^{-1}.mg prot^{-1})*	*State 4 (natomO.min^{-1}.mg prot^{-1})*	*RCR*	*ADP/O*
Sedentary	165.6±6.8	16.7±0.6	9.9±0.4	2.9±0.1
Trained	185.9±9.9*	13.1±0.6	14.1±0.3*	2.3±0.3
Succinate				
Sedentary	179.3±6.4	52.7±2.5	3.4±0.2	2.6±0.1
Trained	224.5±14.4*	48.6±1.8	4.9±0.4*	2.4±0.1

Note: Data are means±SEM for skeletal muscle mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group. Oxidative phosphorylation was measured polarographically at 25ºC in a total volume of 1 ml. Respiration medium and other experimental details are provided in methods. RCR – respiratory control ratio (state 3/state 4); ADP/O - number of nmol ADP phosphorylated by natom O consumed.* vs. sedentary ($p<0.05$).

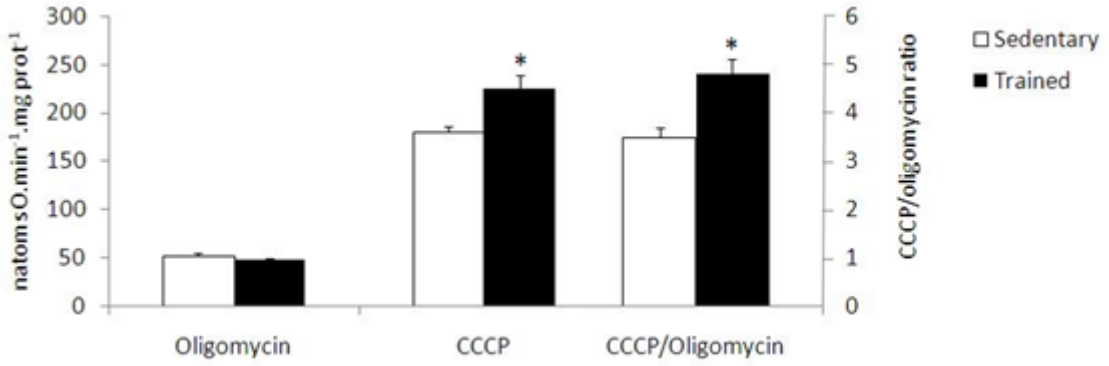

Figure 1 Effect of endurance treadmill training on respiratory rates with CCCP (2µM) and with oligomycin (1.5µg.ml^{-1}) of succinate (10mM) energized *gastrocnemius* muscle mitochondria plus rotenone (4µM) isolated from sedentary control and trained rats. CCCP/oligomycin ratio, an estimate of mitochondrial inner membrane integrity is also presented. Data are means±SEM obtained from different mitochondrial preparations for each experimental group; * vs. sedentary ($p<0.05$).

3.3. Transmembrane electric potential

To attain further insight into the effects of endurance training on mitochondrial capacity to use the protonmotive gradient energy arising from oxidative metabolism, Δψ fluctuations associated with mitochondrial respiration and ADP phosphorylation were also investigated. Considering both trials with complex I and complex II-related substrates, mitochondria isolated from sedentary control and endurance treadmill trained animals did not show significant differences regarding the maximal Δψ, ADP-induced depolarization and repolarization Δψ fluctuations (Table 4). Upon energization with malate plus pyruvate and with succinate, muscle

mitochondria isolated from sedentary and trained animals developed a Δψ around -200 mV and -210mV, respectively. Interestingly, both in presence and absence of rotenone in the reaction medium, muscle mitochondria isolated from trained animals showed a significantly higher Δψ obtained when no exogenous substrates were added (Figure 2, table 4). Moreover, the phosphorylative lag phase was significantly lower in the endurance trained group when both malate plus pyruvate (73.7±2.1 vs. 100.0±8.7 seconds) and succinate (126.8±7.2 vs. 86.7±5.6 seconds) were used, confirming the data obtained for state 3 respiration (Table 3).

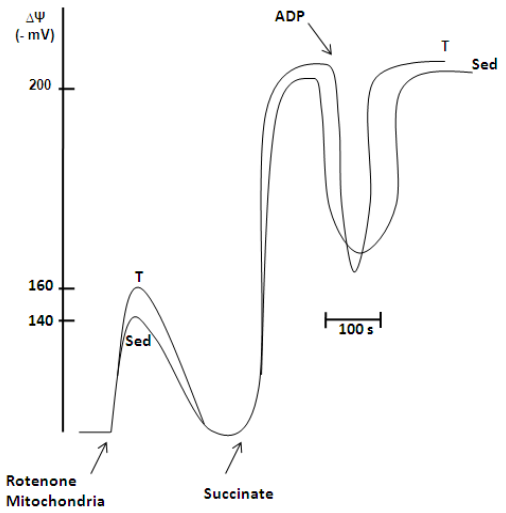

Figure 2 Representative recordings of membrane electric potential (Δψ) of *gastrocnemius* muscle mitochondria measured by a TPP+-selective electrode. Δψ was measured prior (initial small increase in membrane potential) and upon exogenous substrate addition (in this case, succinate) as described in methods section. Depolarization was obtained by adding ADP to the medium. Whenever possible, additions of substrates and ADP were matched in time.

Table 4 Effect of endurance treadmill training on *gastrocnemius* muscle mitochondrial transmembrane potential (Δψ) fluctuations with malate plus pyruvate or succinate as substrates.

	Δψ (-mV)			
Malate/pyruvate	Absence of substrates	Malate+pyruvate energization	ADP Depolarization	Repolarization
Sedentary	150.6±4.9	194.3±2.2	176.4±2.1	196.4±4.2
Trained	181.2±2.8*	199.2±2.2	181.6±2.5	204.0±2.1
Succinate				
Sedentary	139.9±5.4	207.7±3.8	173.7±1.1	207.9±3.1
Trained	158.7±2.4*	213.2±2.8	180.9±3.3	213.4±2.9

Data are means±SEM for skeletal muscle mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group. Table shows the average response of mitochondrial membrane potential developed without any exogenous substrate addition (absence of substrates), with malate (2mM) plus pyruvate (5mM) or succinate (10 mM) (energization), the decrease in membrane potential after ADP addition (depolarization) and the repolarization value after ADP phosphorylation. Mitochondrial transmembrane potential was measured using a TPP^+-selective electrode at 25ºC in a total volume of 1 ml. Reaction medium and other experimental details are provided in methods.* vs. sedentary ($p<0.05$).

3.4. Mitochondrial osmotic swelling

Both the antioxidant and antiapoptotic effect of endurance training on skeletal muscle and the increase in mitochondrial mass with possible altered expression of proapoptotic proteins can modulate the susceptibility to MPTP opening and consequently mitochondrial physiology. Following this idea, the possible effects of endurance treadmill training on the *in vitro* opening of the calcium-induced MPTP was also investigated. Figure 3A represents a typical recording from swelling experiments carried out in mitochondria from sedentary and trained rats. As illustrated, the addition of calcium results in a decrease in the mitochondrial suspension absorbance with three distinct phases. Initially, an increase in absorbance is observed, which most likely results from the formation of opaque calcium crystals inside mitochondria (Andreyev, Fahy, & Fiskum, 1998). Upon MPTP pore opening, a decrease of absorbance with a slow followed by a fast kinetic rate was observed. Incubation of mitochondrial suspension with cyclosporin A, a specific MPTP inhibitor (Broekemeier, Dempsey, & Pfeiffer, 1989), completely inhibits the absorbance decrease after calcium addition, which demonstrate the association with MPTP opening. Figure 3 (panels B-E) shows several end-points measured from the recordings obtained, namely swelling amplitude (the difference between the initial suspension absorbance and the final value upon absorbance stabilization), the average swelling rate, maximal swelling rate and time elapsed until the faster swelling kinetics starts. The results from different parameters when measuring MPTP induction demonstrate that *gastrocnemius* mitochondria isolated from endurance trained rats are more susceptible to calcium-induced PTP opening.

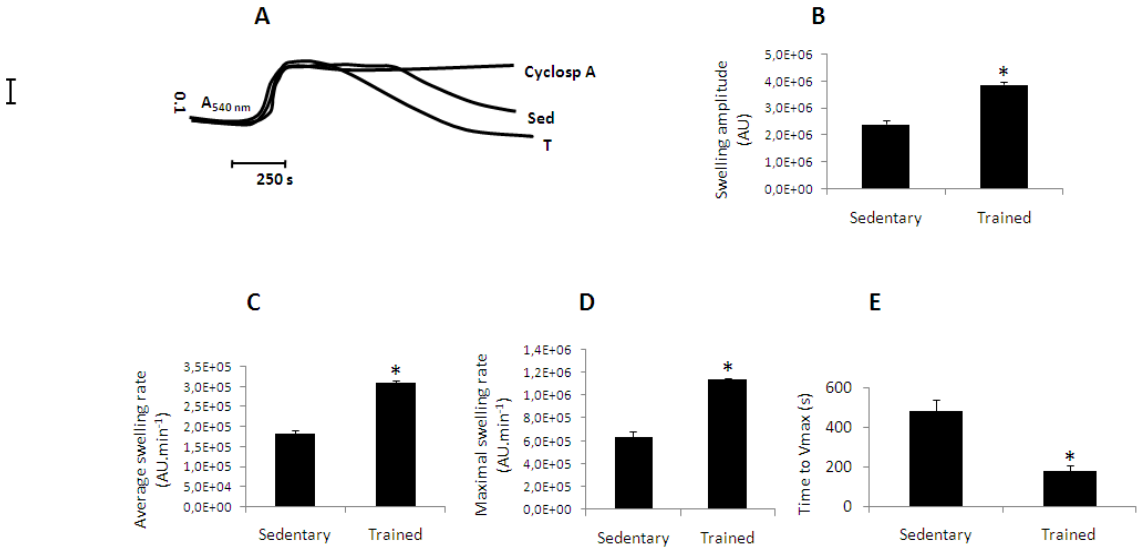

Figure 3 Effect of endurance training on mitochondrial swelling evaluations during MPTP induction in succinate-energized *gastrocnemius* muscle mitochondria. The absorbance of mitochondrial suspension was followed at 540nm. Mitochondria were incubated as described in methods. A 240 μM calcium pulse (960nmol.mg protein^{-1}) was added where indicated to 0.5 mg of mitochondrial protein in order to attain the cyclosporin A-sensitive swelling, indicating that the decreased optical density corresponding to the increased swelling was due to mtPTP opening. Panel A - Typical recording of mitochondrial swelling (decrease of optical density) of different mitochondrial preparations for each experimental group is shown; Panel B - swelling amplitude; Panel C - average swelling rate; Panel D - maximal swelling rate; Panel E – Time to Vmax, meaning the lag phase elapsed until large scale swelling starts are presented. Data are means±SEM; * vs. sedentary ($p<0.05$).

3.5. Calcium accumulation capacity

The increased susceptibility of *gastrocnemius* mitochondria from trained rats to PTP opening is further evidenced by following calcium fluxes through the mitochondrial membrane. Figure 4A represents a typical recording of the fluorometric monitoring of extramitochondrial calcium movements by using the fluorescent probe Calcium Green 5-N. *Gastrocnemius* mitochondria isolated from trained rats were able to initially accumulate a significantly higher amount of calcium (figure 4B) when compared to their sedentary counterparts. However, as can be seen in figure 4C, mitochondria from the trained group are not able to retain calcium inside, which results

into calcium discharge back into the media, and hence into higher calcium release, as pictured. Cyclosporin A was able to prevent calcium release to the external buffer. Caspase-9 activity was selected as a marker of mitochondrial-triggered susceptibility to apoptosis.

The data obtained showed that the 14-wks period of endurance training increased caspase-9-like activity in the endurance trained gastrocnemius muscle.

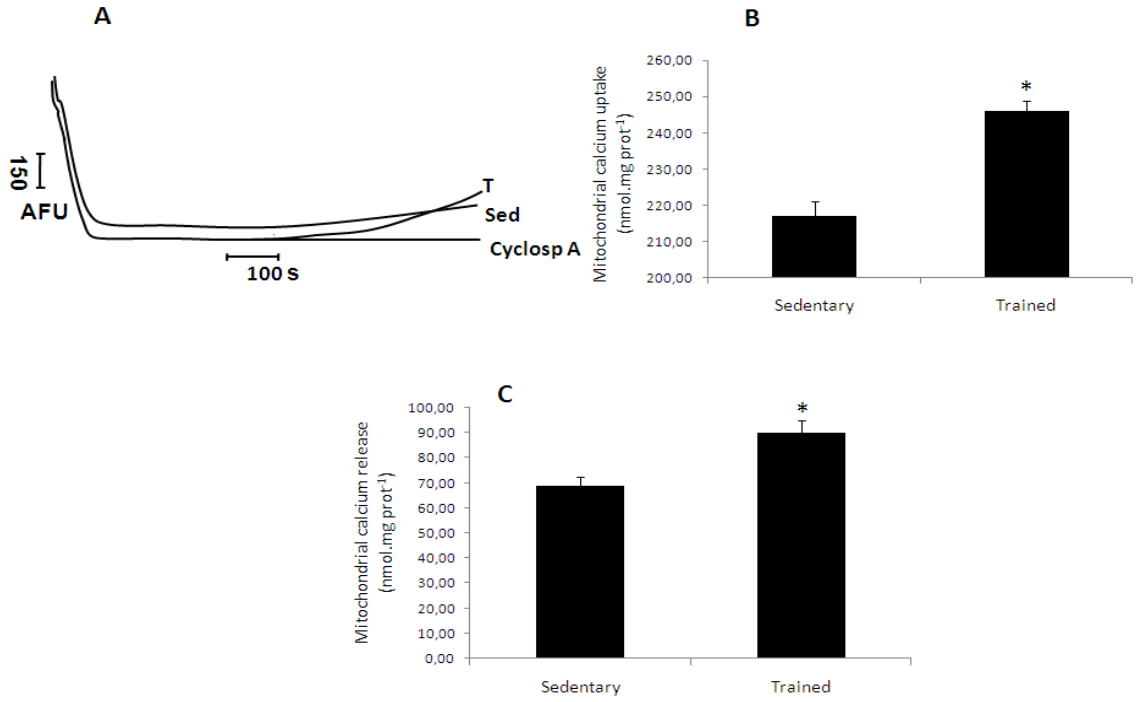

Figure 4 Effect of endurance training on extramitochondrial calcium fluxes in succinate-energized *gastrocnemius* muscle mitochondria. Panel A - Typical measurements of calcium movements using fluorescent calcium sensitive probe Calcium Green 5-N as described in methods. The previous calibration curve is not shown. The reactions were carried out in 2mL of reaction medium supplemented with 0.1 mg protein, 4µM rotenone, 100nM Calcium Green 5-N. Calcium entry into mitochondria (indicated by fluorescence decay) was initiated by 10mM succinate addition. Fluorescence was monitored continuously and the increase in signal indicates calcium release. The release of calcium from mitochondria was inhibited by 1µM cyclosporin A, indicating that the observed release was due to MPTP opening. Calcium fluxes are representatively expressed as arbitrary units of fluorescence (AFU); Panel B – mitochondrial calcium uptake; and Panel C – mitochondrial calcium release. Data are means±SEM; * vs. sedentary ($p<0.05$)

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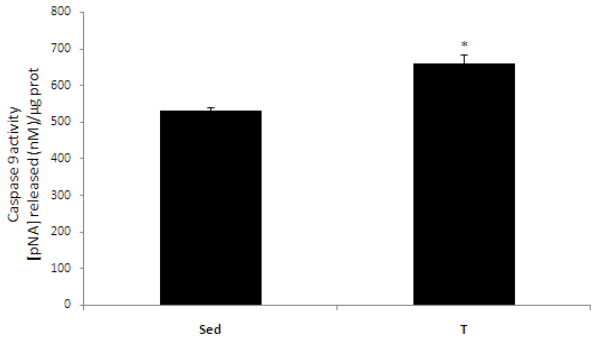

Figure 5 Effect of endurance training on caspase 9 activity in *gastrocnemius* muscle. Caspase-9-like activity was measured by following the cleavage of the colorimetric substrates Ac-LEHD-pNA. The caspase-like activity was expressed by [pNA] released (nM)/µg prot. Data is expressed as means±SEM; * vs. sedentary ($p<0.05$)

4. Discussion

4.1. Overview of principal findings

The present investigation provides new insights into the mechanisms by which endurance treadmill exercise training interacts with mitochondrial bioenergetics in rat *gastrocnemius* muscle. Data from this study suggest for the first time that the enhanced tolerance to permeability transition pore opening is not a primary mechanism for increased *gastrocnemius* mitochondrial function induced by endurance training, which contradicts our initial hypothesis.

4.2. Mitochondrial oxygen consumption and transmembrane potential

It is known that regular endurance training induces numerous physiological adaptations, including alterations in skeletal muscle mitochondrial respiratory function ultimately improving exercise capacity. To confirm that endurance training improves mitochondrial function, *gastrocnemius* mitochondrial respiration associated with transmembrane electrical potential and phosphorylation cycle induced by ADP were measured using complex I and II-linked substrates (tables 3 and 4 and figure 2). The 14 wks of endurance treadmill training improved state 3 and RCR when both energy substrates were used as well as the

uncoupled respiration induced by CCCP. The respiratory data from the present study agree with the general mitochondrial respiratory improvement in aerobically trained muscles largely reported both in humans and animals (Daussin et al., 2008; Garnier et al., 2005; Holloszy, 1967; Tonkonogi & Sahlin, 1997; Tonkonogi, Walsh, Svensson, & Sahlin, 2000; Walsh, Tonkonogi, & Sahlin, 2001; Zoll et al., 2002).

The increase in state 3 respiration in trained animals can be caused by one or more of the following factors: a) a respiratory chain functional improvement through intrinsic up-regulation of its oxido-reductases, b) increased availability of reducing equivalents or c) specific improvements of the mitochondrial phosphorylation system (ATP synthase, phosphate transporter or the adenine nucleotide translocator). The increase in CCCP-induced uncoupled respiration indicates that the maximal rate of electron transfer was improved by endurance training suggesting that mechanisms a) or b) were involved, although the data do not exclude also an improved efficiency of the phosphorylative system in *gastrocnemius* mitochondria from the trained group.

The complementary study of Δψ is essential for an integrated appraisal of the mitochondrial function as it reflects basic useful energetic relationships in the maintenance of cellular homeostasis. For instance, when cytosolic Ca^{2+} concentrations rise due to disturbances of cellular Ca^{2+} homeostasis, mitochondrial act as Ca^{2+} buffers through uptake and accumulation (Gunter, Buntinas, Sparagna, Eliseev, & Gunter, 2000; Gunter & Gunter, 2001; Gunter, Yule, Gunter, Eliseev, & Salter, 2004). In addition, intramitochondrial Ca^{2+} modulates the activity of Ca^{2+}-sensitive dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase), of F_0F_1ATPase, and of the adenine nucleotide translocase (see Gunter, Buntinas, Sparagna, Eliseev, & Gunter, 2000; Gunter & Gunter, 2001; Gunter, Yule, Gunter, Eliseev, & Salter, 2004). 14 wks of endurance treadmill running training did not affect the maximal Δψ generated by malate plus pyruvate or by succinate. Only one previous study using the fluorescent probe rhodamine 123 measured basal Δψ in *gastrocnemius* mitochondria from rats previously submitted to swimming training reporting reduced Δψ in trained group, an effect that is suggested to be independent of UCP modulation (Venditti, Masullo, & Di Meo, 1999). The decreased expression of mitochondrial UCP3 content in endurance trained muscle was also described both in rats

(Boss et al., 1998) and humans (Fernstrom, Tonkonogi, & Sahlin, 2004). Nevertheless, others proposed that endurance training and chronic electric stimulation-induced mitochondrial biogenesis increased muscle mitochondrial UCP content in rats (Ljubicic, Adhihetty, & Hood, 2004) or the sensitivity of basal mitochondrial respiration to UCP-regulating ligands such as non-esterified fatty acids in humans (Tonkonogi, Krook, Walsh, & Sahlin, 2000). Actually, the role of endurance training on muscle mitochondrial permeability and the related mechanisms should be further investigated.

The ratio between the oxygen consumption rates for CCCP/oligomycin may be interpreted as an estimate of inner mitochondrial membrane integrity (Magalhaes et al., 2007b). Our results showed that this ratio is increased in the trained group (Figure 1), indicating a decreased permeability of inner mitochondrial membrane to protons, although the adaptation does not result into a significant increase in Δψ or in lower state 4 respiration.

An interesting finding of the present study is the increase in the developed Δψ previous to substrate addition, this increased Δψ being higher in the trained group (Table 4 and figure 2). As can be depicted from figure 2, when mitochondria are added to the reaction medium, the apparent Δψ increased and decreased in a few seconds. Although other factors can account for this effect, one possibility is that an increased availability of endogenous substrates exists in mitochondria from the trained animals.

4.3. Modulation of the permeability transition pore

Mitochondrial dysfunction can also be triggered by stress-induced opening of the MPTP, which has been involved in calcium dynamics and cell death (Crompton, 1999; Hengartner, 2000). Recent studies have also shown that an increased vulnerability to skeletal muscle MPTP opening develops under various pathophysiological conditions including aging (Chabi et al., 2008), denervation-induced atrophy (Adhihetty, O'Leary, Chabi, Wicks, & Hood, 2007; Csukly et al., 2006), bupivacaine administration (Irwin et al., 2002), and muscle dystrophies (Millay et al., 2008); interestingly, exercise is largely recommended as a powerful strategy, either to prevent and/or to treat mitochondrial-related myopathies (Adhihetty, O'Leary, & Hood, 2008; Lumini, Magalhaes, Oliveira, & Ascensao, 2008; Tarnopolsky & Raha, 2005). The study of the effects of *in vivo* exercise, particularly of endurance

running training, on the susceptibility to MPTP opening in *gastrocnemius* skeletal muscle is clearly of important clinical relevance.

A hallmark of MPTP is the cyclosporine-inhibited large-scale swelling induced by calcium in the presence of phosphate, which occurs through the generation of oxidative stress (Kowaltowski, Castilho, Grijalba, Bechara, & Vercesi, 1996). The unexpected finding of the present study was that the curve profiles of mitochondria from sedentary control and trained groups were different indicating increased susceptibility to the calcium-induced MPTP in the trained group (figure 3A). The results showed that mitochondria from the trained group indeed accumulate a higher amount of calcium, although the initial higher accumulation resulted into a faster and higher calcium release when compared with mitochondria from their sedentary counterparts (figure 4).

Although endurance training-induced skeletal muscle mitochondrial alterations are clearly an advantageous metabolic adaptation from an energy standpoint, mitochondria are also intimately involved in apoptosis. As a higher mitochondrial mass resulting from biogenesis in the endurance trained muscle may also be related with an increase in the concentration of mitochondrial proapoptotic factors, it may be likely that a stimulus for cellular death may result in a magnified apoptotic response. Most of the reports regarding the effect of endurance training or chronic *in situ* stimulation on skeletal muscle apoptotic indexes suggest beneficial adaptations towards an antiapoptotic phenotype (Adhihetty, Ljubicic, & Hood, 2007; Adhihetty, O'Leary, & Hood, 2008; Siu, Bryner, Martyn, & Alway, 2004; Siu, Bryner, Murlasits, & Alway, 2005). Recently, Adhihetty et al. (2007) observed that an artificial model of exercise produced by electrical stimulation of the motor nerve increased the content of 70-kDa HSP and apoptosis repressor with a caspase recruitment domain by 1.3- and 1.4-fold, respectively. Although mitochondrial subfractions revealed some signs suggestive of increased apoptotic susceptibility, the aforementioned increases were accompanied by suppression of reactive oxygen species (ROS) in mitochondria during state 4, a lower amount of mitochondrial cytochrome c and apoptosis inducible factor release upon ROS treatment and increased time to maximal rate of absorbance decrease following stimulation.

Although we have no data to confirm, for instance on myosin isoforms, one possible explanation for the higher susceptibility of *gastrocnemius*

mitochondria isolated from our 14 wks endurance trained group to undergo PTP induced by calcium could be associated to a possible shift toward a more slow oxidative muscle phenotype induced by endurance training. In fact, some studies have reported fiber type differences in mitochondrial properties (Leary et al., 2003) including the resistance to calcium-induced MPTP (Picard et al., 2008). Picard et al., (2008) elegantly demonstrated that mitochondria from slow oxidative *soleus* muscle have higher sensitivity to PTP opening than those from fast glycolitic superficial white *gastrocnemius*. Among the different explanations for the increased MPTP induction in mitochondria from the trained animals, several possibilities may be advanced including, among others, alterations in the expression of putative MPTP complex proteins including cyclophilin D, ANT or the VDAC (Adhihetty, O'Leary, & Hood, 2008; Di Lisa & Bernardi, 2005; Kowaltowski, Castilho, & Vercesi, 2001). Although we did not measure some of these potential factors related to the susceptibility of MPTP opening, the study of Picard et al., (2008) shows that the different resistance to calcium-induced MPTP was not related to the expression of pore components.

One strong possibility that is corroborated by data from the present work is that the increased activity of muscle mitochondrial respiratory chain observed in the trained animals leads to the observed higher calcium entry, which would trigger a faster formation of the MPTP. Also, Picard et al., (2008) found that endogenous mitochondrial matrix calcium levels under baseline conditions were higher in oxidative than in glycolitic fibres, limiting calcium retention capacity before undergoing PTP opening. However, in the present study, mitochondria from the trained group were able to uptake significantly higher amounts of calcium (figure 4 B), which could possible mean that the hypothetical higher basal matrix calcium levels in mitochondria from the trained animals may not limit calcium uptake capacity. Accordingly, early data from Tate et al., (1978) suggested that long-term endurance training resulted in an increased uptake and endogenous mitochondrial calcium content of skeletal muscle. Certainly, further studies regarding the influence of exercise on possible mechanisms behind MPTP modulation in skeletal muscle are required.

We further determined the activity of caspase 9 in the whole tissue homogenate as a marker of apoptotic cell death driven by mitochondrial dysfunction. The results of the present study show that endurance training

increased caspase 9-like activity suggesting that endurance training may predispose *gastrocnemius* mitochondria to increase basal caspase 9 activation. Although the few studies addressing the effect of endurance training on markers of apoptosis in skeletal muscle revealed positive outcomes (Siu, Bryner, Martyn, & Alway, 2004; Siu, Bryner, Murlasits, & Alway, 2005), to the best of our knowledge no one yet determined caspase 9 activity. It is likely that other adaptive antiapoptotic mechanisms independent of caspase 9 activation may be involved in response to endurance exercise training, in order to avoid the loss of muscle cells through apoptotic mechanisms.

5. Summary

In summary, our results support the notion that a program of 14 wks of moderate endurance treadmill running training induces improvements in mitochondrial respiratory function, although we can speculate that one indirect consequence of increased mitochondrial activity is a higher calcium accumulation and enhanced MPTP opening.

References

Adhihetty, P. J., Ljubicic, V., & Hood, D. A. (2007). Effect of chronic contractile activity on SS and IMF mitochondrial apoptotic susceptibility in skeletal muscle. *Am J Physiol Endocrinol Metab, 292*(3), E748-755.

Adhihetty, P. J., O'Leary, M. F., Chabi, B., Wicks, K. L., & Hood, D. A. (2007). Effect of denervation on mitochondrially mediated apoptosis in skeletal muscle. *J Appl Physiol, 102*(3), 1143-1151.

Adhihetty, P. J., O'Leary, M. F., & Hood, D. A. (2008). Mitochondria in skeletal muscle: adaptable rheostats of apoptotic susceptibility. *Exerc Sport Sci Rev, 36*(3), 116-121.

Andreyev, A. Y., Fahy, B., & Fiskum, G. (1998). Cytochrome c release from brain mitochondria is independent of the mitochondrial permeability transition. *FEBS Lett, 439*(3), 373-376.

Ascensao, A., & Magalhaes, J. (2006). Exercise and mitochondrial function in striated muscle. In A. J. Moreno, P. J. Oliveira & M. C. Palmeira (Eds.), *Mitochondrial Pharmacology and Toxicology* (pp. 237-270). Kerala: Transworld Research Network.

Atalay, M., Oksala, N. K., Laaksonen, D. E., Khanna, S., Nakao, C., Lappalainen, J., et al. (2004). Exercise training modulates heat shock protein response in diabetic rats. *J Appl Physiol, 97*(2), 605-611.

Benamar, A., Tallon, C., & Macherel, D. (2003). Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing. *Seed Sci Res, 13*, 35-45.

Boss, O., Samec, S., Desplanches, D., Mayet, M. H., Seydoux, J., Muzzin, P., et al. (1998). Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 in the rat. *Faseb J, 12*(3), 335-339.

Broekemeier, K. M., Dempsey, M. E., & Pfeiffer, D. R. (1989). Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem, 264*(14), 7826-7830.

Brookes, P. S. (2005). Mitochondrial H(+) leak and ROS generation: an odd couple. *Free Radic Biol Med, 38*(1), 12-23.

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol, 287*(4), C817-833.

Cadenas, E. (2004). Mitochondrial free radical production and cell signaling. *Mol Aspects Med, 25*(1-2), 17-26.

Chabi, B., Ljubicic, V., Menzies, K. J., Huang, J. H., Saleem, A., & Hood, D. A. (2008). Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. *Aging Cell, 7*(1), 2-12.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *Biochem J, 341 (Pt 2)*, 233-249.

Csukly, K., Ascah, A., Matas, J., Gardiner, P. F., Fontaine, E., & Burelle, Y. (2006). Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D. *J Physiol, 574*(Pt 1), 319-327.

Daussin, F. N., Zoll, J., Ponsot, E., Dufour, S. P., Doutreleau, S., Lonsdorfer, E., et al. (2008). Training at high exercise intensity promotes qualitative adaptations of mitochondrial function in human skeletal muscle. *J Appl Physiol, 104*(5), 1436-1441.

Desplanches, D., Ecochard, L., Sempore, B., Mayet-Sornay, M. H., & Favier, R. (2004). Skeletal muscle HSP72 response to mechanical unloading: influence of endurance training. *Acta Physiol Scand, 180*(4), 387-394.

Di Lisa, F., & Bernardi, P. (2005). Mitochondrial function and myocardial aging. A critical analysis of the role of permeability transition. *Cardiovasc Res, 66*(2), 222-232.

Estabrook, R. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods Enzymol, 10*, 41-47.

Fernstrom, M., Tonkonogi, M., & Sahlin, K. (2004). Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle. *J Physiol, 554*(Pt 3), 755-763.

Fluck, M., & Hoppeler, H. (2003). Molecular basis of skeletal muscle plasticity--from gene to form and function. *Rev Physiol Biochem Pharmacol, 146*, 159-216.

Garnier, A., Fortin, D., Zoll, J., N'Guessan, B., Mettauer, B., Lampert, E., et al. (2005). Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. *Faseb J, 19*(1), 43-52.

Gore, M., Fiebig, R., Hollander, J., Leeuwenburgh, C., Ohno, H., & Ji, L. L. (1998). Endurance training alters antioxidant enzyme gene expression in rat skeletal muscle. *Can J Physiol Pharmacol, 76*(12), 1139-1145.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J Biol Chem, 177*(2), 751-766.

Gunter, T. E., Buntinas, L., Sparagna, G., Eliseev, R., & Gunter, K. (2000). Mitochondrial calcium transport: mechanisms and functions. *Cell Calcium, 28*(5-6), 285-296.

Gunter, T. E., & Gunter, K. K. (2001). Uptake of calcium by mitochondria: transport and possible function. *IUBMB Life, 52*(3-5), 197-204.

Gunter, T. E., Yule, D. I., Gunter, K. K., Eliseev, R. A., & Salter, J. D. (2004). Calcium and mitochondria. *FEBS Lett, 567*(1), 96-102.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Hollander, J., Fiebig, R., Gore, M., Bejma, J., Ookawara, T., Ohno, H., et al. (1999). Superoxide dismutase gene expression in skeletal muscle: fiber-specific adaptation to endurance training. *Am J Physiol, 277*(3 Pt 2), R856-862.

Holloszy, J. O. (1967). Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem, 242*(9), 2278-2282.

Irwin, W., Fontaine, E., Agnolucci, L., Penzo, D., Betto, R., Bortolotto, S., et al. (2002).

Bupivacaine myotoxicity is mediated by mitochondria. *J Biol Chem, 277*(14), 12221-12227.

Kamo, N., Muratsugu, M., Hongoh, R., & Kobatake, Y. (1979). Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol, 49*(2), 105-121.

Kowaltowski, A. J., Castilho, R. F., Grijalba, M. T., Bechara, E. J., & Vercesi, A. E. (1996). Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca2+ ions. A proposed model for phosphate-stimulated lipid peroxidation. *J Biol Chem, 271*(6), 2929-2934.

Kowaltowski, A. J., Castilho, R. F., & Vercesi, A. E. (2001). Mitochondrial permeability transition and oxidative stress. *FEBS Lett, 495*(1-2), 12-15.

Leary, S. C., Lyons, C. N., Rosenberger, A. G., Ballantyne, J. S., Stillman, J., & Moyes, C. D. (2003). Fiber-type differences in muscle mitochondrial profiles. *Am J Physiol Regul Integr Comp Physiol, 285*(4), R817-826.

Ljubicic, V., Adhihetty, P. J., & Hood, D. A. (2004). Role of UCP3 in state 4 respiration during contractile activity-induced mitochondrial biogenesis. *J Appl Physiol, 97*(3), 976-983.

Lowry, O. H., Rosenbrough, N., Farr, A. L., & Radall, R. J. (1951). Protein measurement with the folin phenol reagent. *J Biol Chem*(193), 265-275.

Lumini, J. A., Magalhaes, J., Oliveira, P. J., & Ascensao, A. (2008). Beneficial effects of exercise on muscle mitochondrial function in diabetes mellitus. *Sports Med, 38*(9), 735-750.

Magalhaes, J., Ascensao, A., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., et al. (2005). Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. *J Appl Physiol, 99*(4), 1247-1253.

Magalhaes, J., Ferreira, R., Neuparth, M. J., Oliveira, P. J., Marques, F., & Ascensao, A. (2007). Vitamin E prevents hypobaric hypoxia-induced mitochondrial dysfunction in skeletal muscle. *Clin Sci (Lond)*.

Millay, D. P., Sargent, M. A., Osinska, H., Baines, C. P., Barton, E. R., Vuagniaux, G., et al. (2008). Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. *Nat Med, 14*(4), 442-447.

Moore, R. L., & Palmer, B. M. (1999). Exercise training and cellular adaptations of normal and diseased hearts. *Exerc Sport Sci Rev, 27*, 285-315.

Naito, H., Powers, S. K., Demirel, H. A., & Aoki, J. (2001). Exercise training increases heat shock protein in skeletal muscles of old rats. *Med Sci Sports Exerc, 33*(5), 729-734.

Oliveira, P. J., Bjork, J. A., Santos, M. S., Leino, R. L., Froberg, M. K., Moreno, A. J., et al. (2004). Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. *Toxicol Appl Pharmacol, 200*(2), 159-168.

Picard, M., Csukly, K., Robillard, M. E., Godin, R., Ascah, A., Bourcier-Lucas, C., et al. (2008). Resistance to Ca2+-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles. *Am J Physiol Regul Integr Comp Physiol, 295*(2), R659-668.

Rajdev, S., & Reynolds, I. J. (1993). Calcium green-5N, a novel fluorescent probe for monitoring high intracellular free Ca2+ concentrations associated with glutamate excitotoxicity in cultured rat brain neurons. *Neurosci Lett, 162*(1-2), 149-152.

Siu, P. M., Bryner, R. W., Martyn, J. K., & Alway, S. E. (2004). Apoptotic adaptations from exercise training in skeletal and cardiac muscles. *Faseb J, 18*(10), 1150-1152.

Siu, P. M., Bryner, R. W., Murlasits, Z., & Alway, S. E. (2005). Response of XIAP, ARC, and FLIP apoptotic suppressors to 8 wk of treadmill running in rat heart and skeletal muscle. *J Appl Physiol, 99*(1), 204-209.

Skulachev, V. P. (1999). Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. *Mol Aspects Med, 20*(3), 139-184.

Tarnopolsky, M. A., & Raha, S. (2005). Mitochondrial myopathies: diagnosis, exercise intolerance, and treatment options. *Med Sci Sports Exerc, 37*(12), 2086-2093.

Tate, C. A., Bonner, H. W., & Leslie, S. W. (1978). Calcium uptake in skeletal muscle mitochondria. II. The effects of long-term chronic and acute exercise. *Eur J Appl Physiol Occup Physiol, 39*(2), 117-122.

Tonkonogi, M., Krook, A., Walsh, B., & Sahlin, K. (2000). Endurance training increases stimulation of uncoupling of skeletal muscle mitochondria in humans by non-esterified fatty acids: an uncoupling-protein-mediated effect? *Biochem J, 351 Pt 3*, 805-810.

Tonkonogi, M., & Sahlin, K. (1997). Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. *Acta Physiol Scand, 161*(3), 345-353.

Tonkonogi, M., & Sahlin, K. (2002). Physical exercise and mitochondrial function in human skeletal muscle. *Exerc Sport Sci Rev, 30*(3), 129-137.

Tonkonogi, M., Walsh, B., Svensson, M., & Sahlin, K. (2000). Mitochondrial function and antioxidative defence in human muscle: effects of endurance training and oxidative stress. *J Physiol, 528 Pt 2*, 379-388.

Venditti, P., Masullo, P., & Di Meo, S. (1999). Effect of training on H(2)O(2) release by mitochondria from rat skeletal muscle. *Arch Biochem Biophys, 372*(2), 315-320.

Walsh, B., Tonkonogi, M., & Sahlin, K. (2001). Effect of endurance training on oxidative and antioxidative function in human permeabilized muscle fibres. *Pflugers Arch, 442*(3), 420-425.

Zoll, J., Sanchez, H., N'Guessan, B., Ribera, F., Lampert, E., Bigard, X., et al. (2002). Physical activity changes the regulation of mitochondrial respiration in human skeletal muscle. *J Physiol, 543*(Pt 1), 191-200.

Study III

Experimental article

Endurance training reverts heart mitochondrial dysfunction, permeability transition and apoptotic signaling in long-term severe hyperglycaemia

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Abstract

The present study analyzed the effects of endurance training against cardiac mitochondrial dysfunction, particularly on the susceptibility to mitochondrial permeability transition pore (MPTP) induction in streptozotocin (STZ)-induced hyperglycaemia.

Twenty-four young male Wistar rats were randomly assigned into sedentary-citrate (SED+CIT), sedentary type-I diabetes (SED+STZ; 50mg/kg), T+CIT (14-wk treadmill running, 60min/day) and T+STZ (injected 4-wks before training). After 18-wk, isolated heart mitochondria were used for *in vitro* oxygen consumption and transmembrane potential ($\Delta\Psi$) assessment. Cyclosporin A (CyclA)-sensitive osmotic swelling and Ca^{2+} fluxes were measured to study MPTP susceptibility. Voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT), cyclophilin D (CypD), transcription factor A (Tfam), Bax, Bcl-2 contents, caspase-3 and -9 activities were determined.

In sedentary group, long-term severe hyperglycaemia decreased state 3, CCCP-induced uncoupling and increased oligomycin-inhibited respiration, state 4 and lag phase with glutamate-malate. A decreased state 3 and state 4 with succinate was observed. Moreover, hyperglycaemia decreased Ca^{2+} uptake and increased CyclA-sensitive Ca^{2+} release and Ca^{2+}-induced mitochondrial swelling. The oxygen consumption and $\Delta\Psi$ parameters impaired by long-term severe hyperglycaemia were reverted by endurance training (SED+STZ vs. T+STZ). Training increased mitochondrial Ca^{2+} uptake and decreased Ca^{2+} release in hyperglycaemic groups. Additionally, endurance training reverted hyperglycaemia-induced CypD elevation, attenuating decrease of ANT, VDAC and Tfam. Moreover, training prevented the STZ-induced elevation in Bax, Bax-to-Bcl-2 ratio, caspase-3 and -9 and the increased Bcl-2.

Endurance training reestablished heart mitochondrial respiratory dysfunction caused by long-term severe hyperglycaemia and reduced the increased susceptibility to MPTP induction probably by modulation of MPTP regulatory proteins.

1.Introduction

Diabetes mellitus (DM) is one of the most common metabolic diseases in the world. The complications associated with DM are often responsible for a decreased quality of life in many patients. Cardiovascular disease remains the major cause of morbidity and mortality in people with DM. Being mitochondria the cellular powerhouses and vital to many other functions including cell signaling and the regulation of calcium homeostasis, heart mitochondrial dysfunction during hyperglycaemia may play an important role in the pathophysiology of cardiac complications during DM. Accordingly, recent and early biochemical, functional and morphological data demonstrated increased generation of reactive oxygen species with consequent augmented levels of oxidative (Dabkowski et al., 2009; Shen, Zheng, Metreveli, & Epstein, 2006) and morphological damage (Bugger et al., 2008; Bugger et al., 2009; Searls, Smirnova, Fegley, & Stehno-Bittel, 2004; Shen et al., 2004), impairment of the mitochondrial phosphorylative system (Boudina et al., 2005; Bugger et al., 2008; Bugger et al., 2009; Dabkowski et al., 2009; Felix, Gillis, Driedzic, Paulson, & Broderick, 2001; Lashin & Romani, 2003; Lashin, Szweda, Szweda, & Romani, 2006) and also an increased susceptibility to the mitochondrial permeability transition (MPT) (Oliveira et al., 2003) in hearts from hyperglycaemic animals, which is associated with the physiopathology of diabetic cardiomyopathy (Boudina & Abel, 2006, 2007; Oliveira, 2005).

MPT is one of the negative conditions that can affect mitochondrial function, caused by the formation of polyprotein pores (the MPT pores, or MPTP), occurring under conditions of mitochondrial calcium overload and increased oxidative stress (Crompton, 1999). Most of the literature published so far supports that the release of pro-apoptotic proteins contained in mitochondria is dependent, at least partially, on the formation and opening of MPTP that span the inner and outer mitochondrial membranes leading to the loss of mitochondrial membrane potential ($\Delta\Psi$), mitochondrial osmotic swelling and rupture of the outer mitochondrial membrane (Crompton, 1999). Despite some reports suggesting that voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) are dispensable for the formation of MPTP opening (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007; Kokoszka et al., 2004), a large body of literature to date supports that one of the classic structures of the MPTP comprises the outer membrane VDAC as well as the inner membrane ANT and matricial cyclophilin D (Crompton, 1999).

Given the variety of possible mechanisms underlying the mitochondrionopathy that characterizes hyperglycaemic hearts, several pharmacological and non-pharmacological therapeutic measures have been studied in order to counteract the deleterious effects of this condition on cardiac mitochondria, including antioxidant supplementation and physical exercise (Green, Brand, & Murphy, 2004; Lumini, Magalhaes, Oliveira, & Ascensao, 2008). Endurance training has been importantly advised as a non-pharmacological strategy against myocardial injury. In fact, it is well described that endurance training improves myocardial tolerance to deleterious stimuli which result from oxidative injury and increased apoptosis and which are known to involve mitochondria in the process (Ascensao, Ferreira, & Magalhaes, 2007). The benefits of endurance training against diabetes-induced cardiac toxicity and dysfunction have been established. Previous works have suggested that the advantage of endurance training on the cardiovascular system of hyperglycaemic rats include the protection of the heart tissue and mitochondria (Lumini, Magalhaes, Oliveira, & Ascensao, 2008). However, the effects of endurance treadmill training on long-term and severe hyperglycaemia-induced MPTP induction and apoptotic signaling were never studied so far. Our hypothesis is that moderate endurance treadmill training reverts the damage to mitochondrial function, decreasing MPTP susceptibility to calcium and apoptotic signaling in hearts from streptozotocin (STZ)-induced hyperglycaemic animals. In the present study, we used STZ-induced rats as a model for human type I diabetes mellitus. STZ-induced hyperglycaemia is obtained after selective destruction of □-cell by STZ, a broad spectrum antibiotic with diabetogenic effects. STZ-treated rats present many characteristics seen in insulin-dependent diabetic human patients such as hypoinsulinemia, hyperglycaemia, ketonuria, and hyperlipidemia. Therefore, this model is of great use to evaluate the alterations promoted by uncontrolled type I diabetes.

2. Methods

2.1 Chemicals

All chemicals were obtained from Sigma, St. Louis, MO, unless otherwise described.

2.2 Animals

Twenty-four Wistar male rats (aged 6-8 wks, weighting 200 g at the beginning of the experiments) were used. During the experimental protocol, animals were housed in collective cages (2 rats per

cage) and were maintained in a room at normal environment (21-22º C; ~50-60% humidity) receiving food and water *ad libitum* in 12 hours light/dark cycles. The animals were randomly divided into four groups (n=6 *per* group): Sedentary citrate (SED+CIT), sedentary type I diabetes (SED+STZ), endurance trained citrate (T+CIT) and endurance trained type I diabetes (T+STZ). 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.

2.3 Induction and characterization of STZ-induced hyperglycaemia

In order to induce hyperglycaemia, two groups were injected intraperitoneally with a single injection of STZ (50 mg/kg), after a 16-h fasting period. The volume used was always 0.5 ml/200 g body weight. Control animals were injected with the same volume of citrate solution. In the following 24h, animals were orally feed with glicosilated serum in order to avoid hypoglycaemia resulting from massive destruction of β-cells and consequent release of intracellular insulin. During this period, weight was measured and blood glucose was determined from the tail vein as described. Values were taken in fasting conditions just before STZ administration and in non-fasting conditions in the weeks after. If feeding blood glucose in the tail vein exceeded 250 mg/dl, animals were considered hyperglycaemic.

2.4 Glycaemia and HbA1C evaluation

Blood glucose concentration was determined immediately after animal sacrifice (Glucometer-Elite, Bayer). The glycated hemoglobin (HbA1C) values were determined in blood collected at the time of animal sacrifice through ionic exchange chromatography (Abbott Imx Glicohemoglobin, Abbott Laboratories, Portugal).

2.5 Endurance training protocol

Four weeks after induction of severe hyperglycaemia, the animals from T+CIT and T+STZ groups 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). The protocol included 5 days of habituation to the treadmill with 10 min of running at 15m/min and 0% grade, with daily increases of 5-10 min until 30 min was achieved. Habituation was followed by one consecutive wk of continuous running (30 min/day) at 15m/min and 0% grade and was gradually increased until 60min/day (20m/min) on the second wk.

The animals from SED+CIT and SED+STZ groups were not exercised but were placed on a non-moving treadmill instead five times per wk (10-30 min/session) with the purpose of habituating animals to 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	60	60	60	60	60	60	60	60	60	60	60	60
Treadmill speed (m/min)	15	20	25	25	25	25	25	25	25	25	25	25	25	25
% Grade (treadmill inclination)	0	0	0	0	0	3	3	6	6	6	6	6	6	6

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

2.6 Animals sacrifice, plasma collection, heart and soleus harvesting and homogenization

Twenty-four hours after the last training session, animals were anaesthetized with 50 mg.kg^{-1} sodium pentobarbital and placed in supine position. After a quick opening of the chest cavity, rat hearts were then rapidly excised, rinsed, carefully dried and weighted. A representative portion of heart ventricles and *soleus* muscle were separated and immediately frozen at 80°C for later determination of caspase 3 and 9 (heart) and citrate synthase (CS, *soleus*) activities, as indexes of apoptosis and training efficacy, respectively.

Heart ventricle portion and *soleus* muscle were separately homogenized in homogenization buffer (20mM Tris, 137mM NaCl, 0.2mM EDTA, 0.5mM EGTA, 1% Triton X-100, 10% Glycerol, pH 7.6) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C 3-5 times for 5 sec at speed low setting, with a final burst at a higher speed setting. Homogenates were centrifuged during 2 min at 2000 x*g*, 4°C, in order to eliminate cellular debris. The resulting supernatant was stored at –80°C for later assays.

2.7 Isolation of rat heart mitochondria

Rat heart mitochondria were prepared using conventional methods of differential centrifugation as follows (Bhattacharya, Thakar, Johnson, & Shanklin, 1991). Briefly, 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.

The minced blood free tissue was then ressuspended in 40ml of isolation medium containing 1mg protease subtilopeptidase A Type VIII (Sigma P-5380) *per* g of tissue and homogenized with a tightly fitted homogenizer. The suspension was incubated for 1 minute (4ºC) and then re-homogenized. The homogenate was then centrifuged at 14,500x*g* for 10 minutes. 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 minutes and the resulting supernatant was centrifuged at 12,000x*g* for 10 minutes. The pellet was resuspended using a paintbrush and re-pellet at 12,000x*g* for 10 minutes. EGTA and defatted BSA were omitted from the final washing medium (250mM sucrose, 10mM Hepes-KOH pH 7.4). Mitochondrial protein content was determined by the Biuret method calibrated with BSA. All isolation procedures were performed at 0-4°C. Considering the relatively greater abundance of intermyofibrillar (IMF) (~80%) compared with subsarcolemmal (SS) (~20%) mitochondria within the cells, a potentially dominant role for the IMF subfraction vs. the SS subfraction in evoking the mitochondrial alterations was possibly observed.

An aliquot of the mitochondrial suspension was taken after isolation and prepared for later semiquantification of proteins by Western Blotting as detailed below. The remaining fresh mitochondrial suspensions were used within 4 hours (maintained on ice at 0-4°C throughout this period) for in vitro assays of mitochondrial oxygen consumption, transmembrane potential, spectrophotometric osmoting swelling and fluorimetric calcium movements. Isolation procedures yielded well-coupled mitochondria: the respiratory control ratio (RCR) of isolated mitochondria varied from 5-6 (with glutamate/malate) or 2-3 (with succinate plus rotenone) for controls, as determined according to the method of Estabrook (1967). Higher RCR is expected with glutamate/malate as substrate for complex I in comparison with succinate as a substrate for complex II.

2.8 Mitochondrial oxygen consumption assays

Mitochondrial respiratory function was measured polarographically, at 25°C, 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/malate to a final concentration of 10 and 5 mM each, respectively, or succinate (10mM) plus rotenone (4 μM). State 3 respiration was determined after adding ADP (150 μM); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. 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 natom O/ml as the value for oxygen solubility at 25°C in doubly distilled water. In an independent assay, basal respiration was also measured in the presence of succinate, where ADP (1mM), oligomycin (1.5 μg) and carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) (2 μM) were subsequently added to inhibit ADP-induced state 3 and to induce uncoupled respiration, respectively.

2.9 Mitochondrial Membrane Potential

Mitochondrial transmembrane potential (Δψ) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+ selective electrode prepared in our laboratory as described by Kamo et al. (1979) and a AgCl reference electrode (Tacussel, Model MI 402). Both the TPP^+ electrode and the reference electrode were inserted into an open vessel with magnetic stirring and connected to a pH meter (Jenway, Model 3305). The signals were fed to a potentiometric recorder (Kipp & Zonen, Model BD 121). No correction factor was used to correct the passive binding contribution of TPP^+ to membrane potential as the purpose of this study was to show the relative changes in the potential, rather than absolute values. As a consequence, a slight overestimation of the Δψ values is anticipated. The Δψ was estimated from the following equation (at 25ºC): $\Delta\psi = 59 \times \log (v/V) - 59 \times \log (10^{\Delta E/59} - 1)$, where v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium, and deflection of the electrode potential from the baseline, respectively. A mitochondrial matrix volume of 1.1 μl/mg of protein was assumed. Due to the exponential nature of the Nernst equation, small differences in matrix volumes and binding corrections have a mild effect on estimated Δψ; therefore several studies have used assumed values reported in the past. We did not consider any different matrix volumes between groups as recent studies reported no differences in these parameters between healthy controls and STZ-induced diabetic rats, sacrificed two months after a single 60

mg/kg dose of STZ treatment (Herlein, Fink, O'Malley, & Sivitz, 2009). Reactions were carried out in 1 ml of reaction buffer containing 65mM KCl, 125mM sucrose, 10mM Tris, 20 µM EGTA, 2.5mM KH_2PO_4, pH 7.4, supplemented with 3 µM TPP^+ and 0.5 mg/ml of protein with the temperature maintained at 25ºC. For measurements of Δψ with complex I-linked substrates, energization was carried out with 10 mM of glutamate and 5 mM of malate and ADP-induced phosphorylation was achieved by adding 200 nmol ADP. For measurements of Δψ with complex II-linked substrates, 10mM succinate supplemented with 4 µM rotenone were added to the medium containing 3 µM TPP^+ and mitochondria. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured for both substrates.

2.10 Determination of Mitochondrial Swelling during MPTP induction

Previous reports on substrate-specific regulation of MPTP showed lower calcium retention capacity with complex I-linked substrates compared with substrates for complex II due to the fact that electron flow though complex I may act as a potent pore sensitizer independently of other regulators such as the redox state of pyridine nucleotides, ΔΨ, pH and ROS production (Fontaine, Eriksson, Ichas, & Bernardi, 1998). Hence, we decided to perform all studies involving MPTP induction with succinate as the substrate. Mitochondrial osmotic volume changes were followed by monitoring the classic decrease of absorbance at 540 nm with a Jasco V-560 spectrophotometer. Swelling amplitude and time until maximal absorbance decrease upon calcium addition were considered as MPTP susceptibility indexes. The reaction was continuously stirred and the temperature was maintained at 25ºC. The assays were performed in 2 ml of reaction medium containing 200 mM sucrose, 10 mM Tris, 10 µM EGTA, 5 mM KH_2PO_4, pH 7.4, supplemented with 4 µM rotenone, 10 mM succinate and a single pulse of 800 nmol of calcium with 0.5 mg/ml protein. Control trials were made by using 1 µM of cyclosporin-A, the selective MPTP inhibitor (Broekemeier, Dempsey, & Pfeiffer, 1989).

2.11 Mitochondria Calcium Fluxes

Extramitochondrial free calcium was measured with the hexapotassium salt of the fluorescent probe Calcium Green 5-N. Muscle mitochondria (0.1 mg/ml) were resuspended in 2 ml reaction buffer containing 200 mM sucrose, 10 mM Tris, 10 µM EGTA, 5 mM KH_2PO_4 (pH 7.4) supplemented with 4 µM rotenone. Prior calibration was made using subsequent calcium pulses to a final concentration of

360 nmoles of calcium/mg prot. Calcium uptake was initiated by adding 10 mM succinate to the reaction chamber. Free calcium was monitored with 100 nM Calcium Green 5-N. Fluorescence was continuously recorded in a water-jacketed cuvet holder at 25°C with a Perkin-Elmer LS-50B fluorescence spectrometer with excitation and emission wavelengths of 506 and 531 nm, respectively. From the fluorescence decrease (i.e. calcium entry to mitochondria) and with proper calibration (as previously described), calcium uptake was determined. After calcium accumulation, fluorescence increased (calcium release from mitochondria). At the end of each experiment, a calibration was performed for calculating the maximum and minimum fluorescence signal, with an excess of calcium and EGTA, respectively. Control trial was made using 1 μM cyclosporin-A. The used dissociation constant for the complex calcium probe was 4 μM (Rajdev & Reynolds, 1993).

2.12 Analysis of ANT, VDAC, Cyp D, Tfam, Bax and Bcl-2 protein levels

Equivalent amounts of proteins, as later confirmed by Ponceau labeling, 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), anti-Tfam A-17 (1:500; sc-2358 goat polyclonal IgG; Santa Cruz Biotechnology) or anti-ANTQ18 (1:1000; sc-9300 goat polyclonal IgG; Santa Cruz Biotechnology) or anti-VDAC/porin (1:1000; ab34726 rabbit polyclonal, IgG; abcam) or anti-Cyp D (1:1000; sc-33068 goat polyclonal IgG; Santa Cruz Biotechnology) 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) or anti-goat IgG antibodies (1:1000; 705-035-147 Jackson ImmunoResearch Laboratories) for 2 hours.

Protein 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.

2.13 Caspase-like activity assay

To measure caspase 3 and 9-like activities, aliquots of heart ventricles homogenate were incubated in a reaction buffer containing 25 mM Hepes (pH 7.4), 10% sucrose; 10 mM DTT, 0.1% CHAPS and 100 µM caspase substrate Ac-LEHD-*p*NA (235400, Calbiochem) for 2 h at 37°C. Caspase-like activity was determined by following the detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate Ac-LEHD-*p*-nitroanilide. The method was calibrated with known concentrations of *p*-nitroanilide (Calbiochem).

2.14 Soleus Citrate Synthase activity

Soleus CS activity was measured using the method proposed by Coore et al. (Coore, Denton, Martin, & Randle, 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.

2.15 Statistical analysis

Mean and standard error of mean were calculated for all variables in each group. Two-way ANOVA was used to test hyperglycaemia and training interaction. No significant interaction was found between the two considered stimuli when two-way analysis of variance was used. One-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. Statistical Package for the Social Sciences (SPSS Inc, version 16.0) was used for all analysis. The significance level was set at 5%.

3. Results

3.1 Characterization of the animals

Body and heart weights, as well as body to heart weight ratio, blood glucose and glycated hemoglobin are depicted in Table 2.

After 18 weeks STZ-treatment, sedentary rats presented a significant decrease in body weight (SED+CIT vs. SED+STZ). Endurance training per se, also decreased body weight (SED+CIT vs. T+CIT). However, body weights of the T+STZ group were significantly higher than in the SED+STZ group. Heart weights in the SED+STZ were lower than in the SED+CIT group while T+STZ animals showed a higher heart weight than SED+STZ group. Heart to body weight ratio was significantly higher in the T+CIT than in the SED+CIT group. Blood glucose was significantly higher in both sedentary and trained

hyperglycaemic groups than normal glycaemic groups. Glycated hemoglobin (HbA1c) followed the same pattern of blood glucose, with higher values in STZ-treated than in non-treated groups.

A significant increase in *soleus* CS activity was observed in trained (T+CIT and T+STZ) compared with sedentary groups (SED+CIT and SED+STZ).

Table 2 Characterization of animals from the different experimental groups

	SED+CIT	SED+STZ	T+CIT	T+STZ
Rat Wt (g)	459.30 ± 12.32	255.50 ± 3.31*	373.33 ± 7.23*	345.33 ± 6.54*†
Heart Wt (g)	1.17 ± 0.03	0.76 ± 0.02*	1.14 ± 0.02	0.92 ± 0.05†
Heart Wt/Rat Wt ($mg.g^{-1}$)	2.56 ± 0.10	2.96 ± 0.11	3.05 ± 0.05*	2.66 ± 0.16
Fasting blood glucose ($mg.dL^{-1}$)	104.8±1.7	590.5±13.4*	101.2±4.5†	580.5±11.3#
HbA1c (% total)	4.37 ± 0.18†	12.63 ± 0.47*	4.03 ± 0.15	12.20 ± 0.27*#
CS ($\mu mol.mg^{-1}.min^{-1}$)	0.013 ± 0.002	0.016 ± 0.003	0.073 ± 0.005*	0.068 ± 0.004*†

Data are means ± SEM for body and heart weights (wt), fasting glycaemia, glicated heamoglobin (HbA1c) and *soleus* citrate synthase (CS) activity; * vs. SED+CIT; † vs. SED+STZ; # vs. T+CIT; ($p<0.05$). Sedentary + citrate (SED+CIT); Sedentary + STZ (SED+STZ); Trained + citrate (T+CIT); Trained + STZ (T+STZ).

3.2 Mitochondrial oxygen consumption

To elucidate if the moderate endurance training could revert the mitochondrial phenotype associated to prolonged severe hyperglycaemia, oxygen consumption was determined with substrates for both Complex I and II (Table 3).

Prolonged and severe hyperglycaemia decreased state 3 and state 4 respiration in sedentary animals (SED+CIT vs. SED+STZ). Fourteen weeks of endurance treadmill training increased per se the RCR as well as state 3 respiratory rate using both substrates.

The training protocol reverted the respiration inhibition by hyperglycaemia, as T+STZ animals showed a higher state 3 respiration with both substrates and an increased RCR with complex I substrates. Training also resulted into increased state 4 respiration with succinate as substrate (T+STZ vs. SED+STZ).

Table 3 Respiratory parameters (state 3 and state 4 rates, respiratory control rate - RCR and ADP/O ratios) of glutamate-malate and succinate-energized heart mitochondria isolated from each experimental group.

Glutamate-Malate	SED+CIT	SED+STZ	T+CIT	T+STZ
State 3	274.84 ± 3.93	198.42 ± 7.45*	294.77 ± 0.59*	301.73 ± 3.61†
State 4	37.19 ± 1.95	29.91 ± 1.22*	31.92 ± 2.00	33.21 ± 1.73
RCR	7.47 ± 0.30	6.85 ± 0.39	9.43 ± 0.64*	9.20 ± 0.42†
ADP/O	3.56 ± 0.29	3.12 ± 0.09	3.84 ± 0.22	3.66 ± 0.10
Succinate				
State 3	394.27 ± 7.10	276.66 ± 7.91*	457.41 ± 5.82*	454.28 ± 20.84†
State 4	117.02 ± 4.09	75.43 ± 3.93*	111.83 ± 3.38	114.10 ± 4.88†
RCR	3.39 ± 0.12	3.70 ± 0.15	4.12 ± 0.16*	3.99 ± 0.10
ADP/O	1.26 ± 0.08	1.45 ± 0.15	1.47 ± 0.03	1.29 ± 0.04

Note: Data are means±SEM for heart mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group energized with glutamate-malate (10mM) (5mM) and succinate (10mM). Oxidative phosphorylation was polarographically measured at 25ºC in a total volume of 0.75 ml. Respiratory rates (state 3 and state 4 are expressed in natomO.min^{-1}.mg prot^{-1}. Respiration medium and other experimental details are provided in the methods section. RCR – respiratory control ratio (state 3/state 4); ADP/O - number of nmol ADP phosphorylated by natom O consumed. * vs. SED+CIT; † vs. SED+STZ ($p<0.05$).

Figure 1 shows respiratory data regarding CCCP-induced uncoupled respiration and oxygen consumption in the presence of oligomycin. As expected, long-term and severe hyperglycaemia induced by STZ-treatment decreased CCCP-induced uncoupled respiration (394.4±6.9 vs. 277.8±7.8 natoms O.min^{-1}.mg prot^{-1}; $p<0.05$ for SED+CIT vs. SED+STZ) which was reverted by training (277.8±7.8 vs. 455.8±21.2 natoms O.min^{-1}.mg prot^{-1}; $p<0.05$ for SED+STZ vs. T+STZ). Oxygen consumption in the presence of oligomycin followed the same trend of state 4, decreasing with hyperglycaemia (115.8±3.8 vs. 74.9±3.7 natoms O.min^{-1}.mg prot^{-1}; $p<0.05$ for SED+CIT vs. SED+STZ) and reverted by training (74.9±3.7 vs. 113.3±4.8 natoms O.min^{-1}.mg prot^{-1}; $p<0.05$ for SED+STZ vs. T+STZ). The CCCP/oligomycin ratio, an indicator of inner membrane integrity (Benamar, Tallon, & Macherel, 2003), was only increased in the T+CIT group (3.4±0.11 vs. 4.2±0.14 natoms O.min^{-1}.mg prot^{-1}; $p<0.05$ for SED+CIT vs. T+CIT).

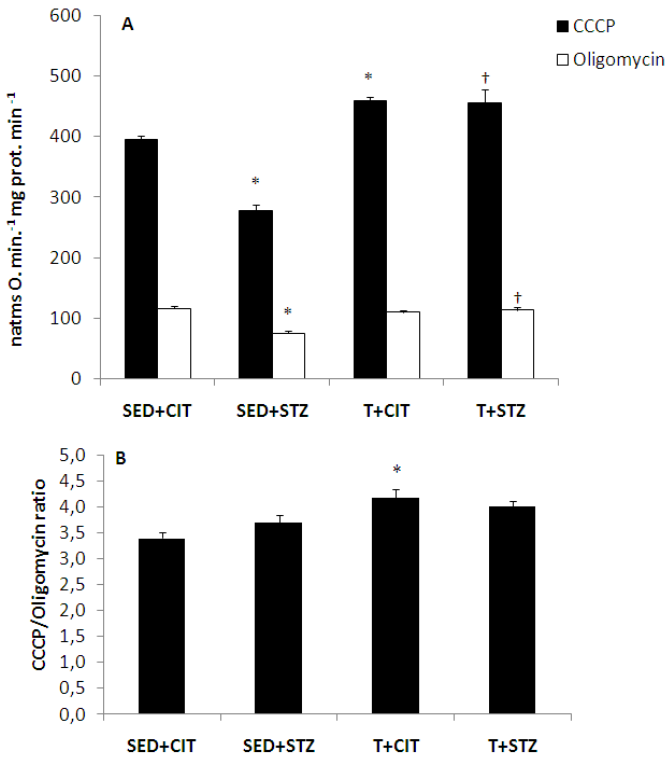

Figure 1 Effect of long-term severe hyperglycaemia and endurance training on respiratory rates with CCCP (2μM) and with oligomycin (1.5μg.ml^{-1}) of succinate (10mM)-energized heart mitochondria (panel A). CCCP/oligomycin ratio, an estimate of mitochondrial inner membrane integrity is also presented (panel B). Data are means±SEM; * vs. SED+CIT; † vs. SED+STZ ($p<0.05$). Sedentary + citrate (SED+CIT); Sedentary + STZ (SED+STZ); Trained + citrate (T+CIT); Trained + STZ (T+STZ).

3.3 Transmembrane electric potential

To attain further insight into the effects of STZ-induced severe hyperglycaemia and endurance training, fluctuations on the transmembrane electric potential (ΔΨ) associated with mitochondrial respiration and the phosphorylation cycle induced by ADP were also investigated.

No significant differences regarding maximal energization, ADP-induced depolarization and repolarization ΔΨ fluctuations were found (Table 4). Significant changes were observed regarding the phosphorylative lag phase but only when glutamate plus malate were used as substrates (SED+STZ vs. all other groups). A non-significant increase in the phosphorylative lag phase in SED+STZ vs. SED+CIT was observed when succinate was used as substrate for

complex II. Endurance training attenuated the increased ADP lag phase observed in the SED+STZ group (SED+STZ vs. T+STZ).

Table 4 Relative mitochondrial transmembrane potential (Δψ) fluctuations (maximal energization, ADP-induced depolarization, and repolarization) and ADP phosphorylation lag phase of glutamate-malate and succinate energized heart mitochondria isolated from each experimental group.

Glutamate-Malate	SED+CIT	SED+STZ	T+CIT	T+STZ
Maximal energization Δψ (% SED+CIT)	100.0 ± 1.37	100.4 ± 0.42	97.6 ± 0,32	100.5 ± 0.36
ADP depolarization Δψ (% SED+CIT)	100.0 ± 1.70	99.7 ± 0.53	96.3 ±0.44	100.0 ± 0.56
Repolarization Δψ (% SED+CIT)	100.0 ± 1.31	100.1 ± 0,58	97.0 ± 0.35	99.7 ± 0.25
Lag phase (s)	70.00 ± 2.06	112.00 ± 2.06*	70.00 ± 2.15	66.00 ± 4.10†
Succinate-rotenone				
Maximal energization Δψ (% SED+CIT)	100.0 ± 0.45	100.6 ± 1.25	99.4 ± 1.05	99.9 ± 1.15
ADP depolarization Δψ (% SED+CIT)	100.0 ± 1.19	96.1 ± 1.28	99.1 ± 2.24	94.0 ± 2.39
Repolarization Δψ (% SED+CIT)	100.0 ± 0.99	104.7 ± 2.78	98.9 ± 1.36	99.8 ± 1.38
Lag phase (s)	75.00 ± 7.47	92.00 ± 1.80	75.00 ± 6.30	84.00 ± 3.10

Note: Data are means±SEM for heart mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group. Table shows the response of mitochondrial membrane potential expressed in percentage of control SED+CIT values developed with glutamate (10mM) - malate (5mM) or succinate (10mM) (energization), the decrease in membrane potential after ADP addition (depolarization), the repolarization value after ADP phosphorylation. Mitochondrial transmembrane potential was measured using a TPP^+-selective electrode at 25ºC in a total volume of 1 mL. Reaction medium and other experimental details are provided in the methods section. * vs. SED+CIT; † vs. SED+STZ ($p<0.05$).

3.4 Induction of the mitochondrial permeability transition

Severe hyperglycaemia has previously been shown to alter the modulation of heart mitochondrial function by increasing the susceptibility to MPTP opening (Oliveira et al., 2003). Possible *in vitro* effects of endurance treadmill training on MPTP opening was previously described but never with complete unanimous conclusions. However, the effects of moderate endurance training against increased susceptibility to cardiac MPTP induction in long-term severe hyperglycaemia was never explored so far. Figure 1 (panels A and B) shows several end-points for MPTP induction, namely swelling amplitude (the difference between the initial suspension absorbance and the final value upon absorbance stabilization) and time elapsed until the faster swelling kinetics starts. The results demonstrate that heart mitochondria isolated from sedentary diabetic rats are significantly more

susceptible to calcium-induced MPTP opening (SED+CIT vs. SED+STZ), showing an increased swelling amplitude (panel A) and a lower time until the maximal rate of absorbance decrease (panel B). However, mitochondria from T+STZ group demonstrated to be significantly less susceptible to calcium-induced MPTP opening (SED+STZ vs. T+TSZ).

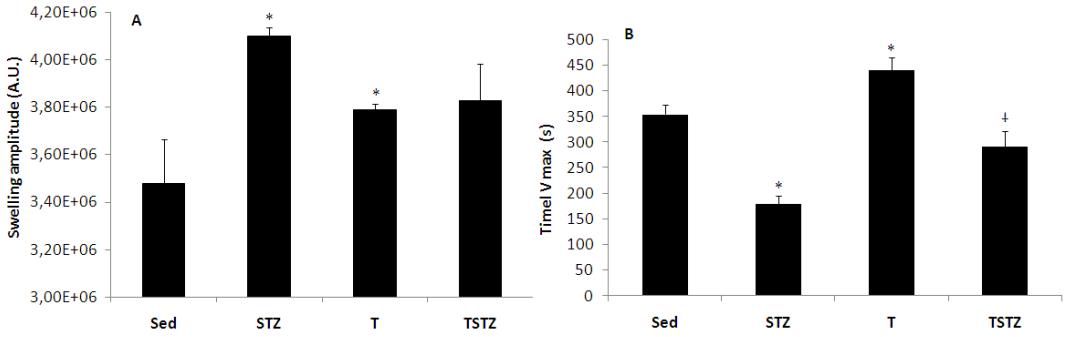

Figure 2 Effect of long-term severe hyperglycaemia and endurance training on MPTP induction in succinate-energized heart mitochondria, as evaluated by mitochondrial swelling. The absorbance of mitochondrial suspension was followed at 540nm. Mitochondria were incubated as described in the methods section. A single 800 nmol calcium pulse was added to 0.5 mg of mitochondrial protein/ml in order to attain the cyclosporin A-sensitive swelling, which indicates that the decreased optical density corresponding to the increased swelling was due to MPTP opening. Panel A - swelling amplitude, Panel B – Time to Vmax, meaning the lag phase elapsed until large scale swelling initiates, are presented. Data are means±SEM; * vs. SED+CIT; † vs. SED+STZ ($p<0.05$).

The decreased susceptibility of heart mitochondria from trained rats to MPTP opening was further confirmed by following calcium fluxes through the mitochondrial membranes. The results showed that mitochondrial calcium uptake from sedentary diabetic group was lower than all other groups. However, endurance training was able to completely revert the decrease in calcium loading capacity as calcium uptake in the T+STZ was higher than in the SED+STZ group. Both trained groups showed a higher calcium buffering capacity, releasing lower amounts of calcium to the external medium. Calcium release in all experiments was inhibited by the presence of cyclosporin-A.

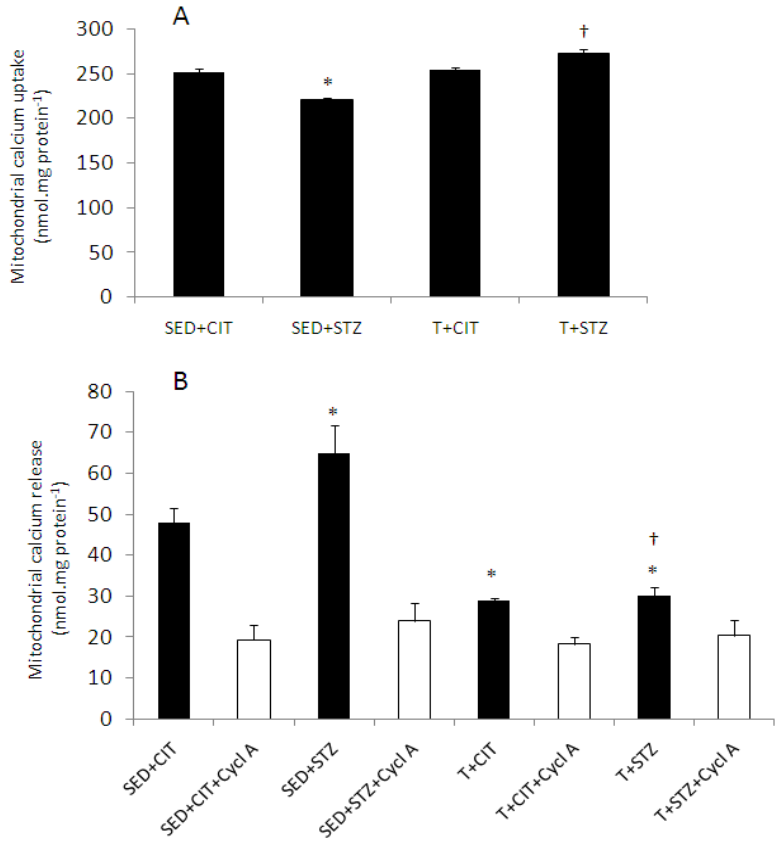

Figure 3 Effect of long-term severe hyperglycaemia and endurance training on extramitochondrial calcium fluxes in succinate-energized heart muscle mitochondria. The reactions were carried out in 2ml of reaction medium supplemented with 0.1 mg/ml protein, 4µM rotenone, 100nM Calcium Green 5-N. Calcium entry into mitochondria was initiated by 10mM succinate addition. MPTP Panel A – mitochondrial calcium uptake; and Panel B – mitochondrial calcium release with and without cyclosporine A in the reaction medium. Data are means±SEM; * vs. SED+CIT; † vs. SED+STZ; ($p<0.05$).

3.5 Protein Immunoassay by Western Blotting

Immunoblotts were then performed in order to semiquantify MPTP proteins such as ANT, VDAC and Cyp D, proteins that are known to be involved on mitochondrial apoptotic signalling including the pro and anti-apoptotic Bcl-2 family proteins, Bax and Bcl-2, respectively, and the key activator of mitochondrial transcription Tfam (Figures 4-6). Eighteen weeks of severe hyperglycaemia induced a significant decrease of ANT, VDAC and Tfam. However, endurance training was able to restore these protein levels (SED+STZ vs. T+STZ) to control values. Regarding Cyp D content, SED+STZ animals showed an increase in Cyp D when compared to SED+CIT, whereas no change was observed in T+STZ.

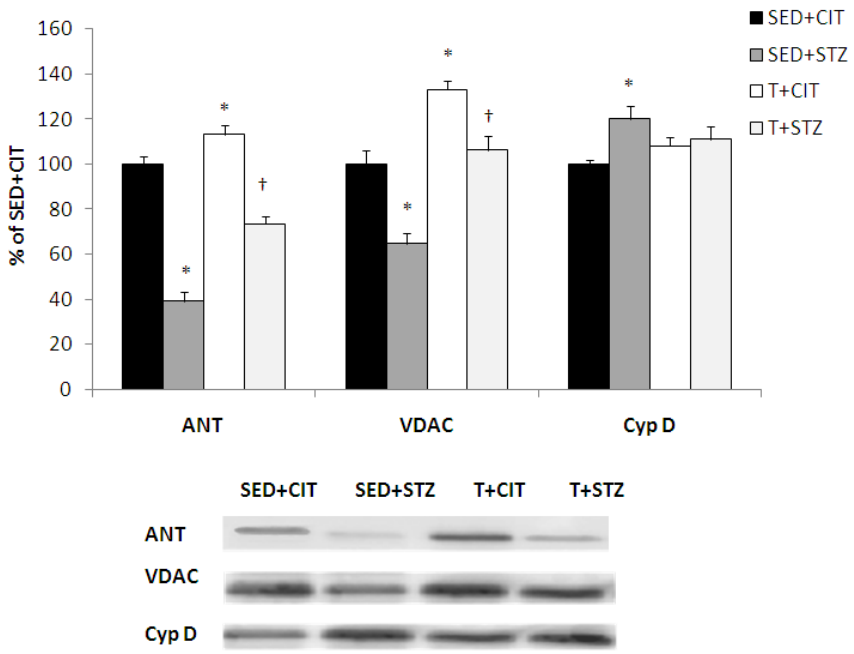

Figure 4 Effect of long-term severe hyperglycaemia and endurance training on mitochondrial protein levels of ANT, VDAC and Cyp D. Representative Western blots of ANT, VDAC and Cyp D are shown for each group. Values are means±SEM and are expressed as percentage of SED+CIT; * vs. SED+CIT; † vs. SED+STZ obtained from 6 independent experiments ($p<0.05$).

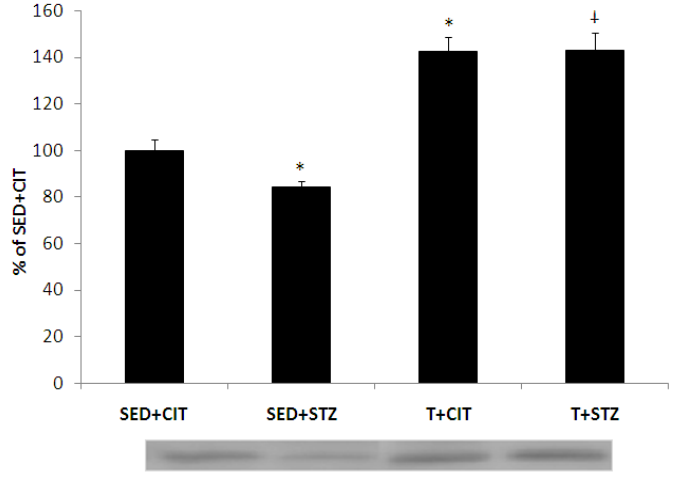

Figure 5 Effect of long-term severe hyperglycaemia and endurance training on mitochondrial protein levels of Tfam. Representative Western blots of Tfam are shown for each group. Values are means±SEM and are expressed as percentage of SED+CIT; * vs. SED+CIT; † vs. SED+STZ obtained from 6 independent experiments ($p<0.05$).

As can be depicted in figure 6, long-term severe hyperglycaemia induced a significant elevation in the amount of the pro-apoptotic protein Bax, a decrease of Bcl-2 and a consequent increased Bax-to-Bcl-2 ratio. Endurance training prevented all the alterations observed (SED+STZ vs. T+STZ).

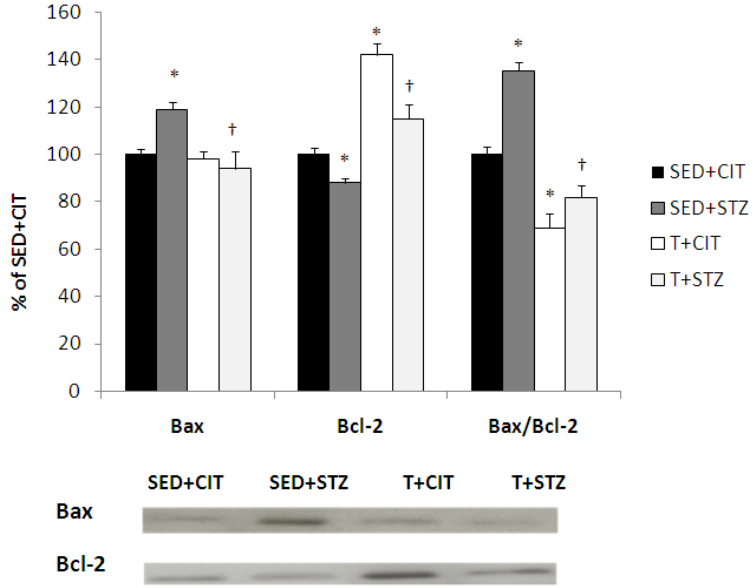

Figure 6 Effect of long-term severe hyperglycaemia and endurance training on the mitochondrial expression of Bax and Bcl-2, and Bax-to-Bcl-2 ratio. Representative Western blots of Bax and Bcl-2 are shown for each group. Values are means±SEM; * vs. SED+CIT; † vs. SED+STZ, obtained from 6 independent experiments ($p<0.05$).

Additionally, endurance training prevented (SED+STZ vs. T+STZ) the elevated activities of caspases-3 and -9 induced by long-term severe hyperglycaemia (SED+CIT vs. SED+STZ) (Figure 7).

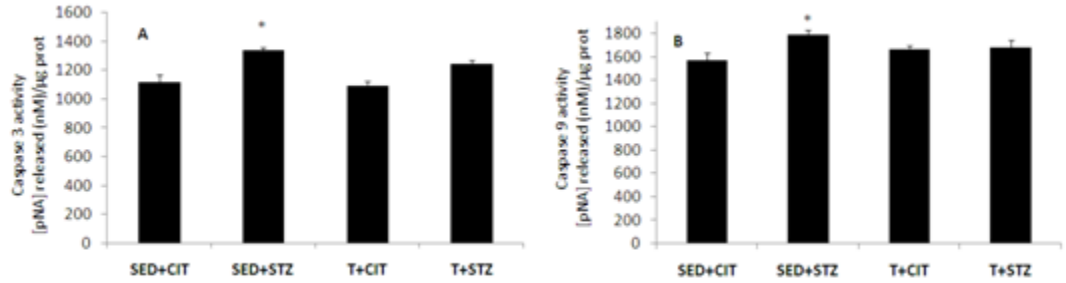

Figure 7 Effect of long-term severe hyperglycaemia and endurance training on heart caspase-3 (panel A) and 9 (panel B)-like activities measured by following the cleavage of the colorimetric substrates Ac-LEHD-pNA. The caspase-like activity was expressed by [pNA] released (nM)/µg prot. Values are means±SEM; * vs. SED+CIT ($p<0.05$).

4. Discussion

4.1 Overview of principal findings

The experiments tested for the first time the hypothesis that respiratory dysfunction and the increased susceptibility to calcium-induced MPTP opening in isolated heart mitochondria from long-term severe hyperglycaemia (see Boudina & Abel, 2007) is reverted by moderate endurance treadmill training. Our findings confirmed this hypothesis, as important classic functional endpoints of mitochondrial bioenergetics and MPTP-related functional and regulatory parameters impaired in sedentary hyperglycaemic group were favourably reverted by training. Moreover, endurance training promoted a more apoptosis resistant phenotype as demonstrated by the reversion of increased caspase-3 and -9 activities as well as Bax/Bcl-2 ratio observed in the sedentary hyperglycaemic group. Taking together, the results are consistent with the notion that the cardiac adaptations induced by chronic exercise in mitochondria contribute to revert the mitochondrial phenotype observed during severe hyperglycaemia.

4.2 Exercise reverts hyperglycaemia-induced altered mitochondrial bioenergetics

Cardiac dysfunction associated with defective mitochondrial function in diabetes has previously been studied (see Boudina & Abel, 2007 for refs). The present work again confirmed that our experimental model of long-term severe hyperglycaemia results into decreased mitochondrial respiration and increased phosphorylative lag phase.

It is known that regular endurance training induces numerous physiological adaptations including alterations in

cardiac mitochondrial respiratory function that ultimately result in improved exercise capacity and cardiac tolerance (Ascensao, Ferreira, & Magalhaes, 2007). Our present results demonstrate that 14 wk of moderate endurance running training reverted the inhibition of mitochondrial respiration caused by long-term severe hyperglycaemia, which is in accordance with previous functional and morphological evidence of protection afforded by training against STZ-induced mitochondrionopathy (Mokhtar, Lavoie, Rousseau-Migneron, & Nadeau, 1993; Searls, Smirnova, Fegley, & Stehno-Bittel, 2004).

The data obtained confirms that STZ-induced hyperglycaemia (SED+CIT vs. SED+STZ) affects also the phosphorylative system, as previously observed by others (Bugger et al., 2008; Bugger et al., 2009; Flarsheim, Grupp, & Matlib, 1996; Lashin & Romani, 2003, 2004; Lashin, Szweda, Szweda, & Romani, 2006; Mokhtar, Lavoie, Rousseau-Migneron, & Nadeau, 1993; Shen, Zheng, Metreveli, & Epstein, 2006; Shen et al., 2004; Tomita et al., 1996), being moderate endurance training able to revert most of the observed changes. Alterations in mitochondrial oxidative phosphorylation induced by severe hyperglycaemia can be originated by several factors: 1) inactivation and/or diminishing gene expression of dehydrogenases, providing limited amounts of reducing equivalents to the mitochondrial electron transport chain (Bugger et al., 2009; Lashin, Szweda, Szweda, & Romani, 2006; Savabi & Kirsch, 1991), 2) increased PKC-dependent aconitase phosphorylation suggested to be responsible for reverse mitochondrial activity of this enzyme (Lin, Brownsey, & MacLeod, 2009), 3) decreased gene expression or activity of electron transport chain complexes or proteins of the phosphorylation system (Bugger et al., 2009; Dabkowski et al., 2009; Lashin, Szweda, Szweda, & Romani, 2006) and 4) depression of ATP synthase gene expression and activity (Bugger et al., 2009; Tomita et al., 1996). As CCCP-induced uncoupled respiration, reflecting the maximal capacity of electron transfer through the electron transport chain, decreases in the SED+STZ group which is in agreement with data from Bugger et al. (2009) and because endurance training reversed these defects, one can argue that exercise training likely prevented the dysfunction of the respiratory chain enzymes and/or the upstream supply of electrons to the respiratory components resulting from the exposure to severe and persistent hyperglycaemic state.

One possible mitochondrial target for hyperglycaemia is the ANT. The ANT is one of the most prominent single protein

components of the inner mitochondrial membrane that under physiological conditions, exchanges cytosolic ADP for mitochondrial ATP, a process that occurs in coordination with proton reflux through the ATP synthase and with oxidative phosphorylation (Halestrap & Brennerb, 2003). In fact, the important role of ANT in the regulation of mitochondrial respiration is compromised if the amount of this protein is limited in either quantity or activity during different pathologies or drug-induced mitochondrial dysfunction (Oliveira & Wallace, 2006; Zhou, Starkov, Froberg, Leino, & Wallace, 2001). Given that in the present study hyperglycaemia induced a decrease in ANT content in SED+STZ group and that training attenuated this effect (Figure 4), it is possible that the decrease in the ANT explains, at least in part, the altered oxidative phosphorylation in the hearts of diabetic animals and the recovery afforded by moderate endurance exercise.

Additionally, Bo et al. (2008) also described an increase in heart mitochondrial ATP synthase after endurance training, so we can speculate that an up-regulation of components of the phosphorylative system can also contribute to increased energy output verified after exercise.

Tfam is the key transcription activator of mitochondrial genes encoding some important mitochondrial proteins. The present model of long-term hyperglycaemia caused a decrease in the expression of Tfam, a condition that was completely reverted and even increased in trained groups (Fig 5). Data from others showed that Tfam unchanged content and decreased activity were associated with an attenuated binding to the promoter sequence of mitochondrial DNA in diabetic hearts (Kanazawa et al., 2002; Nishio, Kanazawa, Nagai, Inagaki, & Kashiwagi, 2004). However, ultrastructural analyses have also revealed increased mitochondrial proliferation in models of type 1 diabetes with concomitant impairments in mitochondrial function (Shen, Zheng, Metreveli, & Epstein, 2006; Shen et al., 2004). These studies suggest that the increased cardiac mitochondrial content in diabetes do not necessarily imply an improvement of mitochondrial bioenergetics. Further studies are needed to elucidate the relationship between modifications in heart mitochondrial content and function in diabetes as well as the interaction with endurance exercise training.

The rate of oxygen consumption during state 4 may reflect the level of inner membrane leakage of protons. In the present study, hyperglycaemia induced a decrease in state 4 respiration without significant effect on the developed

maximal energization (table 4). However, the estimation of $\Delta\Psi$ obtained in the present study is not in accordance with the results of other studies reporting decreases in cardiac mitochondrial $\Delta\Psi$ during the early phase of diabetes (Ferko et al., 2008; Ghosh et al., 2005; Mujkosova, Ferko, Humenik, Waczulikova, & Ziegelhoffer, 2008; Waczulikova et al., 2007). It is possible that methodological differences in the estimation of $\Delta\Psi$ and in the duration of diabetic condition might justify such discrepancies.

Data from literature regarding state 4 respiration in heart mitochondria from type 1 diabetic animals is conflicting, with studies suggesting no change (Bugger et al., 2008; Bugger et al., 2009; Shen, Zheng, Metreveli, & Epstein, 2006; Shen et al., 2004), increase (Lashin & Romani, 2003, 2004; Lashin, Szweda, Szweda, & Romani, 2006) and decrease (Herlein, Fink, O'Malley, & Sivitz, 2009), even with an augmented UCP3 content (Bugger et al., 2008; Herlein, Fink, O'Malley, & Sivitz, 2009). It is important to note that in the present investigation, endurance training returned state 4 values to those similar to control. Further studies are needed to clarify heart mitochondrial basal respiration and proton conductance variations in response to the combination of these two stimuli.

4.3 Training-induced decreased MPTP sensitivity

To the best of our knowledge, there are no data available in literature concerning the effect of exercise training on the modulation of heart MPTP gating in long-term and severe hyperglycaemia. Results of the present study provide for the first time evidence that 14-wks of endurance treadmill training reverted the increased sensitivity of the heart MPTP opening caused by long-term severe hyperglycaemic condition.

The present results regarding MPTP induction assessed eighteen weeks after STZ treatment confirm that heart mitochondria isolated from sedentary animals revealed a decreased ability to accumulate calcium caused by enhanced MPTP induction (Flarsheim, Grupp, & Matlib, 1996; Oliveira et al., 2003; Tanaka, Konno, & Kako, 1992). Hyperglycaemia is often associated with increased oxidative stress (Green, Brand, & Murphy, 2004), a condition known to induce the MPTP via the oxidation of critical thiol residues (Kowaltowski, Castilho, & Vercesi, 2001). In fact, increased free radical generation and oxidative stress with associated respiratory chain complex dysfunction and cardiolipin depletion were already observed in the heart mitochondria from type 1 diabetic animal models (Dabkowski et al., 2009; Shen, Zheng, Metreveli, &

Epstein, 2006). We can speculate that these defects found in mitochondrial electron transport chain complexes may, in fact lead to enhanced reactive oxygen species production by the mitochondrial respiratory chain and/or *vice versa*, a fact that could be exacerbated by the presence of calcium possibly contributing to the increased MPTP induction in the SED+STZ group compared with the SED+CIT. On the other hand, as can be depicted from osmotic swelling and fluorescence experiments (Figs 2 and 3, respectively), endurance training was able to revert the increased sensitivity of heart mitochondria to calcium-induced MPTP opening. The effect of endurance training on the susceptibility to heart MPTP induced by calcium is still controversial with data suggesting no positive (Starnes, Barnes, & Olsen, 2007) and protective outcomes (Kavazis, McClung, Hood, & Powers, 2008; Marcil, Bourduas, Ascah, & Burelle, 2006). However, given the refereed close relationship between increased mitochondrial oxidative stress and the susceptibility to MPTP induction (Kowaltowski, Castilho, & Vercesi, 2001); it is possible that the known decrease heart mitochondrial free radical production found in rats undergoing regular exercise (Judge et al., 2005) may contribute to these protective effects. Moreover, despite the data from Bo et al. (2008) reporting a decrease in $\Delta\Psi$ with endurance training, the absence of differences in the maximal mitochondrial $\Delta\Psi$ developed found in the present study (table 4) led us to think that a possible voltage difference between the experimental groups was not a cause for differential MPTP triggering or alterations in calcium accumulation.

4.4 Effect of training and hyperglycaemia on ANT, VDAC and cyclophylin D levels

The calcium-induced MPT is modulated by a variety of physiological effectors. It is increasingly recognized that the molecular composition of the pore is likely variable (He & Lemasters, 2002; Zoratti, Szabo, & De Marchi, 2005); despite this, the prevailing hypothesis is that the ANT, VDAC and the regulatory matrix Cyp D are the major proteins forming the MPTP complex (Crompton, 1999; Halestrap & Brennerb, 2003). In fact, despite some conflicting results (Chabi et al., 2008; Picard et al., 2008), variations in the level of expression of the MPTP component/regulatory proteins ANT and Cyp D were reported to correlate with the susceptibility of isolated mitochondria to undergo PTP opening and decisively contribute to mitochondrial dysfunction (Csukly et al., 2006; Matas et al., 2009; Oliveira & Wallace, 2006).

As mentioned above, besides its physiological role as adenine nucleotide

exchanger, ANT is also a major component of the MPTP, which places this protein as a candidate target for pathological events and for possible modulation by known protective countermeasures such as exercise. The decrease in the expression of heart mitochondria ANT in STZ-treated rats found in our study (Figure 4) does not agree with other reports in which the activity and content of this protein were not changed (Herlein, Fink, O'Malley, & Sivitz, 2009; Mokhtar, Lavoie, Rousseau-Migneron, & Nadeau, 1993). Possible differences in the duration of hyperglycaemic conditions between studies may justify the discrepant results. However, our results are in the line of others suggesting that the decreased expression of this protein is associated with the susceptibility to MPTP opening (Csukly et al., 2006; Oliveira & Wallace, 2006) as occurred in SED+STZ group. Mitochondrial redox state is known to be closely related with MPTP-opening susceptibility. In fact, strong evidence exist that the oxidation of pyridine nucleotides and critical thiol groups occur under conditions of enhanced oxidative stress, augmenting the mitochondrial sensitivity to pore gating (Kowaltowski, Castilho, & Vercesi, 2001). However, as recent data regarding the effect of type 1 diabetes on heart mitochondrial reactive oxygen species generation and oxidative stress is conflicting (Bugger et al., 2008; Dabkowski et al., 2009), caution should be devoted to the relationship between oxidative stress and ANT-induced MPTP modulation in this model.

Of note and regardless the possible mechanisms, training *per se* increased ANT content (SED+CIT vs. T+CIT) and perhaps more importantly, it did attenuate the decreased expression in long-term severe hyperglycaemic group (SED+STZ vs. T+STZ), which correlates with the alterations regarding MPTP opening.

The literature is conflicting concerning the involvement of VDAC in MPTP. Despite the idea that this protein is part of the pore complex since an interaction between VDAC and ANT has been demonstrated at contact sites between the inner and outer mitochondrial membranes (Crompton, 1999; Halestrap, Clarke, & Javadov, 2004), recent data suggest that the VDAC is dispensable for mitochondrial-dependent cell death (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007). In the present study (Figure 4), the decreased expression of mitochondrial VDAC in sedentary hyperglycaemic group (SED+CIT vs. SED+STZ), which is in accordance with data from 2D proteomic analysis of diabetic rat hearts (Turko & Murad, 2003), was reverted by endurance training (SED+STZ vs. T+STZ).

Another potential factor that may importantly contribute to explain our results regarding MPTP induction is Cyp D, a peptidyl-prolyl *cis-trans* isomerase chaperone-like protein from the immunophilin family located in the mitochondrial matrix that acts as a potent MPTP sensitizer by binding to putative pore components of the inner membrane (Crompton, 1999). This pore sensitizing role was demonstrated by the sevenfold increase in the required amount of calcium to trigger MPTP in mitochondria from *Ppif*$^{-/-}$ mice, which are devoid of Cyp D, being the inhibitory effect of Cyclosporin-A in these mice completely abolished (Basso et al., 2005). Accordingly, Baines et al., (2005) reported that *Ppif* null mice were protected from ischemia/reperfusion-induced cell death *in vivo*, whereas Cyp D overexpressing mice present increased mitochondrial swelling and spontaneous cell death. As can be seen from western blotting data in figure 4, the increased Cyp D observed in heart mitochondria from sedentary hyperglycaemic rats (SED+CIT vs. SED+STZ) was prevented by endurance training (SED+STZ vs. T+STZ). The Cyp D variation profile is in accordance with swelling and fluorescent experiments (Figures 2 and 3, respectively), in which the protective effect of endurance training against the increased susceptibility to calcium-induced MPTP gating was observed. The relationship between mitochondrial Cyp D content and PTP sensitivity found in the present study also agrees with data from others using stress models of MPTP induction such as muscle denervation and cardiac volume overload-induced compensated hypertrophy (Csukly et al., 2006; Matas et al., 2009).

Another hypothesis which seems attractive regarding the role of endurance training on MPTP resistance involves a possible modulation of Akt/PKB-related hexokinase content bound to mitochondria, as hexokinase expression is both known to be regulated by insulin (Kruszynska, Mulford, Baloga, Yu, & Olefsky, 1998) and to be involved in an inhibitory regulation of the MPTP complex (Crompton, 1999). However, despite the described effect of endurance training on the partial normalization of heart PKB-serine^{473} and -threonine^{308} phosphorylation of Zucker diabetic fatty rats (Lajoie et al., 2004), several studies reported no alterations in heart HK activity with endurance training (Stuewe, Gwirtz, Agarwal, & Mallet, 2000; Turcotte & Belcastro, 1991).

4.5 Effect of training and STZ treatment on apoptotic signalling

Mitochondrial (dys)function is increasingly considered a key event in a variety of forms of cell death, including apoptosis.

We analyzed the effects of long term severe hyperglycaemia and endurance training on caspase-3 and -9 activities and on the expression of the pro- and antiapoptotic Bcl-2 family proteins Bax and Bcl-2. The relative expression of the later in the mitochondrial outer membrane is thought to decide the fate of the cell by regulating outer membrane integrity and the consequent release of mitochondrial apoptotic proteins such as cytochome c or the apoptosis-inducing factor (Hengartner, 2000). Moreover, data suggest that Bax, and perhaps its homologs Bak and Bid, may regulate MPTP opening, being these effects antagonized by the anti-apoptotic Bcl-2 family members (Zoratti, Szabo, & De Marchi, 2005). In the present study, hyperglycaemia induced an increase in mitochondrial Bax and Bax-Bcl-2 ratio and a decrease in Bcl-2 in sedentary group (SED+CIT vs. SED+STZ), which were reverted by endurance training (SED+STZ vs. T+STZ) (Figure 6). These results are in accordance with previous reports in both STZ-treated and trained rats, revealing increased pro-apoptotic signaling (Bojunga et al., 2004) and anti apoptotic mitochondrial phenotype (Ascensao et al., 2005b; Siu, Bryner, Martyn, & Alway, 2004), respectively. It is thus possible that the reversion effect of training observed against STZ-induced increased MPTP gating sensitivity could also be favored by changes in the expression of Bcl-2 family proteins.
Our data further demonstrated that the activation of the caspase-9 and caspase-3 apoptotic pathway by STZ treatment was inhibited in trained hearts (Figure 7) and followed the same trend as Bax content and Bax-to-Bcl-2 ratio, suggesting, at least partially, a mitochondrial-mediated origin for caspase-3 activation.

5. Summary

All together, results from the present study indicate for the first time that the decreased respiratory dysfunction and the increased susceptibility to PTP gating of heart mitochondria isolated from rats with long term and severe hyperglycaemia, a model of type 1 diabetes, were reverted by 14 wks of moderate endurance treadmill training. Additionally, other novel finding of the work was that the prevention of MPTP sensitivity was accompanied by possible favorable modulation of known pore regulatory/component proteins and apoptosis. These adaptations could potentially be beneficial to the cardiac tissue in the setting of the diabetic cardiomyopathy, a situation in which exercise training was shown to be protective (Shao et al., 2009). Our data suggests that protection may be mediated, at least in part, by alterations in

the expression of the pore proteins ANT and Cyp D as well as in the pro and antiapoptotic proteins of the Bcl-2 family. Considering that hyperglycaemia can result in a mitochondrial disease with consequent loss of organelle and tissue functions, the pharmacological and non-pharmacological countermeasures such as physical exercise that revert diabetic mitochondrionopathy should be taken into account, due to its positive effects. Despite the absence of available data regarding these effects on human type I diabetes, it is likely that the results obtained could contribute to emphasize the protective role of chronic exercise, for instance against the increased susceptibility to ischemia-reperfusion injury-induced mitochondrial dysfunction, including MPTP and apoptosis, described in diabetes (Bojunga et al., 2004; Galinanes & Fowler, 2004; Oliveira et al., 2003).

References

Ascensao, A., Ferreira, R., & Magalhaes, J. (2007). Exercise-induced cardioprotection - biochemical, morphological and functional evidence in whole tissue and isolated mitochondria. *Int J Cardiol, 117*, 16-30.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., et al. (2005). Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *Am J Physiol Heart Circ Physiol, 289*(2), H722-731.

Baines, C. P., Kaiser, R. A., Purcell, N. H., Blair, N. S., Osinska, H., Hambleton, M. A., et al. (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature, 434*(7033), 658-662.

Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J., & Molkentin, J. D. (2007). Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol, 9*(5), 550-555.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M. A., & Bernardi, P. (2005). Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem, 280*(19), 18558-18561.

Benamar, A., Tallon, C., & Macherel, D. (2003). Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing. *Seed Sci Res, 13*, 35-45.

Bhattacharya, S. K., Thakar, J. H., Johnson, P. L., & Shanklin, D. R. (1991). Isolation of skeletal muscle mitochondria from hamsters using an ionic medium containing ethylenediaminetetraacetic acid and nagarse. *Anal Biochem, 192*(2), 344-349.

Bo, H., Jiang, N., Ma, G., Qu, J., Zhang, G., Cao, D., et al. (2008). Regulation of mitochondrial uncoupling respiration during exercise in rat heart: role of reactive oxygen species (ROS) and uncoupling protein 2. *Free Radic Biol Med, 44*(7), 1373-1381.

Bojunga, J., Nowak, D., Mitrou, P. S., Hoelzer, D., Zeuzem, S., & Chow, K. U. (2004). Antioxidative treatment prevents activation of death-receptor- and mitochondrion-dependent apoptosis in the hearts of diabetic rats. *Diabetologia, 47*(12), 2072-2080.

Boudina, S., & Abel, E. D. (2006). Mitochondrial uncoupling: a key contributor

to reduced cardiac efficiency in diabetes. *Physiology (Bethesda), 21*, 250-258.

Boudina, S., & Abel, E. D. (2007). Diabetic cardiomyopathy revisited. *Circulation, 115*(25), 3213-3223.

Boudina, S., Sena, S., O'Neill, B. T., Tathireddy, P., Young, M. E., & Abel, E. D. (2005). Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity. *Circulation, 112*(17), 2686-2695.

Broekemeier, K. M., Dempsey, M. E., & Pfeiffer, D. R. (1989). Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem, 264*(14), 7826-7830.

Bugger, H., Boudina, S., Hu, X. X., Tuinei, J., Zaha, V. G., Theobald, H. A., et al. (2008). Type 1 diabetic akita mouse hearts are insulin sensitive but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. *Diabetes, 57*(11), 2924-2932.

Bugger, H., Chen, D., Riehle, C., Soto, J., Theobald, H. A., Hu, X. X., et al. (2009). Tissue-Specific Remodeling of the Mitochondrial Proteome in Type 1 Diabetic Akita Mice. *Diabetes*.

Chabi, B., Ljubicic, V., Menzies, K. J., Huang, J. H., Saleem, A., & Hood, D. A. (2008). Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. *Aging Cell, 7*(1), 2-12.

Coore, H. G., Denton, R. M., Martin, B. R., & Randle, P. J. (1971). Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones. *Biochem J, 125*(1), 115-127.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *Biochem J, 341 (Pt 2)*, 233-249.

Csukly, K., Ascah, A., Matas, J., Gardiner, P. F., Fontaine, E., & Burelle, Y. (2006). Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D. *J Physiol, 574*(Pt 1), 319-327.

Dabkowski, E. R., Williamson, C. L., Bukowski, V. C., Chapman, R. S., Leonard, S. S., Peer, C. J., et al. (2009). Diabetic cardiomyopathy-associated dysfunction in spatially distinct mitochondrial subpopulations. *Am J Physiol Heart Circ Physiol, 296*(2), H359-369.

Estabrook, R. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods Enzymol, 10*, 41-47.

Felix, C., Gillis, M., Driedzic, W. R., Paulson, D. J., & Broderick, T. L. (2001). Effects of propionyl-L-carnitine on isolated mitochondrial function in the reperfused diabetic rat heart. *Diabetes Res Clin Pract, 53*(1), 17-24.

Ferko, M., Habodaszova, D., Waczulikova, I., Mujkosova, J., Kucharska, J., Sikurova, L., et al. (2008). Endogenous protective mechanisms in remodeling of rat heart mitochondrial membranes in the acute phase of streptozotocin-induced diabetes. *Physiol Res, 57 Suppl 2*, S67-73.

Flarsheim, C. E., Grupp, I. L., & Matlib, M. A. (1996). Mitochondrial dysfunction accompanies diastolic dysfunction in diabetic rat heart. *Am J Physiol, 271*(1 Pt 2), H192-202.

Fontaine, E., Eriksson, O., Ichas, F., & Bernardi, P. (1998). Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation By electron flow through the respiratory chain complex i. *J Biol Chem, 273*(20), 12662-12668.

Galinanes, M., & Fowler, A. G. (2004). Role of clinical pathologies in myocardial injury following ischaemia and reperfusion. *Cardiovasc Res, 61*(3), 512-521.

Ghosh, S., Pulinilkunnil, T., Yuen, G., Kewalramani, G., An, D., Qi, D., et al. (2005). Cardiomyocyte apoptosis induced by short-term diabetes requires mitochondrial GSH depletion. *Am J Physiol Heart Circ Physiol, 289*(2), H768-776.

Green, K., Brand, M. D., & Murphy, M. P. (2004). Prevention of mitochondrial oxidative damage as a therapeutic strategy

in diabetes. *Diabetes, 53 Suppl 1*, S110-118.

Halestrap, A. P., & Brennerb, C. (2003). The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. *Curr Med Chem, 10*(16), 1507-1525.

Halestrap, A. P., Clarke, S. J., & Javadov, S. A. (2004). Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovasc Res, 61*(3), 372-385.

He, L., & Lemasters, J. J. (2002). Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function? *FEBS Lett, 512*(1-3), 1-7.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Herlein, J. A., Fink, B. D., O'Malley, Y., & Sivitz, W. I. (2009). Superoxide and respiratory coupling in mitochondria of insulin-deficient diabetic rats. *Endocrinology, 150*(1), 46-55.

Judge, S., Jang, Y. M., Smith, A., Selman, C., Phillips, T., Speakman, J. R., et al. (2005). Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. *Am J Physiol Regul Integr Comp Physiol, 289*(6), R1564-1572.

Kamo, N., Muratsugu, M., Hongoh, R., & Kobatake, Y. (1979). Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J Membr Biol, 49*(2), 105-121.

Kanazawa, A., Nishio, Y., Kashiwagi, A., Inagaki, H., Kikkawa, R., & Horiike, K. (2002). Reduced activity of mtTFA decreases the transcription in mitochondria isolated from diabetic rat heart. *Am J Physiol Endocrinol Metab, 282*(4), E778-785.

Kavazis, A. N., McClung, J. M., Hood, D. A., & Powers, S. K. (2008). Exercise induces a cardiac mitochondrial phenotype that resists apoptotic stimuli. *Am J Physiol Heart Circ Physiol, 294*(2), H928-935.

Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E., Cai, J., Jones, D. P., et al. (2004). The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature, 427*(6973), 461-465.

Kowaltowski, A. J., Castilho, R. F., & Vercesi, A. E. (2001). Mitochondrial permeability transition and oxidative stress. *FEBS Lett, 495*(1-2), 12-15.

Kruszynska, Y. T., Mulford, M. I., Baloga, J., Yu, J. G., & Olefsky, J. M. (1998). Regulation of skeletal muscle hexokinase II by insulin in nondiabetic and NIDDM subjects. *Diabetes, 47*(7), 1107-1113.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(259), 680-685.

Lajoie, C., Calderone, A., Trudeau, F., Lavoie, N., Massicotte, G., Gagnon, S., et al. (2004). Exercise training attenuated the PKB and GSK-3 dephosphorylation in the myocardium of ZDF rats. *J Appl Physiol, 96*(5), 1606-1612.

Lashin, O., & Romani, A. (2003). Mitochondria respiration and susceptibility to ischemia-reperfusion injury in diabetic hearts. *Arch Biochem Biophys, 420*(2), 298-304.

Lashin, O., & Romani, A. (2004). Hyperglycemia does not alter state 3 respiration in cardiac mitochondria from type-I diabetic rats. *Mol Cell Biochem, 267*(1-2), 31-37.

Lashin, O. M., Szweda, P. A., Szweda, L. I., & Romani, A. M. (2006). Decreased complex II respiration and HNE-modified SDH subunit in diabetic heart. *Free Radic Biol Med, 40*(5), 886-896.

Lin, G., Brownsey, R. W., & MacLeod, K. M. (2009). Regulation of mitochondrial aconitase by phosphorylation in diabetic rat heart. *Cell Mol Life Sci, 66*(5), 919-932.

Locke, M., Noble, E. G., & Atkinson, B. G. (1990). Exercising mammals synthesize stress proteins. *Am J Physiol, 258*(4 Pt 1), C723-729.

Lumini, J. A., Magalhaes, J., Oliveira, P. J., & Ascensao, A. (2008). Beneficial effects of exercise on muscle mitochondrial function in diabetes mellitus. *Sports Med, 38*(9), 735-750.

Marcil, M., Bourduas, K., Ascah, A., & Burelle, Y. (2006). Exercise training induces respiratory substrate-specific decrease in Ca2+-induced permeability transition pore opening in heart mitochondria. *Am J Physiol Heart Circ Physiol, 290*(4), H1549-1557.

Matas, J., Young, N. T., Bourcier-Lucas, C., Ascah, A., Marcil, M., Deschepper, C. F., et al. (2009). Increased expression and intramitochondrial translocation of cyclophilin-D associates with increased vulnerability of the permeability transition pore to stress-induced opening during compensated ventricular hypertrophy. *J Mol Cell Cardiol, 46*(3), 420-430.

Mokhtar, N., Lavoie, J. P., Rousseau-Migneron, S., & Nadeau, A. (1993). Physical training reverses defect in mitochondrial energy production in heart of chronically diabetic rats. *Diabetes, 42*(5), 682-687.

Mujkosova, J., Ferko, M., Humenik, P., Waczulikova, I., & Ziegelhoffer, A. (2008). Seasonal variations in properties of healthy and diabetic rat heart mitochondria: Mg2+-ATPase activity, content of conjugated dienes and membrane fluidity. *Physiol Res, 57 Suppl 2*, S75-82.

Nishio, Y., Kanazawa, A., Nagai, Y., Inagaki, H., & Kashiwagi, A. (2004). Regulation and role of the mitochondrial transcription factor in the diabetic rat heart. *Ann N Y Acad Sci, 1011*, 78-85.

Oliveira, P. J. (2005). Cardiac mitochondrial alterations observed in hyperglycaemic rats - what can we learn from cell biology? *Curr Diabetes Rev, 1*(1), 11-21.

Oliveira, P. J., Seica, R., Coxito, P. M., Rolo, A. P., Palmeira, C. M., Santos, M. S., et al. (2003). Enhanced permeability transition explains the reduced calcium uptake in cardiac mitochondria from streptozotocin-induced diabetic rats. *FEBS Lett, 554*(3), 511-514.

Oliveira, P. J., & Wallace, K. B. (2006). Depletion of adenine nucleotide translocator protein in heart mitochondria from doxorubicin-treated rats--relevance for mitochondrial dysfunction. *Toxicology, 220*(2-3), 160-168.

Picard, M., Csukly, K., Robillard, M. E., Godin, R., Ascah, A., Bourcier-Lucas, C., et al. (2008). Resistance to Ca2+-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles. *Am J Physiol Regul Integr Comp Physiol, 295*(2), R659-668.

Rajdev, S., & Reynolds, I. J. (1993). Calcium green-5N, a novel fluorescent probe for monitoring high intracellular free Ca2+ concentrations associated with glutamate excitotoxicity in cultured rat brain neurons. *Neurosci Lett, 162*(1-2), 149-152.

Savabi, F., & Kirsch, A. (1991). Alteration of the phosphocreatine energy shuttle components in diabetic rat heart. *J Mol Cell Cardiol, 23*(11), 1323-1333.

Searls, Y. M., Smirnova, I. V., Fegley, B. R., & Stehno-Bittel, L. (2004). Exercise attenuates diabetes-induced ultrastructural changes in rat cardiac tissue. *Med Sci Sports Exerc, 36*(11), 1863-1870.

Shao, C. H., Wehrens, X. H., Wyatt, T. A., Parbhu, S., Rozanski, G. J., Patel, K. P., et al. (2009). Exercise training during diabetes attenuates cardiac ryanodine receptor dysregulation. *J Appl Physiol, 106*(4), 1280-1292.

Shen, X., Zheng, S., Metreveli, N. S., & Epstein, P. N. (2006). Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy. *Diabetes, 55*(3), 798-805.

Shen, X., Zheng, S., Thongboonkerd, V., Xu, M., Pierce, W. M., Jr., Klein, J. B., et al. (2004). Cardiac mitochondrial damage and biogenesis in a chronic model of type 1 diabetes. *Am J Physiol Endocrinol Metab, 287*(5), E896-905.

Siu, P. M., Bryner, R. W., Martyn, J. K., & Alway, S. E. (2004). Apoptotic adaptations from exercise training in skeletal and cardiac muscles. *Faseb J, 18*(10), 1150-1152.

Starnes, J. W., Barnes, B. D., & Olsen, M. E. (2007). Exercise training decreases reactive oxygen species generation but does not attenuate Ca2+-induced dysfunction in rat heart mitochondria. *J Appl Physiol*.

Stuewe, S. R., Gwirtz, P. A., Agarwal, N., & Mallet, R. T. (2000). Exercise training enhances glycolytic and oxidative enzymes in canine ventricular myocardium. *J Mol Cell Cardiol, 32*(6), 903-913.

Tanaka, Y., Konno, N., & Kako, K. J. (1992). Mitochondrial dysfunction observed in situ in cardiomyocytes of rats in experimental diabetes. *Cardiovasc Res, 26*(4), 409-414.

Tomita, M., Mukae, S., Geshi, E., Umetsu, K., Nakatani, M., & Katagiri, T. (1996). Mitochondrial respiratory impairment in streptozotocin-induced diabetic rat heart. *Jpn Circ J, 60*(9), 673-682.

Turcotte, R. A., & Belcastro, A. N. (1991). Biochemical adaptation of cardiac and skeletal muscle to physical activity. *Int J Biochem, 23*(2), 221-226.

Turko, I. V., & Murad, F. (2003). Quantitative protein profiling in heart mitochondria from diabetic rats. *J Biol Chem, 278*(37), 35844-35849.

Waczulikova, I., Habodaszova, D., Cagalinec, M., Ferko, M., Ulicna, O., Mateasik, A., et al. (2007). Mitochondrial membrane fluidity, potential, and calcium transients in the myocardium from acute diabetic rats. *Can J Physiol Pharmacol, 85*(3-4), 372-381.

Zhou, S., Starkov, A., Froberg, M. K., Leino, R. L., & Wallace, K. B. (2001). Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. *Cancer Res, 61*(2), 771-777.

Zoratti, M., Szabo, I., & De Marchi, U. (2005). Mitochondrial permeability transitions: how many doors to the house? *Biochim Biophys Acta, 1706*(1-2), 40-52.

CHAPTER IV

GENERAL DISCUSSION

GENERAL DISCUSSION

Even in an autoimmune disease like T1DM, several signs of degenerative disorder with changes in mitochondrial DNA (mtDNA) and mitochondrial regulatory proteins in both skeletal muscle (Karakelides et al., 2007) and heart (Shen et al., 2004) are observed suggesting mitochondrial dysfunction. Studies with diabetic mitochondria isolated from skeletal and cardiac muscles have consistently shown respiratory dysfunction (Bonnard et al., 2008; Bugger et al., 2009; Dabkowski et al., 2009; Herlein, Fink, O'Malley, & Sivitz, 2009) as well as metabolic and morphological disturbances at different levels of cellular organization in both animal and human (Herlein, Fink, O'Malley, & Sivitz, 2009; Karakelides et al., 2007; Petersen, Dufour, Befroy, Garcia, & Shulman, 2004; Robitaille et al., 2007; Rouyer et al., 2007; Short, Nair, & Stump, 2004).

Streptozotocin-induced diabetes in rat has long been used in medical research to produce an animal model for T1DM as it's simple and relatively inexpensive when compared to genetic manipulated animals (Rees & Alcolado, 2005). T1DM in humans is characterized by the destruction of the pancreatic β-cells, frequently connected with immune-mediated damage. Although the damage may occur silently over many years, there is little surviving of β-cell mass and the disorder progresses to absolute insulinopaenia. Streptozotocin is a nitrosurea derivative that acts as a powerful alkylating agent interfering with glucose transport (Wang & Gleichmann, 1998), glucokinase function (Zahner & Malaisse, 1990) and induce multiple DNA strand breaks destroying pancreatic β-cell. Thus, it creates an insulin-deprived animal model of T1DM with high blood glucose levels which is the common feature to all diabetic states and it is at the core of DM dysfunction and complications. Even if it is not the most representative or the most relevant model of DM, T1DM is an interestingly model to study the pathophysiological aetiology or the effects of hyperglycaemia-induced dysfunction at a mechanistic level.

One of the phenomenon that is implicated in several models of necrotic and apoptotic cell death in various tissues [for review see (Duchen, Verkhratsky, & Muallem, 2008; Lemasters, Theruvath, Zhong, & Nieminen, 2009)] and it is associated with the pathophysiology of several diseases, including diabetes (Duchen, 2004) is the susceptibility to MPTP-opening.

The pioneer studies of Crofts and Chapell (Chappell & Crofts, 1965; Crofts & Chappell, 1965), more than 40 years ago, showed that energized mitochondria exposed to high

calcium concentrations could undergo massive swelling. Since then, many studies have been developed focusing on the structure and function of what became known as the MPTP. Even so, it's structure and function are yet unclear despite it has been implicated in cellular death pathways and in the progression of several disease states like diabetes (Duchen, Verkhratsky, & Muallem, 2008). Many studies have assessed the role of the MPTP on diabetes-induced dysfunction in several tissues. Despite studies using brain (Moreira, Santos, Moreno, Seica, & Oliveira, 2003), liver (Ferreira, Seica et al., 2003), kidney (Ferreira, Oliveira et al., 2003), testis and heart (Oliveira, Esteves, Rolo, Palmeira, & Moreno, 2004; Oliveira et al., 2003) diabetic mitochondria, not much is known regarding the effect of this severe hyperglycaemic condition on skeletal muscle or about the effects of exercise, particularly on the possible modulation of the MPTP.

Exercise has been used as a therapeutic measure against diabetic dysfunction (for review see Lumini, Magalhaes, Oliveira, & Ascensao, 2008) and in the treatment of muscle mitochondriopathies (for review see Taivassalo & Haller, 2005). In fact, there are evidences in the literature that both exercise (Moran, Delgado, Gonzalez, Manso, & Megias, 2004) and the severity and duration of DM dysfunction (Ferreira, Seica et al., 2003) seem to be related with time-dependent mitochondrial adaptations. So, it was challenging and relevant to explore the effects of a prolonged endurance exercise training program on mitochondrial function of both skeletal and cardiac muscle, both highly energy demanding tissues, which we initially thought would improve mitochondrial function and protect against chronic model of hyperglycaemia-induced MPTP-induction.

The results of the studies comprising in this thesis lead to interesting and in some cases unexpected conclusions that seem to sustain the notion that both long-term severe hyperglycaemia (study I) and treadmill endurance exercise training (study II-III) can induce tissue specific mitochondrial adaptations.

To the best of our knowledge, it was the first time that both $\Delta\psi$ and oxygen consumption parameters were analysed in skeletal muscle mitochondria from diabetic and trained rats. Data from study I reinforces the idea that hyperglycaemic conditions is associated with mitochondrial OXPHOS system. There are many different mechanisms that could explain these data and some of these were explored in this work. In fact, one possible mechanism related with hyperglycaemia induced respiratory dysfunction could be the activity and concentration of the ANT as the decrease in ANT protein

concentration has been correlated with the inhibition of mitochondrial respiration and increased ability to directly induce or regulate the MPTP (Oliveira & Wallace, 2006). This hypothesis is not generally accepted as some authors consider ANT to be less critical in determining the kinetics of the pore opening (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007). However, data from study III seems to reinforce the first hypothesis as ANT decrease was associated with both respiratory dysfunction and an increased susceptibility to MPTP. Although this was not investigated in skeletal muscle due to sample limitations and methological problems, it could also explain, at least in part, the altered oxidative phosphorylation and the decrease in ADP production reported by others (el Midaoui, Tancrede, & Nadeau, 1996). Another possibility could arise from the decreased availability of endogenous substrates in mitochondria of the diabetic animals. However, data from our lab (data not shown) seem to demonstrate the opposite, as STZ skeletal muscle mitochondria develop higher transmembrane Δψ without any substrate addition than their normoglycaemic counterparts. Another interesting result comes from the work of Bo and colleagues (2008). They described an increase in the activity of heart mitochondrial ATP synthase with endurance training, so one can speculate that an up-regulation of components of the phosphorylative system could also contribute to the increased energy output verified after exercise in heart mitochondria of trained normoglycaemic and diabetic animals (study III). Skeletal muscle and heart mitochondrial membrane integrity improved with training although only in the normoglycaemic group (studies II and III) and decreased with hyperglycaemia (study I). Both skeletal muscle and heart mitochondrial uncoupled respiration induced by CCCP was elevated by endurance exercise training reflecting an improvement in the rate of electron transfer (studies II and III). As in heart mitochondria from sedentary diabetic animals CCCP-uncoupled respiration has decreased and because exercise training reversed these defects, one can argue that training likely prevented the dysfunction of the respiratory chain enzyme complexes and/or the upstream supply of electrons to the respiratory components (study III). 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. Mitochondria have several accurate membrane-linked systems that interact independently or coordinated to keep sufficiently low intracellular oxygen concentrations and to diminish the lifetime of one-electron oxygen reductants and ultimately lead to a decrease in ROS production. These include UCP's, ANT (Korshunov, Korkina, Ruuge, Skulachev, & Starkov, 1998;

Simonyan & Skulachev, 1998) and ATP-sensitive K^+ channels (Ferranti, da Silva, & Kowaltowski, 2003). UCP's uncouple oxidative phosphorylation, reduce aerobic ATP production and dissipate energy as heat, thereby playing an important role in the regulation of human energy expenditure. Moreover, low UCP3 content in type I fibers have been associated with higher exercise capacity but not to skeletal muscle mitochondrial efficiency (Mogensen, Bagger, Pedersen, Fernstrom, & Sahlin, 2006). Increasing evidence concerning the role of ANT arises from the fact that these dysfunctions were reverted by endurance exercise training in both tissues and this protein was elevated by training in the heart (study III). However Fernstrom et al. (2004) did not find any ANT changes in human skeletal muscle mitochondria with exercise. On the other hand, one of the most prominent and described adaptation of endurance training is the rapid induction of mitochondrial biogenesis, a process that is considered a favorable adaptation from an energy standpoint and probably translated into improvements in respiratory endpoints. In fact, the increased respiratory capacity with endurance training was evidenced by the increase in state 3 and RCR with both malate-pyruvate and succinate in study II, suggesting an improvement in mitochondrial coupling and phosphorylative capacity. Further research is needed to better understand the explanatory mechanisms for the possible up-regulation of mitochondrial respiration with chronic exercise.

An enhancement in ROS generation would result in inhibition of mitochondrial electron transport and should produce redox changes in the electron carriers with consequent impairments to the respiratory system. Although not determined in study I, one could suspect from the large body of data in the literature that the hyperglycaemic condition-induced increased ROS production (Brownlee, 2005; Gao et al., 2010; Mehta, Rasouli, Sinha, & Molavi, 2006; Rolo & Palmeira, 2006) is probably a key player in the observed alterations. However, this is not consensual given that some authors (Kayali et al., 2004) reported decreases in mitochondrial oxidative damage parameters in the liver, pancreas and kidney in a model of severe T1DM but with no significant changes in skeletal muscle while others have shown that both skeletal muscle and diabetic heart tissues have increased ROS production (Bonnard et al., 2008; Ye, Metreveli, Ren, & Epstein, 2003). Moreover, data from study I revealed that STZ mitochondria were less susceptible to calcium induced-MPTP opening. Although unexpected in face of some above mentioned studies, this was not a unique case as liver mitochondria from STZ-treated rats had previously evidenced an increased resistance to MPTP-opening (Ferreira, Seica et al., 2003).

Apparently with surprise came the fact that endurance training had an inverse effect (study II), resulting in an increased susceptibility to calcium induced MPTP-opening in trained normoglycaemic skeletal muscle mitochondria (Sed vs. T).

In order to investigate other possible mechanism like mitochondrial intrinsic apoptotic activation, caspase 9-like activity was assessed. Accordingly, the increased susceptibility to MPTP-opening was associated with the decrease and the increase in caspase 9-like activity observed in studies I and II, respectively. Interestingly, Picard et al (2008) found that mitochondria from oxidative muscles were more prone to MPTP induction than mitochondria from glycolitic fibers. In a somewhat speculative reasoning, it is possible that an endurance exercise training program would likely induce some phenotypic shifts both in white and red fibers that might possibly contribute to the observed increased MPTP susceptibility. However, further studies are needed to better clarify this issue, including the analysis of how red and white muscle portions respond to exercise regarding this particular phenomenon related to MPTP susceptibility.

Another hypothesis which seems attractive, involves possible constituents of the MPTP. These include a possible reduction in the levels of hexokinase which has been involved in the formation MPTP complex (Crompton, 1999) and has been shown to be depressed in diabetic skeletal muscle (Kruszynska, Mulford, Baloga, Yu, & Olefsky, 1998; Pendergrass et al., 1998) and increased by continuous exercise bouts (Peter, Jeffress, & Lamb, 1968). Further studies are needed to determine other MPTP constituents and modulators that were not evaluated due to sample restrictions. Additional information comes from possible MPTP constituents in study III. Endurance training seems to modulate some pore constituents like ANT,,VDAC and Cyp-D as well as the expression of the pro- and antiapoptotic Bcl-2 family proteins Bax and Bcl-2 which is thought to regulate the outer membrane integrity and the consequent release of mitochondrial apoptotic proteins (Hengartner, 2000). In study III, hyperglycaemia induced an increase in mitochondrial Bax and Bax-Bcl-2 ratio and a decrease in Bcl-2 in sedentary group which were reverted by endurance training .

Most of the mitochondrial effects of calcium require its entry across the double membrane into the matrix. While in skeletal muscle hyperglycaemia and endurance training seem to increase calcium uptake capacity, only exercise increase calcium accumulation capacity in heart. Although, skeletal muscle mitochondria from hyperglycaemic animals were less susceptible to permeability transition, diabetic heart

mitochondria showed an increased vulnerability to MPTP-opening as previously described by (Oliveira et al., 2003).

As ANT is diminished in hyperglycaemic heart mitochondria and one of the contributors for the possible protective modulation exerted by endurance exercise (as it was increased by training, see study III), ANT is also a possible candidate target proteins related to the deleterious consequences of hyperglycaemia. Moreover, these findings seem concurrent with others suggesting that the decreased expression of this protein, as occurred in sedentary hyperglycaemic heart, is associated with the susceptibility to MPTP opening (Csukly et al., 2006; Oliveira & Wallace, 2006). Despite the suggestion that VDAC is not essential for the mitochondrial-dependent cell death through MPTP (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007), VDAC seems to have an indirect role in MPTP formation due to the fact that it binds to calcium and has a role in the regulation of the mitochondrial calcium homoeostasis (Gincel, Zaid, & Shoshan-Barmatz, 2001).

Cyp-D, which was increased by hyperglycaemia in heart mitochondria (study III) constitutes another possible candidate to explain our results. Cyp-D is refereed as sensitizer of the MPTP (Crompton, 1999) as mice with Cyp-D ablated mitochondria not only require higher amounts of calcium to trigger MPTP (Basso et al., 2005), but also are more protected from ischemia/reperfusion-induced injury in the heart (Baines et al., 2005). Furthermore, the expression of both ANT and Cyp D were reported to correlate with the susceptibility of isolated mitochondria to undergo MPTP opening (Csukly et al., 2006; Matas et al., 2009; Oliveira & Wallace, 2006). This suggests that heart mitochondria from STZ-induced diabetic rats with an increase in Cyp-D would be more prone to undergo PTP-opening has was observed in study III.

Very recently, Williamson and colleagues (Williamson et al., 2010) showed that diabetic interfibrillar (IMF) mitochondria had an enhanced apoptotic propensity compared to subsarcolemmal (SS). Despite the differences in the isolation procedure here used, a mixed suspension of mitochondria is obtained with IMF of greater abundance (~80%) when compared with SS (~20%). These data points to a potentially dominant role for the IMF sub fraction in evoking the mitochondrially-driven alterations and is concurrent what was observed in study III.

CHAPTER IV

CONCLUSIONS

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Based on the results of the different studies which comprised in this dissertation, it seems reasonable to highlight the following conclusions:

a) Eighteen weeks of severe hyperglycaemia leads to an impaired mitochondrial respiratory function in *gastrocnemius* muscle and to an increased resistance to calcium-dependent MPTP opening.
b) Fourteen weeks of endurance treadmill training did not afford protection to *gastrocnemius* mitochondria against MPTP-opening, despite the observed improvement of respiratory endpoints.
c) STZ-induced diabetes resulted in defects on heart mitochondrial respiratory coupling which were attenuated or reverted by endurance exercise training.
d) Long-term severe hyperglycaemia increases heart mitochondrial susceptibility to MPTP-opening, being this effect attenuated or reverted by moderate endurance training.
e) The modulator effect of endurance exercise training on MPTP regulatory proteins seems to play a key role on the increased susceptibility of heart mitochondria to undergo MPTP.
f) The cross tolerance effect of endurance training against heart mitochondrial dysfunction of severe diabetic animals seems to be closely associated with apoptotic signaling.
g) From the results obtained, it seems that mitochondrial adaptations to both hyperglycaemia and training are tissue specific.

In summary, the findings of the studies presented in this work seem to suggest a tissue specific tolerance to MPTP-opening as well as different adaptive mechanisms to chronic endurance exercise and hyperglycaemia in mitochondria of skeletal and cardiac muscle. Moreover, the data obtained suggest that factors affecting MPTP dynamics might be involved in the well-described training-induced cardioprotection against deleterious insults in DM.

CHAPTER IV

FUTURE DIRECTIONS

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Despite the limitations presented by these studies, particularly in skeletal muscle due to time, methodological and sample limitations, at light of the evidenced presented by these and other studies already mentioned it seems reasonable to assume the role of not one but several pathways. Our data suggests that protection may be mediated, at least in part, by alterations in the expression of the pore proteins ANT and Cyp D as well as in the pro and antiapoptotic proteins of the Bcl-2 family. Interaction of these structural and regulatory proteins in the modulation of MPTP needs to be clarified. Differences in mitochondrial subpopulations need to be further explored as well as the different susceptibility between glycolitic and oxidative muscles particularly in more ecological DM models like in T2DM in order to develop new therapeutic strategies to manage and treat other pathologies like DM.

It will be of interest to analyze how the combination of these stimuli can interact with the expression of other important proteins involved in the dysfunction that characterizes pathological conditions and in the mostly enhanced protective phenotype associated with exercise.

REFERENCES

REFERENCES

Adhihetty, P. J., Ljubicic, V., & Hood, D. A. (2006). Effect of chronic contractile activity on SS and IMF mitochondrial apoptotic susceptibility in skeletal muscle. *Am J Physiol Endocrinol Metab*.

Adhihetty, P. J., O'Leary, M. F., Chabi, B., Wicks, K. L., & Hood, D. A. (2007). Effect of denervation on mitochondrially mediated apoptosis in skeletal muscle. *J Appl Physiol, 102*(3), 1143-1151.

Alessio, H. M. (1993). Exercise-induced oxidative stress. *Med Sci Sports Exerc, 25*(2), 218-224.

Antonetti, D. A., Reynet, C., & Kahn, C. R. (1995). Increased expression of mitochondrial-encoded genes in skeletal muscle of humans with diabetes mellitus. *J Clin Invest, 95*(3), 1383-1388.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., et al. (2005b). Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *Am J Physiol Heart Circ Physiol, 289*(2), H722-731.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., et al. (2006). Endurance training limits the functional alterations of rat heart mitochondria submitted to in vitro anoxia-reoxygenation. *Int J Cardiol, 109*(2), 169-178.

Baines, C. P., Kaiser, R. A., Purcell, N. H., Blair, N. S., Osinska, H., Hambleton, M. A., et al. (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature, 434*(7033), 658-662.

Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J., & Molkentin, J. D. (2007). Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol, 9*(5), 550-555.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M. A., & Bernardi, P. (2005). Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem, 280*(19), 18558-18561.

Befroy, D. E., Petersen, K. F., Dufour, S., Mason, G. F., de Graaf, R. A., Rothman, D. L., et al. (2007). Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. *Diabetes, 56*(5), 1376-1381.

Bo, H., Jiang, N., Ma, G., Qu, J., Zhang, G., Cao, D., et al. (2008). Regulation of mitochondrial uncoupling respiration during exercise in rat heart: role of reactive oxygen species (ROS) and uncoupling protein 2. *Free Radic Biol Med, 44*(7), 1373-1381.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M. A., Morio, B., et al. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest, 118*(2), 789-800.

Boudina, S., & Abel, E. D. (2007). Diabetic cardiomyopathy revisited. *Circulation, 115*(25), 3213-3223.

Brookes, P. S. (2005). Mitochondrial H(+) leak and ROS generation: an odd couple. *Free Radic Biol Med, 38*(1), 12-23.

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol, 287*(4), C817-833.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature, 414*(6865), 813-820.

Brownlee, M. (2005). The pathobiology of diabetic complications: a unifying mechanism. *Diabetes, 54*(6), 1615-1625.

Brustovetsky, N., Tropschug, M., Heimpel, S., Heidkamper, D., & Klingenberg, M. (2002). A large Ca2+-dependent channel formed by recombinant ADP/ATP carrier from Neurospora crassa resembles the mitochondrial permeability transition pore. *Biochemistry, 41*(39), 11804-11811.

Buchanan, J., Mazumder, P. K., Hu, P., Chakrabarti, G., Roberts, M. W., Yun, U. J., et al. (2005). Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology, 146*(12), 5341-5349.

Bugger, H., Chen, D., Riehle, C., Soto, J., Theobald, H. A., Hu, X. X., et al. (2009). Tissue-Specific Remodeling of the Mitochondrial Proteome in Type 1 Diabetic Akita Mice. *Diabetes*.

Cadenas, E. (2004). Mitochondrial free radical production and cell signaling. *Mol Aspects Med, 25*(1-2), 17-26.

Chappell, J. B., & Crofts, A. R. (1965). Calcium Ion Accumulation and Volume Changes of Isolated Liver Mitochondria. Calcium Ion-Induced Swelling. *Biochem J, 95*, 378-386.

Chen, V., & Ianuzzo, C. D. (1982). Metabolic alterations in skeletal muscle of chronically streptozotocin-diabetic rats. *Arch Biochem Biophys, 217*(1), 131-138.

Chow, L. S., Greenlund, L. J., Asmann, Y. W., Short, K. R., McCrady, S. K., Levine, J. A., et al. (2007). Impact of endurance training on murine spontaneous activity, muscle mitochondrial DNA abundance, gene transcripts, and function. *J Appl Physiol, 102*(3), 1078-1089.

Ciminelli, M., Ascah, A., Bourduas, K., & Burelle, Y. (2006). Short term training attenuates opening of the mitochondrial permeability transition pore without affecting myocardial function following ischemia-reperfusion. *Mol Cell Biochem, 291*(1-2), 39-47.

Crofts, A. R., & Chappell, J. B. (1965). Calcium Ion Accumulation and Volume Changes of Isolated Liver Mitochondria. Reversal of Calcium Ion-Induced Swelling. *Biochem J, 95*, 387-392.

Crompton, M. (1999). The mitochondrial permeability transition pore and its role in cell death. *Biochem J, 341 (Pt 2)*, 233-249.

Csukly, K., Ascah, A., Matas, J., Gardiner, P. F., Fontaine, E., & Burelle, Y. (2006). Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D. *J Physiol, 574*(Pt 1), 319-327.

Dabkowski, E. R., Williamson, C. L., Bukowski, V. C., Chapman, R. S., Leonard, S. S., Peer, C. J., et al. (2009). Diabetic cardiomyopathy-associated dysfunction in spatially distinct mitochondrial subpopulations. *Am J Physiol Heart Circ Physiol, 296*(2), H359-369.

Davies, K. J., Quintanilha, A. T., Brooks, G. A., & Packer, L. (1982). Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun, 107*(4), 1198-1205.

Di Lisa, F., & Bernardi, P. (2009). A CaPful of mechanisms regulating the mitochondrial permeability transition. *J Mol Cell Cardiol, 46*(6), 775-780.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev, 82*(1), 47-95.

Duchen, M. R. (2004). Roles of mitochondria in health and disease. *Diabetes, 53 Suppl 1*, S96-102.

Duchen, M. R., Verkhratsky, A., & Muallem, S. (2008). Mitochondria and calcium in health and disease. *Cell Calcium, 44*(1), 1-5.

Dumas, J. F., Simard, G., Flamment, M., Ducluzeau, P. H., & Ritz, P. (2009). Is skeletal muscle mitochondrial dysfunction a cause or an indirect consequence of insulin resistance in humans? *Diabetes Metab, 35*(3), 159-167.

el Midaoui, A., Tancrede, G., & Nadeau, A. (1996). Effect of physical training on mitochondrial function in skeletal muscle of normal and diabetic rats. *Metabolism, 45*(7), 810-816.

Fernstrom, M., Tonkonogi, M., & Sahlin, K. (2004). Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle. *J Physiol, 554*(Pt 3), 755-763.

Ferranti, R., da Silva, M. M., & Kowaltowski, A. J. (2003). Mitochondrial ATP-sensitive K+ channel opening decreases reactive oxygen species generation. *FEBS Lett, 536*(1-3), 51-55.

Ferreira, F. M., Oliveira, P. J., Rolo, A. P., Santos, M. S., Moreno, A. J., da Cunha, M. F., et al. (2003). Cholestasis induced by chronic treatment with alpha-naphthyl-isothiocyanate (ANIT) affects rat renal mitochondrial bioenergetics. *Arch Toxicol, 77*(4), 194-200.

Ferreira, F. M., Seica, R., Oliveira, P. J., Coxito, P. M., Moreno, A. J., Palmeira, C. M., et al. (2003). Diabetes induces metabolic adaptations in rat liver mitochondria: role of coenzyme Q and cardiolipin contents. *Biochim Biophys Acta, 1639*(2), 113-120.

Flarsheim, C. E., Grupp, I. L., & Matlib, M. A. (1996). Mitochondrial dysfunction accompanies diastolic dysfunction in diabetic rat heart. *Am J Physiol, 271*(1 Pt 2), H192-202.

Fontaine, E., Ichas, F., & Bernardi, P. (1998). A ubiquinone-binding site regulates the mitochondrial permeability transition pore. *J Biol Chem, 273*(40), 25734-25740.

Gakh, O., Park, S., Liu, G., Macomber, L., Imlay, J. A., Ferreira, G. C., et al. (2006). Mitochondrial iron detoxification is a primary function of frataxin that limits oxidative damage and preserves cell longevity. *Hum Mol Genet, 15*(3), 467-479.

Gao, C. L., Zhu, C., Zhao, Y. P., Chen, X. H., Ji, C. B., Zhang, C. M., et al. (2010). Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. *Mol Cell Endocrinol, 320*(1-2), 25-33.

Gincel, D., Zaid, H., & Shoshan-Barmatz, V. (2001). Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function. *Biochem J, 358*(Pt 1), 147-155.

Green, K., Brand, M. D., & Murphy, M. P. (2004). Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. *Diabetes, 53 Suppl 1*, S110-118.

Gross, M. D., Harris, S., & Beyer, R. E. (1972). The effect of streptozotocin-induced diabetes on oxidative phosphorylation and related reactions in skeletal muscle mitochondria. *Horm Metab Res, 4*(1), 1-7.

Gunter, T. E., & Gunter, K. K. (2001). Uptake of calcium by mitochondria: transport and possible function. *IUBMB Life, 52*(3-5), 197-204.

Gunter, T. E., Buntinas, L., Sparagna, G., Eliseev, R., & Gunter, K. (2000). Mitochondrial calcium transport: mechanisms and functions. *Cell Calcium, 28*(5-6), 285-296.

Gunter, T. E., Yule, D. I., Gunter, K. K., Eliseev, R. A., & Salter, J. D. (2004). Calcium and mitochondria. *FEBS Lett, 567*(1), 96-102.

Halestrap, A. P. (1991). Calcium-dependent opening of a non-specific pore in the mitochondrial inner membrane is inhibited at pH values below 7. Implications for the protective effect of low pH against chemical and hypoxic cell damage. *Biochem J, 278 (Pt 3)*, 715-719.

Halestrap, A. P. (2009a). What is the mitochondrial permeability transition pore? *J Mol Cell Cardiol*.

Halestrap, A. P. (2009b). What is the mitochondrial permeability transition pore? *J Mol Cell Cardiol, 46*(6), 821-831.

Halestrap, A. P., & Pasdois, P. (2009). The role of the mitochondrial permeability transition pore in heart disease. *Biochim Biophys Acta*.

Halestrap, A. P., Woodfield, K. Y., & Connern, C. P. (1997). Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J Biol Chem, 272*(6), 3346-3354.

Harris, D. A. (1993). Regulation of the mitochondrial ATP synthase in rat heart. *Biochem Soc Trans, 21 (Pt 3)*(3), 778-781.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Herlein, J. A., Fink, B. D., O'Malley, Y., & Sivitz, W. I. (2009). Superoxide and respiratory coupling in mitochondria of insulin-deficient diabetic rats. *Endocrinology, 150*(1), 46-55.

Herrero, P., Peterson, L. R., McGill, J. B., Matthew, S., Lesniak, D., Dence, C., et al. (2006). Increased myocardial fatty acid metabolism in patients with type 1 diabetes mellitus. *J Am Coll Cardiol, 47*(3), 598-604.

Hidaka, S., Kakuma, T., Yoshimatsu, H., Sakino, H., Fukuchi, S., & Sakata, T. (1999). Streptozotocin treatment upregulates uncoupling protein 3 expression in the rat heart. *Diabetes, 48*(2), 430-435.

Hojlund, K., Wrzesinski, K., Larsen, P. M., Fey, S. J., Roepstorff, P., Handberg, A., et al. (2003). Proteome analysis reveals phosphorylation of ATP synthase beta -subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. *J Biol Chem, 278*(12), 10436-10442.

Holloszy, J. O. (1967). Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem, 242*(9), 2278-2282.

Hood, D. A. (2001). Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J Appl Physiol, 90*(3), 1137-1157.

Hood, D. A., Balaban, A., Connor, M. K., Craig, E. E., Nishio, M. L., Rezvani, M., et al. (1994). Mitochondrial biogenesis in striated muscle. *Can J Appl Physiol, 19*(1), 12-48.

Hunter, D. R., & Haworth, R. A. (1979). The Ca2+-induced membrane transition in mitochondria. I. The protective mechanisms. *Arch Biochem Biophys, 195*(2), 453-459.

International Diabetes Federation. (2003). *Diabetes Atlas 2003* (2nd ed.). Brussels: International Diabetes Federation.

Inzucchi, S. E. (2005). Classification and diagnosis of diabetes. In S. E. Inzucchi (Ed.), *The diabetes mellitus manual: A primary care companion to Ellenberg and Rifkin's* (6th ed., pp. 1-15): MacGraw-Hill.

Ji, L. L., Gomez-Cabrera, M. C., & Vina, J. (2006). Exercise and hormesis: activation of cellular antioxidant signaling pathway. *Ann N Y Acad Sci, 1067*, 425-435.

Judge, S., Jang, Y. M., Smith, A., Selman, C., Phillips, T., Speakman, J., et al. (2005). Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. *Am J Physiol Regul Integr Comp Physiol*. 289(6), R1564-1572.

Karakelides, H., Asmann, Y. W., Bigelow, M. L., Short, K. R., Dhatariya, K., Coenen-Schimke, J., et al. (2007). Effect of insulin deprivation on muscle mitochondrial ATP production and gene transcript levels in type 1 diabetic subjects. *Diabetes, 56*(11), 2683-2689.

Kayali, R., Cakatay, U., Telci, A., Akcay, T., Sivas, A., & Altug, T. (2004). Decrease in mitochondrial oxidative protein damage parameters in the streptozotocin-diabetic rat. *Diabetes Metab Res Rev, 20*(4), 315-321.

Kelley, D. E., He, J., Menshikova, E. V., & Ritov, V. B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes, 51*(10), 2944-2950.

Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E., Cai, J., Jones, D. P., et al. (2004). The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature, 427*(6973), 461-465.

Korshunov, S. S., Korkina, O. V., Ruuge, E. K., Skulachev, V. P., & Starkov, A. A. (1998). Fatty acids as natural uncouplers preventing generation of O2.- and H2O2 by mitochondria in the resting state. *FEBS Lett, 435*(2-3), 215-218.

Krauskopf, A., Eriksson, O., Craigen, W. J., Forte, M. A., & Bernardi, P. (2006). Properties of the permeability transition in VDAC1(-/-) mitochondria. *Biochim Biophys Acta, 1757*(5-6), 590-595.

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol Rev, 87*(1), 99-163.

Kruszynska, Y. T., Mulford, M. I., Baloga, J., Yu, J. G., & Olefsky, J. M. (1998). Regulation of skeletal muscle hexokinase II by insulin in nondiabetic and NIDDM subjects. *Diabetes, 47*(7), 1107-1113.

LaMonte, M. J., Blair, S. N., & Church, T. S. (2005). Physical activity and diabetes prevention. *J Appl Physiol, 99*(3), 1205-1213.

Lashin, O. M., Szweda, P. A., Szweda, L. I., & Romani, A. M. (2006). Decreased complex II respiration and HNE-modified SDH subunit in diabetic heart. *Free Radic Biol Med, 40*(5), 886-896.

Lemasters, J. J., Theruvath, T. P., Zhong, Z., & Nieminen, A. L. (2009). Mitochondrial calcium and the permeability transition in cell death. *Biochim Biophys Acta, 1787*(11), 1395-1401.

Lin, G., Brownsey, R. W., & MacLeod, K. M. (2009). Regulation of mitochondrial aconitase by phosphorylation in diabetic rat heart. *Cell Mol Life Sci, 66*(5), 919-932.

Lumini, J.A., Magalhaes, J., Oliveira, P. J., & Ascensao, A. (2008). Beneficial effects of exercise on muscle mitochondrial function in diabetes mellitus. *Sports Med, 38*(9), 735-750.

Marcil, M., Bourduas, K., Ascah, A., & Burelle, Y. (2006). Exercise training induces respiratory substrate-specific decrease in Ca2+-induced permeability transition pore opening in heart mitochondria. *Am J Physiol Heart Circ Physiol, 290*(4), H1549-1557.

Matas, J., Young, N. T., Bourcier-Lucas, C., Ascah, A., Marcil, M., Deschepper, C. F., et al. (2009). Increased expression and intramitochondrial translocation of cyclophilin-D associates with increased vulnerability of the permeability transition pore to stress-induced opening during compensated ventricular hypertrophy. *J Mol Cell Cardiol, 46*(3), 420-430.

Mehta, J. L., Rasouli, N., Sinha, A. K., & Molavi, B. (2006). Oxidative stress in diabetes: a mechanistic overview of its effects on atherogenesis and myocardial dysfunction. *Int J Biochem Cell Biol, 38*(5-6), 794-803.

Mitchell, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol Rev Camb Philos Soc, 41*(3), 445-502.

Mitchell, P. (1976). Possible molecular mechanisms of the protonmotive function of cytochrome systems. *J Theor Biol, 62*(2), 327-367.

Mogensen, M., Bagger, M., Pedersen, P. K., Fernstrom, M., & Sahlin, K. (2006). Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency. *J Physiol, 571*(Pt 3), 669-681.

Mokhtar, N., Lavoie, J. P., Rousseau-Migneron, S., & Nadeau, A. (1993). Physical training reverses defect in mitochondrial energy production in heart of chronically diabetic rats. *Diabetes, 42*(5), 682-687.

Mootha, V. K., Lindgren, C. M., Eriksson, K. F., Subramanian, A., Sihag, S., Lehar, J., et al. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet, 34*(3), 267-273.

Moran, M., Delgado, J., Gonzalez, B., Manso, R., & Megias, A. (2004). Responses of rat myocardial antioxidant defences and heat shock protein HSP72 induced by 12 and 24-week treadmill training. *Acta Physiol Scand, 180*(2), 157-166.

Moreira, P. I., Santos, M. S., Moreno, A. M., Proenca, T., Seica, R., & Oliveira, C. R. (2004). Effect of streptozotocin-induced diabetes on rat brain mitochondria. *J Neuroendocrinol, 16*(1), 32-38.

Moreira, P. I., Santos, M. S., Moreno, A. M., Seica, R., & Oliveira, C. R. (2003). Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid-beta exposure. *Diabetes, 52*(6), 1449-1456.

Morino, K., Petersen, K. F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., et al. (2005). Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. *J Clin Invest, 115*(12), 3587-3593.

Murphy, E., & Steenbergen, C. (2006). Preconditioning: The Mitochondrial Connection. *Annu Rev Physiol*.

Murray, A. J., Panagia, M., Hauton, D., Gibbons, G. F., & Clarke, K. (2005). Plasma free fatty acids and peroxisome proliferator-activated receptor alpha in the control of myocardial uncoupling protein levels. *Diabetes, 54*(12), 3496-3502.

Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., et al. (2005). Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature, 434*(7033), 652-658.

Noble, E. G., & Ianuzzo, C. D. (1985). Influence of training on skeletal muscle enzymatic adaptations in normal and diabetic rats. *Am J Physiol, 249*(4 Pt 1), E360-365.

Novgorodov, S. A., Gudz, T. I., Jung, D. W., & Brierley, G. P. (1991). The nonspecific inner membrane pore of liver mitochondria: modulation of cyclosporin sensitivity by ADP at carboxyatractyloside-sensitive and insensitive sites. *Biochem Biophys Res Commun, 180*(1), 33-38.

O'Leary, M. F., & Hood, D. A. (2008). Effect of prior chronic contractile activity on mitochondrial function and apoptotic protein expression in denervated muscle. *J Appl Physiol, 105*(1), 114-120.

Oliveira, P. J., & Wallace, K. B. (2006). Depletion of adenine nucleotide translocator protein in heart mitochondria from doxorubicin-treated rats--relevance for mitochondrial dysfunction. *Toxicology, 220*(2-3), 160-168.

Oliveira, P. J., Esteves, T. C., Seica, R., Moreno, A. J., & Santos, M. S. (2004). Calcium-dependent mitochondrial permeability transition is augmented in the kidney of Goto-Kakizaki diabetic rat. *Diabetes Metab Res Rev, 20*(2), 131-136.

Oliveira, P. J., Esteves, T., Rolo, A. P., Palmeira, C. M., & Moreno, A. J. (2004). Carvedilol inhibits the mitochondrial permeability transition by an antioxidant mechanism. *Cardiovasc Toxicol, 4*(1), 11-20.

Oliveira, P. J., Rolo, A. P., Seica, R., Sardao, V., Monteiro, P., Goncalves, L., et al. (2002). Impact of diabetes on induction of the mitochondrial permeability transition. *Rev Port Cardiol, 21*(6), 759-766.

Oliveira, P. J., Seica, R., Coxito, P. M., Rolo, A. P., Palmeira, C. M., Santos, M. S., et al. (2003). Enhanced permeability transition explains the reduced calcium uptake in cardiac mitochondria from streptozotocin-induced diabetic rats. *FEBS Lett, 554*(3), 511-514.

Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2006). Mitochondrial Oxidative Stress: Implications for Cell Death. *Annu Rev Pharmacol Toxicol*.

Palmeira, C. M., Ferreira, F. M., Santos, D. L., Ceica, R., Suzuki, K., & Santos, M. S. (1999). Higher efficiency of the liver phosphorylative system in diabetic Goto-Kakizaki (GK) rats. *FEBS Lett, 458*(2), 103-106.

Palmeira, C. M., Santos, D. L., Seica, R., Moreno, A. J., & Santos, M. S. (2001). Enhanced mitochondrial testicular antioxidant capacity in Goto-Kakizaki diabetic rats: role of coenzyme Q. *Am J Physiol Cell Physiol, 281*(3), C1023-1028.

Patti, M. E., Butte, A. J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., et al. (2003). Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A, 100*(14), 8466-8471.

Pendergrass, M., Koval, J., Vogt, C., Yki-Jarvinen, H., Iozzo, P., Pipek, R., et al. (1998). Insulin-induced hexokinase II expression is reduced in obesity and NIDDM. *Diabetes, 47*(3), 387-394.

Peter, J. B., Jeffress, R. N., & Lamb, D. R. (1968). Exercise: effects on hexokinase activity in red and white skeletal muscle. *Science, 160*(824), 200-201.

Petersen, K. F., Dufour, S., Befroy, D., Garcia, R., & Shulman, G. I. (2004). Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med, 350*(7), 664-671.

Picard, M., Csukly, K., Robillard, M. E., Godin, R., Ascah, A., Bourcier-Lucas, C., et al. (2008). Resistance to Ca2+-induced opening of the permeability transition pore differs in mitochondria from glycolytic and oxidative muscles. *Am J Physiol Regul Integr Comp Physiol, 295*(2), R659-668.

Rahimi, R., Nikfar, S., Larijani, B., & Abdollahi, M. (2005). A review on the role of antioxidants in the management of diabetes and its complications. *Biomed Pharmacother, 59*(7), 365-373.

Rees, D. A., & Alcolado, J. C. (2005). Animal models of diabetes mellitus. *Diabet Med, 22*(4), 359-370.

Ritov, V. B., Menshikova, E. V., He, J., Ferrell, R. E., Goodpaster, B. H., & Kelley, D. E. (2005). Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. *Diabetes, 54*(1), 8-14.

Roberts, C. K., Won, D., Pruthi, S., Lin, S. S., & Barnard, R. J. (2006). Effect of a diet and exercise intervention on oxidative stress, inflammation and monocyte adhesion in diabetic men. *Diabetes Res Clin Pract*.

Robitaille, M., Dube, M. C., Weisnagel, S. J., Prud'homme, D., Massicotte, D., Peronnet, F., et al. (2007). Substrate source utilization during moderate intensity exercise with glucose ingestion in Type 1 diabetic patients. *J Appl Physiol, 103*(1), 119-124.

Rolo, A. P., & Palmeira, C. M. (2006). Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol, 212*(2), 167-178.

Roussel, D., Chainier, F., Rouanet, J., & Barre, H. (2000). Increase in the adenine nucleotide translocase content of duckling subsarcolemmal mitochondria during cold acclimation. *FEBS Lett, 477*(1-2), 141-144.

Rouyer, O., Zoll, J., Daussin, F., Damge, C., Helms, P., Talha, S., et al. (2007). Effect of angiotensin-converting enzyme inhibition on skeletal muscle oxidative function and exercise capacity in streptozotocin-induced diabetic rats. *Exp Physiol, 92*(6), 1047-1056.

Santos, M. S., Santos, D. L., Palmeira, C. M., Seica, R., Moreno, A. J., & Oliveira, C. R. (2001). Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. *Diabetes Metab Res Rev, 17*(3), 223-230.

Savabi, F., & Kirsch, A. (1991). Alteration of the phosphocreatine energy shuttle components in diabetic rat heart. *J Mol Cell Cardiol, 23*(11), 1323-1333.

Scheffler, I. E. (2007). *Mitochondria* (2nd ed.). Hoboken: Wiley&Sons.

Schrauwen, P., & Hesselink, M. K. (2004). Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. *Diabetes, 53*(6), 1412-1417.

Sen, C. K. (1995). Oxidants and antioxidants in exercise. *J Appl Physiol, 79*(3), 675-686.

Shen, X., Zheng, S., Thongboonkerd, V., Xu, M., Pierce, W. M., Jr., Klein, J. B., et al. (2004). Cardiac mitochondrial damage and biogenesis in a chronic model of type 1 diabetes. *Am J Physiol Endocrinol Metab, 287*(5), E896-905.

Short, K. R., Nair, K. S., & Stump, C. S. (2004). Impaired mitochondrial activity and insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med, 350*(23), 2419-2421; author reply 2419-2421.

Short, K. R., Vittone, J. L., Bigelow, M. L., Proctor, D. N., Rizza, R. A., Coenen-Schimke, J. M., et al. (2003). Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes, 52*(8), 1888-1896.

Simoneau, J. A., & Kelley, D. E. (1997). Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. *J Appl Physiol, 83*(1), 166-171.

Simonyan, R. A., & Skulachev, V. P. (1998). Thermoregulatory uncoupling in heart muscle mitochondria: involvement of the ATP/ADP antiporter and uncoupling protein. *FEBS Lett, 436*(1), 81-84.

Siu, P. M., Bryner, R. W., Martyn, J. K., & Alway, S. E. (2004). Apoptotic adaptations from exercise training in skeletal and cardiac muscles. *FASEB J, 18*(10), 1150-1152.

Skulachev, V. P. (1999a). Anion carriers in fatty acid-mediated physiological uncoupling. *J Bioenerg Biomembr, 31*(5), 431-445.

Skulachev, V. P. (1999b). Mitochondrial physiology and pathology; concepts of programmed death of organelles, cells and organisms. *Mol Aspects Med, 20*(3), 139-184.

Sreekumar, R., Halvatsiotis, P., Schimke, J. C., & Nair, K. S. (2002). Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes, 51*(6), 1913-1920.

Starnes, J. W., Barnes, B. D., & Olsen, M. E. (2007). Exercise training decreases reactive oxygen species generation but does not attenuate Ca2+-induced dysfunction in rat heart mitochondria. *J Appl Physiol*.

Sun, Z., Liu, L., Liu, N., & Liu, Y. (2008). Muscular response and adaptation to diabetes mellitus. *Front Biosci, 13*, 4765-4794.

Taivassalo, T., & Haller, R. G. (2005). Exercise and training in mitochondrial myopathies. *Med Sci Sports Exerc, 37*(12), 2094-2101.

Tanveer, A., Virji, S., Andreeva, L., Totty, N. F., Hsuan, J. J., Ward, J. M., et al. (1996). Involvement of cyclophilin D in the activation of a mitochondrial pore by Ca2+ and oxidant stress. *Eur J Biochem, 238*(1), 166-172.

Tomita, M., Mukae, S., Geshi, E., Umetsu, K., Nakatani, M., & Katagiri, T. (1996). Mitochondrial respiratory impairment in streptozotocin-induced diabetic rat heart. *Jpn Circ J, 60*(9), 673-682.

Tsujimoto, Y., & Shimizu, S. (2007). Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis, 12*(5), 835-840.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J Physiol, 552*(Pt 2), 335-344.

Van Der Lee, K. A., Willemsen, P. H., Van Der Vusse, G. J., & Van Bilsen, M. (2000). Effects of fatty acids on uncoupling protein-2 expression in the rat heart. *FASEB J, 14*(3), 495-502.

Venditti, P., Masullo, P., & Di Meo, S. (1999). Effect of training on H(2)O(2) release by mitochondria from rat skeletal muscle. *Arch Biochem Biophys, 372*(2), 315-320.

Walter, L., Nogueira, V., Leverve, X., Heitz, M. P., Bernardi, P., & Fontaine, E. (2000). Three classes of ubiquinone analogs regulate the mitochondrial permeability transition pore through a common site. *J Biol Chem, 275*(38), 29521-29527.

Wang, Y., Ebermann, L., Sterner-Kock, A., Wika, S., Schultheiss, H. P., Dorner, A., et al. (2009). Myocardial overexpression of adenine nucleotide translocase 1 ameliorates diabetic cardiomyopathy in mice. *Exp Physiol, 94*(2), 220-227.

Wang, Z., & Gleichmann, H. (1998). GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. *Diabetes, 47*(1), 50-56.

Williamson, C. L., Dabkowski, E. R., Baseler, W. A., Croston, T. L., Alway, S. E., & Hollander, J. M. (2010). Enhanced apoptotic propensity in diabetic cardiac mitochondria: influence of subcellular spatial location. *Am J Physiol Heart Circ Physiol, 298*(2), H633-642.

Yaguzhinsky, L. S., Yurkov, V. I., & Krasinskaya, I. P. (2006). On the localized coupling of respiration and phosphorylation in mitochondria. *Biochim Biophys Acta, 1757*(5-6), 408-414.

Ye, G., Metreveli, N. S., Ren, J., & Epstein, P. N. (2003). Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. *Diabetes, 52*(3), 777-783.

Young, M. E., Patil, S., Ying, J., Depre, C., Ahuja, H. S., Shipley, G. L., et al. (2001). Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. *FASEB J, 15*(3), 833-845.
Zahner, D., & Malaisse, W. J. (1990). Kinetic behaviour of liver glucokinase in diabetes. I. Alteration in streptozotocin-diabetic rats. *Diabetes Res, 14*(3), 101-108.

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REVIEW ARTICLE

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Beneficial Effects of Exercise on Muscle Mitochondrial Function in Diabetes Mellitus

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Contents

Abstract

The physiopathology of diabetes mellitus has been closely associated with a variety of alterations in mitochondrial histology, biochemistry and function. Generally, the alterations comprise increased mitochondrial reactive oxygen and nitrogen species (RONS) generation, resulting in oxidative stress and damage; decreased capacity to metabolize lipids, leading to intramyocyte lipid accumulation; and diminished mitochondrial density and reduced levels of uncoupling proteins (UCPs), with consequent impairment in mitochondrial function. Chronic physical exercise is a physiological stimulus able to induce mitochondrial adaptations that can counteract the adverse effects of diabetes on muscle mitochondria. However, the mechanisms responsible for mitochondrial adaptations in the muscles of diabetic patients are still unclear. The main mechanisms by which exercise may be considered an important non-pharmacological strategy for preventing and/or attenuating diabetes-induced mitochondrial impairments may involve (i) increased mitochondrial biogenesis, which is dependent on the increased expression of some important proteins, such as the 'master switch'

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ORIGINAL ARTICLE

Long-term hyperglycaemia decreases gastrocnemius susceptibility to permeability transition

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ABSTRACT

Background Hyperglycaemia-resulting in mitochondrial bioenergetics' complications is associated with skeletal muscle dysfunction. The aim of this work was to analyse the effect of long-term severe hyperglycaemia on *gastrocnemius* mitochondrial bioenergetics, with special relevance on the susceptibility to mitochondrial permeability transition pore (MPTP) opening.

Methods Sixteen adult (6- to 8-week-old) male Wistar rats were randomly divided into two groups (n = 8/group): control and diabetic. A single dose (50 mg kg^{-1}) of streptozotocin (STZ) was administrated i.p. to induce hyperglycaemia. *In vitro* mitochondrial oxygen consumption rates, membrane potential ($\Delta\psi$) fluctuations, MPTP induction as followed by osmotic swelling and extramitochondrial calcium movements and caspase 9-like activity were evaluated 18 weeks after STZ treatment.

Results STZ treatment induced an increase in state 4 and a decrease in the respiratory control ratio with complex I substrates ($P < 0{\cdot}05$), whereas no differences were observed using complex II substrates. In both conditions, no significant differences were observed when measuring maximal $\Delta\psi$, although STZ treatment increased $\Delta\psi$ during ADP-induced depolarization when succinate was used. The most critical result was that muscle mitochondria isolated from STZ-treated rats showed a decrease susceptibility to MPTP induction by calcium, as followed by two different experimental protocols. Interestingly, the protection was accompanied by a decrease in muscle caspase 9-like activity.

Conclusions These data demonstrate that 18 weeks of STZ treatment lead to a decrease in *gastrocnemius* mitochondrial respiratory control ratio and to decreased calcium-dependent mitochondrial MPTP. Results from this and other works suggest that mitochondrial effects of hyperglycaemia are time and organ specific.

Keywords Apoptosis, bioenergetics, diabetes, mitochondrial function, skeletal muscle, streptozotocin.

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Introduction

Diabetes mellitus is a group of common metabolic and degenerative diseases clinically characterized by a phenotype of increased blood glucose, decreased insulin secretion and/or resistance and impairments in carbohydrate, fat and protein metabolism, associated with a variety of pathophysiological complications [1], responsible for the decreased quality of life of millions of people all over the world and for 5·2% of the total number of deaths worldwide in 2000 [2]. Type I diabetes is characterized by an absolute insulin deficiency, requiring daily insulin replacement. To understand the physiological and pathological changes of the disease, animal models of diabetes of hyperglycaemia are important research tools, allowing a tight control over experimental conditions, which is difficult with humans [3]. In this study, we used streptozotocin (STZ)-induced rats as a model for human type I diabetes mellitus. STZ-induced hyperglycaemia is obtained after selective destruction of β-cell by STZ, a broad spectrum antibiotic with diabetogenic effects. STZ-treated rats present many

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Endurance training improves gastrocnemius mitochondrial function despite increased susceptibility to permeability transition

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ABSTRACT

The aim of the present work was to test the hypothesis that moderate endurance treadmill training ameliorates gastrocnemius mitochondrial bioenergetics and increases the tolerance to the calcium-induced mitochondrial permeability transition pore (MPTP) opening. Twelve adult (6–8 week old) male Wistar rats were randomly divided into two groups ($n = 6$\per group): sedentary and trained (14 week of endurance treadmill running, 60 min/day). Several end-points for *in vitro* gastrocnemius mitochondrial function including oxygen consumption, transmembrane electric potential and susceptibility to calcium-induced MPTP opening were evaluated. Caspase-9 activity was measured in the intact tissue. Endurance training induced significant increases in state 3 and in respiratory control ratio both with complex I and II-linked substrates (malate + pyruvate and succinate, respectively). Increased CCCP-induced uncoupled respiration with succinate as substrate was also observed ($p < 0.05$). No differences were found regarding state 4 and ADP/O ratio with both substrates. In addition, training significantly decreased the phosphorylative lag phase, whereas no changes were observed on maximal transmembrane electric potential, ADP-induced depolarization and repolarization potential ($p < 0.05$). Interestingly and as opposed to our hypothesis, muscle mitochondria isolated from trained rats were more susceptible to MPTP induction by calcium, although in an initial phase muscle mitochondria isolated from trained rats had an increased calcium uptake. Interestingly, we also verified that endurance training increased the activity of caspase 9. The data obtained confirms that endurance training results in a general improvement in the gastrocnemius mitochondrial respiratory function, although mitochondrial and cellular alterations during training also result in increased calcium-induced MPTP opening.

1. Introduction

Skeletal muscle has a high reliance on oxidative metabolism, with mitochondria being critical organelles involved in ATP synthesis, cell osmotic regulation, modulation of redox status and pH control, signal transduction and in the establishment of calcium homeostasis (Brookes et al., 2004; Cadenas, 2004; Brookes, 2005). Mitochondria also provide an important contribution for the regulation of cell fate through its role on the regulation of cellular death, either by directly triggering apoptosis or acting by amplifying the apoptotic signals (Skulachev, 1999). The release of pro-apoptotic proteins contained in mitochondria has been referred to be dependent, at least partially, on the formation and opening of pores that span the inner and outer mitochondrial membranes leading to the loss of mitochondrial membrane potential ($\Delta\psi$), to mitochondrial osmotic swelling and rupture of the outer mitochondrial membrane, in a phenomenon denominated the mitochondrial permeability transition. The pores that originate the mitochondrial permeability transition are termed mitochondrial permeability transition pores (MPTP). It is well established that the susceptibility to MPTP opening occurs under conditions of increased oxidative stress and Ca^{2+} overload (Crompton, 1999; Kowaltowski et al., 2001). In fact, despite the recognized buffering role of mitochondria in the regulation of cellular calcium homeostasis both under basal and stressful conditions, the synergetic effect of increased free radical production and deregulated calcium levels lead to a physiopathological condition favoring MPTP opening. Although some debate exists on the role of the MPTP in apoptosis, most of the literature published so far supports the role of the MPTP in the facilitation of

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