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AGING AND MITOCHONDRIAL DYSFUNCTION IN MICE SKELETAL MUSCLE

The Influence of Lifelong Sedentary Behaviors

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To my Parents

Para a Aní e João Pedro

To Aní and João Pedro

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Resumo

O principal objectivo deste trabalho passou por estudar as alterações induzidas pela idade e pelo sedentarismo ao longo da vida na massa muscular esquelética e em mitocondrias isoladas a partir do músculo-esquelético de ratinhos machos C57BL/6, associando as alterações observadas com parâmetros de stress oxidativo. Com vista a alcançar este objectivo foram realizados vários estudos (artigo III, IV e V) cada um deles com diferentes abordagens metodológicas. No artigo III e IV os principais objectivos foram estudar as alterações mitocondriais associadas à idade em mitocondrias isoladas de musculo esquelético de ratinhos C57BL/6 utilizando procedimentos metodológicos tradicionais e originais, nomeadamente ao nível da normalização dos dados mitocondriais e dos testes *in vitro* de determinação da respiração mitocondrial. Para este efeito, procedeu-se à análise de vários parâmetros de funcionalidade respiratória mitocondrial (i.e. Estado 3 e 4 da respiração mitocondrial, Ratio de Controlo Respiratório (RCR), ratio ADP fosforilado por Oxigénio consumido (ADP/O)), variáveis bioquímicas (actividade da Citrato Sintetase) e determinação de alguns marcadores bioquímicos de lesão oxidativa (actividade da Aconitase, Grupos Carbonilo, Grupos Sulfidrilicos Proteicos e Malondealdeído) em suspensões mitocondriais isoladas a partir de músculo-esquelético de animais com diferentes idades (3 e 18 meses). Juntamente com os testes tradicionais de determinação da funcionalidade respiratória mitocondrial foi utilizado um teste *in vitro* que consistiu na estimulação das mitocondrias com adições consecutivas de ADP com vista a avaliar a capacidade mitocondrial para restabelecer a sua homeostasia entre estimulações sucessivas de ADP. O número de mitocondrias em cada suspensão mitocondrial, calculado por microscopia electrónica de transmissão, foi usado para normalizar os dados funcionais e bioquímicos obtidos da suspensão mitocondrial. No artigo V o principal objectivo foi investigar o impacto da idade e do sedentarismo ao longo da vida na massa muscular e na funcionalidade mitocondrial de ratinhos. Com vista a alcançar este objectivo, animais com dois meses de idade foram aleatoriamente divididos em três grupos (Jovens-J; Velhos Sedentários-VS; Velhos Activos-VA). Os animais Jovens foram sacrificados após uma semana de quarentena e os animais dos grupos VS e VA foram colocados individualmente em gaiolas padrão ou em gaiolas equipadas com roda de actividade, respectivamente, até ao sacrifício (25 meses). O peso corporal e o peso dos músculos dos membros posteriores foi determinado em todos os grupos. A funcionalidade respiratória mitocondrial (i.e. Estado 3 e 4 da Respiração Mitocondrial, RCR, ADP/O) e marcadores bioquímicos de lesão oxidativa (actividade da Aconitase, Grupos Carbonilo e Grupos Sulfidrilicos Proteicos) foram determinados em suspensões mitocondriais. Os principais resultados do presente trabalho suportam a tese de que o envelhecimento está associado a uma diminuição da função respiratória de mitocondrias isoladas a partir de músculo-esquelético de ratinhos C57BL/6. Adicionalmente, o sedentarismo ao longo da vida exacerba a diminuição observada na funcionalidade respiratória mitocondrial. Os nossos dados demonstram igualmente que o declínio na função mitocondrial relacionado com a idade e com o sedentarismo ao longo da vida está associado com níveis mais elevados de lesão oxidativa mitocondrial. Globalmente, os resultados dos nossos estudos permitem-nos referir que para além do processo de envelhecimento por si, o sedentarismo ao longo da vida é determinante na etiologia da sarcopenia e perda de função mitocondrial do músculo-esquelético.

Abstract

The main objective of this work was to analyze the influence of age and lifelong sedentariness on skeletal muscle mass and on isolated skeletal muscle mitochondrial status of C57BL/6 strain male mice, and to associate those alterations with oxidative stress markers. In order to accomplish that goal, several studies have been performed (paper III, IV and V) comprising, each one, different experimental approaches. In paper III and IV the main objectives were to investigate the influence of age on the status of mitochondria isolated from mice skeletal muscle using traditional and novel methodological procedures, namely concerning the mitochondrial data normalization and the *in vitro* mitochondrial respiratory tests. In order to accomplish that, several mitochondrial respiratory functional measures (ie, State 3 and 4 respiration, Respiratory Control Ratio (RCR) and number of nanomoles of ADP phosphorylated by nanomoles of O2 consumed (ADP/O)), biochemical analyses (Citrate Synthase activity) and assessment of biochemical markers of oxidative damage (Aconitase activity, Protein Carbonyl derivatives, Sulfhydryl groups, and Malondialdehyde) were measured in isolated mitochondrial suspensions from skeletal muscle of animals with different ages (3 and 18 months). Along with traditional tests of mitochondrial function, an *in vitro* repetitive ADP-stimulation test was used to evaluate the mitochondrial capacity to re-establish the homeostatic balance between successive ADP stimulations. The number of mitochondria per mitochondrial suspension, calculated by transmission electron microscopy, was used to normalize functional and biochemical data. In study V the main objective was to investigate the impact of age and lifelong sedentariness on skeletal muscle mass and mitochondrial function of mice. To reach this purpose, two month old animals were randomly divided into three groups (young-Y; old sedentary-OS; old active-OA). Young animals were sacrificed after one week of quarantine and OS and OA groups were individually placed into standard cages and in cages with running wheels, respectively, until sacrifice (25 months). Body weights and hindlimb skeletal muscle wet weights were obtained from all groups. Mitochondrial respiratory functional measures (i.e., state 3 and 4 respiration, RCR and ADP/O) and biochemical markers of oxidative damage (Aconitase activity, Protein Carbonyl derivatives, Sulfhydryl groups) were measured in isolated mitochondrial suspensions. The main findings of the experimental work support the thesis that aging is associated with a diminished respiratory function of skeletal muscle mitochondria of C57BL/6 strain male mice. Additionally, lifelong sedentariness exacerbates the reduction in skeletal muscle mitochondrial respiratory function with age. Moreover, our data demonstrates that the age- and sedentary-related decline in mitochondrial function is associated with higher levels of mitochondrial oxidative damage. The overall data allow us to state that beyond aging *per se*, lifelong sedentariness is a major determinant in the etiology of sarcopenia and muscular mitochondrial dysfunction.

Résumé

Le principal objectif de ce travail c'était d'étudier les altérations induites par l'âge et par la sédentarité tout au long de la vie dans la masse musculaire squelettique et à l'état de mitochondries isolées à partir du muscle-squelettique de petites souris mâles C57BL/6, en rapprochant les altérations observées avec des paramètres de stress oxydative. Pour atteindre cet objectif, plusieurs études ont été réalisés (article III, IV et V) avec différentes approches méthodologiques. Dans l'article III et IV, les principaux objectifs étaient d'étudier les altérations dans les mitochondries en rapport avec l'âge, dans des mitochondries isolées de muscle squelettique de petites souris C57BL/6, en utilisant des approches méthodologiques traditionnelles et originales, nommément au niveau de la normalisation des données mitochondriales et des tests *in vitro* de détermination de la respiration des mitochondries. Ainsi, on a fait une analyse de plusieurs paramètres de la fonction respiratoire mitochondriale (i.e. État 3 et 4 de la respiration mitochondriale, Ratio de Contrôle Respiratoire (RCR), ratio ADP fosforilé par l'oxygène consommé (ADP/O)), des variables biochimiques (activité de la Citrato Sintetase) et la détermination de quelques marqueurs biochimiques de lésion oxydative (activité de l'Aconitase, Groupes Carbonyle, Groupes Sulfhydryl Protéiques Malondéaldeído) dans des suspensions des mitochondries isolées à partir du muscle-squelettique d'animaux de différents âges (3 et 18 mois). En même temps que les tests traditionnels de détermination de la fonctionnalité respiratoire des mitochondries, on a utilisé un test *in vitro* qui a compris la stimulation des mitochondries par des ajouts consécutifs de l'ADP, afin d'évaluer la capacité des mitochondries à rétablir son homéostasie entre des successives stimulations d'ADP. Le nombre de mitochondries dans chaque suspension mitochondriale déterminée par microscopie électronique de transmission a été utilisé pour normaliser les données fonctionnelles et biochimiques obtenues de la suspension mitochondriale. Dans l'article V, le principal objectif c'était d'étudier l'impact de l'âge et de la vie sédentaire tout au long de la vie dans la masse musculaire et dans la fonctionnalité mitochondriale de petites souris. Pour atteindre ce but, des animaux âgés de 2 mois ont été partagés, au hasard, en 3 groupes (Jeunes – J ; Vieux Sédentaires – VS ; Vieux Actifs – VA). Les animaux jeunes ont été sacrifiés après une semaine de quarantaine et les animaux des groupes VS et VA ont été placés individuellement, dans des cages-types ou dans des cages équipées avec des roues d'activité, respectivement, jusqu'au sacrifice (25 mois). Le poids corporel et le poids des muscles des membres d'arrière a été déterminé dans toutes les groupes. La fonction respiratoire mitochondriale (i.e. État 3 et 4 de la Respiration mitochondriale, RCR, ADP/O) et les marqueurs biochimiques de la lésion oxydative (activité de l'Aconitase, Groupes Carbonyle et Groupes Sulfhydryl Protéiques) ont été déterminés en suspensions mitochondriales. Les principaux résultats de cette étude appuient la théorie selon laquelle le vieillissement est associé à une diminution de la fonction respiratoire des mitochondries isolées à partir du muscle squelettique des petites souris C57BL/6. En plus, la vie sédentaire tout au long de la vie aggrave la diminution observée dans la fonction respiratoire mitochondriale. Nos données montrent, également, que le déclin dans la fonction mitochondriale en rapport avec l'âge est associé à des niveaux plus hauts de lésion oxydative mitochondriale. Globalement, les résultats de nos études permettent de mentionner que, par ailleurs le processus de vieillissement en soi, la vie sédentaire tout au long de la vie est déterminant dans l'étiologie de la sarcopenie et dans la perte de la fonction mitochondriale du muscle-squelettique.

List of Abbreviations

8OH-dG	8-hydroxy 2-deoxyguanosine
ACON	Aconitase
ADP	Adenosine di-phosphate
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocase
ATP	Adenosine tri-phosphate
Ca^{2+}	Calcium
CaMK	Ca^{2+}-calmodulin-dependent protein kinases
CAT	Catalase
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
Cit. *c*	Cytochrome *c*
COX	Cytochrome *c* oxidase
CS	Citrate synthase
CuZnSOD	Copper-zinc superoxide dismutase
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
Fe^{2+}	Iron
FMN	Flavin-mononucleotide GSH- Glutathione
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
Hsp60	Heat-shock protein 60
Hsp70	Heat-shock protein 70
LMPCR	Ligation-mediated polymerase chain reactions
MDA	Malondialdehyde
MMP	Mitochondrial membrane permeability
mnSOD	Manganese superoxide dismutase
MPP	Matrix processing peptidase
mRNA	Messenger ribonucleic acid

mtDNA	Mitochondrial DNA
MSF	Mitochondrial import stimulation factor
mtTFB	Mitochondrial transcription factor B
NAD^+	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NF-kB	Nuclear transcription factor-kB
NRF-1	Nuclear respiratory factor-1
NRF-2	Nuclear respiratory factor-2
$O_2^{\bullet-}$	Superoxide
PKC	Protein kinase C
PPAR-α	Peroxisome proliferator-activated receptors-α
PPAR-γ	Peroxisome proliferator-activated receptors-γ
PUFAs	Polyunsaturated fatty acids
$QH^{\bullet-}$	Ubisemiquinone radical
QH_2	Ubiquinol
QPCR	Quantitative polymerase chain reactions
RCR	Respiratory control ratio
ROS	Reactive oxygen species
RRF	Ragged red fibers
rRNA	Ribosomal RNA
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase
Sp1	Specificity protein 1
TBARS	Thiobarbituric acid reactive substances
TCA	Trycarboxilic acid cycle
TEM	Transmission electron microscopy
Tfam	Mitochondrial transcription factor A
Tim	Translocases of the inner membrane
Tom	Translocases of the outer membrane
tRNA	Transfer RNA
VDAC	Voltage-dependent anion channels
ΔpH	*Trans*-membrane proton gradient
$\Delta\Psi_m$	Membrane potential

This thesis is based on the following papers which are referred to in the text by their Roman numerals:

Paper I

Figueiredo PA, Mota MP, Appell HJ, Duarte J. (2006) Ceasing of muscle function with aging: is it the consequence of intrinsic muscle degeneration or a secondary effect of neuronal impairments? *Eur Rev Aging Phys Act*, 3(2): 75-83.

Paper II

Figueiredo PA, Mota MP, Appell HJ, Duarte J. (2008) The role of mitochondria in aging of skeletal muscle. *Biogerontology*, 9 (2): 67-84.

Paper III

Figueiredo PA, Ferreira RM, Appell HJ, Duarte JA (2008) Age-induced morphological, biochemical and functional alterations in isolated mitochondria from murine skeletal muscle. *J Gerontol A Biol Sci Med Sci*, 63 (3): 350-359.

Paper IV

Figueiredo PA, Powers SK, Ferreira RM, Appell HJ, Duarte JA (2009) Aging impairs skeletal muscle mitochondrial bioenergetic function. *J Gerontol A Biol Sci Med Sci*, 64: 21-33.

Paper V

Figueiredo PA, Powers SK, Ferreira RM, Amado F, Appell HJ, Duarte JA (2009) Impact of lifelong sedentary behavior on mitochondrial function of mice skeletal muscle. *J Gerontol A Biol Sci Med Sci* (**accepted for publication**).

General Introduction

General Introduction

At birth living creatures have only one certainty: the inevitability of dying. Strong possibility also exists that they will go through the processes of growth, development and aging. From all of the above, aging is the most feared since it is commonly associated to disability, disease, loss of functional autonomy and ultimately to increased probability of death (Adhihetty & Hood, 2003; Doherty, 2003; Lenaz et al., 2000).

Considering the notion that the global conditions of life and advances in science have contributed to the control and treatment of many diseases, the population of both developed countries and the majority of developing countries has increased its life expectancy in recent years. This global trend has lead to the increasing interest from researchers and institutions in the search for solutions aiming to minimize the effects of aging on the population. In fact, the quest for *"How and why do we age?"* is probably among the most exciting questions in science in order to understand its mechanisms with the prospect of delaying age-related functional decline and ensure a continuous functional capacity and autonomy in the last decades of life.

In the context of this work, aging is defined as a loss of redundancy in redundant organisms due to an age-related and irreversible accumulation of damaged components resulting from the interaction between genetic and stochastic factors (Arslan, Csermely, & Soti, 2006; Gavrilov & Gavrilova, 2004, 2006; Goto, Naito, Kaneko, Chung, & Radak, 2007; Rattan, 2006). This leads to a diminished functionality of organs, systems and tissues of an individual, with a reduced capacity to withstand internal and/or external stimuli and therefore with an increased susceptibility to disease and death (Adhihetty & Hood, 2003; Gems & Partridge, 2008; Lenaz et al., 2000; Ventura, Genova, Bovina, Formiggini, & Lenaz, 2002).

The aging process occurs at different rates among different tissues of the same individual and the functional manifestations also vary (Cristofalo, Gerhard, & Pignolo, 1994), however one undoubtedly important component is the loss of mobility, due in part to the loss of muscle mass, strength and endurance

(Adhihetty & Hood, 2003; Andersen, 2003; Booth, Weeden, & Tseng, 1994; Cristofalo et al., 1994; Doherty, 2003; Lexell, Taylor, & Sjostrom, 1988; Proctor, Balagopal, & Nair, 1998; Vandervoort, 2002). In fact, one of the most adversely affected tissues with increasing age is skeletal muscle (Booth et al., 1994; Carmeli, Coleman, & Reznick, 2002; Doherty, 2003; Roubenoff, 2001; Terman & Brunk, 2004) presenting progressive and deleterious changes associated with structural disorganization and dysfunction. The decline in skeletal muscle mass and function with age, also known as sarcopenia, occurs in all individuals to some degree as a consequence of aging, but it can be accelerated by a variety of factors including physical inactivity/sedentary lifestyles, poor nutrition and chronic illness (Carmeli et al., 2002; Doherty, 2003; Porter, Vandervoort, & Lexell, 1995; Roubenoff, 2001; Vandervoot & Symons, 2001).

A number of mechanisms have been proposed in an attempt to explain the changes in total muscle mass and strength with advancing ages and it has been suggested that skeletal muscle morphological and functional alterations with age do not only result from intrinsic factors within the muscle fibers but are also influenced by the aging phenomena of other organs and systems which support skeletal muscle functionality, such as the endocrine, cardiovascular and neural systems (Roos, Rice, & Vandervoort, 1997; Terman & Brunk, 2004).

With an integrative point of view and independently of the underlying mechanisms these deleterious age-related repercussions can be seen as a result from the accumulation of damaged redundant elements, diminishing the functional reserve capacity of a given tissue or system, giving rise to the loss of maximal function associated with advancing age and compromising the ability to withstand additional homeostatic disturbances (Gavrilov & Gavrilova, 2004, 2006; Rattan, 2006). Moreover, the inability to repair the inflicted damage will result in a loss of cell viability with a progressive reduction of cell number and/or function over time and will *per se* compromise the redundancy of tissues leading to a reduced reserve capacity and maximal functionality of organs and systems (Gavrilov & Gavrilova, 2004, 2006; Rattan, 2006).

Taking into consideration a variety of harmful conditions that are experienced during the entire lifespan, the occurrence of oxidative stress at basal and

stressful situations has been pointed out as one major reason for the increased damage to cells and its components with age, namely proteins, lipids and DNA (Cadenas & Davies, 2000; Golden, Hinerfeld, & Melov, 2002; Merry, 2004; Shigenaga, Hagen, & Ames, 1994). In this context, it has been suggested that mitochondria constitutes the major source of reactive oxygen species (ROS) under physiological conditions and might also be among the most adversely affected organelles with advanced age (Kwong & Sohal, 2000; Salvioli, Bonafe, Capri, Monti, & Franceschi, 2001; Wallace, 1999) with the consequent accumulation of oxidatively damaged components (Searcy, 2003; Stadtman, 2002). Bearing in mind the deleterious repercussions of oxidative stress to skeletal muscle fibers, it has been suggested that a reduction in mitochondrial number and/or activity with age might account for the increased muscle fatigability, reduced endurance capacity and loss of strength seen at old ages (Bua, McKiernan, Wanagat, McKenzie, & Aiken, 2002; Cadenas & Davies, 2000; Conley, Jubrias, & Esselman, 2000). In fact, skeletal muscle mitochondrial dysfunction has been associated with increased age (Cadenas & Davies, 2000; Kwong & Sohal, 2000; Passos, von Zglinicki, & Kirkwood, 2007; Salvioli et al., 2001; Short et al., 2005) and the age-related oxidative damage to mitochondrial DNA, lipids and proteins has been suggested by several authors as one possible mechanism behind this loss of function (Short et al., 2005; Short & Nair, 2001; Van Remmen, Hamilton, & Richardson, 2003; Van Remmen & Richardson, 2001), resulting in a progressive reduction of mitochondrial bioenergetic capacity, leading to cellular energy deficits and compromising overall cellular functionality (Nicholls, 2004; Shigenaga et al., 1994).

However, despite it is well established that aging results in loss of skeletal muscle mass and contractile dysfunction, data in the literature about age-related mitochondrial dysfunction remains controversial. Several techniques have been used to determine the impact of age on mitochondrial function. *In vitro* assays involve the isolation of intact mitochondria from tissue samples and the measurement of several respiratory parameters in the presence of various substrates (Rasmussen & Rasmussen, 2000; Rasmussen et al., 2001; Tonkonogi & Sahlin, 2002) as well as the assessment of mitochondrial

enzymatic activities (Rasmussen, Krustrup, Kjaer, & Rasmussen, 2003a); *in vivo* techniques involve the utilization of nuclear magnetic resonance technology to determine the average rate of ATP production in several muscle groups (Conley et al., 2000; Kent-Braun & Ng, 2000). The most widely studied is the respiratory chain capacity of mitochondria isolated from different tissues and in this context, several reports suggest that mitochondria isolated from aged skeletal muscle exhibit impaired functionality (Drew et al., 2003; Mansouri et al., 2006; Shigenaga et al., 1994; Sugiyama, Takasawa, Hayakawa, & Ozawa, 1993; Tonkonogi et al., 2003), whereas other investigations have failed to demonstrate age-related changes in mitochondrial phenotypes in humans (Barrientos et al., 1996; Rasmussen et al., 2003a; Rasmussen, Krustrup, Kjaer, & Rasmussen, 2003b) and other animals (Beyer et al., 1984; Kerner, Turkaly, Minkler, & Hoppel, 2001) arguing that mitochondrial capabilities are not affected by chronological age. This contradictory results could be due to several methodological issues, namely (a) experimental design and protocol, (b) number of groups and animals age, (c) sedentariness and/or physical activity levels, and (d) mitochondrial populations under study (Short et al., 2005; Short & Nair, 2001).

In this context, it should be emphasized that the procedures inherent to mitochondria isolation and subsequent assessment of its functional capacity may themselves bias the results. In fact, the homogenization process, considered to be one of the most important steps in the process of mitochondrial isolation (Rasmussen et al., 2003a) may leave some non-mitochondrial proteins in the final suspensions leading to different degrees of contamination. Taking into account that mitochondrial biochemical and functional data is frequently normalized to the total protein content in suspension, different degrees of contamination with non-mitochondrial proteins will bias the specific activities of isolated mitochondria and compromise the comparisons between various age groups (Rasmussen et al., 2003a). In addition, and bearing in mind the specificity and sensitivity of the *in vitro* assays usually used to assess the age-related alterations in skeletal muscle mitochondrial functionality, it may be argued that the *in vivo* bioenergetic

function of skeletal muscle mitochondria appears to be very different from the metabolic stress transitorily imposed by the traditional *in vitro* tests; thus, this routinely used *in vitro* tests might not be sensitive or specific enough to detect age-related alterations in mitochondrial function. Animals' age might also constitute an additional problem since the occurrence of age-related concomitant disease in very old animals might bias the results (Miller & Nadon, 2000). Another important confounding variable in aging research is the physical activity levels. In fact, it has been proposed that the decreased respiratory function of skeletal muscle mitochondria with age is not so apparent when the levels of physical activity of the subjects are taken into account (Brierley, Johnson, James, & Turnbull, 1997). Accordingly, Waters et al. (2003) found an age-related decline in mitochondrial function which appeared to be attenuated when corrected for physical activity levels. In contrast, several authors have demonstrated that the loss of mitochondrial respiratory function with increasing age is not solely an effect of physical deconditioning, constituting *per se* a central element of biological aging (Conley et al., 2000; Short et al., 2005; Tonkonogi et al., 2003). Furthermore, it is well described that regular physical exercise influence skeletal muscle mitochondrial content even in the aged skeletal muscle (Hood, 2001; Menshikova et al., 2006) constituting a mean to counteract the notion that skeletal muscle mitochondrial density is diminished with age (Hood, 2001; Menshikova et al., 2006; Rooyackers, Adey, Ades, & Nair, 1996); however, little is known about the impact of lifelong behaviors, namely sedentariness and active lifestyles on the functionality of individual mitochondria. In fact, an interesting question arises whether the specific activity of skeletal muscle mitochondria is altered by lifelong voluntary physical activity since several reports suggests that the increased skeletal muscle fiber respiratory capacity in response to chronic physical activity is attributable to the expansion of mitochondrial mass, rather than to changes in its specific activity (Tonkonogi & Sahlin, 2002).

Taking into consideration the aforementioned scientific background, the main purpose of this thesis was to analyze the alterations induced by age and lifelong

sedentariness on skeletal muscle mass and on isolated skeletal muscle mitochondrial morphological, biochemical and functional status of C57BL/6 strain male mice, and to associate those alterations with markers of oxidative stress.

In order to achieve it, several review and experimental papers were prepared constituting two main sections of this document. The working hypotheses inherent to this work were enunciated on each of the following manuscripts.

Theoretical Background

Paper I

With this review we intended to assess mainly the age-related skeletal muscle morphological alterations and their influence on skeletal muscle function. Additionally, attention was also focused on age-associated morphological and functional alterations at the level of the nervous system as an attempt to highlight a potential extrinsic mechanism to explain the loss of skeletal muscle function with age.

Paper II

The main purpose of this review was to analyze the mechanisms behind the production of mitochondrial ROS, antioxidant defenses, oxidative stress and damage. Moreover, we have also discussed the mechanisms responsible for the eventual loss of mitochondrial function with age and whether this loss might be interpreted as a cause or a consequence of skeletal muscle dysfunction with advancing age.

Experimental Work

Paper III

The main purposes of this study were:

(i) To estimate the amount of mitochondria in mitochondrial suspensions obtained from skeletal muscle of young and mature animals; (ii) to correlate the calculated number of mitochondria with the total protein determination and Citrate Synthase (CS) activity of the mitochondrial suspensions, and (iii) to

perform a functional characterization of skeletal muscle mitochondria of young and mature animals normalizing the data to the number of mitochondria.

Paper IV

The main purposes of this manuscript were:

(i) To study the influence of age on the functional status of mitochondria isolated from skeletal muscle and its association with markers of oxidative stress, and (ii) to develop a standardized *in vitro* test that mimic the *in vivo* metabolic stress imposed to mitochondria alternating, successively, between high metabolic activity and rapid oxygen consumption (state 3 respiration) and reduced metabolic activity with low rates of oxygen consumption (state 4 respiration), thereby studying their capacity to withstand several successive ADP stimulations and re-establish their respiratory homeostatic balance.

Paper V

The main purpose of this study was:

(i) To investigate the impact of age and lifelong sedentariness on both skeletal muscle mass and mitochondrial function and its association with markers of oxidative stress.

Theoretical Background

Paper I

Ceasing of Muscle Function with Aging: Is it the Consequence of Intrinsic Muscle Degeneration or a Secondary Effect of Neuronal Impairments?

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ACADEMIC LITERATURE REVIEW

Ceasing of muscle function with aging: is it the consequence of intrinsic muscle degeneration or a secondary effect of neuronal impairments?

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Abstract Aging is associated with a significant decline in neuromuscular function leading to an eventual loss of independence and mobility of senescent people. Age-related sarcopenia, characterised by a reduction in muscle mass and strength, is considered one of the most striking features of aging at the level of the skeletal muscle. Morphological alterations in skeletal muscle can be considered as one of the consequences responsible for muscle weakness in the aged population. Beyond 60 years of age, human muscle undergoes a process of continuous denervation and reinnervation, due to an accelerating loss of motor units. It appears evident that phenotypic alterations in muscle depend on the motor drives provided by the nervous system. Because the peripheral nerves, the neuromuscular junction and motor neurons exhibit degenerative features during advanced age, sarcopenia does not seem to intrinsically develop, but is rather a secondary effect of impaired neuronal function. It is therefore recommended that elderly subjects undergo an exercise program that is aimed towards the improvement of coordinative skills and of muscle strength.

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Keywords Skeletal muscle aging · Sarcopenia · Neuropathy · Functional impairment

Introduction

Aging is an inevitable biological process characterised by the progressive deterioration of numerous physiological functions leading eventually to reductions in functional capacities [1, 11, 23, 47]. There is an enormous biological variability in the aging process not only among individuals with similar chronological ages but also among the several organs and systems of the same individual [16]. In fact, the rate of aging varies dramatically between tissues like neural, cardiac, skeletal muscle and other long-living post-mitotic cells, becoming dramatically altered during senescence. In contrast, bone marrow cells and other short-living cells undergo only slight or almost undetectable changes [57, 58]. It is also evident that aging of a particular physiological system will have a deleterious influence on the aging of other physiological systems, so overall aging must be understood and interpreted as a multifactorial process.

In this context, it is believed that skeletal muscle is a tissue with high susceptibility to the aging process [8, 13, 23, 43, 52], which will inevitably undergo progressive and deleterious changes associated with structural disorganization and functional decline. It is well known that the human aging process is associated with a significant decline in muscular function and physical performance [10, 23, 25, 56]. The reduction in muscle mass and strength with advanced age is considered by many authors [1, 3, 8, 16, 23, 27, 35, 47, 63] to result in dramatic reductions in the functional capacity of the elderly, being probably a frequent and most important cause of ceasing independence and

mobility in senescence. Age-related morphological alterations in skeletal muscle can be considered responsible for the decrements in muscle strength in aged individuals [2, 29, 49, 53, 63]. Muscle weakness in the aged muscle is correlated with a loss in muscle mass [23], documented by a decrease in the cross-sectional area of the whole muscle and individual muscle fibres as well as by a reduced number of muscle fibres and declining capillary density [25, 36, 44]. The mechanisms of aging-related sarcopenia are complex and hitherto not completely understood. Whilst it may be considered a part of normal changes within the final third of the human life span [63], it must also be recognised that other factors such as a sedentary lifestyle, inadequate nutrition and diseases deleteriously influence muscle performance [13, 23, 52]. Like the underlying causes of aging, the biology of sarcopenia remains elusive. A number of mechanisms have been proposed in an attempt to explain the changes in total muscle mass and strength with aging. These include a variety of systemic and/or local factors, such as lack of regular physical activity, changes in protein metabolism, with a deficit of protein synthesis vs degradation, alterations in the endocrine milieu, an impairment of neuromuscular function, altered gene expression, deficits in satellite cell recruitment, oxidative stress, age-related accumulations of mitochondrial abnormalities (e.g., mitochondrial DNA mutations; electron transport system abnormalities) and apoptosis [10, 38].

Whilst the structural alterations observed in old muscles at first glance appear to resemble an intrinsic process of muscle aging, one has to consider that muscle structure and therefore function is triggered extrinsically by the nervous system. Consequently, neuronal alterations occurring in senescence should also contribute substantially to the eventual loss of muscle function at advanced age.

It is a fact that aging is also associated with neurological changes which directly affect voluntary force production [49]. One of the major features of aging in skeletal muscle is the rearrangement of the pattern of innervation. The loss of fast motor neurons leave populations of denervated fibres within the aging muscle, which partly may be compensated by sprouting of the remaining neurons to supply some of the denervated muscle fibres [8]. It is believed that age-related muscle denervation accounts for the muscle weakness encountered in the aged muscle [62]. Considering that motor neurons control muscle growth and fibre type properties by regulating muscle gene expression by local (release of neural factors) and general mechanisms (neuronal electrical activity) [55], it should be suggested that skeletal muscle phenotypic alterations with age are strongly linked to respective alteration of the neural system.

In this review, we will focus our attention mainly on the age-related muscle morphological alterations and their influence on skeletal muscle function, as well as on the age-related morphological and functional alterations at the level of the nervous system.

Aging of skeletal muscle

Clinical manifestation of muscle aging

It has been demonstrated that total muscle cross sectional area decreases by ~40% between the ages of 20 and 60 years [21, 43, 63]. Total cross sectional areas have been determined in several limb muscles by various imaging techniques, for example by ultrasound scanning and computed tomography, magnetic resonance imaging (MRI) and direct measurements of whole muscle cross sections in cadaver specimens [23, 36]. For example, Young et al. [66, 67] reported 25 to 35% reductions in the total quadriceps cross sectional area for older (70–79 years old) men and women as compared to young controls (21–28 years old).

Age-related decrements in strength have been well documented in multiple cross-sectional studies under isometric and dynamic conditions [23, 43]. Some recent reports are consistent with the fact that healthy people of 70 to 80 years exhibit 20–40% less strength compared with younger subjects, as assessed during isometric and concentric strength tests of knee extensor muscles [23, 44]. Even greater losses have been reported for subjects in the ninth decade of life (50% or more). These age-related reductions are relatively similar in both sexes, for proximal and distal muscles, and in upper and lower extremities [49]. One peculiar finding is that these decreases are less remarkable during isokinetic eccentric contractions [49]. This could be related to slower contractile properties and increased or altered connective tissue contents and properties favouring the idea of more muscle stiffness in older adults [23].

Morphological changes of muscle fibres

The decline in total muscle cross sectional area during aging is most likely due to decreases in total fibre number, and to a lesser extent to a reduction in fibre size [36]. There is some variability concerning the reductions in muscle fibre cross sectional area, with values ranging from 20 to 50% for type II and 1 to 25%, for type I muscle fibres [3, 21, 28, 32, 35, 43, 49]. The ranges of variation may be associated with sampling variability, potential sampling biases in muscle biopsies, factors concerning somewhat older or younger control groups and the muscles used for analyses [23, 59]. Moreover, the age-related fibre atrophy is very inhomogeneous when looking at the fibre pool as a whole. In muscle biopsies from very old subjects (>85 years), Andersen [3] observed that in some areas the

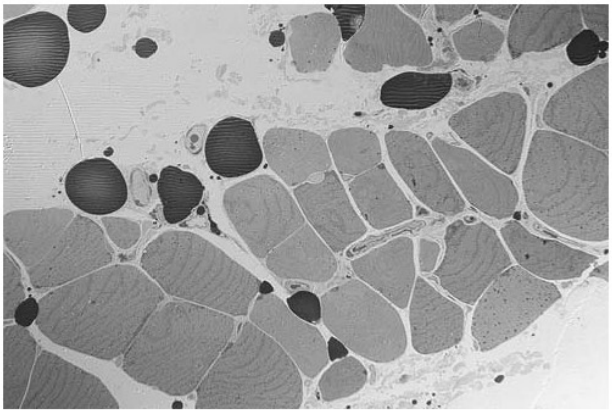

Fig. 1 Light micrograph from a transverse section of gluteus medius of a 65-year-old woman evidencing several muscle fibres with a great heterogeneity in fibre size and shape. Note also the enlargement of the interstitial space, which is infiltrated by many adipocytes and connective tissue (original magnification: 400×)

fibres had almost "normal" size, and in their close vicinity, he found areas where the fibres were small, or even extremely small (cf. Fig. 1). Age-related atrophy might be fibre type specific because numerous studies have pointed out that in vastus lateralis, tibialis anterior and biceps brachii muscles, the cross sectional area of type II fibres are significantly reduced with aging, whilst those of type I fibres are less affected [3, 21, 28, 32, 35, 43, 49]. The reductions in fibre size appear moderate compared with the reductions in total muscle volume, suggesting that reductions in muscle fibre number may play a crucial role when considering the gross muscle atrophy with aging [27].

In this matter, the total number in muscle fibres starts to decrease at about 25 years of age and progresses eventually at an accelerated rate [35]. Lexell et al. [35], using whole muscle cross sections from vastus lateralis muscle obtained post-mortem, reported about 50% less type I and type II fibres by the ninth decade compared with 20 years old controls. So the picture emerges that preferentially type II fibres do atrophy with advanced age, but both fibre types experience losses in numbers more or less equally.

The changes skeletal muscle is undergoing with aging are well documented at the structural and ultrastructural level. In the elderly human muscle, fibres from the vastus lateralis muscle cut transversely exhibit a flattened appearance (cf. Fig. 1), in contrast to the spherical shape of the fibre types from young muscles [3]. This flattening of the fibres is more pronounced among the type II fibres and it was suggested that this change in shape could be the very first sign of a programmed cell death [3]. This may also depend on the pattern of activation. An age-related type II disuse can be assumed because very old people are not subjected to work demanding force and power and therefore do not (or very seldomly) activate a great percentage of type II fibres, thus they slowly atrophy due to lack of stimulation. This is also reflected by the notion that old muscles become slower.

When ultrastructural characteristics of aging rat anterior tibial muscle (35 44 months) were determined [29], a reduction of and abnormalities within the myofibrils were the main causes of atrophy at the cellular level. The myofibrils become thinner and partly desintegrated, and the structure of the sarcomeres becomes incomplete with occasional losses of the Z-line (cf. Figs. 2 and 3). Many of the atrophied fibres demonstrate a condensation and deformation of nuclei, with the nuclear membrane deeply indented (cf. Fig. 4). Some nuclei often appear shrunken and hyperchromatic. Also, a diminished number and size of mitochondria were reported by Kaminska and colleagues [29] in aged rats (cf. Figs. 2, 4 and 5). Lipofuscin is frequently observed (cf. Fig. 4) often accumulating beneath the sarcolemma in moderately and severely atrophied fibres; fragmentation of muscle fibres and alterations in the appearance of the basement membrane are other morphological features described by the above-mentioned authors. Also, in very old rats (27 months old), there is ultrastructural evidence of myofibrillar loss and an increase in intermyofibrillar spaces [33] (cf. Figs. 2 and 5), which is known to influence the specific tension (tension per cross sectional area) during muscle contraction [5].

However, these features are not unequivocally described in the literature. A study on senescent human vastus lateralis muscle [51] did not report any ultrastructural

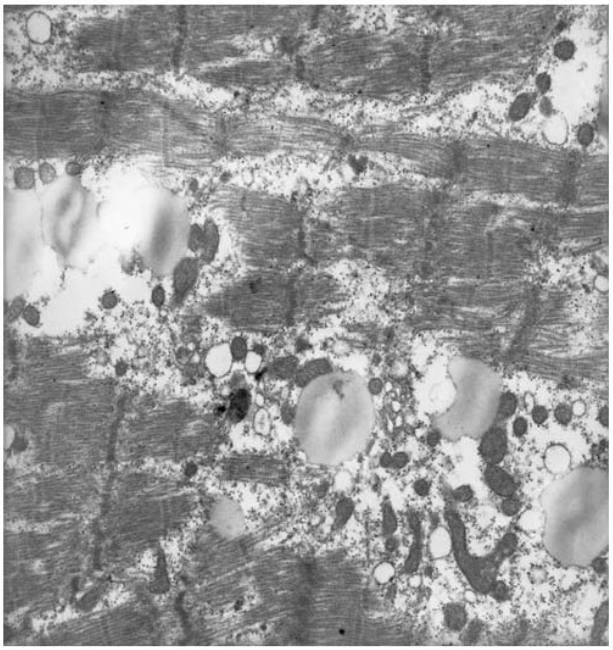

Fig. 2 Transmission electron micrograph from a longitudinal section of the gluteus medius muscle of a 70-year-old man, evidencing several lipid inclusions, small volume mitochondria and myofibril disarrangement (original magnification: 12,500×)

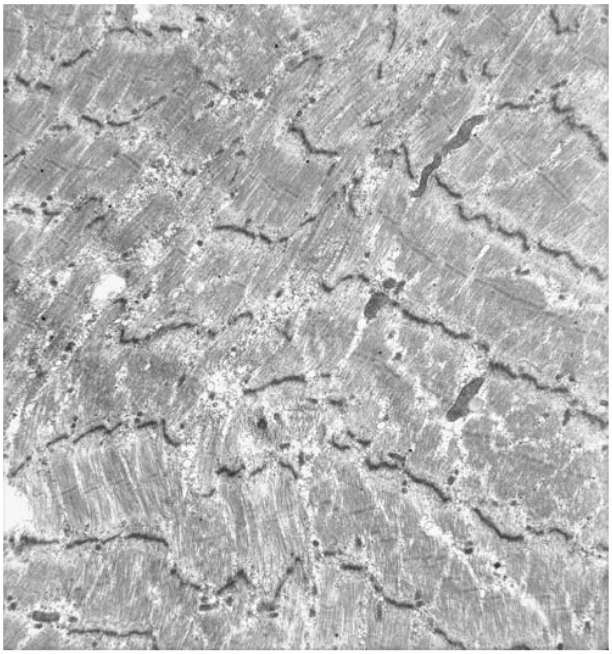

Fig. 3 Transmission electron micrograph of a longitudinal section of the gluteus medius muscle of a 70-year-old man, showing marked alterations of the myofibrillar pattern (original magnification: 8,000×)

differences between sedentary young and older men. In contrast, the works of Scelsi et al. [54] and Tomonaga [61] suggested that older sedentary individuals exhibited more frequently signs of muscle damage in several muscles, namely, myofibrillar degeneration with Z-line streaming. In elderly men, Andersen [3] and Larsson et al. [33] found an increased incidence of a scalloped appearance of the sarcolemma in muscle fibres expressing fast myosin isoforms. This could be the consequence of a degenerative process which preferentially affects type II muscle fibres [3]. Ansved and Edstrom [5] also reported a pronounced folding of the sarcolemma in aged rat muscles and suggested that this might be related to a decreased calibre of myofibrils in response to a loss of innervation.

Alterations in the interstitial space

The reduction in muscle cross sectional area with age is accompanied by an augmentation of non-contractile structures in the interstitial space [42, 48]. Rice et al. [48] reported large age-related increases in non-muscle tissue (fat and connective tissue, cf. Figs. 1 and 5) for the arm flexors (27%), arm extensors (45%) and foot plantar flexors (81%). Overend et al. [42] found reductions in the cross-sectional area of the quadriceps (27%) and hamstrings (18%) muscles of elderly men (65 77 years old), and concomitant increases in non-muscle tissue of 59% (quadriceps) and 127% (hamstrings). This coincidence shows that the reductions in active muscle mass with age are much greater than the data of total muscle cross sectional area suggest.

The findings from animal experiments regarding age-related changes in capillary density are not unequivocal. The number of capillaries can change because of several factors including development, aging and variations in muscle activity [31]. Most studies did not indicate severe age-related influences on skeletal muscle capillary density [14, 41]; however, others have reported either a decrease [18] or an increase [17] in skeletal muscle capillary density with aging. Capillary luminal diameter is an important factor in determining the functional potential for peripheral blood flow and supply [31]. Whilst tail-suspended rats show a diminished capillary luminal size in their atrophied muscles [30], there are no significant differences in capillary luminal diameter in old rats, indicating that capillary size remains unaltered in atrophied muscles due to aging [31].

A question that is now emerging is whether the age-related changes in muscle tissue previously mentioned are due to intrinsic alterations at the level of the muscle fibres themselves or whether at least some of these alterations are the consequence of extrinsic factors resulting from the aging process of other organs and systems.

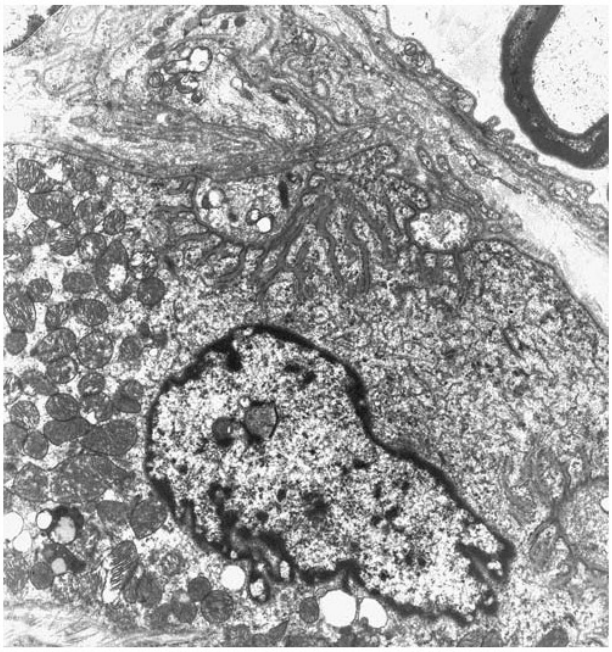

Fig. 4 Transmission electron micrograph from a transversal section of the gluteus medius muscle of a 68-year-old man, illustrating a neuromuscular junction with marked signals of mitochondrial degeneration. The nucleus has a scalloped surface and there are lipofuscin granules between the small mitochondria (original magnification: 5,400×)

Fig. 5 Transmission electron micrograph from a longitudinal section of the vastus lateralis muscle of a 68-year-old man, showing a muscle fibre containing condensed mitochondria of small size and the interstitial space enriched with collagen fibres (original magnification: 24,000×)

Aging of motor neurons

The function of skeletal muscle intimately depends on the central and peripheral nervous system. Motor neurons control muscle contraction through the well-characterised process of excitation contraction coupling; in addition, motor neurons control muscle growth and fibre type properties by regulating muscle gene expression [55]. These neural effects on skeletal muscle involve two distinct mechanisms; a humoral mechanism mediated by the release of neural factors (e.g. agrin and neuregulin) from the nerve terminals and a neurophysiological mechanism mediated by the pattern of electrical nerve discharge [55]. It is suggested that the control by the electrical activity per se, rather than the release of neural factors, is the predominant mechanism because motor neuron silencing, without any structural interruption of the neuromuscular connections, has dramatic effects on muscle fibre size and fibre type profile, similar to those observed after experimental denervation. Moreover, direct electrostimulation of denervated muscles can prevent muscle atrophy and modulate contractile properties and fibre type profiles, according to the impulse pattern applied [55]. In addition, the loss of specific force found in senescence may partly be explained by a reduced pool of muscle fibres with intact innervation resulting from age-associated spontaneous denervation [62].

Alterations at the neuromuscular junction

The neuromuscular junction is the synaptic link between motor neurons and muscle fibres [45] and some of the aging-related modifications in neuromuscular activity may result from morphological and physiological alterations at the neuromuscular junction [20]. Some studies have reported an age-related remodelling of the neuromuscular junction in locomotor muscles, such as fragmentation, altered patterns of terminal branching, retraction of nerve terminals and enlarged or denuded postsynaptic areas [15]. When examining age-related ultrastructural alterations in neuromuscular junctions, Wokke et al. [65] found an increased complexity at the postsynaptic side, namely, an increased extension and spreading of the postsynaptic membrane with a concomitant flattening of synaptic folds. Prakash and Sieck [45] found an increased number of nerve terminals in the diaphragm, particularly in type IIx and IIb fibres, and an enlarged end-plate area, which may result from an increased number of nerve terminals. Taken together, these changes are assumed to represent the process of denervation and reinnervation, which may compromise motor drive and should contribute to muscle weakness [45].

The synaptic deterioration observed in aged animals has also been proposed to be partly due to changes in the activity of motor neurons [6, 20]. It is known that alterations in neuronal activity induce morphological and physiological adaptations at the neuromuscular junction. Chronic exercise results in larger pre- and post-synaptic dimensions in the neuromuscular junction [4, 19], and complete muscular disuse by denervation elicits morphological patterns of remodelling, like nerve terminal sprouting and expansion of the endplate [46]. Muscle unloading in aged rats leads also to a marked morphological remodelling of synaptic contact zones with increased numbers of presynaptic vesicles [20] (cf. Fig. 6). These structural findings might be interpreted as a yet obsolete attempt to compensate for insufficient motor drives towards the aging muscle fibres.

Motor unit enlargement by sprouting of new nerve terminals is an important compensatory mechanism against the loss of working motor units during aging and also in neuromuscular diseases [26]. With advanced age, an increased instability of motor innervation occurs, which has been established in the muscles of mice older than 15 months [7]. This instability could be the result of a loss of terminal branches of the motor nerve and of a reduced density of postsynaptic acetylcholine receptors. At 12 18 months of age (in mice), the motor neurons appear to compensate some of the eventual losses of synaptic sites by sprouting and thereby adding new sites; however, this compensatory addition appears to decrease dramatically

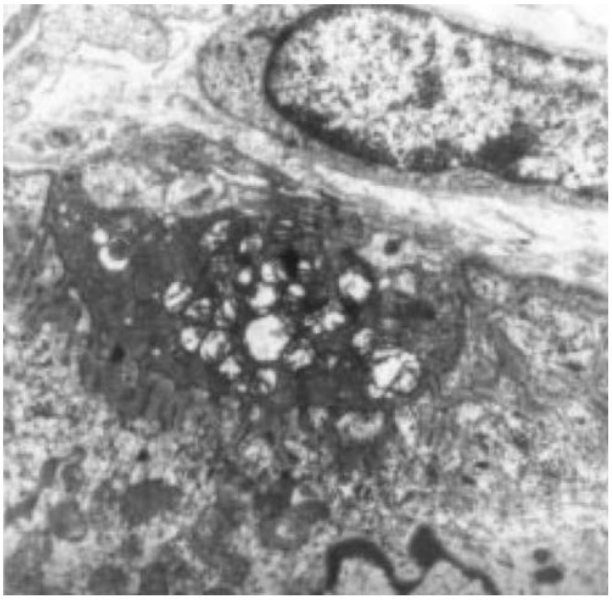

Fig. 6 Transmission electron micrograph of the neuromuscular junction in soleus muscle of an old rat showing degenerating mitochondria and a very high density of synaptic vesicles (original magnification: 24,000×)

with age. At 24 36 months, the vast majority of neuromuscular junctions in the sternomastoid muscle of mice [6] undergo significant losses of pre- and post-synaptic sites. This coincides with an increased incidence of synaptic transmission failure towards the muscle fibres when the motor nerves are stimulated at relatively low frequencies (10 to 20 Hz).

Motor unit remodelling

Fibre type grouping has become evident in a number of studies examining either whole muscle preparations or muscle biopsies of elderly subjects [3, 24, 34]. Grouping describes the phenomenon that fibres of identical type seem to concentrate in clusters rather than being distributed in the random fashion commonly observed in younger muscles [3]. According to Andersen [3], the mechanisms for grouping of the fibre types in aging human muscle still need to be completely clarified. However, the most intriguing explanation seems to be that fibre type grouping arises from a continuous process of denervation and partial reinnervation that is claimed to accelerate with advanced aging and is secondary to a chronic neuropathic process. This may reach a phase where the compensatory reinnervation does not keep pace anymore with the denervation and thereby muscle fibres or even complete motor units get lost, subsequently being replaced by fat and fibrous tissue [36]. This could explain why muscles from elderly individuals comprise a much smaller portion of parenchymal tissue and a much higher portion of stromal fat or connective tissue than younger controls [35, 48].

One of the major features of aging skeletal muscle is the rearrangement of the innervation. Motor unit remodelling corresponds to the natural cycle of synaptic turnover at the neuromuscular junction by the process of denervation, axonal sprouting and reinnervation of muscle fibres [9]. During aging, type II fibres are selectively denervated and reinnervated by collateral sprouting of axons belonging to slow motor units, which might result from their faster axonal growth or their superiority in establishing permanent connections with both type I and type II muscle fibres [8, 49], thereby eventually changing the contractile characteristics of the latter ones. According to Carmeli and Reznick [12] this theory can account for many of the observed functional and morphological changes in skeletal muscle, especially the well known age-related slowing of muscles.

Observations reported in the literature show that the morphological alterations of motor units are consistent with a chronic neuropathic process [21, 22]. The most evident change is a decrease in the total number of motor units [21]. Loss of motor units has been estimated to amount 1% of their total number per year, beginning during the third decade of life and increasing rapidly in rate beyond the age of 60 [49]. This, however, is not accompanied by an equal loss of muscle fibres; therefore, each motor neuron innervates more muscle fibres in the aged than in younger muscles [49]. This has been shown indirectly by EMG techniques documenting an increase in motor unit size in the vastus lateralis, tibialis anterior and biceps brachii muscles from subjects older than 60 years (reviewed by [63]). Based on experiments in senescent rats, there is a general consensus that age related reductions in the number of motor units range from 40 75% with greater losses among the largest motor units with the lowest oxidative capacities (reviewed by [21]). These results are in accordance with the phenomenon of fibre grouping and suggest an early preferential degeneration of the fast motor units with age [21].

The number of excitable motor units is considered to be reduced with advanced age, particularly in the seventh decade of life [22]. This has so far been explained with mechanisms within the peripheral nervous system. The question arises as to whether also a loss of motor neurons appears in the spinal cord.

Tomlinson and Irving [60] have estimated the number of motor neurons in the lumbosacral segments (L1-S3) of 47 individuals aged 13 95 years; their findings suggest an average loss of motor neurons from the second to tenth decade of life of approximately 25%, and several subjects above 60 years even had only half the number of motor units young or middle-aged adults had. The loss of motor neurons is accompanied by a reduction in both, numbers

and diameters of motor axons in the ventral roots [37]. Functionally, the integrity of the peripheral motor system is often assessed by measuring the maximal motor conduction velocity of a given nerve. Axonal conduction velocity decreases with age presumably reflecting a variety of changes in the nerve fibres, such as a dropout of the largest and fastest conducting motor fibres, segmental demyelination and reduced internodal length [22, 64]. This progressive loss and/or related dysfunction of motor nerves should play an important role in muscle aging and atrophy. It should also be noted that although nerve cells may still remain existing in aged people, they become dysfunctional because of accumulating biochemical changes shown by the presence of lipofuscin, which does apparently precipitate during neuronal degeneration [21, 40]. Lipofuscin is a pigment derived from oxidation of lipids or lipoproteins and is accepted to be the most reliable and widespread cytological sign correlated with neuronal aging [21].

Concluding remarks

Although the mechanisms for age-related decline in skeletal muscle mass and function still remain to be fully understood in many details, the functional consequences of sarcopenia are well known. These include muscle weakness, a reduced endurance capacity and an increased fatigability resulting in an eventual loss of mobility, independence and in increased fragility in many older adults [38, 50, 53].

Considering all the above referred findings, it is suggested that skeletal muscle morphological and functional alterations with age do not only result from intrinsic factors within the muscle fibres but are also influenced by aging phenomena of the nervous system, especially of its peripheral components. In this sense, the alterations found in muscle fibres represent secondary effects based on the aging process of the nervous system.

This concept should not only be useful to explain eventual losses in strength encountered at advanced age, but should also explain the increased inability of elderly subjects to perform properly simple tasks of daily life. A loss of motor units and concomitantly larger motor units lead to an impairment of coordination in general. Moreover, alterations found in peripheral nerves and at the neuromuscular junction might incline the neuromuscular system to impairments in reflex responses necessary for, e.g. maintenance of posture and balance. These impaired coordinative skills frequently result in an increased susceptibility to fall among elderly subjects. It therefore should be a preventive challenge to subject the elderly to a mild exercise program with the aim to improve muscle strength and especially coordination.

In this matter, it seems reasonable to expect that interventions aimed to retard or prevent the loss of skeletal muscle mass and strength with age, like physical activity, should exert a major influence on neural adaptations. In fact, bearing in mind that aged skeletal muscle has a reduced adaptative potential [47] with a diminished number and reduced proliferative capacity of satellite cells [59], we can assume that the benefits of physical activity in the aged muscle, particularly strength training, are probably and mainly due to neural adaptations which might retard or prevent the consequences of the aging process primarily at the level of the nervous system, with beneficial secondary effects on the functional properties of skeletal muscle.

In addition, age-related alterations of skeletal muscle are accompanied by decreases in the metabolic rate, bone density and insulin sensitivity [39]. For these reasons, strategies for preserving muscle mass with advancing age should not only be important to maintain functional independence, but also to prevent some age associated chronic diseases.

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Paper II

The role of mitochondria in aging of skeletal muscle

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REVIEW ARTICLE

The role of mitochondria in aging of skeletal muscle

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Abstract Aging can be characterized as a time dependent decline of maximal functionality that affects tissues and organs of the whole body. Such is induced by the progressive loss of redundant components and leads to an increased susceptibility to disease and risk of death. Regarding the aging of skeletal muscle, it has been pointed out that mitochondria is a key factor behind the loss of redundancy and functionality, since this organelle has a major role in cellular homeostasis particularly at the level of the bioenergetic status. Decreased activities of the mitochondrial electron transport chain complexes and an increased release of reactive oxygen species from mitochondria are well documented with age; it is suggested that the mitochondrial loss of function results from the increased oxidative damage to proteins, lipids, and DNA of this organelle. However, it is important to be aware that the mitochondrial loss of function could also be a consequence, rather than a cause, of the cellular deterioration with age, which compromises mitochondrial biogenesis, mitochondrial protein turnover and autophagocytosis of damaged mitochondria. In this review several topics will be addressed regarding the age-related loss of skeletal muscle redundancy associated with mitochondrial dysfunction, emphasizing hypotheses for underlying mechanisms. In addition, we discuss some of the cellular mechanisms that can be pointed out as being responsible for the age-related mitochondrial dysfunction.

Keywords Cell damage · Mitochondrial dysfunction · Oxidative stress · Redundancy · Skeletal muscle aging

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Introduction

Aging can be defined as an irreversible, progressive, and time dependent decline of overall body functions, resulting from the interaction of genetic and stochastic factors. These lead to a diminished adaptative capacity to withstand internal and external stimuli, together with an increased susceptibility to disease and risk of death (Lenaz et al. 2000; Ventura et al. 2002; Adhihetty and Hood 2003).

Several theories tried to explain biological aging (Medvedev 1990) by means of specific mechanisms which are influenced by a restricted number of

variables, but without giving an integrative approach towards all conditioning factors that potentially enhance the complexity of this phenomenon. Recently, in a review paper about the theories of biological aging, Rattan (2006) have questioned the rationale for the existence of more than 300 aging theories, qualifying them as "hypothesis" or "aspect theories". In fact, the author suggests that although the large body of data concerning the aging process underlies the multifaceted, diverse and complex nature of aging, this can not be viewed as an obstacle to the pursuit of an unified theory of biological aging by gerontologists. In this context, it is imperative to have an integrative approach to this issue, since it is documented that both, genetic and stochastic factors influence the phenomenon of aging given that its rate is different not only among organs and tissues of the same subject but also between individuals exposed to the same environment (Harman 1998; Kwong and Sohal 2000; Harman 2001; Rattan 2006).

It is assumed that complex biological systems, like the human body, are composed by redundant components at various levels of organization that are not essential for the homeostatic maintenance under basal conditions, but are crucial to withstand additionally demanding and stressful situations. The number of redundant components in a biological system, independent of its level of organization, will determine the functional reserve capacity of that system. Moreover, the age-related damage of some redundant components will not necessarily induce the death of the system; instead, it will lead to the accumulation of defects, giving rise to the loss of maximal function associated with advancing age (Gavrilov and Gavrilova 2006). This decrease in the functional reserve capacity of the system, resulting from the accumulation of damaged redundant elements, will compromise the ability to withstand additional homeostatic disturbances (Fig. 1) (Gavrilov and Gavrilova 2004, 2006). Indeed, at the molecular level, it is suggested that increased levels of damage with age may have further deleterious consequences, such as altered gene expression, loss of the potential for cell division, tissue disorganization and increased vulnerability to stress. It is therefore the loss of redundancy that results in an altered homeodynamic status with a consequent inefficiency of cells and tissues to repair the inflicted damage (Gavrilov and Gavrilova 2006; Rattan 2006).

With this integrative point of view, independent of the underlying mechanisms, aging can be seen as a loss of redundancy in redundant organisms, due to an age-related and irreversible accumulation of damaged components resulting from the interaction between genetic and stochastic factors. In fact, the damage accumulation does not exclusively depend on the

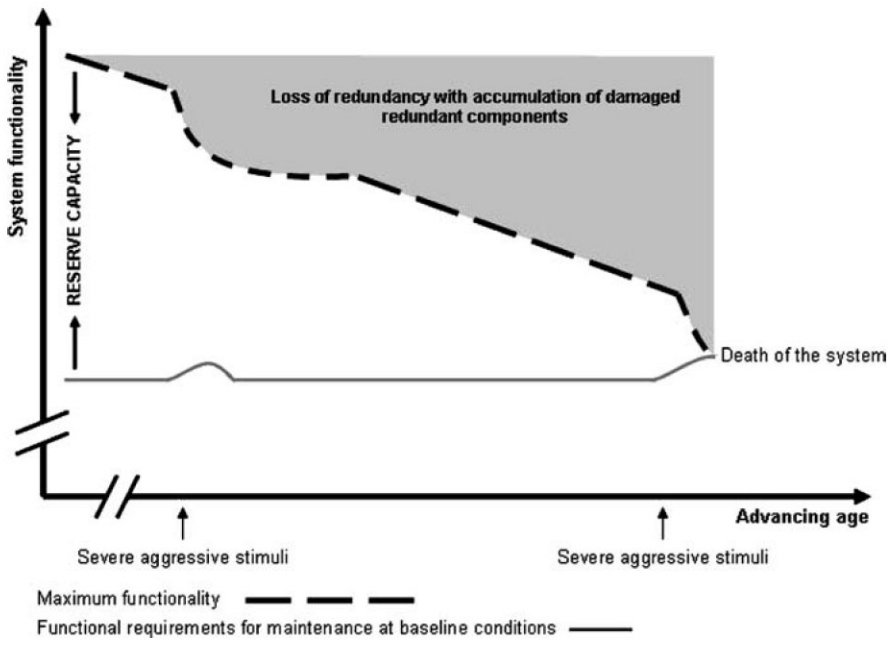

Fig. 1 Hypothetical loss of redundancy with increasing age. This loss of redundancy is a consequence of the system inability to repair the damage that is continuously inflicted by environmental exposure, diminishing the functional reserve capacity to levels near those required for basal functioning. However, the rate loss of redundant components may be exacerbated by the occasional exposition to more aggressive stimuli

exposure to environmental stimuli, but also on the genetically conditioned susceptibility to those stimuli and, moreover, on the genetically conditioned repair capacity (Butler et al. 2003; Warner 2005). The inability to repair the inflicted damage will result in a loss of cell viability, with a progressive reduction of cell number and/or function over time, and will per se compromise the redundancy of tissues leading to a reduced reserve capacity and maximal functionality of organs and systems (Gavrilov and Gavrilova 2004, 2006; Rattan 2006). Post-mitotic tissues such as nervous tissue and striated muscle are preferentially prone to the adverse effects of advancing age (Kwong and Sohal 2000; Sastre et al. 2003; Terman and Brunk 2004a, b). Skeletal muscle seems to be most affected by the irreversible loss of function with age, which is usually explained by the intrinsic mechanisms of the tissue itself, but also aggravated by the aging of other organs and systems which support skeletal muscle functionality, such as the endocrine, cardiovascular, and neural systems (Terman and Brunk 2004a, b; Figueiredo et al. 2006). In this context, the age-related loss of redundancy in systems responsible for maintaining the eutrophic state in skeletal muscle will also favour the loss of redundancy of muscle components, which ultimately occurs at the cellular and molecular level.

Regarding a variety of harmful environmental conditions that are experienced during the entire lifespan, the occurrence of oxidative stress at basal and stressful situations has been pointed out as one major reason for the increasing damage to cells and its components with age, namely proteins, lipids, and deoxyribonucleic acid (DNA) (nuclear and mitochondrial) (Shigenaga et al. 1994; Cadenas and Davies 2000). It has been suggested that mitochondria constitutes the major source of reactive oxygen species (ROS) and might also be a prime target of ROS with the consequent accumulation of oxidatively damaged components (Stadtman 2002). Considering the harmful repercussions of oxidative stress to myofibres, the loss of redundancy inflicted by these endogenously produced oxidants could be the likely cause of skeletal muscle mitochondrial dysfunction with age (Cadenas and Davies 2000). This can lead eventually to several age-related pathological conditions in this tissue, namely insulin resistance (Petersen et al. 2003), exercise intolerance (Conley et al. 2000), and sarcopenia (Bua et al. 2002; Tonkonogi et al. 2003). Indeed, dysfunctional mitochondria will be unable to meet cellular ATP demands, diminishing the oxidative capacity of the myofibre, disrupting cellular energetics, and compromising the cellular ability to adapt to physiological stress imposed to skeletal muscle across the entire lifespan (Shigenaga et al. 1994). Bearing this in mind, this review intends to analyse the mechanisms behind the production of mitochondrial ROS, antioxidant defences, oxidative stress and damage, as they might be seen as potential means to explain the loss of redundant components of skeletal muscle. Moreover, it seems also important to discuss the mechanisms responsible for the age-related loss of mitochondrial redundancy and to discuss whether this loss might be interpreted as a consequence rather than a cause of skeletal muscle dysfunction with advancing age.

Mitochondria as a source of molecular damage

The mitochondrial electron transport chain (ETC) plays an important role for energy production in aerobic organisms. Electrons from NADH (the reduced form of nicotinamide adenine dinucleotide) and $FADH_2$ (the reduced form of flavin adenine dinucleotide), produced during several metabolic processes, flow down the ETC and are coupled to the establishment of an electrochemical gradient across the mitochondrial inner membrane (Barja 1999). The ETC is located in the inner mitochondrial membrane and has five protein complexes: NADH dehydrogenase (Complex I), succinate dehydrogenase (SDH) (Complex II), cytochrome bc1 complex (Complex III), cytochrome *c* oxidase (COX) (Complex IV), and ATP synthase (Complex V) (Mandavilli et al. 2002). Complexes I, III and IV pump protons across the inner mitochondrial membrane into the intermembrane space, creating an electrochemical gradient, which is then utilized by complex V for ATP generation.

Production of reactive oxygen species

In addition to the ATP synthesis, the ETC is also a significant source of ROS under physiological conditions, thereby identifying mitochondria as the primary cellular source of these compounds (Brookes

et al. 2004; Brookes 2005). The ETC consumes more than 90% of the oxygen taken up by the cell, and up to 5% of that is converted into superoxide ($O_2^{\cdot-}$) even during a normal physiological state (Leeuwenburgh and Heinecke 2001). The primary ROS generated in the mitochondria is $O_2^{\cdot-}$ which is then converted to hydrogen peroxide (H_2O_2) by spontaneous dismutation or by superoxide dismutase (SOD) (Brookes 2005). Hydrogen peroxide in turn is broken down into water by glutathione peroxidase (GPx) or catalase (CAT). If this does not occur, H_2O_2 can undergo Fenton's reaction in the presence of divalent cations such as iron (Fe^{2+}) to produce hydroxyl radicals ($HO^{\cdot}$), which can even be more harmful to the mitochondrial biomolecules (Stadtman 1992). This basal rate of $O_2^{\cdot-}$ production may be altered by several factors, such as pathological conditions (Droge 2002), aging (Barja 2002) and altered metabolic demands (Vasilaki et al. 2006).

Complexes I and III are considered to be the primary sites for $O_2^{\cdot-}$ production (Barja 1999). The relative contribution of every site to the overall $O_2^{\cdot-}$ production varies from organ to organ and also depends on whether mitochondria are actively respiring (state 3) or the respiratory chain is highly reduced (state 4) (Barja 1999). Superoxide generated in the matrix is also eliminated in that compartment, while a part of the $O_2^{\cdot-}$ produced in the intermembrane space may be carried out into the cytoplasm via voltage-dependent anion channels (VDAC) (Han et al. 2003); however, the intermembrane space contains both, CuZnSOD (a copper- and zinc-containing SOD) (Okado-Matsumoto and Fridovich 2001) and cytochrome *c*, which can be reduced by $O_2^{\cdot-}$ and pass on the electrons to complex IV (Mailer 1990). It is assumed that the main mitochondrial $O_2^{\cdot-}$ sources are at the ubisemiquinone radical ($QH^{\cdot-}$) of the Q cycle at the complex III, facing the intermembrane space (Brookes 2005), although some $O_2^{\cdot-}$ may also be released to the matrix from this site (Han et al. 2001; St-Pierre et al. 2002; Turrens 2003). Another mitochondrial $O_2^{\cdot-}$ source seems to be located at the complex I, facing the matrix (Turrens and Boveris 1980), although the exact site where $O_2^{\cdot-}$ is produced within the complex I is still unclear, with the flavin-mononucleotide (FMN) cofactor, the various Fe–S clusters, and the Q binding sites proposed as potential sources of ROS (Turrens 2003; Brookes 2005) (Fig. 2).

The primary factor governing mitochondrial ROS generation is the redox state of the respiratory chain (Brookes 2005). The energy released as electron flow through the respiratory chain is converted into a *trans*-membrane proton gradient (ΔpH) and to a membrane potential ($\Delta\psi_m$), which can inhibit the pumps by feedback when sufficiently high (Brookes 2005). This gradient in turn is dissipated through the ATP synthase complex (complex V) and is responsible for ATP synthesis. In the absence of adenosine diphosphate (ADP), the movement of H^+ through ATP synthase ceases and the H^+ gradient increases causing the electron flow to slow down and the respiratory chain to become more reduced (state 4 respiration). As a result, the relative physiological steady-state concentration of $O_2^{\cdot-}$ increases. In this context there is a widespread notion that mitochondria only produce oxygen radicals in state 4, which results from the observation that H_2O_2 production

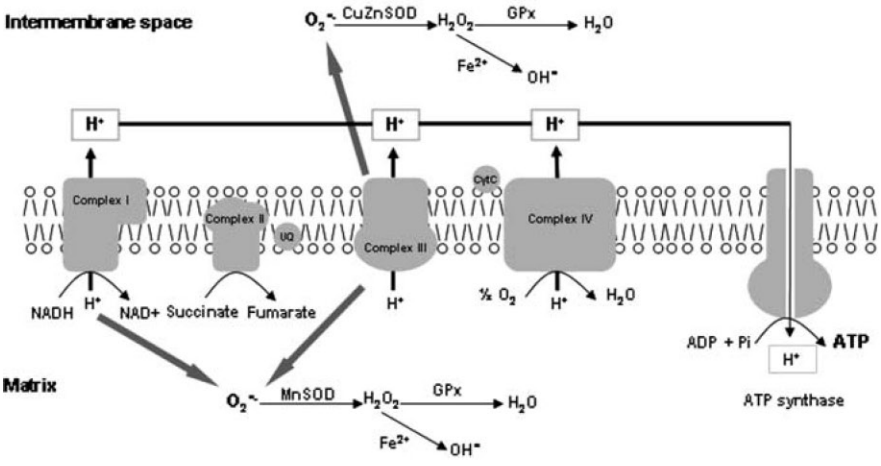

Fig. 2 ROS release by the electron transport chain. ROS are released into the mitochondrial matrix by complexes I and III. ROS is released into the intermembrane space from complex III. UQ—ubiquinone; Cyt.C—Cytochrome *c*

with complex II-linked substrates is virtually stopped after the addition of ADP in order to cause the transition from state 4 to state 3 (reviewed in Barja 1999). However, when pyruvate/malate is used instead of succinate, the addition of ADP no longer stops the H_2O_2 production, suggesting that complex I continues to produce oxygen radicals in state 3 (Barja 1999). In this case, transition from state 4 to state 3 in pyruvate/malate supplemented mitochondria does not significantly increase H_2O_2 generation, although the oxygen consumption significantly increases during the transition between respiratory states (Herrero and Barja 1998). In fact, it is suggested that free radical leaking is smaller in state 3 than in state 4, and this can be attributed to the lower reduction of the ETC complexes in state 3 in relation to state 4 (Barja 1999). Accordingly, Barja (1999) has suggested that this diminished free radical leak compensates for the increased electron flow in state 3, avoiding a massive increase in ROS production in the aerobically active state. This at the same time suggests that there would be an exercise-induced oxidative stress in the tissue, resulting from moderate increases in the mitochondrial production of oxygen radicals during state 4 to state 3 transitions.

Antioxidant defences and oxidative stress

As already mentioned, mitochondria in vitro convert 1–5% of the oxygen molecules consumed into superoxide anions (Shigenaga et al. 1994; Leeuwenburgh and Heinecke 2001). Given that this estimation was made on isolated mitochondria in the presence of high, non-physiological concentrations of oxygen, the in vivo rate of $O_2^{\cdot-}$ production might be considerably lower (Finkel and Holbrook 2000). However, independent of the absolute amount of ROS generation, several protective antioxidant systems are evolved in order to counteract this oxidant production.

Cellular antioxidant systems have been traditionally divided into two categories: enzymatic and non-enzymatic. Primary antioxidant enzymes include SOD which hastens the dismutation of superoxide to H_2O_2, GPx, and CAT, both converting H_2O_2 to water (Beckman and Ames 1998). Non-enzymatic antioxidant defences include hydrophilic radical scavengers such as ascorbate, urate, and glutathione (GSH), and lipophilic radical scavengers, namely tocopherols, flavonoids, carotenoids, and ubiquinol (Beckman and Ames 1998). Non-enzymatic antioxidants, like vitamin E (α-tocopherol), vitamin C and β-carotene directly scavenge superoxide and $HO^{\cdot}$, as well as singlet oxygen (Yu 1994). Glutathione and other antioxidants of low molecular weight play an important role in maintaining sufficient substrate levels for GPx and keeping vitamin E and vitamin C in the reduced state (Meister and Anderson 1983).

Mitochondria contain ~10–12% of the total GSH content in a cell but due to their relatively small matrix volume the concentration of GSH in the mitochondrial matrix is somewhat higher than in the cytoplasm. Since mitochondria lack enzymes for GSH biosynthesis, the intramitochondrial pool of GSH is replenished by a rapid net uptake of GSH from the cytoplasm (Ji et al. 1992; Leeuwenburgh et al. 1997). GSH provides the substrate for GPx and glutathione S-transferase (GST), which are important enzymes for the removal of cytotoxic hydroperoxides and xenobiotics (Leeuwenburgh et al. 1997). In fulfilling these functions, GSH is oxidized to GSSG that cannot be exported to the cytosol and must be reduced back to GSH in the mitochondrial matrix (Leeuwenburgh et al. 1994). This reduction is catalyzed by glutathione reductase (GR), which is present in the mitochondrial matrix and utilizes the reduced form of intramitochondrial NADPH as a source of reducing equivalents (reviewed in Andreyev et al. 2005).

Glutathione peroxidase, which is probably the best studied mitochondrial antioxidant enzyme, plays an important role in the decomposition of H_2O_2 produced in mitochondria. In fact, GPx activity seems to exceed that of any competing H_2O_2 scavenger in mitochondria (Cadenas and Davies 2000). Despite mostly present in the peroxisomes, CAT might also play a role in the decomposition of mitochondrial H_2O_2 to H_2O (Cadenas and Davies 2000).

The mitochondrial matrix contains a specific form of SOD, manganese-containing superoxide dismutase (mnSOD), which eliminates the superoxide produced in the matrix (Turrens 2003) by facilitating the dismutation of the superoxide radical to H_2O_2; it thereby protects the mitochondrial iron–sulfur cluster-containing enzymes from superoxide attack (Gardner et al. 1995). The expression of this antioxidant enzyme is further increased by agents that cause oxidative stress in a process mediated by the

oxidative activation of the nuclear transcription factor-kB (NF-kB) (Warner et al. 1996). In the mitochondrial intermembrane space, the antioxidant activity is controlled by three different mechanisms, namely (i) the existence of CuZnSOD, (ii) the presence of cytochrome *c*, which can be alternatively reduced by superoxide or by the respiratory chain and consequently can transfer electrons to the terminal oxidase (COX), and (iii) the spontaneous dismutation of superoxide, which is facilitated by the lower pH in this compartment, resulting from the pumping of H^+ coupled to electron transfer (reviewed in Turrens 2003).

In addition to cytochrome *c*, ubiquinol (QH_2) has also been shown to act as a reducing agent in the elimination of peroxides in the presence of succinate (Eto et al. 1992). The lipophilic radical scavenger α-tocopherol, present in mitochondrial membranes, also has a role in interfering with the propagation of free-radical mediated chain reactions, thereby protecting membrane lipids from peroxidation (Andreyev et al. 2005).

As a consequence of their biological functions, mitochondria are always exposed to ROS production and have a complex antioxidant defence system to counteract it. Oxidative stress occurs when the homeostatic balance between oxidant and antioxidant capacities in a determined biological system is disturbed and the redox state becomes more pro-oxidizing (Droge 2002). Thus, under conditions of oxidative stress where mitochondrial ROS production exceeds the antioxidant capacity, mitochondria may suffer from oxidative damage to their biomolecules. Since the removal and repair of altered structures may not be completely efficient, the oxidizing products might accumulate in this organelle (Matsuo and Kaneko 2000) (Fig. 3) leading to a reduction in the number of redundant mitochondrial elements.

Age-related mitochondrial oxidative damage

Oxidative damage and modifications of proteins is one hallmark of aging in biological systems (Stadtman 2002). Oxidative damage to a specific protein, especially at the active site, can induce a progressive loss of a particular biochemical function (Sohal 2002). An increase in the oxidation levels of mitochondrial proteins with age has been demonstrated mainly by the determination of the content of protein carbonyl derivatives and by analysing the loss of protein sulfhydril groups (Floyd et al. 2001; Sohal 2002; Stadtman 2002). Other methods, like the determination of oxidation products of specific amino acids, e.g. tyrosine oxidation products (dityrosine or nitrotyrosine) or oxidation of methionine to

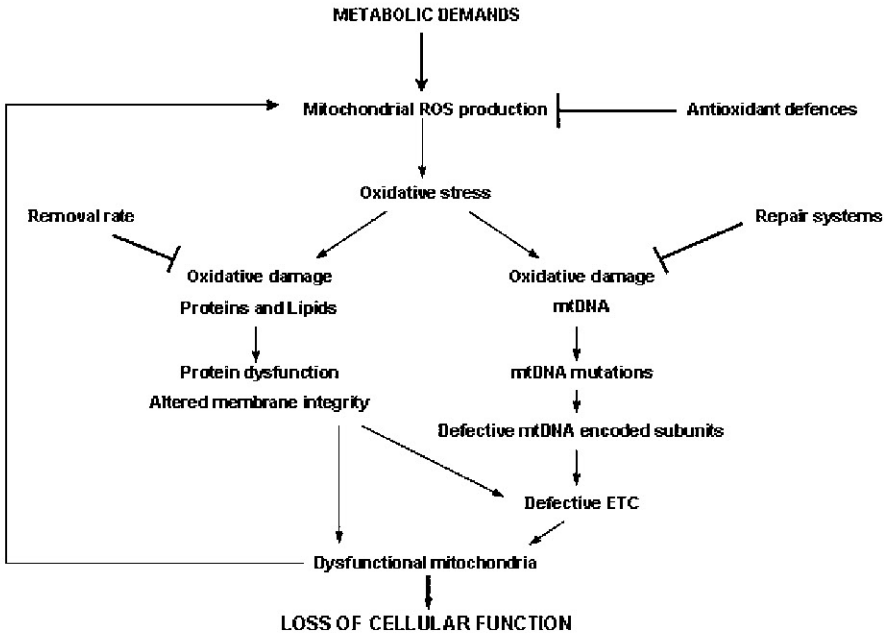

Fig. 3 Role of the continuous ROS production as a source of damage to mitochondrial components

methionine sulphoxide, had also been used (reviewed in Van Remmen and Richardson 2001).

Increased amounts of oxidized mitochondrial proteins with age, as measured by the content of protein carbonyls, have been experimentally demonstrated (Sohal et al. 1993; Yan et al. 1997; Yan and Sohal 1998) and reflect the imbalance between its rate of formation and subsequent removal (Floyd et al. 2001). It has recently been suggested that age-related mitochondrial protein oxidation might be protein-specific rather than being a random process (Van Remmen and Richardson 2001; Sohal 2002). In fact, western blot analysis of protein carbonyls in flight muscle mitochondria of house flies revealed an increase in protein carbonyls that appears to be limited to the adenine nucleotide translocase (ANT) in mitochondrial membranes (Yan and Sohal 1998) and to aconitase (ACON) in the mitochondrial matrix (Yan et al. 1997). In this context, ROS may act in a random fashion, but the sensitivity and proximities of potential targets may differ (Sohal 2002). The degree of oxidative damage to protein with age depends on many factors including the nature and relative location of the source of an oxidant or free radical, the proximity of the radical-oxidant to a protein target, and the nature and concentrations of available antioxidant enzymes and compounds (Grune et al. 2001). In addition, the factors that affect the selectivity of oxidative damage to proteins include the presence of a metal binding site, the molecular conformation, the rate of proteolysis, and the relative abundance of amino acids residues susceptible to metal-catalysed oxidation (Sohal 2002). This selective oxidative damage, namely related to ANT and ACON, can have a significant functional importance. In fact, damage to specific proteins has been hypothesized to constitute an important mechanism linking oxidative stress and age-related loss of function (Sohal 2002). ANT oxidation and functional inactivation are associated with increased age (Yan and Sohal 1998), which can ultimately lead to a decrease in the maximal ADP-stimulated state 3 respiration and to a consequent increase in mitochondrial H_2O_2 generation due to an enhanced reduction of ETC-related mitochondrial complexes (Sohal 2002). Similarly, the oxidative inactivation of ACON, which is particularly sensitive to the reaction with superoxide due to the iron–sulfur clusters [4Fe–4S] at its active site (Floyd et al. 2001), may slow down the tricarboxylic acid cycle (TCA), resulting in decreases in the electron flow to oxygen and in diminished oxidative phosphorylation. Furthermore, ACON oxidative inactivation can lead to an accumulation of reduced metabolites, such as NADH (Yan et al. 1997).

Components of mitochondrial membranes can be particularly sensitive to oxidative damage by oxygen free radicals continuously generated by the ETC, because of their rich content of unsaturated fatty acids with carbon-carbon double bonds (Van Remmen and Richardson 2001; Paradies et al. 2002). Peroxidation of membrane lipids has been suggested to be one of the major causes of decreased mitochondrial membrane function (Paradies et al. 2002). In fact, peroxidation alters the structure of membrane lipids, which can disrupt the structural organization of the lipid double layer, altering membrane fluidity and permeability (Oh-ishi et al. 2000; Valls et al. 2005). Lipid peroxidation reactions are generally free radical-driven chain reactions in which one radical can induce the oxidation of a comparatively large number of substrate molecules, particularly the highly vulnerable polyunsaturated fatty acids (PUFAs) (Abuja and Albertini 2001).

It is widely accepted that lipid peroxidation increases with age (Spiteller 2001). A significant increase in the peroxidation products (malondialdehyde—MDA) has been reported in rat liver mitochondria (Valls et al. 2005). This increase was accompanied by a decreased activity in the antioxidant enzymes SOD and CAT as well as by a diminished membrane potential. The decline in the mitochondrial membrane potential could be related with an increased oxidative damage to the mitochondrial membranes (Valls et al. 2005). Also, in mice skeletal muscle mitochondria, Faist et al. (1998) have demonstrated an increased mitochondrial formation of thiobarbituric acid reactive substances (TBARS) with age.

Cardiolipin, a phospholipid of the inner mitochondrial membrane, is particularly rich in unsaturated fatty acids (90% composed by linoleic acid) and is in close proximity to the mitochondrial production site for ROS, which makes it a target candidate for oxidative attack and damage (Paradies et al. 2002). Cardiolipin peroxidation can be particularly harmful to the mitochondria, because this lipid plays an

important role in the function of a number of major integral inner membrane proteins, including anion carriers and complexes of the respiratory chain (Hoch 1992). In this context, the results by Paradies et al. (2002) indicate that cardiolipin is specifically required for the activity of the mitochondrial complex I. These authors have also demonstrated that ROS generation leads to a marked loss in the cardiolipin content and that this loss is related with a diminished complex I activity. It has been reported in this context that cardiolipin is specifically required for several mitochondrial biological processes, namely ETC complexes I, III and IV activity, and ANT functioning (Yan and Sohal 1998; Van Remmen and Richardson 2001; Paradies et al. 2002).

Oxidative stress can significantly induce oxidative damage to the DNA molecule through breaks of DNA strands and modifications of the bases (Van Remmen et al. 2003). The $HO^{\cdot}$ is a major radical in oxidative DNA damage, causing a variety of base modifications as well as a fragmentation of the deoxyribose sugar, resulting in a variety of modified products (Van Remmen et al. 2003). Hydrogen peroxide and the superoxide anion can also contribute to strand breaks and base modifications. The DNA molecule has a high concentration of phosphate groups that make it negatively charged and readily able to bind metal ions (iron or copper). Such would facilitate the generation of $HO^{\cdot}$ by interaction of H_2O_2 and superoxide anions with metal ions by the Haber Weiss or Fenton reaction (Van Remmen et al. 2003).

Mitochondrial DNA (mtDNA) is located in the mitochondrial matrix in close association with the inner mitochondrial membrane. Mitochondrial DNA encodes 37 genes, all related to ATP production (Cao et al. 2001). More specifically, mtDNA codes for 13 proteins participating in the ETC, 22 transfer RNAs (tRNAs), and 2 ribosomal RNA (rRNAs) (Stevnsner et al. 2002). Each mammalian cell contains several hundred to more than one thousand mitochondria, each of which carries several copies of mtDNA (Mandavilli et al. 2002). The mutant mtDNAs usually co-exist with the wild-type mtDNA within a cell (named heteroplasmy), and the degree of heteroplasmy often varies in different tissues of the same individual (Wei and Lee 2002). Three types of heteroplasmy can occur in a tissue, namely *intercellular*, in which mutant and wild-type mtDNA occur in different cells, *intracellular* and *intermitochondrial*, in which mutant and wild-type mtDNAs occur in different mitochondria in the same cell, and *intracellular* and *intramitochondrial*, in which mutant and wild-type mtDNAs occur in the same organelle (Attardi 2002). Since most mtDNA mutations are recessive, it is suggested that the coexistence of both, wild-type and mutant mtDNA in the same organelle, could lead to the complementation of the mutation (Attardi 2002). It has been suggested in this context that mtDNA mutations start to occur after the fourth decade of life and that they accumulate with age in post-mitotic tissues (reviewed in Wei and Lee 2002).

Due to its proximity to the generation site of mitochondrial ROS, mtDNA is particularly vulnerable to oxidative damage and has been shown to experience a higher level of oxidative damage compared to nuclear DNA (nDNA) (Wei and Lee 2002; Barja 2004). In addition, mtDNA is a 16.5 kb molecule and is not protected by histone proteins as is the case for nDNA (Van Remmen and Richardson 2001). Despite this apparently enhanced susceptibility, it is now recognized that mitochondria have extensive DNA repair systems for the excision of oxidized bases but it is also assumed that the large number of mtDNA deletions might not be totally repairable (Linnane et al. 2007).

In the context of oxidative damage to mitochondrial biomolecules, it has been suggested that oxidative damage to mtDNA could have more implications for age-related mitochondrial dysfunction than damage to protein or lipids, because damage to mtDNA can be propagated as mitochondria and cells divide, thus allowing the physiological consequences of the damage to be amplified (Van Remmen and Richardson 2001; Van Remmen et al. 2003). This assumption is supported by results obtained with mtDNA mutator mice in which the dysfunction of oxidative phosphorylation that parallels their shortened lifespan mainly arises from the replication process of damaged mtDNA (reviewed in Linnane et al. 2007).

Oxidative damage to mtDNA has been extensively studied using various techniques including HPLC-electrochemical detection of 8-hydroxy 2-deoxyguanosine (8OH-dG), southern analysis, quantitative polymerase chain reactions (QPCR), and ligation-mediated polymerase chain reactions (LMPCR)

(reviewed in Mandavilli et al. 2002). The most widely used is the determination of the levels of 8OH-dG, a biomarker of oxidative DNA damage (Barja et al. 1994; Barja and Herrero 2000).

Several studies have shown age-related increases in the levels of 8OH-dG in mtDNA isolated from different mammalian species (Barja and Herrero 2000). Oxidative damage to mtDNA has also been shown to accumulate with age in human diaphragm muscle and brain (Lenaz 1998). However, others (Drew et al. 2003) have failed to demonstrate an accumulation of lesions in the mitochondrial genome with age. This lack of concordance might be explained by the fact that DNA extraction procedures could artificially oxidise the DNA molecule, which is particularly important in the case of mtDNA, since it appears that mtDNA is more susceptible to oxidation during the fractionation of the organelle sample and subsequent DNA extraction (Beckman and Ames 1999). On the other hand, another possibility might be related to some degree of contamination of mtDNA by nDNA, leading to an underestimation of the amount of 8OH-dG present in the mitochondria (Van Remmen et al. 2003). Oxidative stress has also been shown to lead to increased levels of DNA deletions (Shigenaga et al. 1994), and the proportion of mutant mtDNA has been shown to correlate with the 8OH-dG content of mtDNA (reviewed in Wei and Lee 2002). However, the exact mechanism by which oxidative stress causes mtDNA mutations is poorly understood. In fact, even though numerous reports suggested that ROS damage was a major contributor to mtDNA dysfunction there is no convincing evidence in support of such a view (reviewed in Linnane et al. 2007). Moreover, despite several mtDNA deletions have been reported to increase with age (Cortopassi and Wang 1995), the frequency of even the most common deletion (4,977 bp) is estimated to be less than 2%, which may not have any physiological relevance (Van Remmen and Richardson 2001). It should moreover be taken into account that ROS-induced mtDNA mutations and deletions with age, i.e. nucleotide modifications including base oxidations and DNA strand breaks, could take place very fast. Since mtDNA is constantly repaired for oxidative lesions (Larsen et al. 2005) the true levels of mtDNA oxidative damage may be very low and most difficult to detect in aged tissue (Dirks et al. 2006).

Functional repercussions

Various aspects of mitochondrial function have been shown to be compromised with age (Short and Nair 2001; Nicholls 2002). The most widely studied is the respiratory chain capacity of mitochondria isolated from different tissues of humans (Tonkonogi et al. 2003; Menshikova et al. 2006) and animals (Kwong and Sohal 2000; Drew et al. 2003) of different age groups, provided by measures of oxygen consumption in the presence (state 3) or absence (state 4) of ADP.

Several techniques have been applied to determine the extent to which mitochondrial oxidative capacity is affected by age. In vitro assays involve the isolation of mitochondria from tissue samples (e.g. skeletal muscle) and the measurement of several respiratory parameters, e.g. respiratory states of mitochondria, respiratory control ratio (RCR), ADP/O, among others, in the presence of various substrates (Rasmussen and Rasmussen 2000; Rasmussen et al. 2001; Tonkonogi and Sahlin 2002) as well as the measurement of mitochondrial enzymatic activities (Rasmussen et al. 2003). In vivo techniques involve the utilization of nuclear magnetic resonance technology to determine the average rate of ATP formation in several muscle groups (Conley et al. 2000; Kent-Braun and Ng 2000).

Recently, Short et al. (2005), studying skeletal muscle mitochondria, have demonstrated that older people have significantly higher levels of oxidative damage to DNA and that mtDNA abundance decreases with age. The latter finding was associated with a lower content of messenger ribonucleic acid (mRNA) transcripts that encode mitochondrial proteins. The authors have also reported a lower mitochondrial protein content and a diminished citrate synthase (CS) activity in older people, as well as a continuous decline in mitochondrial capacity for oxidative phosphorylation with advancing age. It was suggested that this diminished mitochondrial function with age is due to a combination of a reduced mitochondrial content together with a functional impairment in the remaining mitochondrial population. Likewise, Mansouri et al. (2006) have reported a significant reduction in ATP production in mitochondria from old mice, as well as a reduction in the activities of both, complex I and complex V of the mitochondrial respiratory chain.

By using nuclear magnetic resonance, Waters et al. (2003) found an age-related decline in mitochondrial function in healthy exercising elderly which appeared to be somewhat attenuated with higher levels of physical activity. Considering this, it has been proposed that the decreased respiratory function of skeletal muscle mitochondria with age is not so apparent when the levels of physical activity of the subjects are taken into account (Brierley et al. 1997); however, when corrected for physical activity levels, a decline in mitochondrial function has been reported in several studies (Conley et al. 2000; Tonkonogi et al. 2003; Short et al. 2005).

Accordingly, Tonkonogi et al. (2003) have demonstrated that muscle oxidative power measured in skinned muscle fibres and in isolated mitochondria is lower in elderly subjects, and this age-related alteration in mitochondrial function is not solely an effect of physical deconditioning, but constitutes an intrinsic element of biological aging. Diminished mitochondrial function with age has also been shown in various animal studies (Kwong and Sohal 2000; Ventura et al. 2002; Drew et al. 2003). This mitochondrial dysfunction with age is suggested to be tissue-specific. When assessing the age-related alterations in mitochondrial ATP content and production rate, Drew et al. (2003) found that mitochondria isolated from *gastrocnemius* muscle of rats demonstrated a ~50% reduction in ATP content and in maximal ATP production rate; in contrast, these authors did not report any alterations in the mitochondria of aged hearts. They have suggested that, since aged cardiac muscle exhibits smaller increases in mtDNA deletions compared with those exhibited by skeletal muscle mitochondria (Short and Nair 2001), it is possible that ROS-induced damage is relatively lower in myocardium compared to skeletal muscle. It is therefore assumed that mtDNA deletions are tissue specific (Barazzoni et al. 2000; Short and Nair 2001) and that this interferes with the functionality of the complexes of the mitochondrial ETC (Van Remmen and Richardson 2001; Drew et al. 2003; Van Remmen et al. 2003).

Consequently, the activities of the mitochondrial ETC complexes have been reported to decrease with age, namely the activities of the complexes I, III and IV, while complex II activity appears to be unchanged with age (Van Remmen and Richardson 2001). This could be related with the fact that many of the subunits comprising complexes I, III and IV of the ETC are encoded by mtDNA, while complex II is encoded by nDNA (Mandavilli et al. 2002).

Some protein complexes of the ETC may be more prone to oxidative damage. Complex I has been shown to be particularly sensitive to oxidative damage because of its Fe–S clusters (Van Remmen and Richardson 2001), and its activity has been reported to decrease with age in mouse skeletal muscle mitochondria (Desai et al. 1996; Kumaran et al. 2004; Mansouri et al. 2006). Furthermore, because the age-dependent decline of the glutamate–malate supported respiration was found to be more evident than that of the succinate-supported respiration, it has been suggested that mutation(s) in the seven genes of NADH dehydrogenase (complex I) encoded by mtDNA may be involved in this age associated decline of respiratory function (Wei and Lee 2002). The extent of mtDNA mutation strongly correlates with the progressive decrease of COX activity in aging human muscle (reviewed in Wei and Lee 2002). Complex IV activity (COX) has also been shown to decrease with age, both in humans (Tonkonogi et al. 2003; Kumaran et al. 2004) and rodents (Desai et al. 1996; Paradies et al. 1997; Kumaran et al. 2004). Cytochrome *c* oxidase is composed of 13 subunits, three of which are encoded by mtDNA (Mandavilli et al. 2002), and the appearance of COX negative fibres in aged skeletal muscle has been proposed to be related with increased mitochondrial dysfunction at advanced ages (Van Remmen and Richardson 2001).

Peroxidation of the phospholipids of mitochondrial membranes could also play a role in the increased mitochondrial dysfunction in aging. Age-related alterations in the fluidity of the mitochondrial membrane have been reported (Oh-ishi et al. 2000), which can have a considerable impact on the activity of the respiratory chain as well as on the generation of the electrochemical proton gradient (Kwong and Sohal 2000). This would result in increased state 4 respiratory rates and probably in decreased RCRs (Rasmussen et al. 2003). Nakahara et al. (1998) have reported an increase in state 4 respiration rates in liver mitochondria of senescent accelerated mice with a concomitant decrease in state 3 respiratory rates and in RCR. These results were suggested to arise from greater respiratory uncoupling due to membrane damage, resulting from increased levels of oxidative

damage and leading to altered functions of the inner mitochondrial membrane. Decreased state 3 respiration and RCR was also reported by Faist et al. (1998) in skeletal muscle of senile mice. The authors did not find any significant alteration in state 4 respiratory rates; however, the mitochondrial content of TBARS was significantly increased with age, probably due to pronounced lipid peroxidation.

In summary, there is strong evidence pointing out an age-related increase in the levels of oxidative stress and oxidative damage continuously imposed on mitochondrial biomolecules, which becomes progressively more apparent with advancing age (Terman and Brunk 2004a, b). Since the cellular capacity for repair is not completely efficient, this increased damage might lead to accumulation of dysfunctional proteins, impaired membrane integrity and increased levels of mutant mtDNA, which will proliferate in an irreversible way by means of mitochondrial and cellular division. Consequently, the number of redundant components of intact mitochondria will be reduced, compromising the maximal mitochondrial function and consequently the maximal energetic capacity of skeletal muscle fibres (Carmeli et al. 2002; Gavrilov and Gavrilova 2006). Provided that this progressive loss of mitochondrial redundancy may not limit its capacity to supply the cellular energetic demands at basal metabolic conditions, such might limit the functionality of myofibres during situations with higher energetic requirements.

Mitochondria as redundant cellular components

Mitochondrial homeostasis is a very important feature in cell survival, and an imbalance in the production or degradation of various mitochondrial components can lead to accumulation of non-assembled subunits, to mitochondrial dysfunction and even to cell death (Bota 2001). An important factor for protein maintenance under conditions of oxidative stress and damage is the enzymatic reversal of the oxidative modifications and/or protein degradation (Stadtman 1992; Carmeli et al. 2002).

Mitochondrial decay has been observed with aging in several tissues, including skeletal muscle, in both animals and humans (Shigenaga et al. 1994). This mitochondrial dysfunction, proposed to result from oxidative damage, has been associated with a reduced capacity for oxidative phosphorylation in muscle, most likely due to a decrease in mitochondrial content and/or function (Short et al. 2005). Although the loss of mitochondrial redundancy affects the maximal availability of energy for the cellular environment, it is important to note that the mitochondrial production of ROS and consequent extrusion to the cytosolic space may also play a role in the oxidative damage to cells (McArdle and Jackson 2000; Dizdaroglu et al. 2002; Droge 2002; Leeuwenburgh 2003), contributing to the loss of cellular redundancy. Moreover, considering the role of ANT on the mitochondrial membrane permeability (MMP) complex and the age-related specific oxidative damage imposed on these proteins (Yan and Sohal 1998), leading to opening of large pores in the inner and outer membranes (Yan and Sohal 1998; Scheffler 2001), the extrusion of cytochrome *c* to the cytosolic space (Floyd et al. 2001) may also constitute a potential mitochondrial mechanism to explain the progressive loss of skeletal muscle redundancy, since cytochrome *c* is a central participant in caspase(s)-mediated apoptotic events (Dirks and Leeuwenburgh 2002). In fact, the activation of these proteolytic caspases may be partly responsible for the initiation of the degradation of muscle protein and for a loss of myonuclei, leading ultimately to the loss of nuclear domains with muscle fibre atrophy (Allen et al. 1999; Leeuwenburgh 2003).

Age-related mitochondrial phenotypic alterations

It is widely accepted that the number of skeletal muscle mitochondria is diminished with age (Rooyackers et al. 1996; Hood 2001; Menshikova et al. 2006). Several age-related alterations in mitochondrial morphology have been reported, namely vacuolization of the matrix, shortened cristae, enlargement and loss of dense organelles (Shigenaga et al. 1994). However, except for the changes in the number and size of mitochondria (Ozawa 1997), little is known about the relation between mitochondrial morphology and aging.

In conventional electron micrographs, mitochondria appear in the cells as rods or spheres as these images stem from very thin sections. However, it is now assumed that mitochondria build up a giant interconnected reticulum that is highly dynamic and

is maintained and reconstructed by permanent events of fusion and fission (Westermann 2002). The size of individual mitochondria, normally described in the range from 0.5 to 3 μm in diameter, varies from cell to cell and even within the same cell type (Ozawa 1997). A number of physiological conditions are known to influence mitochondrial mass within a cell, namely altered energy demands (Menshikova et al. 2006) and aging (Lyons et al. 2006). Several studies have demonstrated age-related decreases in mitochondrial content (Conley et al. 2000; Lyons et al. 2006; Menshikova et al. 2006), and Coleman et al. (1987) have reported the occasional appearance of "giant" mitochondria in senescent cardiomyocytes. Several pathologies associated with premature aging (e.g. mitochondrial myopathy) are characterized by the appearance of ragged red fibres (RRF) which contain typical accumulations of pathologically altered mitochondria (reviewed in Ozawa 1997). Ragged red fibres were also detected in normal human muscle as an age-related alteration (Muller-Hocker 1990), and it has been suggested that their occurrence supports the concept of mtDNA mutations (Sastre et al. 2003). An absence of COX, which is partly encoded by the mitochondrial genome, is the most common abnormal ETC phenotype and is often associated with an increase in SDH activity (Lee et al. 1998). Succinate dehydrogenase is entirely encoded by the nuclear genome and it has therefore been suggested that SDH overexpression is the result of a nuclear up-regulation of mitochondrial biogenesis in an attempt to compensate the mitochondrial deficit (Sastre et al. 2003). The combination of COX absence and SDH overexpression phenotypes in a skeletal muscle fibre is typical for the RRF phenotype observed in some premature aging syndromes and also in normal human aged skeletal muscle (Sastre et al. 2003).

The extent to which the oxidative capacity of skeletal muscle can be affected by age-related alterations in its mitochondrial content and/or function has been addressed by several authors (Conley et al. 2000; Menshikova et al. 2006). Conley et al. (2000), using nuclear magnetic resonance techniques, demonstrated that the loss of oxidative capacity per muscle volume not only reflects the diminished mitochondrial volume but also a reduced capacity of the mitochondria themselves. Recently, Menshikova et al. (2006) have reported a diminished mitochondrial content in aged human skeletal muscle when compared with younger counterparts. In addition, the authors demonstrated that exercise was able to significantly increase the mitochondrial content and function also in aged human skeletal muscle.

Mitochondrial biogenesis

Regular physical exercise is known to influence skeletal muscle mitochondrial content (Hood 2001), even in the aged skeletal muscle (Menshikova et al. 2006). Interestingly, mitochondrial biogenesis can also be induced by a retrograde response mechanism, which is seen as a cellular attempt to surpass the age-related mitochondrial dysfunction (Passos et al. 2007). Mitochondrial biogenesis implies the cellular processes involved in the synthesis and degradation of the organelle (Hood 2001). Mitochondrial homeostasis is controlled by multiple pathways and can be disturbed by impairment of mitochondrial degradation or deregulation of mitochondrial biogenesis (Passos et al. 2007). The understanding of the mechanisms responsible for the decreased number of mitochondria with age as well as the mechanisms responsible for the age-related accumulation of defective mitochondria (Kowald and Kirkwood 2000) are of crucial importance, and the existing concepts are consistent with the idea that the loss of mitochondrial content and function with age might be interpreted as a loss of redundancy within the myofibre. Several studies have demonstrated that human cells containing mutated mtDNA and/or defective mitochondria possess a lower respiratory function and exhibit higher rates of ROS production (Cortopassi and Wong 1999; Esposito et al. 1999). In addition, the extent to which aged skeletal muscle can chronically adapt to physical activity with regard to mitochondrial number, size, and functionality is also fundamental, as this organelle has recently been concerned not only in the context of a diminished capacity for oxidative phosphorylation with age, but also in age-related sarcopenia (Leeuwenburgh 2003; Dirks and Leeuwenburgh 2005).

Contractile activity initiates a series of physiological and biochemical events which trigger mitochondrial biogenesis (Hood 2001). The synthesis of mitochondrial proteins involves the regulated expression of genes originating from nuclear and

mitochondrial genomes. Several transcription factors have been considered in mediating the physiological and metabolic adaptations connected with the expression of these genes. Such include the nuclear respiratory factor-1 and -2 (NRF-1, NRF-2), two peroxisome proliferator-activated receptors (PPAR-α; PPAR-γ), specificity protein 1 (Sp1), and the products of the immediate early genes *c-jun* and *c-fos* (Hood 2001). A considerable amount of evidence implicates alterations of intracellular calcium (Ca^{2+}) and ATP turnover as the initial triggers eliciting the activation of signalling cascades which provoke changes in gene expression (Hood 2001; Adhihetty et al. 2003).

In addition to its crucial role in muscle contraction, Ca^{2+} is recognized as an important second messenger of intracellular signals capable to induce alterations in gene expression (Adhihetty et al. 2003). Elevations in intracellular Ca^{2+} concentrations can activate a number of kinases and phosphatases, namely Ca^{2+}-calmodulin-dependent protein kinases (CaMK), protein kinase C (PKC), and calcineuriun (reviewed in Hood 2001), which will translocate their signals to the nucleus to alter the rate of gene transcription. In this context, the mechanism of mammalian retrograde response in what concerns with mitochondrial biogenesis, despite not being fully characterized, is proposed to be Ca^{2+}-dependent; it has been shown that mitochondrial dysfunction and consequent elevation of cytosolic free calcium seems to induce mitochondrial biogenesis through the activation of Ca^{2+}-calmodulin-dependent protein kinases IV (CaMKIV) (Passos et al. 2007). Alterations in the cellular energy status brought about either by exercise or by mitochondrial uncoupling with the result of a decreased ATP/ADP ratio may be also involved in the induction of mitochondrial biogenesis (Hood 2001). In fact, a signal transduction pathway for mitochondrial biogenesis has been linked with the activation of the AMP-activated protein kinase (AMPK) driven by decreases in phosphocreatine and ATP contents (Adhihetty et al. 2003).

The mitochondrial transcription factor A (Tfam) is the best-known controller of mitochondrial regulation and transcription in mammals (Duguez et al. 2002). Tfam expression is well correlated with alterations in mitochondrial transcriptional activation and oxidative capacity. Gordon et al. (2001) have demonstrated that increases in Tfam mRNA induced by contractile activity are followed by the enhancement of mitochondrial protein and Tfam import from the cytosolic space, which is accompanied by increased mitochondrial transcript levels and an elevated COX enzyme activity. Other transcription factors, like the mitochondrial transcription factor B (mtTFB) and p43, which is a matrix-localized receptor for the thyroid hormone, have also been addressed (reviewed in Hood et al. 2003; Adhihetty et al. 2003).

During mitochondrial biogenesis, organelle synthesis is dependent on the incorporation of nuclear-encoded matrix and membrane proteins (Adhihetty et al. 2003). These nuclear-encoded precursor proteins are targeted to the mitochondria by molecular chaperones, the most important among these are the cytosolic heat-shock protein 70 (Hsp70) and the mitochondrial import stimulation factor (MSF) (Hood 2001; Adhihetty et al. 2003; Hood et al. 2003). Due to the lipophilic nature of the mitochondrial membranes, the import of the nuclear-encoded proteins in appropriate form can only be achieved by protein aqueous channels. These are the translocases of the outer membrane, the so-called Tom complex that serves as an elaborate subunit network to allow the entrance of pre-proteins to the intermembrane space of mitochondria, and the translocases of the inner membrane, the Tim complex that enables the transfer to the matrix (reviewed in Hood 2001; Hood et al. 2003). Both, Tom and Tim protein complexes are located in areas of close contact between the outer and inner membranes, facilitating the transfer of precursors from one complex to another (Hood et al. 2003).

In this context, cardiolipin is also an important contributor for protein translocation across the inner mitochondrial membrane (Hood et al. 2003). It is suggested that this phospholipid (integrated in the inner membrane) orientates the precursor protein into the correct position, ensuring a proper interaction between the immature protein and the Tim44-mtHsp70 complex (Takahashi and Hood 1993). After arrival of the precursor protein and before it is functionally activated, a mitochondrial matrix processing peptidase (MPP) converts it into a mature protein, which binds heat-shock protein 60 (Hsp60) and its chaperonin cpn10, in order to be refolded into its active conformation (Hood 2001).

Contractile activity has been shown to influence both, the mRNA and/or the protein contents of

several transcription factors associated with mitochondrial biogenesis as well as the increased expression of some components of the protein import machinery (reviewed in Hood 2001; Adhihetty et al. 2003; Hood et al. 2003). Furthermore, an increased cardiolipin content described in response to exercise in elderly human skeletal muscle (Menshikova et al. 2006) can also be an important factor in the functionality of the protein import machinery.

Physical exercise has been associated with increased mitochondrial biogenesis in aged individuals (Menshikova et al. 2006) however the extent to which exercise-induced mitochondrial biogenesis will increase the fraction of mutant mitochondria is as yet poorly understood. In addition, a retrograde response, characterized by a nuclear response to mitochondrial dysfunction, with an increased mitochondrial biogenesis, may promote the proliferation of abnormal mitochondria from damaged precursors leading to a greater fraction of dysfunctional mitochondria (Passos et al. 2007). These proliferation of abnormal mitochondria usually described with advanced age can be associated with alterations in the activity of the intramitochondrial proteases, namely the ATP-dependent Lon protease (Bota 2001) and/or with the preferential propagation of defective mitochondria (Terman and Brunk 2004b). It has interestingly been suggested that mutated mitochondria may acquire a replicative advantage over "normal" ones (Terman and Brunk 2004b). In this context, the mitochondrial Lon protease is assumed to play a key role in the clearance of damaged mitochondrial components. Hence, an age-related decline in the activity of this proteolytic system may underlie the accumulation of oxidatively modified and dysfunctional proteins (e.g. oxidatively modified ACON (Bota 2001)), which may be considered as another factor to explain the diminished mitochondrial functionality (Bulteau et al. 2006). In fact, an age-related decreased activity of Lon protease has been reported in mouse liver mitochondria, together with an accumulation of oxidatively modified proteins, which may affect the ability of aging mitochondria to respond to additional stress and compromise cell viability (Bakala et al. 2003). However, it is important to highlight that the accumulation of mutant mitochondria seen at advanced ages may also be the consequence of a decreased degradation of mitochondria due to a diminished autophagic cellular capacity (Kowald and Kirkwood 2000).

Concluding remarks

Complex biological systems, such as the human body, are composed by redundant components, which are crucial to sustain demanding and stressful situations. The age-related loss of redundancy in a particular tissue due to an increased and irreversible accumulation of damage will ultimately lead to a decreased maximal function of the tissue. In this context, aging can be considered as a loss of redundancy in redundant organisms.

Mitochondria are widely accepted to represent the major site of ROS production as well as the prime target of ROS with the consequent age-related increases in oxidative stress levels. It is well documented that the age-related increases in oxidative stress levels and oxidative damage to mitochondrial biomolecules will have deleterious effects on maximal mitochondrial function, which is a consequence of loss of mitochondrial redundancy. Nevertheless, besides being involved in the self-inflicted damage of their components, mitochondria might also have an important role in cellular damage with advancing age.

In addition to the age-related accumulation of oxidatively damaged components, the age-related loss of cellular and mitochondrial adaptative and regenerative capacity must also be taken into consideration. In fact, the inability to maintain mitochondrial homeostasis is a very important feature in cell survival, and an imbalance between the production and removal of damaged components can lead to mitochondrial dysfunction and even cell death (Bota 2001).

Despite the age-related loss of cellular functionality can have major repercussions at the level of mitochondrial redundancy, the diminished mitochondrial function with advancing age will also have an important role in the loss of overall cellular functionality. As we get older, mitochondria and their age-related dysfunction are neither a simple cause nor a consequence of muscular loss of function, but it is rather an organelle that must be seen as an important mediator of multiple cellular processes, not only at the level of the maintenance of cellular homeostasis but also when considering altered metabolic demands, apoptosis and other key regulatory processes of cellular function.

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Experimental Work

Age-Induced Morphological, Biochemical, and Functional Alterations in Isolated Mitochondria from Murine Skeletal Muscle

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Age-Induced Morphological, Biochemical, and Functional Alterations in Isolated Mitochondria From Murine Skeletal Muscle

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Several in vitro studies about age-associated skeletal muscle mitochondrial dysfunction are somewhat conflicting, and this might be related to different normalization procedures. The objective of this study was to normalize the functional and biochemical data per number of mitochondria present in a mitochondrial suspension. Functional and biochemical parameters were obtained in mitochondrial suspensions from murine skeletal muscle of different ages. Mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode. Biochemical analyses included determination of citrate synthase (CS) activity and total protein content in the mitochondrial suspension. Electron microscopy analysis of the suspensions allowed calculation of the number of mitochondria per milligram of protein. Our results conclude that advanced age is associated with mitochondrial dysfunction; moreover, from the correlation between morphological and biochemical data, it is evident that CS activity in the mitochondrial suspensions is a more accurate marker of mitochondrial mass than is total protein content.

Key Words: Aging—Citrate synthase—Respiratory function.

AGING is characterized by a diminished functionality of organs, systems, and tissues of an individual, with a reduced capacity to withstand internal and/or external stimuli and therefore with an increased susceptibility to disease and death (1–3). At the cellular level, aging is also associated with an overall increased dysfunction, ranging from energetic deficits (2,4) to impaired stress tolerance (5,6). This statement suggests that the phenomenon of aging occurs at various levels of organization, from cells to tissues or complete organs. In this context, and regarding skeletal muscle, morphological degenerative alterations of mitochondria have been strongly associated with advancing age (7,8). These age-related alterations could be interpreted as a consequence or, as suggested by others (9,10), as the main cause for the loss of the overall cellular functionality, with an increased susceptibility to damage and death, that parallels chronological age (11–13). The supporters of the mitochondrial theory of aging state that age-related oxidative damage to mitochondria will result in a progressive reduction of mitochondrial bioenergetic capacity, leading to cellular energy deficits and compromising overall cellular functionality (7,10,13,14). However, despite the existence of morphological alterations, data in the literature about age-related mitochondrial dysfunction are somewhat uncertain, not only considering the maximal adenosine 5′-triphosphate (ATP) production rate but also when taking into account the activity of the complexes of the respiratory chain; whereas several authors have reported age-related decreases in mitochondrial function, both in humans (15) and animals (16), others (17,18) have failed to demonstrate that. In fact, some studies argue that mitochondrial capabilities in skeletal muscle are not affected by chronological age and exclude the possibility of a substantial or even moderate decrease in mitochondrial activities concerning the central bioenergetic reactions (18).

In this context, differences in (a) experimental design, (b) number of groups and age comparisons, (c) physical activity levels, and (d) mitochondrial populations are the main factors usually reported in the literature (15,19) to explain these inconclusive results among studies. Nevertheless, it is important to highlight that the methodological errors inherent to the process of tissue preparation and mitochondrial extraction for in vitro studies (18) might also bias the results. In fact, isolation of intact mitochondria from skeletal muscle presents several limitations, because the mitochondria are scarce and surrounded by the myofibrillar structures (20). The homogenization procedure, one of the most important steps in the process of isolation of mitochondria from other components (17), may leave some nonmitochondrial proteins in the mitochondrial suspension. Indeed, a major reason for this lack of consensus regarding the age-related mitochondrial dysfunction could be the assumption of some authors that the mitochondrial suspensions obtained from young and aged muscles contained equivalent amounts of nonmitochondrial protein and thereby similar degrees of contamination. For instance, in aged skeletal muscle, collagen is a likely candidate for excess contamination, as collagen is known to be more abundant in old muscles (21). Taking into account that the mitochondrial biochemical and functional status is frequently reported per total protein

content in suspension, different degrees of contamination with nonmitochondrial proteins will bias the specific activities of isolated mitochondria and compromise the comparisons between various age groups (17).

Bearing this in mind, reference enzymatic activities have been used to normalize enzymatic and functional activities of mitochondria (22,23). Citrate synthase (CS) activity has been widely used as a marker of mitochondrial mass based on the supposition that, being located in the matrix, this enzyme activity would be little affected by the homogenization procedure in intact mitochondria (17). However, despite the fact that the data in the literature assume that changes in mitochondrial content parallel changes in enzyme maximum velocity (V_{max}) (24), there is to our knowledge no morphological study that has validated the utilization of a matrix enzyme, such as CS, as a marker for the morphologically preserved mitochondrial content assessed by transmission electron microscopy (TEM) examination.

Following the above-mentioned considerations, the aim of this study was to estimate the amount of mitochondria in mitochondrial suspensions obtained from young and mature animals. Moreover, expecting different mitochondrial concentrations, we intended as well to correlate the protein content, the concentration of mitochondria, and CS activity to verify which one of the two biochemical parameters (protein content vs CS activity) is the more representative value of the total mitochondrial content in the suspension. A further objective of this study was to perform a functional characterization of skeletal muscle mitochondria normalizing the data to the calculated number of mitochondria used to perform the functional assessment. We hypothesized that, when compared to protein content, CS activity should constitute a better marker of mitochondrial mass in the suspension, thus permitting an augmented accuracy of the results among different groups. Moreover, we also expected that skeletal muscle mitochondrial function was diminished with increased age when data is expressed per number of mitochondria.

Methods

Experimental Design

Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, 1996). Sixteen male C57BL/6 mice were divided into two groups according to their ages: young group (YG, 3 months old; $n = 8$) and mature group (MG, 18 months old; $n = 8$). All animals were kept at constant temperature (21°C–25°C) on a 12-hour light/dark cycle with normal activity until death. Mice were provided with food and water ad libitum and were killed after 1 week of quarantine. The local Ethics Committee had approved the study.

Skeletal Muscle Extraction and Mitochondria Isolation

The animals were killed by cervical dislocation, and the hind-limb muscles (soleus, gastrocnemius, tibialis anterior, and quadriceps) were excised for preparation of isolated mitochondria. Skeletal muscle mitochondria were prepared by conventional methods of differential centrifugation, as previously described by Tonkonogi and Sahlin (25). Briefly, muscles were immediately minced in ice-cold isolation medium containing 100 mM sucrose, 0.1 mM EGTA, 50 mM Tris/HCl, 100 mM KCl, 1 mM KH_2PO_4, and 0.2% bovine serum albumin, pH 7.4. Minced blood-free tissue was rinsed and suspended in 10 mL of fresh medium containing bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma, St. Louis, MO) at 0.2 mg/mL 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. After the extraction of 1 mL of this homogenate for biochemical assessment of CS activity and total protein content in skeletal muscle, the remaining homogenate was fractionated by centrifugation at 700 *g* for 10 minutes. The resulting pellet was removed, and the supernatant suspension was centrifuged at 10,000 *g* for 10 minutes. The supernatant was decanted, and the pellet was gently resuspended in isolation medium (1.3 mL/100 mg initial tissue) and centrifuged at 7000 *g* for 3 minutes. The supernatant was discarded, and the pellet, containing the mitochondrial fraction, was carefully resuspended (0.4 μL/mg initial tissue) in a medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris, and 0.1 mM EDTA, pH 7.4. All mitochondrial isolation procedures were performed at 0°C–4°C. The mitochondrial suspensions were used within 2 hours after the excision of the muscles and were maintained on ice (0°C–4°C) throughout this period.

One aliquot from the mitochondrial suspension was used for biochemical analysis. The remaining mitochondrial suspension was processed for measurement of mitochondrial respiratory activity and for morphological analysis.

Biochemical Analysis in Skeletal Muscle Homogenate and Mitochondrial Fraction

Total protein determination.—Total protein concentration in skeletal muscle homogenate and in the mitochondrial suspension was spectrophotometrically estimated according to Lowry and colleagues (26) and with the biuret method, respectively, using bovine serum albumin as standard.

CS determination.—CS activity was measured according to Coore and colleagues (27) by spectrophotometric (412 nm) measurement of the amount of 5,5-dithiobis (2-nitrobenzoate) that reacted with acetyl-coenzyme A (CoA) upon release from the reaction of acetyl-CoA with oxaloacetate. CS activity was assessed in skeletal muscle homogenate and in the whole mitochondrial suspension after treatment with 0.1% Triton X-100.

Mitochondrial density (mg/g muscle wet wt) was estimated according to Kemer and colleagues (28) with the division of CS activity of skeletal muscle homogenate (U/g wet wt) by the CS specific activity in isolated mitochondrial suspension (U/mg mitochondrial protein). The recovery of mitochondria was measured as CS activity

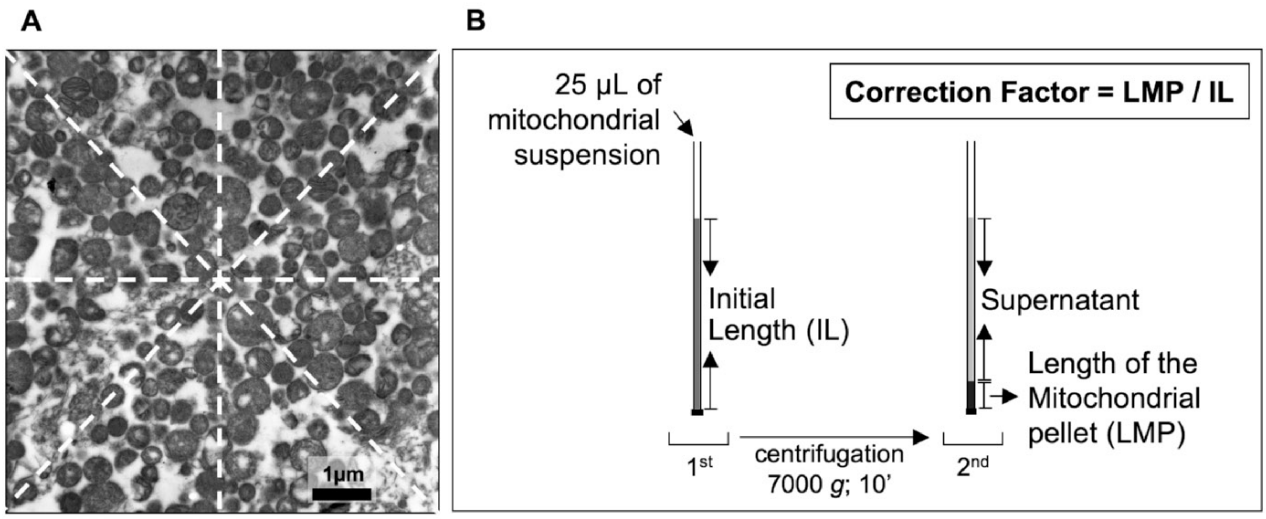

Figure 1. Methodological procedures to quantify the mitochondrial concentration in each suspension. **A,** Number of mitochondria per micrometer was assessed by counting the mitochondria that underlie the four lines crossing the center of the micrograph; the final number of mitochondria per micrometer was established as the mean value of the four counts. The number of mitochondria per square micrometer was evaluated by counting the total mitochondria present in each micrograph. **B,** Assessment of the correction factor for centrifugation-induced compaction, to adjust the data drawn from the microscopic evaluation of the pellet to the real volume of mitochondrial suspension.

in the mitochondrial suspension relative to that in the skeletal muscle tissue homogenate.

Measurement of Mitochondrial Respiratory Activity

Mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode (Hansatech DW 1; Norfork, U.K.). All assays were conducted in a 0.75-mL closed thermostated (25°C) and magnetically stirred glass chamber containing 0.5 mg of protein in a reaction buffer of 225 mM mannitol, 75 mM sucrose, 10 mM Tris, 10 mM KCl, 10 mM K_2HPO_4, and 0.1 mM EDTA, pH 7.5, in accordance with Tonkonogi and colleagues (29). After a 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) or succinate (10 mM) plus rotenone (4 µM). State 3 respiration was determined after adding adenosine diphosphate (ADP) to a final concentration of 200 µM; state 4 respiration was measured as the rate of oxygen consumption in the absence of ADP phosphorylation. The RCR, i.e., the ratio between state 3 and state 4 respiration, and ADP/O were calculated according to Estabrook (30), using 235 nmol O_2/mL as the value for the solubility of oxygen at 25°C. To quantify mitochondrial inner membrane permeability and the maximal rate of uncoupled oxidative phosphorylation, oligomycin (final concentration of 1.5 µg/mL) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; 2 µM), respectively, were added during state 3 respiration with saturated amounts of ADP (final concentration of 1 mM).

Mitochondrial Preparation for TEM

For morphological and morphometric characterization, 100 µL of the mitochondrial suspension was centrifuged at 7000 *g* for 10 minutes, and the resulting pellet was fixed with 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide, dehydrated in graded alcohol, and embedded in LR White. Ultrathin sections mounted on copper grids (300 mesh) were contrasted with uranyl acetate and lead citrate for TEM (Zeiss EM 10A; Carl Zeiss, Oberkochen, Germany) analysis. To obtain a global characterization of the pellet, several grids were prepared (5–8 grids per animal, each one having 3–4 sectioning cuts) from different zones ranging the whole pellet.

Morphometric Analysis of the Mitochondrial Pellet

Morphometric analysis was performed in at least 50 photos per mitochondrial pellet using a morphometric processing program (ImageJ; NIH Image). In each photograph, the areas of mitochondria, their number per micrometer and square micrometer were determined. Taking into account the magnification of each micrograph, the number of mitochondria per micrometer was assessed by counting the mitochondria that overlie four lines that crossed the center of the micrograph, traced horizontally ($n = 1$), vertically ($n = 1$), and obliquely ($n = 2$) (Figure 1A), being the final number of mitochondria per micrometer established as the mean value of the four counts; the number of mitochondria per square micrometer was evaluated by counting the total mitochondria present in each micrograph. For each pellet, the mean number of mitochondria per micrometer and square micrometer was calculated from all analyzed micrographs, and their product was used to calculate the mitochondrial concentration in the pellet (number per cubic micrometer with further adjustment to the number per milliliter).

Afterward, to estimate the mitochondrial concentration in the mitochondrial suspension from the data obtained in the pellet, we assessed the degree of compaction induced by the

Table 1. Body Weights, Skeletal Muscle Wet Weights, and CS Activity in Muscle Homogenate and Isolated Mitochondria From Young and Mature Animals

Measured Parameters	Young	Mature
Body weight, g	24.17 ± 1.77	37.08 ± 1.32*
Dissected skeletal muscle weight (soleus, gastrocnemius, tibialis anterior, and quadriceps), g wet wt	1.90 ± 0.11	1.76 ± 0.14*
Ratio dissected muscle weight/body weight	0.07 ± 0.002	0.04 ± 0.007*
Skeletal muscle CS activity, nmol/min/mg	19.67 ± 5.10	11.57 ± 2.31*
Skeletal muscle mitochondrial density, mg/g muscle wet wt	11.15 ± 4.07	4.53 ± 1.07*
Isolated mitochondria CS activity, nmol/min/mg	254.10 ± 26.9	383.62 ± 10.5*
Recovered mitochondria, %	19.65 ± 5.38	48.97 ± 9.05*

Notes: Data are mean ± standard deviation of eight separate experiments in young and mature animals. Skeletal muscle mitochondrial density was estimated by the division of citrate synthase (CS) activity of skeletal muscle homogenate (U/g wet wt) by the CS specific activity in isolated mitochondrial suspension (U/mg mitochondrial protein). Recovered mitochondria were calculated from the fraction of muscle CS activity recovered in the isolated mitochondria.

*Significantly different from young animals ($p < .05$).

centrifugation procedure using the following method: From each suspension, a small quantity (25 μL) was introduced in a glass capillary tube the tip of which was then closed. Subsequently, the sample was centrifuged at 7000 *g* for 10 minutes and the pellet length was measured (mm, with a 0.1 mm-graded ruler) and divided by the length of the glass capillary tube occupied by the 25 μL of mitochondrial suspension (Figure 1B). This ratio, representing the correction factor necessary to expand each pellet to the length occupied by 25 μL of mitochondrial suspension, was multiplied by the respective mitochondrial concentration calculated from the TEM analysis, to estimate the real number of mitochondria per volume of suspension.

Statistical Analysis

Means and standard deviations were calculated for all variables in both groups. Independent samples *t* test was used to analyze the differences in the variables between groups. The Spearmen correlation coefficient was used to analyze the correlations between total protein determination, CS activity, and concentration of mitochondria using simultaneously the data from all animals. The Statistical Package for the Social Sciences (SPSS version 10.0; Chicago, IL) was used for all analyses. Significance was taken as $p \leq .05$.

Results

Body weights and skeletal muscle CS activity are shown in Table 1. A reduction in skeletal muscle CS activity is apparent in the mature animals, suggesting an impaired skeletal muscle oxidative capacity and mitochondrial mass in the older animals. Likewise, mitochondrial density (mg mitochondrial protein/g skeletal muscle wet wt) was significantly lower in the mature animals.

Skeletal muscle mitochondria were isolated with high levels of structural integrity, both in the young and mature animals as documented by the RCR values (Table 3) and by their morphological appearance in the electron microscopy analysis. When analyzing our mitochondrial suspensions, we found that the morphometric results shown in Table 2 clearly indicate a higher number of these organelles in the suspension of the mature animals, which is in agreement with the CS activity of isolated mitochondria and with the mitochondrial recovery data in both groups (Table 1). Despite the differences in mitochondrial concentration, we did not find significant differences in the mitochondrial areas between the young and the mature animals (YG = 0.16 ± 0.12 μm^2 and MG = 0.11 ± 0.09 μm^2). The qualitative analysis of the electron micrographs revealed the existence of nonmitochondrial material in both groups (Figure 2) suggesting some degree of contamination in the mitochondrial fractions, which was more pronounced in the younger animals. Despite the differences observed in TEM analysis, the total protein content in the mitochondrial suspension was not significantly different between the groups (Table 2).

Table 2. Characterization of the Mitochondrial Suspensions Obtained From Both Age Groups

Measured Parameters	Young	Mature
Protein content, mg/mL	13.86 ± 2.09	15.50 ± 3.77
CS activity, nmol/min/mL	3489.60 ± 343.07	5792.66 ± 594.34*
Mitochondria, $n \times 10^{10}$/mL	2.74 ± 1.2	12.28 ± 3.7*

Notes: Data are mean ± standard deviation. Biochemical data represent eight experiments for each group. The morphometric data represent the counts in at least 50 micrographs of each mitochondrial pellet. After the counts, the correction factor of each sample was applied to estimate the concentration of mitochondria in the suspension (number of mitochondria/mL).

*Significantly different from young animals ($p < .05$).

CS = citrate synthase.

Concerning the correlation between the protein content in the mitochondrial fraction, CS activity of isolated mitochondria, and the morphometric data, strong correlations were found between CS activity and the number of mitochondria ($r = 0.815$; $p < .05$) and a somewhat lower, yet significant correlation was obvious between protein content and CS activity ($r = 0.608$; $p < .05$) as well as between protein content and the number of mitochondria ($r = 0.324$; $p < .05$).

Characterization of Skeletal Muscle Mitochondria

Biochemical data concerning CS activity normalized to the number of mitochondria is presented in Figure 3. We found a significantly diminished CS activity in the mitochondria isolated from the mature animals when data was expressed per mitochondrium, suggesting that isolated mitochondria from mature animals present a reduced oxidative capacity when compared to their younger counterparts.

Functional data obtained from the isolated mitochondria of young and mature animals, namely the state 3 and state 4 respiratory rates as well as the RCR and the ADP/O values are shown in Tables 3 and 4.

It appears obvious that mature animals evidence a significant impairment in the respiratory rates of isolated skeletal muscle mitochondria energized with malate + pyruvate

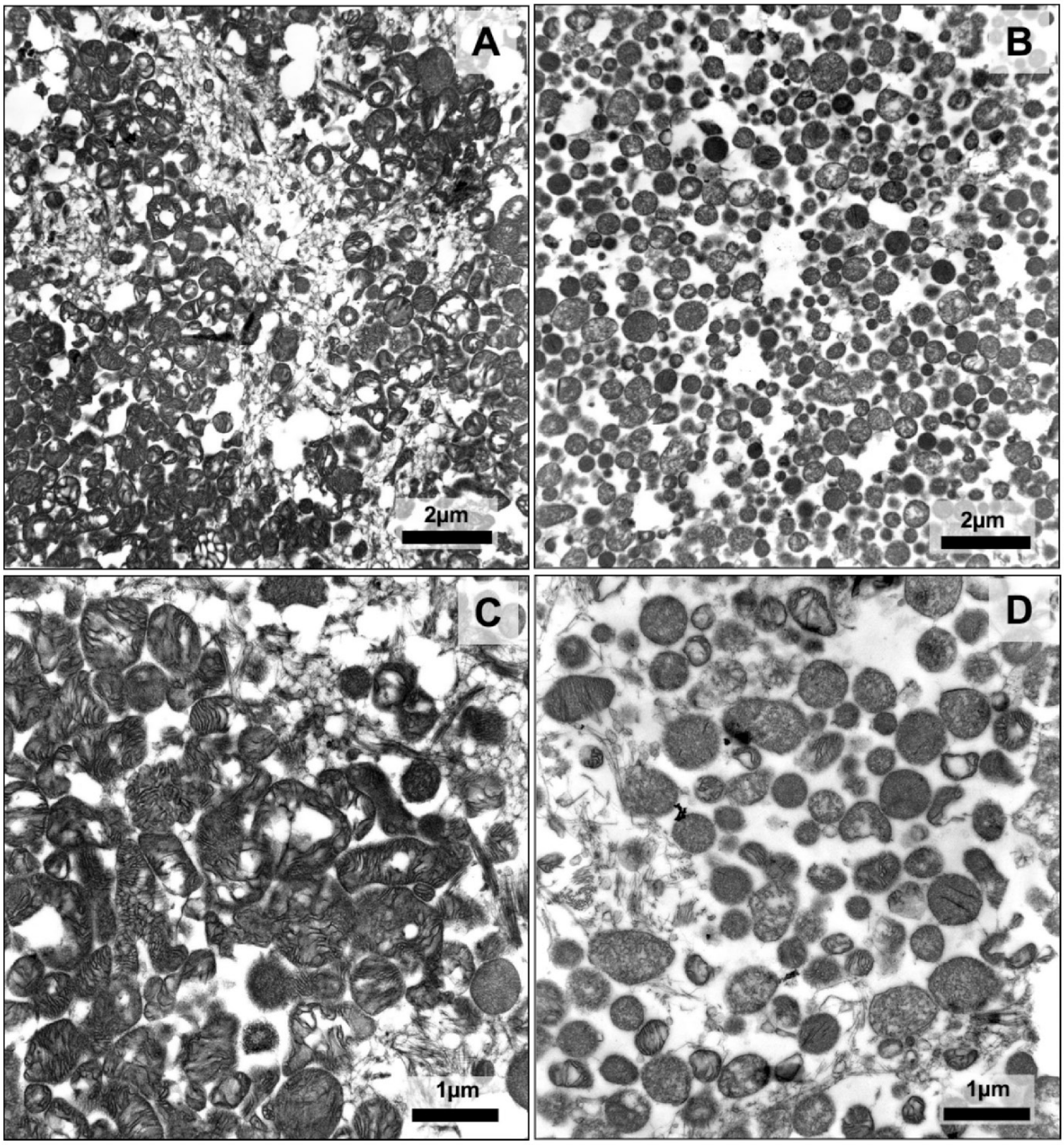

Figure 2. Electron micrographs of mitochondrial pellets obtained from skeletal muscle of young (**A** and **C**) and mature animals (**B** and **D**). **A** and **C,** Note that mitochondria from young animals present a normal structure with high variability in their shape and dimensions. Their cristae are numerous, clearly visible, and well defined. In the intermitochondrial space, several membrane-like cellular debris as well as various kinds of grouped filamentous structures are visible, suggestive for myofibril-like structures. **B** and **D,** Mitochondria from mature animals also present a high variability of dimensions; however, their shape appears more constant and regular, but their cristae are less apparent and not well defined. The intermitochondrial space also reveals the existence of some membranous and filamentlike cellular debris.

(M+P) and succinate + rotenone (S+R), irrespective of whether data are expressed per protein content, per CS activity, or per mitochondrium. This diminished state 3 respiration in the mature animals is somewhat evident with the complex I–linked substrates (–57.68% and –52.20% variation with complex I substrates and complex II substrates, respectively, with data normalized to CS activity). In contrast, state 4 respiration has not been significantly

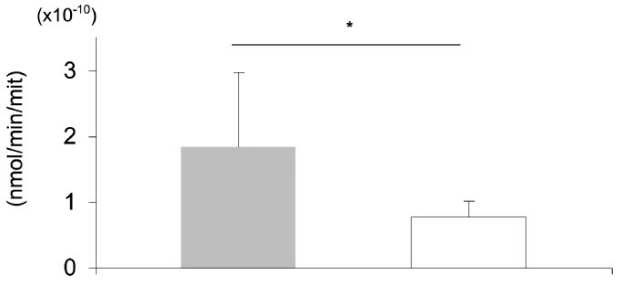

Figure 3. Effect of age on citrate synthase activity when expressed per mitochondrium. Data are mean ± standard deviation and are expressed as nmoL/min/mitochondria. *Filled bar:* young animals; *open bar:* mature animals. *Significantly different from young animals ($p < .05$).

affected by increased age. The RCR values decreased significantly in the older animals, when using both M+P and S+R as respiratory substrates. On the contrary, the ratio of ADP to oxygen consumption showed no alterations with age. Moreover, the RCR data, as well as the state 3 and state 4 respiratory rates with M+P and S+R, suggest that the functional impairment is mainly targeted at the state 3 respiratory rates. These data are further supported by the results of the oligomycin-inhibited state 3 respiration and CCCP-induced uncoupled respiration, shown in Figure 4.

In fact, mitochondria from the mature animals energized with M+P and stimulated with excess ADP showed no significant alterations of the respiratory rate in the presence of oligomycin (state 4) when compared with their younger counterparts. In contrast, uncoupled mitochondrial respiration with CCCP was significantly diminished in the mature animals.

Discussion

The main findings of the present study clearly indicate that mitochondria isolated from skeletal muscle of mature mice present an altered morphological, biochemical, and functional status when compared with younger animals. The TEM analysis of the mitochondrial pellets revealed distinct degrees of impurity in both groups—the young and the mature animals—throughout the complete pellet (Figure 2). In fact, different degrees of nonmitochondrial material were evident in the mitochondrial pellets of both groups, which could lead to an overestimation of the presumed mitochondrial protein determination. Micrographs obtained from the mitochondrial pellets of the young animals (Figure 2) presented some myofibril-like structures and other nonmitochondrial material between the mitochondria. In contrast, the existence of nonmitochondrial material in the micrographs obtained from the older animals was not so evident. In this context, despite the fact that collagen fibers have been identified as potential candidates for an excess contamination of the skeletal muscle mitochondrial suspensions drawn from older animals (21), the analysis of the TEM micrographs did not substantiate this assumption. Moreover, our morphometric results showed clearly that, for the same amount of total protein, the mitochondrial suspensions originated from the mature animals had a higher number of mitochondria when compared with the mitochondrial suspensions from the younger animals (Table 2). These results are supported by the higher mitochondrial recovery data in the mature animals (19.65 ± 5.38% and 48.97 ± 9.05% for young and mature animals, respectively) when compared with their younger counterparts. In fact, it has been suggested that the abundance of myofibrillar proteins may complicate the extraction of mitochondria from skeletal muscle and thereby reduce the mitochondrial yield (31), which could explain the lower mitochondrial yields observed in the younger animals. In contrast, the higher number of mitochondria in the suspensions obtained from the mature animals could not be explained by differences in the sedimentation coefficients of the mitochondria, because it had been demonstrated that the ranges of particle size and density are essentially unchanged with age (18). Although it is claimed that mitochondria are somewhat bigger in aged tissues (32), our results failed to demonstrate such when considering the mitochondrial areas. Moreover, the determination of the protein content in the mitochondrial suspensions did not reveal any significant

Table 3. Functional Data Obtained From Skeletal Muscle Mitochondria Isolated From Young and Mature Animals With Complex I–Linked Substrates, Pyruvate (5 mM), and Malate (2 mM)

Measured Parameters	Young	Mature
State 3		
(nmol O_2/min/mg)	160.9 ± 15.06	104.8 ± 10.55*
(nmol O_2/CS)	0.638 ± 0.07	0.270 ± 0.02*
(nmol O_2/min/mit)	$11.5 \times 10^{-8} \pm 6.2 \times 10^{-8}$	$2.15 \times 10^{-8} \pm 0.7 \times 10^{-8}$*
State 4		
(nmol O_2/min/mg)	18.1 ± 2.05	16.9 ± 1.72
(nmol O_2/CS)	0.071 ± 0.004	0.044 ± 0.004
(nmol O_2/min/mit)	$13.1 \times 10^{-9} \pm 8.0 \times 10^{-9}$	$3.46 \times 10^{-9} \pm 1.2 \times 10^{-9}$
RCR	8.9 ± 0.82	6.2 ± 0.96*
ADP/O	2.1 ± 0.54	1.9 ± 0.13

Notes: Data are mean ± standard deviation and represent eight separate experiments in each group. State 3 and state 4 respiratory rates are expressed as nmol O_2 consumed/min/mg of protein, as nmol O_2 consumed/citrate synthase (CS) activity, and as nmol O_2 consumed/min/mitochondria (mit).

*Significantly different from young animals ($p < .05$).

RCR = respiratory control ratio; ADP/O = number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed.

Table 4. Functional Data Obtained From Skeletal Muscle Mitochondria Isolated From Young and Mature Animals With Complex II–Linked Substrates, Succinate (10 mM), and Rotenone (4 μM)

Measured Parameters	Young	Mature
State 3		
(nmol O_2/min/mg)	187.6 ± 24.33	136.9 ± 10.97*
(nmol O_2/CS)	0.747 ± 0.138	0.357 ± 0.026*
(nmol O_2/min/mit)	$13.6 \times 10^{-8} \pm 7.7 \times 10^{-8}$	$2.80 \times 10^{-8} \pm 0.8 \times 10^{-8}$*
State 4		
(nmol O_2/min/mg)	91.7 ± 3.29	80.8 ± 4.54
(nmol O_2/CS)	0.364 ± 0.043	0.210 ± 0.011
(nmol O_2/min/mit)	$6.64 \times 10^{-8} \pm 3.7 \times 10^{-8}$	$1.65 \times 10^{-8} \pm 0.5 \times 10^{-8}$
RCR	2.0 ± 0.19	1.7 ± 0.18*
ADP/O	0.9 ± 0.31	0.9 ± 0.05

Notes: Data are mean ± standard deviation and represent eight separate experiments in each group. State 3 and state 4 respiratory rates are expressed as nmol O_2 consumed/min/mg of protein, as nmol O_2 consumed/citrate synthase (CS) activity, and as nmol O_2 consumed/min/mitochondria (mit).

*Significantly different from young animals ($p < .05$).

RCR = respiratory control ratio; ADP/O = number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed.

differences between the groups, which is in contrast with the morphometric results and the mitochondrial CS activity data, but is consistent with the idea of some degree of contamination in the suspensions. The higher content of mitochondria in the suspension obtained from the mature animals was confirmed by a correspondingly higher CS activity and supports the utilization of this enzyme as a marker of mitochondrial mass. In fact, it is suggested that CS activity, being located in the matrix, would be little affected by the homogenization procedure in intact mitochondria, and it can therefore be assumed as a good choice for a reference activity in mitochondrial assays (17). To our knowledge, this study is the first to demonstrate, with the utilization of TEM analysis, that when compared with the total protein content, CS activity is a better marker for the mitochondrial fraction in suspension obtained from skeletal muscle tissue. This issue could be particularly important when comparatively analyzing different groups (e.g., young vs old, trained vs untrained).

The correlation coefficients between the CS activity and the morphometric data indicate statistically significant and relevant correlation coefficients between the mitochondrial CS activity and the number of mitochondria, which reinforces the utilization of CS activity as a marker of mitochondrial content. Likewise, we have also found significant correlation coefficients, although lower, between protein content and CS activity, as well as between protein content and the morphometric data, which may be explained by the fact that, despite not statistically significant, the total protein content of the mitochondrial suspensions follows the same tendency as the CS activity and the morphometric results, suggesting that the majority of proteins in the mitochondrial suspensions certainly is of mitochondrial origin. Nevertheless, when expressed per mitochondrium, CS activity was significantly lower in the mature animals. In accordance with the well-described role of CS as an indicator of bioenergetic oxidative capacity, these data suggest that individual mitochondria from the mature animals have a diminished oxidative capacity. Considering this, CS activity could also present some limitations when studying different age groups; however, despite the above-mentioned limitation, our results strongly suggest that the utilization of CS as a marker of mitochondrial mass, to establish a reference activity, would be a more accurate approach than the simple utilization of the total protein content of a determined mitochondrial suspension.

Isolation of intact skeletal muscle mitochondria is a very delicate process in which any artificial damage will induce structural alterations and functional impairments, namely related with the activities associated with oxidative phosphorylation, specifically the state 3 respiration and the RCR values (33). The TEM qualitative analysis concerning the structural integrity of the mitochondria population isolated from the skeletal muscles of young and mature animals revealed that they were isolated with high integrity, and this was further supported by the RCR values (Table 3), indicating that the organelle damage induced by the isolation procedure was negligible. In fact, RCR values obtained both in the young and in the older animals were comparable with other data reported elsewhere on mitochondria isolated from skeletal muscle of different ages (34). However, the mitochondria isolated from skeletal muscle of mature animals, when compared with mitochondria from the younger animals, presented an altered morphological appearance, with regard to their shape and cristae ultrastructure. In this context, several age-related alterations in mitochondrial morphology have been reported, like matrix vacuolization and shortened cristae (7). In addition, when considering the mitochondrial areas, as previously mentioned, our results have failed to demonstrate differences between groups.

The functional assessment of skeletal muscle mitochondria in both groups revealed a general decline in the functionality of this organelle in the mature animals, which is in accordance with several studies demonstrating that mitochondrial function is compromised with advancing age, both in humans (15,34) and mice (16). In the present study, mitochondria from the older animals exhibited a significant decline in the state 3 respiratory rate, both with complex I- and complex II-linked substrates (Tables 3 and 4), which

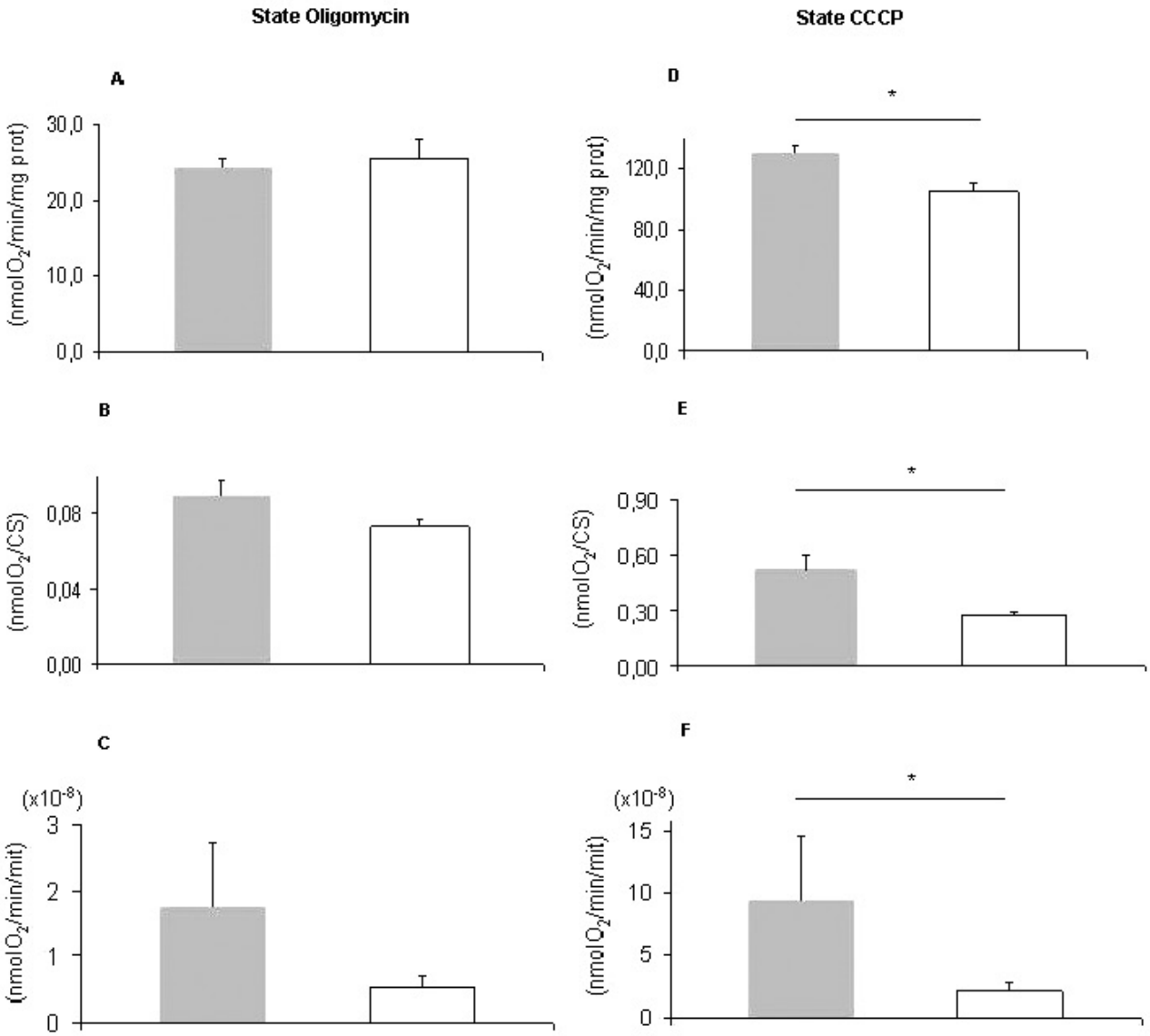

Figure 4. Effect of age on oligomycin-inhibited state 3 respiration (**A–C**) and carbonyl cyanide m-chlorophenylhydrazone (CCCP)-induced uncoupled respiration (**D–F**) in skeletal muscle mitochondria isolated from young and mature animals. Data are mean ± standard deviation and are expressed as nmol O_2 consumed/min/mg of protein (**A** and **D**), as nmol O_2 consumed/CS activity (**B** and **E**), and as nmol O_2 consumed/min/mitochondria (**C** and **F**). Respiration was induced with pyruvate (5 mM) and malate (2 mM) as energizing substrates and saturated ADP concentration (1 mM) to initiate state 3 respiration. State 3 was inhibited after the addition of oligomycin (final concentration 1.5 μg/mL) and CCCP (final concentration 2 μM) to uncouple mitochondrial respiration. *Filled bars:* young animals; *open bars:* mature animals. *Significantly different from young animals ($p < .05$).

indicates that the maximal rate of mitochondrial oxygen consumption was affected by age. The functional impairment at the level of state 3 respiration was slightly higher in the complex I-linked substrate assay, which suggests that complex I-mediated respiration was more affected than succinate-supported respiration. In fact, the activities of mitochondrial electron transport chain (ETC) complexes—namely complex I, III, and IV—have been reported to decrease with age, whereas complex II appears to be unaffected (13). This could be related to the fact that many of the subunits of complex I, III, and IV of the mitochondrial ETC are encoded by mitochondrial DNA (mtDNA), whereas complex II is encoded by nuclear DNA (35). In this context, the age-related increases in oxidative stress and mitochondrial oxidative damage levels, supported by several studies (16,36), can induce significant oxidative damage to the mtDNA that could have serious implications in age-related mitochondrial heteroplasmy and dysfunction (7,13,37). In addition, complex I activity might be affected because of its particular vulnerability to oxidative attack, due to its higher content of Fe-S clusters (13). In contrast to the above-mentioned state 3 respiration data, we have failed to

demonstrate significant differences in state 4 respiration between mitochondria isolated from both groups. In this respect, it appears that the permeability of the inner mitochondrial membrane was not affected by age.

To further confirm the above-mentioned results, we have performed an additional mitochondrial assay, where we used oligomycin and CCCP in mitochondria previously energized with M+P and stimulated with ADP. This assay avoids the possible interference of the permeability to protons through the ATP synthase on state 4 conditions, and ensures that the variations in membrane permeability do not interfere with the inhibition of the respiratory chain, because the permeability in the presence of CCCP is always maximal (38). In this context, the maximal rate of uncoupled respiration was significantly reduced in the mature animals confirming the state 3 respiration data and further supporting that increased age is associated with a decreased maximal functional capacity of the respiratory chain. In contrast, the respiration in the presence of oligomycin was not altered by age in these animals, suggesting that the integrity of the inner mitochondrial membrane was not affected, which is in accordance with the state 4 respiration results previously presented.

Concerning the RCR values, mitochondria from mature animals presented significantly decreased RCR when compared with their younger counterparts, which is consistent with the idea that advancing age might affect the coupling between the ETC and oxidative phosphorylation. Bearing in mind that RCR is a functional variable that represents the functional and structural integrity of respiring mitochondria (38), this result suggests that mitochondria from mature animals are less functional than those from the younger animals. This is mainly a consequence of the decrease in state 3 respiration in the mitochondria from the older animals, as state 4 was unchanged. In contrast, the mitochondrial phosphorylation efficiency (ADP/O ratio) did not appear to be affected by age, which indicates that oxidative phosphorylation was not altered in the mitochondria from the mature animals. Therefore, we might hypothesize that the functional impairments in the mitochondria from aged animals were mainly related to decreased activities of the complex I– and complex II-supported respiration, with little or no functional impairments at the level of the ATP synthase complex.

Summary

Our data suggest that increased age is associated with morphological, biochemical, and functional alterations in isolated skeletal muscle mitochondria. Moreover, the CS activity measured in the mitochondrial suspensions appears to be a more accurate marker of the mitochondrial mass when compared with the determination of the protein content.

Acknowledgments

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Aging Impairs Skeletal Muscle Mitochondrial Bioenergetic Function

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This study investigated the influence of age on the functional status of mitochondria isolated from skeletal muscle of C57BL/6 mice aged 3 and 18 months. We hypothesized that skeletal muscle mitochondria isolated from aged animals will exhibit a decreased respiratory function. Mitochondrial respiratory functional measures (ie, State 3 and 4 respiration, respiratory control ratio and number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondrion) and biochemical markers of oxidative damage (aconitase activity, protein carbonyl derivatives, sulfhydryl groups, and malondialdehyde) were measured in isolated mitochondrial suspensions. Along with traditional tests of mitochondrial function, an in vitro repetitive ADP-stimulation test was used to evaluate the mitochondrial capacity to reestablish the homeostatic balance between successive ADP stimulations. The number of mitochondria per mitochondrial suspension, calculated by transmission electron microscopy, was used to normalize functional and biochemical data. Our results confirm the existence of an age-associated decline in mitochondrial function of mixed skeletal muscle, which is significantly correlated with higher levels of mitochondrial oxidative damage.

Key Words: Aging—Mitochondria—Respiratory function.

AGING is characterized by a progressive decline in cellular function and a decrease in the cell's ability to maintain homeostasis. In this regard, it is clear that mitochondria have a critical role in maintaining cellular homeostasis as mitochondria are involved in cellular bioenergetics, production of reactive oxygen species (ROS), the maintenance of calcium homeostasis, and the induction of apoptotic processes (1,2). Several reports suggest that mitochondria might be among the most adversely affected organelles with advanced age (3,4). For example, a decline in mitochondrial bioenergetic potential and an increase in the oxidative damage to mitochondrial biomolecules occur with aging in many tissues (5–8). An age-related decline in mitochondrial bioenergetic function could contribute to a loss of cell viability, leading to an increase in cellular necrosis and/or apoptosis (9–12).

Although it is well established that aging results in loss of skeletal muscle contractile dysfunction and mass, the role that mitochondrial damage has in this phenomenon remains controversial. For example, although several reports suggest that mitochondria isolated from aged skeletal muscle exhibit impaired morphological, biochemical, and functional alterations (13–18), other investigators have failed to demonstrate age-related changes in mitochondrial phenotypes in humans (21,22,25) and other animals (23,24). These conflicting results could be due to several methodological issues associated with the study of isolated mitochondria along with differences in the ages of the animals investigated. A key methodological issue relates to the procedures used to isolate mitochondria (21). For example, protocols designed to isolate intact mitochondria from muscle do not produce a mitochondrial fraction with high purity, leading to contamination with nonmitochondrial proteins. Therefore, normalization of mitochondrial functional measures to the protein content of the mitochondrial suspension could bias these results. In an effort to avoid this pitfall, several authors have measured the level of mitochondrial matrix enzymes, such as citrate synthase (CS), as a marker of mitochondrial mass (21,26). However, even when mitochondrial data are expressed per CS activity, conflicting results still persist (21); this discrepancy could be due, in part, to CS contamination produced by mitochondrial damage associated with the isolation procedures.

Another problem that could contribute to the conflicting results is related to the sensitivity and specificity of the in vitro assays used to evaluate mitochondrial function. Indeed, measurements performed in energized mitochondria in vitro are typically standardized to evaluate the maximal functionality of several components within isolated mitochondria, such as the electron transport chain (ETC) complexes or the ATP synthase and the integrity of the inner membrane. The main objective of these assays is to acutely stimulate the previously energized mitochondria and thereby to assess their maximal functionality during a brief period of time. However, considering the in vivo physiology of muscle contraction, it is predicted that prolonged alterations of the ATP-to-ADP ratio resulting from the continuous actin–myosin interaction (27–29) would impose a continuous overload to the mitochondria with a prolonged State 3. Therefore, the in vivo bioenergetic function of skeletal muscle mitochondria appears to be very different from the metabolic stress transitorily imposed by the traditional in vitro

tests. It follows that these routinely used in vitro mitochondrial tests might not be sensitive or specific enough to detect age-related alterations in skeletal muscle mitochondrial function.

A final issue that could contribute to the variance in the literature concerning the impact of age on mitochondrial function is the wide variation in animal ages between studies. That is, age per se might also constitute an additional problem because the occurrence of age-related concomitant disease in very old animals will bias the results (30). Therefore, it is important to study aging itself without the influence of other confounding factors, such as diseases.

Therefore, the objective of the present study was to investigate the influence of age on the functional status of mitochondria isolated from skeletal muscle avoiding the aforementioned confounding factors that may influence the results. To avoid the methodological issues associated with the isolation of mitochondria, we normalized all mitochondrial functional and biochemical measures to the number of mitochondria quantified by transmission electron microscopy in the final mitochondrial suspension. Moreover, to mimic the bioenergetic function of skeletal muscle mitochondria during muscular contractions, we have developed a maximal standardized in vitro protocol that involves successive ADP stimulations of mitochondrial State 3 respiration. Finally, to avoid the pitfalls associated with studying old animals that are diseased, our experimental design incorporated mature animals. We tested the hypothesis that compared with young animals, mitochondria isolated from aged mature animals will exhibit a decreased respiratory function. If our experiments reveal that aging results in a decline in mitochondrial function, we also predict that this age-related decline will be associated with increased mitochondrial oxidative stress.

METHODS

Experimental Design

Sixteen male C57BL/6 mice were divided into two groups according to their ages: young group (3 months old; $n = 8$) and aged mature group (18 months old; $n = 8$). Based on the survival curves for these animals (31), 18-month-old animals can be classified as both aged and mature. The option to use aged mature instead of old senescent animals was based on the assumption that the occurrence of any age-related concomitant disease might bias the results. Mice were provided with food and water ad libitum and were sacrificed after 1 week of quarantine. All animals were maintained at a constant temperature (21°C–25°C) on a daily light schedule of 12 hours of light versus dark until sacrifice. Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR 1996). The local ethics committee approved the study, and experiments complied with national laws.

Animal Killing, Skeletal Muscle Extraction and Mitochondria Isolation

Animals were killed by cervical dislocation, and the hindlimb muscles (soleus, gastrocnemius, tibialis anterior, and quadriceps) were excised for preparation of isolated mitochondria by conventional methods of differential centrifugation, as previously described by Tonkonogi and Sahlin (32). Briefly, after weighing, the muscles were immediately minced in ice-cold isolation medium containing 100 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 50 mM Tris–HCl, 100 mM KCl, 1 mM KH_2PO_4, and 0.2% bovine serum albumin (BSA), pH 7.4. The 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, St Louis, MO) and stirred for 2 minutes. The sample was then homogenized with a tightly fitted Potter–Elvehjen homogenizer and a Teflon pestle. After homogenization, three volumes (30 mL) of Nagarse-free isolation medium were added to the homogenate followed by centrifugation at 700*g* for 10 minutes. The resulting pellet was removed, and the supernatant was resuspended and centrifuged at 10,000*g* for 10 minutes. The supernatant was decanted, and the pellet was gently resuspended in isolation medium (1.3 mL/100 mg initial tissue) and centrifuged at 7,000*g* for 3 minutes. The supernatant was discarded, and the final pellet, containing the mitochondrial fraction, was gently resuspended (0.4 μL/mg initial tissue) in a medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris, and 0.1 mM EDTA, pH 7.4. Total protein concentration in the mitochondrial suspension was estimated spectrophotometrically with the biuret method using BSA as standard. All mitochondrial isolation procedures were performed at 0°C–4°C. The mitochondrial suspensions were studied within 2 hours after the excision of the muscles and were maintained on ice (0°C–4°C) throughout this period.

One aliquot from the final mitochondrial suspension was used for measurement of total protein concentration and CS activity in the mitochondrial suspension. A second aliquot was processed for morphological analysis. Another aliquot was used for measurement of mitochondrial respiratory function using standard in vitro methods. The remaining mitochondrial suspension was used to assess functional and biochemical parameters during an in vitro ADP-consecutive stimulation test that incorporates one, three, and six ADP pulses interspersed by a 90-second recovery between stimulation trying to mimic the prolonged cellular metabolic demands during exercise. The utilization of this in vitro–simulated exercise test was designed to evaluate the capacity of mitochondria to reestablish their homeostatic balance between consecutive ADP stimulations as function of time. All the biochemical parameters were assessed in the whole content

of the oxygen chamber following treatment with 0.1% Triton X-100.

Biochemical Analysis in Mitochondrial Fraction

Total protein determination.—Total protein concentration in the mitochondrial suspension was determined using the biuret method with BSA as the standard.

CS assay.—CS activity was measured according to Coore and coworkers (33) by spectrophotometric (412 nm) measurement of the amount of 5,5-dithiobis (2-nitrobenzoate) that reacted with acetyl-CoA upon release from the reaction of acetyl-CoA with oxaloacetate. CS activity was assessed in the mitochondrial suspension after treatment with 0.1% Triton X-100.

Mitochondrial Preparation for Transmission Electron Microscopy (TEM)

Mitochondrial preparation for TEM analyses and further morphometric characterization has been previously described (18). Briefly, 100 μL of the mitochondrial suspension was centrifuged at 7,000*g* for 10 minutes, and the resulting pellet was fixed with 2.5% glutaraldehyde, post-fixed with 2% osmiumtetroxide, deyhdrated in graded alcohol, and embedded in LR White. Ultrathin sections mounted on copper grids (300 Mesh) were contrasted with uranyl acetate and lead citrate for transmission electron microscopy (Zeiss EM 10A) analysis. In order to obtain a global characterization of the pellet, several grids were prepared (five to eight grids per animal each containing three to four sections) from different zones ranging through the whole pellet.

Morphometric Analysis of the Mitochondrial Pellet

Morphometric analysis was performed as described elsewhere (18) in at least 50 photos per mitochondrial pellet using a morphometric processing program (ImageJ, NIH Image). In each photograph, the number of mitochondria per micrometer and square micrometer were determined. Taking into account the magnification of each micrograph, the number of mitochondria per micrometer was assessed by counting the mitochondria which were situated under the four lines that crossed the center of the micrograph, traced horizontally ($n = 1$), vertically ($n = 1$), and obliquely ($n = 2$) (Figure 1A), resulting in the final number of mitochondria per micrometer as the mean value of the four counts; the number of mitochondria per square micrometer was evaluated by counting the total mitochondria present in each micrograph. For each pellet, the mean number of mitochondria per micrometer and square micrometer was calculated from all analyzed micrographs and their product was used to calculate the mitochondrial concentration in the pellet (number per cubic micrometer with further adjustment to the number per milliliter).

Afterward, in order to estimate the mitochondrial concentration in the mitochondrial suspension from the data obtained in the pellet, we assessed the degree of compression induced by the centrifugation procedure using the following method. In each suspension, a small quantity (25 μL) was introduced in a glass capillary tube followed by closure of one end of the tube. Subsequently, the sample was centrifuged at 7,000*g* for 10 minutes and the pellet size was measured (mm, with a 0.1-mm graded ruler) and related to the length of the glass capillary tube occupied by the 25 μL of mitochondrial suspension (Figure 1B). This relation, representing the correction factor necessary to expand each pellet to the length occupied by 25 μL of mitochondrial suspension, was multiplied to the respective mitochondria concentration calculated from the TEM analysis, in order to estimate the real number of mitochondria per volume of suspension. The number of total mitochondria per unit of mitochondrial suspension was then used to normalize all functional and biochemical data.

Measurement of Mitochondrial Respiratory Activity

Traditional tests.—After the determination of the total protein concentration in the mitochondrial suspension (estimated spectrophotometrically with the biuret method using BSA as standard), the mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode (DW 1, Hansatech, Norfork, UK). All assays were conducted in a 0.75-mL closed, magnetically stirred, and temperature-controlled (25°C) glass chamber. Mitochondria were added to the chamber (0.5 mg of protein) in a reaction buffer of 225 mM mannitol, 75 mM sucrose, 10 mM Tris, 10 mM KCl, 10 mM K_2HPO_4, and 0.1 mM EDTA, pH 7.5 (34). After a 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) or succinate (10 mM) plus rotenone (4 μM). State 3 respiration was determined after adding ADP to a final concentration of 200 μM; State 4 respiration was measured as the rate of oxygen consumption in the absence of ADP phosphorylation. The respiratory control ratio (RCR), that is, the ratio between State 3 and State 4 respiration, and number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondrion (ADP/O) were calculated according to Estabrook (19), using 235 nmol O_2/mL as the value for the solubility of oxygen at 25°C. To quantify mitochondrial inner membrane permeability and the maximal rate of uncoupled oxidative phosphorylation, oligomycin (final concentration of 1.5 μg/mL) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; 2 μM), respectively, were added during State 3 respiration with saturated amounts of ADP (final concentration of 1 mM). This protocol avoids interference of the permeability to protons through the ATP synthase during State 4 respiration (state oligomycin)

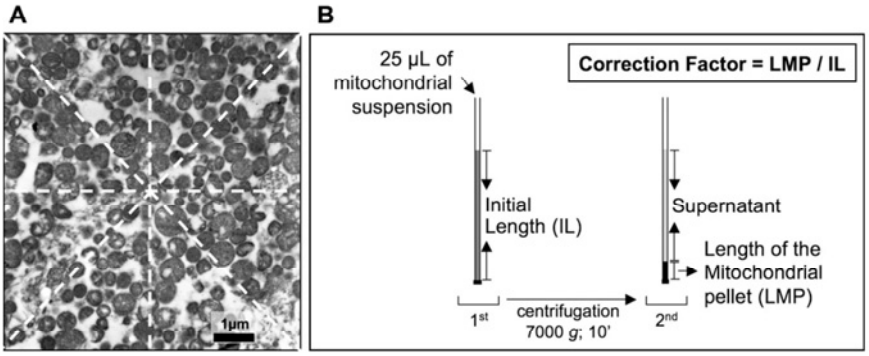

Figure 1. Methodological procedures to quantify the mitochondrial concentration in each suspension. (**A**) The number of mitochondria per micrometer was assessed by counting the mitochondria that were situated under the four lines crossing the center of the micrograph; the final number of mitochondria per micrometer was established as the mean value of the four counts. The number of mitochondria per square meter was evaluated by counting the total mitochondria present in each micrograph. (**B**) Assessment of the correction factor for centrifugation-induced compaction, in order to adjust the data drawn from the microscopic evaluation of the pellet to the real volume of the mitochondrial suspension.

and ensures that the variations in membrane permeability do not interfere with the inhibition of the respiratory chain, because the permeability in the presence of CCCP is always maximal (20).

Consecutive ADP-stimulation test.—In order to mimic the in vivo metabolic stress imposed to mitochondria during muscular exercise, we have performed a consecutive ADP-stimulation test in a Clark-type oxygen electrode (Hansatech DW 1). This in vitro test consisted of four assays (Figure 2)—Assay I: after a 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30 seconds of stabilization; Assay II: after a 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30 seconds of stabilization and the addition of one pulse of ADP followed by 1 minute 30 seconds; Assay III: after a 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30 seconds of stabilization and the addition of three consecutive pulses of ADP interspersed by 1 minute 30 seconds each; Assay IV: after a 1-minute equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30 seconds of stabilization and the addition of six consecutive pulses of ADP interspersed by 1 minute 30 seconds each.

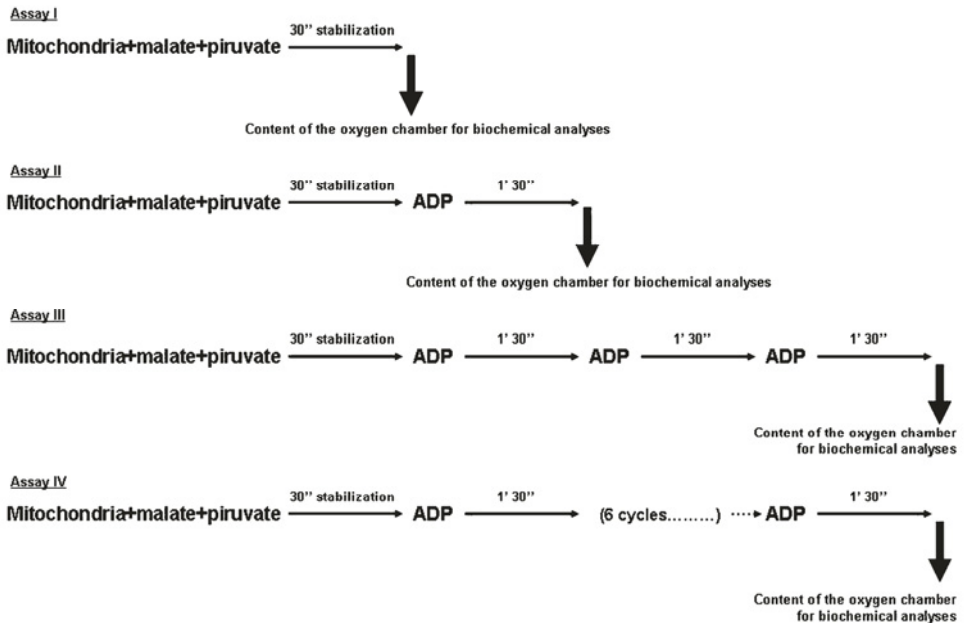

Figure 2. Repetitive ADP-stimulation test. See text for detailed description.

In Assays II, III, and IV, mitochondrial respiratory function was assessed by analyzing several functional parameters (ie, State 3 and State 4 respiratory rates, the RCR and ADP/O) in each of the ADP cycles. At the end of each of the four assays, the content of the oxygen chamber was collected and used for biochemical analysis (Figure 2). The content of the oxygen chamber at the end of Assay I represents the mitochondrial basal condition (prestimulation); the content of the oxygen chamber after Assays II, III, and IV corresponds to different degrees of ADP stimulation. In all assays a certain quantity of the oxygen chamber (500 μL) was used for the determination of protein carbonyl derivatives, total mitochondrial sulfhydryl (SH) groups, and malondialdehyde (MDA). Furthermore, a portion of the oxygen chamber (250 μL) was centrifuged for 15 minutes at 10,000*g* in order to determine the content of extramitochondrial cytochrome *c*. Aconitase (ACON) activity was also quantified in Assay I in order to indirectly assess the age-related superoxide radical generation at basal conditions.

ACON activity.—The activity of ACON was assayed because ACON activity is redox sensitive and diminished ACON activity has been interpreted as an index of superoxide radical generation and mitochondrial oxidative damage (35). ACON activity was measured spectrophotometrically by monitoring the formation of *cis*-aconitate at 240 nm after the addition of 20 mM isocitrate at 25°C, according to Krebs and Holzach (36). One unit of activity was defined as the amount of enzyme necessary to produce 1 μM *cis*-aconitate per minute (molar extinction coefficient [ε] at 240 nm (ε_{240}) = 3.6 $mM^{-1} \cdot cm^{-1}$).

Protein carbonyl derivatives.—For the protein carbonyl derivatives assay, a certain volume (vol) of the oxygen chamber containing 20 μg of protein was derivatized with dinitrophenylhydrazine. Briefly, the sample was mixed with 1 vol of 12% sodium dodecyl sulfate plus 2 vol of 20 mM dinitrophenylhydrazine prepared in 10% trifluoroacetic acid, followed by 30 minutes of dark incubation, after which 1.5 vol of 2 M Tris/18.3% β-mercaptoethanol was added. A negative control was simultaneously prepared for each sample. After the derivatized proteins were diluted in Tris-buffered saline (TBS) to obtain a final concentration of 0.001 μg/μL, a 100-μL volume was slot blotted into a Hybond-polyvinylidene difluoride membrane. Immunodetection of carbonyls was then performed using rabbit polyclonal anti dinitrophenyl: 2000; V0401 DakoCytomation, Freiburg, Germany) as the primary antibody and anti-rabbit IgG peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, UK) as the second antibody (1:2000 dilution). The bands were visualized by treating the immunoblots with enhanced chemiluminescence (ECL) chemiluminescence reagents (Amersham Pharmacia Biotech), according to the supplier's instructions, followed by exposure to X-ray films (Kodak Biomax Light Film, Sigma). The films were analyzed with QuantityOne Software version 4.3.1 (Bio-Rad, Hercules, CA).

Total mitochondrial SH groups.—The oxidatively modified SH groups, including glutathione (GSH) and other SH-containing proteins, were quantified by spectrophotometric measurement according to the method proposed by Hu (37). Briefly, after 1-minute centrifugation at 1,000*g*, 50 μL of supernatant was added to a medium containing 150 μL of 0.25 M Tris, 790 μL of methanol, and 10 μL of 10 mM 5,5′-dithio-bis (2-nitrobenzoic acid). The colorimetric assay was performed at 414 nm against a blank test. Total SH content was calculated using the following molar extinction coefficient: ε_{414} = 13.6 $mM^{-1} \cdot cm^{-1}$.

MDA.—Nonspecific lipid peroxidation was measured by determining the levels of lipid peroxides as the amount of thiobarbituric acid reactive substances (TBARS) formed according to Rohn and colleagues (38), with some modifications. Mitochondrial protein (0.5 mg) obtained from the oxygen chamber was incubated at 25°C, in 500 μL of a medium consisting of 175 mM KCl and 10 mM Tris, pH 7.4. Samples of 50 μL were taken and mixed with 450 μL of a TBARS reagent (1% thiobarbituric acid, 0.6 N HCl, and 0.0056% butylated hydroxytoluene). The mixture was heated at 80–90°C during 15 minutes and cooled down in ice for 10 minutes before centrifugation at 1,500*g* for 5 minutes. Lipid peroxidation was estimated by the appearance of TBARS spectrophotometrically quantified at 535 nm. The amount of TBARS formed was calculated using a molar extinction coefficient (ε) of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ (39).

Extramitochondrial cytochrome c.—After centrifugation, the content of extramitochondrial cytochrome *c* in the supernatant was determined. Equivalent amounts of proteins were electrophoresed on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, followed by blotting on a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech). After blotting, nonspecific binding was blocked with 5% nonfat dry milk in TTBS (TBS with Tween 20) and the membrane was incubated with anti–cytochrome *c* (1:1,000; Pharmingen) antibody for 2 hours at room temperature, washed, and incubated with secondary horseradish peroxidase–conjugated anti-mouse (Amersham Pharmacia Biotech) for 2 hours. The membrane was then washed and developed with Western blotting chemiluminescence reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions, followed by exposure to X-ray films (Kodak Biomax Light Film, Sigma). The films were analyzed with QuantityOne Software (Bio-Rad).

Statistical Analysis

Means and standard deviations were calculated for all variables in both groups. An independent *t* test was used to

Table 1. Body Weights, Skeletal Muscle Wet Weights, and CS Activity in Isolated Mitochondria From Young and Mature Animals

	Young	Mature
Body weight (g)	25.57 ± 0.92	36.64 ± 2.34*
Dissected skeletal muscle weight (soleus, gastrocnemius, tibialis anterior, and quadriceps) (g wet weight)	1.92 ± 0.09	1.78 ± 0.11*
Ratio dissected muscle weight-to-body weight	0.075 ± 0.002	0.048 ± 0.008*
Isolated mitochondria CS activity (nmol/min/mg)	379.50 ± 34.9	524.08 ± 40.5*

Notes: Data are mean ± standard deviation of eight separate experiments in young and mature animals. CS = citrate synthase.

*Significantly different from young animals ($p < .05$).

Table 2. Characterization of the Mitochondrial Suspensions Obtained in Young and Mature Animals

	Young	Mature
Protein content (mg/mL)	15.47 ± 3.04	16.94 ± 3.89
CS activity (nmol/min/mL)	5871.17 ± 489.23	8878.67 ± 682.76*
Mitochondria ($n \times 10^{10}$/mL)	2.36 ± 1.10	7.87 ± 3.20*

Notes: Data are mean ± standard deviation. Biochemical data represent eight experiments in young and mature animals. The morphometric data represent the counts in at least 50 micrographs of each mitochondrial pellet. After the counts, the correction factor of each sample was applied in order to estimate the concentration of mitochondria in the suspension (number of mitochondria per milliliter). CS = citrate synthase.

*Significantly different from young animals ($p < .05$).

analyze the differences between groups. One-way analysis of variance was used to analyze the differences between ADP stimulations in each group. If significant differences existed ($p < .05$), post hoc comparisons were performed using Scheffe test. The Spearman correlation coefficient was used to analyze the correlations between total protein determination, CS activity, and concentration of mitochondria as well as between mitochondrial functional and biochemical data. The Statistical Package for the Social Sciences (SPSS version 10.0) was used for all analyses. Significance was established a priori as $p < .05$.

Results

Body weights, skeletal muscle wet weights, and CS activity in isolated mitochondria are shown in Table 1. Compared with young animals, a reduction in both skeletal muscle wet weight and the ratio of muscle weight-to-body weight is apparent in the mature animals, suggesting the existence of sarcopenia. When analyzing the mitochondrial suspensions obtained from young and mature animals (Table 2), we observed a significantly higher number of mitochondria in the suspensions of the mature animals; this finding agrees with the CS activity of isolated mitochondria shown in Table 1. In contrast, total protein content in the mitochondrial suspension was not significantly different between the groups (Table 2). Likewise, we did not find significant differences in the mitochondrial areas between the young and the mature animals (data not shown). With regard to the correlation coefficients, strong correlations existed between CS activity and the number of mitochondria ($r = .885$; $p < .05$). The correlations between the protein content and CS activity ($r = .664$; $p < .05$) and between the protein content and the number of mitochondria ($r = .384$; $p < .05$) were also significant.

Age-Related Mitochondrial Functional Alterations

Traditional in vitro tests.—The data of the age-related changes of respiratory function of skeletal muscle mitochondria are presented in Tables 3 and 4. Our results reveal that older animals exhibit a significant impairment in the mitochondrial respiratory rates when energized with both malate + pyruvate (M + P) and succinate + rotenone (S + R) as substrates.

Interestingly, State 4 respiratory rates were unchanged with age. To assess the mitochondrial oxidative phosphorylation efficiency, we calculated the ADP/O ratio normalized to the number of mitochondria. Our results indicate that when expressed per mitochondrion, younger animals reveal higher oxidative phosphorylation efficiency when compared with the aged and mature animals. As for the RCR, our results were comparable with other data reported elsewhere on mitochondria isolated from skeletal muscle of different ages, with older animals presenting lower values when using both M + P and S + R as respiratory substrates (16,40).

The results of our study suggest that the age-related functional impairment of mitochondria is mainly targeted at the State 3 respiratory rates, this assumption being further supported by the results of the oligomycin-inhibited State 3 respiration and CCCP-induced uncoupled respiration (Table 5).

Mitochondria isolated from the older animals, when energized with M + P and stimulated with excess ADP, showed no significant alterations in the respiratory rate in the presence of oligomycin when compared with the younger animals (Table 5). In contrast, uncoupled mitochondrial

Table 3. Functional Data Obtained From Skeletal Muscle Mitochondria Isolated From Young and Mature Animals With Complex I–Linked Substrates, Pyruvate (5 mM) and Malate (2 mM)

	Young	Mature
State 3 (nmol O_2/min/mit)	$13.3 \times 10^{-8} \pm 7.2 \times 10^{-8}$	$2.35 \times 10^{-8} \pm 0.79 \times 10^{-8}$*
State 4 (nmol O_2/min/mit)	$1.4 \times 10^{-8} \pm 0.8 \times 10^{-8}$	$0.44 \times 10^{-8} \pm 0.15 \times 10^{-8}$
RCR	9.4 ± 0.6	5.2 ± 0.8*
ADP/O per mit	$2.66 \times 10^{-9} \pm 0.4 \times 10^{-9}$	$0.78 \times 10^{-9} \pm 0.1 \times 10^{-9}$*

Notes: Data are mean ± standard deviation and represent eight separate experiments in each group. State 3 and State 4 respiratory rates are expressed as nanomoles of O_2 consumed per minute per mitochondrion. RCR = respiratory control ratio; ADP/O, number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondrion; mit = mitochondria.

*Significantly different from young animals ($p < .05$).

Table 4. Functional Data Obtained From Skeletal Muscle Mitochondria Isolated From Young and Mature Animals With Complex II–Linked Substrates, Succinate (10 mM) and Rotenone (4 μM)

	Young	Mature
State 3 (nmol O_2/min/mit)	$15.9 \times 10^{-8} \pm 9.0 \times 10^{-8}$	$3.6 \times 10^{-8} \pm 1.1 \times 10^{-8}$*
State 4 (nmol O_2/min/mit)	$7.2 \times 10^{-8} \pm 4.1 \times 10^{-8}$	$2.3 \times 10^{-8} \pm 0.7 \times 10^{-8}$*
RCR	2.2 ± 0.1	1.5 ± 0.1*
ADP/O per mit	$1.15 \times 10^{-9} \pm 0.2 \times 10^{-9}$	$0.33 \times 10^{-9} \pm 0.03 \times 10^{-9}$*

Notes: Data are mean ± standard deviation and represent 8 separate experiments in each group. State 3 and State 4 respiratory rates are expressed as nanomoles of O_2 consumed per minute per mitochondrion. RCR = respiratory control ratio; ADP/O = number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondrion; mit = mitochondria.

* Significantly different from young animals ($p < .05$).

Table 5. Effect of Age on Olygomycin-Inhibited State 3 Respiration and CCCP-Induced Uncoupled Respiration in Skeletal Muscle Mitochondria Isolated From Young and Mature Animals

	Young	Mature
State Olygomycin (nmol O_2/min/mit)	$1.7 \times 10^{-8} \pm 0.9 \times 10^{-8}$	$0.5 \times 10^{-8} \pm 0.1 \times 10^{-8}$
State CCCP (nmol O_2/min/mit)	$9.4 \times 10^{-8} \pm 5.2 \times 10^{-8}$	$2.14 \times 10^{-8} \pm 0.6 \times 10^{-8}$*

Notes: Data are mean ± standard deviation and represent 8 separate experiments in each group. Results are expressed as nanomoles of O_2 consumed per minute per mitochondrion. Respiration was induced with pyruvate (5 mM) and malate (2 mM) as energizing substrates and saturated ADP concentration (1 mM) to initiate State 3 respiration. State 3 was inhibited after the addition of olygomycin (final concentration 1, 5 μg/mL) and CCCP (final concentration 2 μM) to uncouple mitochondrial respiration. CCCP = m-chlorophenylhydrazone; mit = mitochondria.

* Significantly different from young animals ($p < .05$).

respiration with CCCP was significantly diminished in the older animals.

Repetitive ADP-stimulation test.—To mimic the bioenergetic demand that occurs in skeletal muscle mitochondria during repeated bouts of exercise, we designed a repetitive ADP simulation test. This test consisted of exposing separate aliquots of mitochondria that were exposed to one, three, or six repetitive ADP stimulation "cycles"; mitochondrial oxygen consumption was measured during each trial. Following each of these protocols, each sample of mitochondria was removed from the oxygen chamber and biochemically analyzed for biomarkers of oxidative damage.

State 3 respiratory rate was acutely diminished with the consecutive ADP stimulations, in both the young and aged animals (Figure 3). In the aged animals, State 3 respiratory rates were significantly lower in all ADP cycles. Figure 4 shows a typical representation of the mitochondrial oxygen consumption in Assay IV for one young (Figure 4A) and one aged (Figure 4B) animal. Notice that isolated mitochondria from aged animals were unable to reestablish State 4 conditions in the fourth, fifth, and sixth ADP cycles.

In both experimental groups, the ADP/O ratio remained unaltered between the ADP cycles; nonetheless, compared with aged animals, the ADP/O ratio was significantly higher in the younger animals. In both young and aged animals, the RCR decreased as a function of each successive ADP stimulation trial. Compared with young animals the RCR was significantly lower in the aged animals in all ADP cycles.

Age-Related Mitochondrial Biochemical Alterations

In order to assess the age-related alterations in oxidative stress and damage, we determined the activity and content of several oxidative stress biomarkers in mitochondria obtained from young and mature animals (Table 6) at basal conditions (Assay I).

Our results reveal a significantly diminished ACON activity in the mature animals; this observation is consistent with an augmented inactivation probably mediated by an increased superoxide production (41). We further found increased levels of oxidative damage in mitochondria isolated from the mature animals, more specifically at the level of proteins, with increased contents of protein carbonyls and a reduced expression of SH groups. However, our MDA results do not corroborate this assumption because its levels were lower in the mature animals, suggesting that nonspecific lipid peroxidation was not increased in the older animals.

Repetitive ADP-stimulation test.—When stimulated with consecutive ADP cycles, mitochondria from both young and aged animals showed a significant increase in the MDA levels and protein carbonyl and extramitochondrial cytochrome *c* content (Figure 5). In contrast, these changes did not occur in either experimental group following a single ADP stimulation (data not shown).

When correlating the data from mitochondrial respiratory activity with oxidative damage biomarkers, strong correlations were found between State 3 respiratory rate and the content of SH and carbonyl groups ($r = .967$ and $r = -.533$, respectively; $p < .05$) and State 4 respiratory rate with MDA levels ($r = .827$; $p < .05$).

Discussion

Overview of the Principal Findings

Our results support the hypothesis that aging is associated with diminished respiratory function in skeletal muscle mitochondria. Indeed, when mitochondria were acutely stimulated with ADP, both State 3 respiration and the RCR were significantly lower in skeletal muscle mitochondria from aged animals compared with young animals. Similarly, when mitochondria were repeatedly stimulated with the addition of ADP (ie, simulated exercise), the functional decline induced by the consecutive ADP-stimulation test was significantly greater in mitochondria from aged animals.

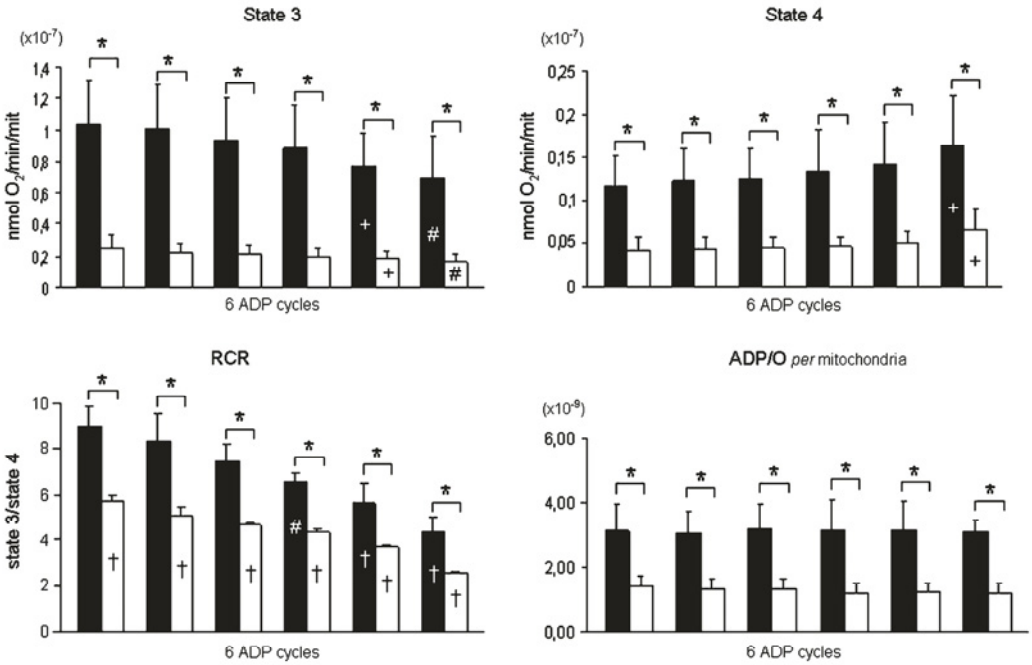

Figure 3. Functional data obtained from skeletal muscle mitochondria isolated from young and mature animals with Complex I–linked substrates, pyruvate (5 mM) and malate (2 mM), in the repetitive ADP-stimulation test. Data are mean ± standard deviation. State 3 and State 4 respiratory rates are expressed as nanomoles of O_2 consumed per mitochondrion. Filled bars: young animals; open bars: mature animals. *Age differences ($p < .05$); +significantly different from first cycle; #significantly different from first and second cycle; †significantly different from all ADP cycles ($p < .05$).

We also predicted that this age-related decline in mitochondrial respiratory function would be associated with higher levels of mitochondrial oxidative damage. Our findings are consistent with this postulate as aging was associated with a decline in mitochondrial SH groups, diminished ACON activity, and increased mitochondrial protein oxidation. A brief discussion of these and related issues follows.

Aging Impairs Skeletal Muscle Mitochondrial Respiratory Function

The impact of aging on skeletal muscle mitochondrial function remains controversial. Although some studies indicate that mitochondria isolated from aged skeletal muscle exhibit impaired respiratory function (13–17), other investigations report no age-related changes in mitochondrial State 3 respiratory function (21–25). We reasoned that these opposing findings could result from both methodological issues associated with investigating isolated mitochondria along with the wide variation in animal ages across studies. To avoid the methodological problems associated with studying isolated mitochondria (ie, normalization errors resulting from contamination of mitochondrial suspension with nonmitochondrial proteins), we normalized mitochondrial functional and biochemical measures to the number of intact mitochondria present in our suspension. Further, to mimic the bioenergetic function of skeletal muscle mitochondria during repeated muscular contractions (ie, muscular

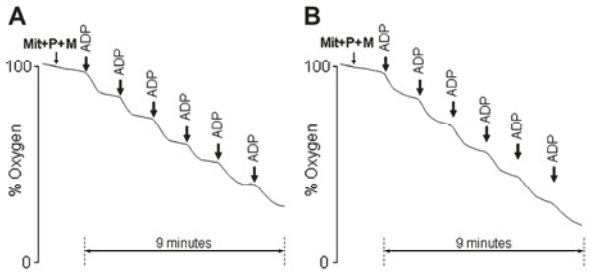

Figure 4. Schematic representation of the Assay IV (6 ADP cycles) from the repetitive ADP-stimulation test in one young animal (**A**) and one mature animal (**B**).

Table 6. Biochemical Data Obtained From Skeletal Muscle Mitochondria Isolated From Young and Mature Animals

	Young	Mature
Aconitase (U/mit)	$3.8 \times 10^{-5} \pm 2.2 \times 10^{-5}$	$0.6 \times 10^{-5} \pm 0.2 \times 10^{-5}$*
Carbonyls (OD/mit)	$1.3 \times 10^{-3} \pm 0.6 \times 10^{-3}$	$6.0 \times 10^{-3} \pm 2.0 \times 10^{-3}$*
SH groups (nmol/mit)	$1.5 \times 10^{-9} \pm 0.8 \times 10^{-9}$	$0.3 \times 10^{-9} \pm 0.1 \times 10^{-9}$*
MDA (nmol/mit)	$6.8 \times 10^{-12} \pm 4.6 \times 10^{-12}$	$3.5 \times 10^{-12} \pm 1.4 \times 10^{-12}$

Notes: Data are mean ± standard deviation. Biochemical data represent eight experiments for each group. All data are expressed per mitochondrion. Protein carbonyl derivatives are expressed as optical density (OD) arbitrary units per mitochondrion. mit = mitochondria; SH = sulfhydryl; MDA = malondialdehyde.

* Significantly different from young animals ($p < .05$).

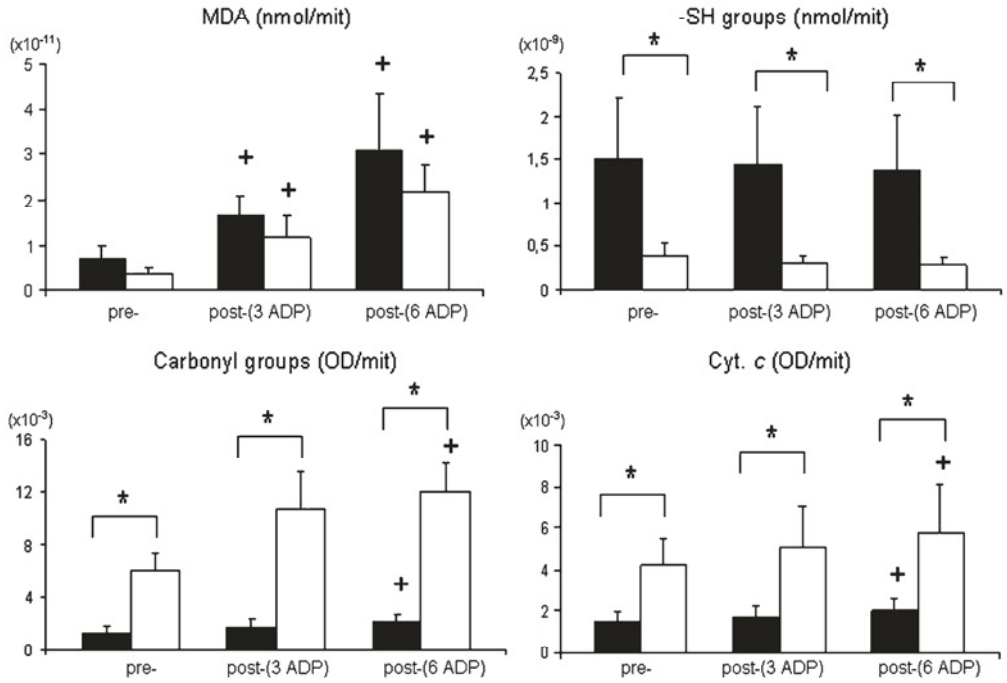

Figure 5. Acute biochemical alterations induced by the repetitive ADP-stimulation test in skeletal muscle mitochondria isolated from young and mature animals. Data are mean ± standard deviation. All data are expressed per mitochondrion. Data from protein carbonyl derivatives and cytochrome *c* are expressed as optical density arbitrary units per mitochondrion. Filled bars: young animals; open bars: mature animals. *Age differences ($p < .05$); +significantly different from prestimulation ($p < .05$).

exercise), we employed a maximal in vitro protocol using standardized and successive ADP stimulations of mitochondrial State 3 respiration. Finally, to avoid the potential problems associated with studying very old and possibly diseased animals, our experimental design incorporated aged, mature animals. Our results clearly support the notion that compared with young animals, skeletal muscle mitochondria isolated from aged animals exhibit a decreased respiratory function; these findings agree with other studies demonstrating that mitochondrial function is diminished with age, both in humans (8,16) and in mice (17,18).

Traditional tests of mitochondrial function.—Our results from traditional in vitro functional tests reveal that mitochondria from aged animals exhibit an impaired State 3 respiratory rate with both Complex I– and Complex II–linked substrates (Tables 3 and 4), indicating that the maximal rate of mitochondrial oxygen consumption was diminished by increased age. Decreased State 3 respiratory rate and RCR was also reported in skeletal muscle of senile mice (40) as well as in liver mitochondria of senescene-accelerated mice (42). These results were further confirmed by the maximal rate of uncoupled respiration that was significantly reduced in the aged animals, supporting the concept of an age-related reduction in the maximal functional capacity of the respiratory chain. The functional impairment observed during State 3 respiration was similar in the Complex I– and Complex II–linked substrate assays (77.11% and 73.94%, respectively), indicating that both Complex I–supported and succinate-supported respiration were similarly affected with increased age. In this context, Desai and colleagues (43) also reported a remarkable decrease (50–75%) in the activity of ETC complexes (ie, Complex I, III, and IV) in the gastrocnemius muscle of 20-month-old mice compared with 10-month-old mice. Despite published data suggesting that age-related decreases in the activities of mitochondrial ETC complexes are mainly targeted at the level of the Complex I, III, and IV (44), our findings indicate that mitochondrial respiration with both Complex I– and Complex II–linked substrates is diminished with increasing age.

Moreover, mitochondria from aged animals exhibited significantly decreased RCR when compared with their younger counterparts; this observation suggests that when analyzed through traditional tests the coupling between the ETC and oxidative phosphorylation is impaired by increased age. Interpreting RCR as a measure of the functional integrity of mitochondria (20), our results reveal that mitochondria from aged animals exhibit impaired function compared with mitochondria from younger animals. This age-related decrease in RCR was a consequence of the decrease in State 3 respiration, as State 4 respiration did not differ between groups. Moreover, our findings disclose that mitochondrial phosphorylation efficiency (ADP/O) was significantly diminished by age, suggesting that ATP synthase complex activity was depressed in mitochondria from the aged animals.

The magnitude of the age-related reduction in skeletal muscle mitochondrial respiratory function in the current experiments is large compared with the literature (17,40). We hypothesize that this is due to the fact that we normalized our respiratory data to the number of mitochondria. Indeed, mitochondrial functional data are frequently normalized to the protein content and this normalization standard may be biased by contamination with nonmitochondrial proteins. In fact, we have previously demonstrated (18) that when normalizing the mitochondrial State 3 respiratory rate of young and mature animals to the protein content or to the number of mitochondria, we observe differences of 27.02% and 79.41%, respectively, which supports our hypothesis that the normalization to the total protein content attenuates the age-related mitochondrial alterations.

Simulated muscular exercise: the repetitive ADP-stimulation test.—To investigate the capacity of aged skeletal muscle mitochondria to reestablish their homeostatic balance between successive bouts of exercise, we developed a mitochondrial respiration test comprising a series of repetitive ADP challenges. Mitochondrial function declined following each successive ADP stimulation cycle, and the pattern of functional decline was similar across the two age groups (Figure 3). Nonetheless, compared with mitochondria from aged animals, skeletal muscle mitochondria from young animals exhibited superior functional responses during each ADP stimulation cycles. Specifically, mitochondria from young animals exhibited higher State 3 respiratory rates, RCR, and ADP/O ratios compared with aged animals in each repetitive ADP cycle. In addition, State 4 respiration increased and the RCR decreased in both age groups at the completion of the simulated exercise test. Interestingly, this increase in State 4 respiration was greater in young animals compared with aged animals. The mechanism to explain this observation is unclear as State 4 respiration is governed by several factors including the integrity of the inner membrane and the content of uncoupling proteins along with ATP synthase complexes (23).

Note that although the mitochondrial RCR decreased during the simulated exercise test in the young animals, the RCRs remained relatively high and were indicative of fully coupled and functional mitochondria. Indeed, throughout the simulated exercise test, the mitochondria from younger animals were able to phosphorylate the ADP added in each ADP cycle (Figure 4). In contrast, from the fourth cycle onward, mitochondria isolated from aged animals were unable to phosphorylate all the ADP added, suggesting that mitochondria from mature animals were incapable of maintaining ATP homeostasis in response to this energetic challenge. This decreased ADP phosphorylation capacity could be due to impaired ETC function (eg, depressed proton pumping), thereby diminishing the proton electrochemical gradient and compromising the generation of ATP. Finally, the ADP/O, normalized to the number of mitochondria, was not altered between the ADP cycles for both the young and the aged animals, suggesting that the efficiency of ADP phosphorylation was not acutely changed.

Collectively, our data indicate an age-related dysfunction of mitochondria isolated from mixed skeletal muscle; however, it is important to highlight that our final mitochondrial suspensions were obtained from different muscle phenotypes containing specific mitochondrial characteristics (45–48). Moreover, it might also be expected that the relative contribution of each used muscle to the final mitochondrial suspension has been different among hindlimb muscles. Therefore, and bearing in mind the well-known variability of age-related alterations among skeletal muscles and fiber types (49–52), the extrapolation of our results to a specific muscle or fiber must be done with caution.

Age-Related Decline in Mitochondrial Respiratory Function Is Associated With Oxidative Damage

We also predicted that the observed age-related decline in mitochondrial function would be associated with mitochondrial oxidative stress as evidenced by an increase in oxidative biomarkers. Our results support this hypothesis as mitochondria isolated from skeletal muscle of aged animals possessed significantly fewer SH groups, lower ACON activity, and higher levels of protein carbonyls during baseline measurements. Collectively, these results are consistent with the notion that the pro-oxidant/antioxidant balance was shifted in favor of pro-oxidants in mitochondria isolated from old animals. These results agree with data from several studies that have reported age-related increases in oxidative stress and levels of mitochondrial oxidative damage (15,17).

This age-related increase in mitochondrial oxidative damage could have several functional consequences. First, our data reveal a marked age-related decrease in the activity of the tricarboxylic acid cycle (TCA) enzyme, ACON. This enzyme is particularly sensitive to a reaction with superoxide and consequently to oxidative damage, due to the iron–sulfur clusters (4Fe-4S) in its active site (53). Further, it has been suggested that age-related mitochondrial protein oxidation can also occur in the adenine nucleotide translocase (ANT) (41,54). Indeed, oxidative damage to one or both of these proteins could impair mitochondrial function and has been hypothesized to constitute an important mechanism linking aging and oxidative stress with mitochondrial dysfunction (7). For example, impaired ACON activity can diminish TCA cycle flux, leading to decreased electron flow within the respiratory chain and, therefore, decreased oxidative phosphorylation (41). Further, oxidative damage to the ANT could promote a decrease in State 3 respiration and increased mitochondrial H_2O_2 production (7). Moreover, in agreement with previous reports (41,53–56), our results reveal an age-related increase in mitochondrial oxidative damage (ie, diminished SH groups and increased protein carbonyl levels). This is significant because oxidative damage to

mitochondrial complexes could negatively impact electron transport and compromise mitochondrial respiration. Moreover, published reports indicate an age-related increase in mitochondrial membrane lipid peroxidation (40,57), and this oxidative injury could contribute to the decreased mitochondrial membrane function observed with increased age (58). Nonetheless, in the current study, we failed to observe an age-related change in mitochondrial levels of MDA. This result could be due to the lack of specificity of the MDA assay or simply because the levels of mitochondrial lipid peroxidation were not significantly increased at 18 months of age in our animals.

During the repetitive ADP-stimulation test, at the end of three (Assay III) and six ADP stimulations (Assay IV), an increase in the extramitochondrial cytochrome *c* content was observed, indicating enhanced outer mitochondrial membrane permeability, both for the young and the mature animals. Because the content of extramitochondrial cytochrome *c* was significantly higher in the older animals, not only at rest but also after stimulation, it is feasible that this enhanced permeabilization of the outer mitochondrial membrane of aged animals after repeated stimulation will favor the occurrence of apoptotic phenomenon in aged animals (12,59). Moreover, an increase in the lipid peroxidation levels at the end of Assay IV was also evident in both young and mature animals, suggesting an enhanced phospholipid membrane dysfunction with alterations in membrane fluidity. This result could have a significant impact on the activity of the respiratory chain as well as in the generation of the electrochemical proton gradient (3), resulting in increased State 4 respiratory rates and decreased RCR. Likewise, an increase in the protein oxidative damage in response to the acute and repeated ADP stimulation was obvious particularly in the mitochondria from the mature animals, which may be explained by a (i) higher production of ROS resulting from the acute stimulation or (ii) an impaired antioxidant capacity. This concept may partly explain the acute functional impairment imposed by the consecutive ADP stimulations, resulting in a progressive inability of mature animals to reestablish their homeostatic status.

Nonetheless, future experiments will be required to determine if oxidative damage to key mitochondrial proteins are the sole explanation for age-related declines in respiratory function; this remains an important area for future research.

Summary and perspectives

The current experiments were performed in an effort to resolve the controversy associated with the question, "does aging impair skeletal muscle mitochondrial function!" Although this query has been addressed in numerous studies, the results of previous investigations have failed to produce a consistent answer. We postulated that these conflicting findings are due to both methodological issues associated with the study of isolated mitochondria and the use of very old animals in several studies. Therefore, our experimental approach was designed to avoid these previous experimental design shortcomings. Specifically, our experiments incorporated a unique "simulated exercise" protocol to investigate mitochondrial function in vitro. Compared with traditional in vitro assays of mitochondrial function, we predict that our repetitive ADP stimulation protocol provides a more physiological assessment of the in vivo bioenergetic demands of skeletal muscle mitochondria during muscular exercise. Further, to avoid problems associated with normalization of mitochondrial functional measures (eg, contamination of sample with nonmitochondrial proteins), we developed a novel method of standardizing mitochondrial function to the number of intact mitochondria in the experimental sample. Finally, to avoid the occurrence of age-related concomitant disease in very old animals, we used aged and mature animals in our experiments as these animals are not likely to possess old age-related diseases that could influence our experimental results. Hence, our study design provides a robust experimental approach to the study of age-related changes in mitochondrial function in skeletal muscle.

Our findings are consistent with the hypothesis that aging is associated with diminished respiratory function in skeletal muscle mitochondria. Indeed, our results indicate that State 3 functional impairments in the mitochondria of aged animals are present with both Complex I and Complex II substrates. Moreover, compared with aged animals, mitochondria isolated from skeletal muscle of young animals exhibited higher functional responses during repetitive ADP stimulation cycles designed to simulate repeated bouts of muscular exercise. Indeed, mitochondria from young animals exhibited higher State 3 respiratory rates, RCR, and ADP/O ratios compared with aged animals during each repetitive ADP cycle. We speculate that this mitochondrial dysfunction could be related with an age-dependent increased oxidative damage to the mitochondrial biomolecules. Our results, however, only provide evidences that a functional limitation exists at the level of isolated mitochondria obtained from mixed skeletal muscle types, giving a merely overall view of age-related skeletal muscle mitochondrial dysfunction.

Additionally, although our results indicate that aging is associated with impaired mitochondrial function in skeletal muscle, our investigation does not identify the mechanisms responsible for this age-related decline in mitochondrial function. Our speculation that oxidation of mitochondrial proteins contributed to the observed respiratory dysfunction evolved from our finding that aging is associated with increased mitochondrial protein damage and the knowledge that oxidative modification of mitochondrial proteins can impair respiratory function (7,13,41,44,54,58). Future cause and effect experiments using mitochondrial targeted antioxidants could prove useful in determining if mitochondrial

oxidative stress is a primary cause of age-related impairment in mitochondrial function.

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Impact of lifelong sedentary behavior on mitochondrial function of mice skeletal muscle

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Running head: Lifelong sedentariness favors skeletal muscle aging

Abstract

This study investigated the impact of lifelong sedentariness on skeletal muscle mass and mitochondrial function. Thirty C57BL/6 strain mice (2 months) were randomly divided into three groups (young-Y; old sedentary-OS; old active-OA). Young animals were sacrificed after one week of quarantine and OS and OA groups were individually placed into standard cages and in cages with running wheels, respectively, until sacrifice (25 months). Body weights and hindlimb skeletal muscle wet weights were obtained from all groups. Mitochondrial respiratory functional measures (i.e., state 3 and 4 respiration, RCR and ADP/O) and biochemical markers of oxidative damage (aconitase activity, protein carbonyl derivatives, sulfhydryl groups) were measured in isolated mitochondrial suspensions. Our results reveal that lifelong sedentary behavior has a negative impact on the age-related loss of skeletal muscle mass and on the isolated mitochondrial function of mixed skeletal muscle of mice, which is associated with an increased oxidative damage to mitochondrial biomolecules.

Keywords: voluntary activity; muscle aging; sarcopenia; running wheel; oxidative stress

Introduction

Aging diminishes both cellular number and function in all organ systems. One of the most adversely affected tissues with increasing age is skeletal muscle (1-5). Indeed, one of the hallmarks of aging is a decrease in both skeletal muscle fiber number and fiber cross sectional area, known as sarcopenia. Moreover, advanced age is associated with a progressive impairment in muscle fiber structure and contractile function (6). This age-related skeletal muscle weakness leads to a loss of independence, decreased quality of life, and an increased risk of morbidity due to falls (4, 7). Intrinsic mechanisms that contribute to aging-induced sarcopenia include a depressed capacity for cellular repair, impaired gene expression, reduced protein synthesis, and compromised mitochondrial function (7-9). Extrinsic mechanisms to muscle fibers, such as the impairment of neuromuscular function and alterations in the endocrine milieu may also contribute to sarcopenia (4, 7).

Beyond the aforementioned intrinsic and extrinsic mechanisms, it is feasible that several behavioural factors influence sarcopenia and the age-related damage to skeletal muscle mitochondria (2, 3, 10, 11). Of these factors, a sedentary lifestyle is potentially one of the most deleterious contributors to sarcopenia and skeletal muscle dysfunction (3, 12). Because physical exercise provides numerous positive adaptations to skeletal muscle including mitochondrial biogenesis (13-16), a lifelong program of regular exercise is widely recommended (16-18). Although physical exercise may provide potential benefits to retard the age-related skeletal muscle dysfunction, the existing literature does not provide a clear picture of the physiological impact of a sedentary lifestyle versus a lifelong active lifestyle on skeletal muscle mass and mitochondrial function. Indeed, most published reports using animal models to investigate this important issue suffer from one or more shortcomings. For example, some studies have incorporated only short durations of exercise programs (19-21), and other studies used forced exercise modalities such as treadmill running or swimming (19, 20, 22-24). A major disadvantage of a forced exercise program is that this training paradigm imposes significant

psychological stress on the animals that may promote deleterious adaptations (25, 26). Clearly, a program of forced exercise does not mimic the normal intermittent exercise routine of rodents in the wilderness.

Indeed, free access to a running wheel is a more appropriate approach in a rodent model of lifelong exercise to satisfy the animals' physiological requirements for voluntary activity. With a voluntary model of exercise, physical activity is not externally imposed and the animals can run intermittently and freely. This type of methodological approach has been associated with positive physiological adaptations in adult and aged animal models, namely augmented VO_2máx. (25), cardiac hypertrophy (27), increases in skeletal muscle mitochondrial enzyme expression (27, 28), decreases in the basal level of H_2O_2 production by isolated mitochondria from cardiac muscle (29), up-regulation of telomere-stabilizing proteins, reduced cellular senescence and apoptotic cell death in cardiomyocytes (30) and a shift from IIb to IIa myosin heavy chain (MHC) expression (27, 28). In addition, this type of lifelong voluntary physical activity (LVPA) has been associated with increases in mean lifespan (31-33). However, although several studies have examined the role of LVPA on survival rates, little is known about the effects of LVPA on aging of skeletal muscle; this void forms the basis for the current experiments.

Therefore, this study investigated the impact of a lifelong sedentary lifestyle on both skeletal muscle mass and mitochondrial function. To achieve this objective, two markedly different models of physical activity were compared in mice; the physical activity in one group of animals was restricted to the space of each cage whereas the second group of animals had free access to a running wheel for voluntary exercise. We tested the hypothesis that compared to voluntary active behavior, a sedentary lifestyle would have negative effects on skeletal muscle mass and mitochondrial function.

Methods

Animals and lifelong interventions

Thirty C57BL/6 strain mice aged two months were randomly divided into three groups (young-Y; old sedentary-OS; old active-OA). One week after arrival in the laboratory, all Y animals (n=10) were sacrificed whereas animals assigned to the OS (n=10) and OA (n=10) groups were individually placed into standard cages 355x235x190 mm (Ref. 2150E, Tecniplast, Italy) and in cages equipped with running wheels with 364x258x350 mm (Ref. 1284L0106, Tecniplast, Italy), respectively. Both OS and OA animals were sacrificed at an age of 25 months. The running wheels (25 cm in diameter) in the cages of the OA animals were equipped with a magnetic switch and a revolution counter to monitor the running distance for each animal throughout the duration of the experimental protocol.

Body weights of all mice were recorded weekly. Animals from both groups were maintained at constant environmental temperatures (21º–25°C) on a daily light schedule of 12 h of light *vs.* dark until sacrifice. Mice were provided with food and water *ad libitum*. Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, 1996). The local Ethics Committee had approved the study and the experiments were complied with the current national laws.

Animals sacrifice, skeletal muscle extraction and mitochondria isolation

At the time of sacrifice (25 months), there were 8 and 10 animals in OS and OA group, respectively. Note that two animals from the OS group died during the experimental protocol (with twenty and twenty two months of age, respectively) resulting in an n=8 in this experimental group. The animals were sacrificed by cervical dislocation, and the hindlimb muscles (*soleus*, *gastrocnemius*, *tibialis anterior*, and *quadriceps*) were excised and weighed prior to mitochondria isolation. The muscles were cleared of fat and connective tissue and wet muscle weight was determined as the difference between the weight of a given volume of cold isolation medium without the muscle sample and the weight of

the same volume of cold isolation medium with the muscle sample. Afterwards, skeletal muscle mitochondria were isolated and prepared by conventional methods of differential centrifugation, as previously described by Tonkonogi and Sahlin (34). Briefly, muscles were immediately minced in ice-cold isolation medium containing 100 mM sucrose, 0.1 mM EGTA, 50 mM Tris•HCl, 100 mM KCl, 1 mM 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 min. 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. After the extraction of 1 mL of this homogenate for biochemical assessment of CS activity and total protein content in skeletal muscle, the remaining homogenate was fractionated by centrifugation at 700*g* for 10 min. The resulting pellet was removed, and the supernatant suspension was centrifuged at 10,000*g* during 10 min. The supernatant was decanted, and the pellet was gently resuspended in isolation medium (1.3 ml/100 mg initial tissue) and centrifuged at 7,000*g* for 3 min. The supernatant was discarded, and the final pellet, containing the mitochondrial fraction, was gently resuspended (0.4 µl/mg initial tissue) in a medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris, and 0.1 mM EDTA, pH 7.4. Total protein concentration in the mitochondrial suspension was estimated spectrophotometrically with the biuret method using bovine serum albumin as standard. All mitochondrial isolation procedures were performed at 0–4°C. The mitochondrial suspensions were used within 2 h after the excision of the muscles and were maintained on ice (0–4°C) throughout this period.

One aliquot from the final mitochondrial suspension was used for mitochondrial biochemical analysis at non stimulated conditions. All the biochemical parameters were assessed in the whole mitochondrial suspension after treatment with 0.1% Triton X-100. Another aliquot was processed for morphological analysis. The remaining mitochondrial suspension was used for mitochondrial *in vitro* stimulation using traditional methods for measurements of the mitochondrial respiratory activity and a simulated exercise test with one,

three and six ADP pulses interspersed by 1:30 minute each in which functional and biochemical data were obtained. The utilization of this *in vitro* test was an attempt to assess the capacity of mitochondria to re-establish their homeostatic balance between consecutive ADP-stimulations as a function of time.

Biochemical analysis in skeletal muscle homogenate and mitochondrial fraction

Total protein determination. Total protein concentrations in skeletal muscle homogenate and in the mitochondrial suspension were determined using the Lowry et al. protocol (35) and with the biuret method, respectively. In both assays, bovine serum albumin served as the standard.

Citrate synthase determination. Citrate synthase activity was measured according to Coore et al. (36). In brief, the CoASH released from the reaction of acetyl-CoA with oxaloacetate was measured by its reaction with DTNB at 412 nm (ε = 13.6 mM^{-1} • cm^{-1}). CS activity was assessed in skeletal muscle homogenate and in the whole mitochondrial suspension after treatment with 0.1% Triton X-100.

Mitochondrial density (mg/g muscle wet wt) was estimated according to Kerner et al. (37). Briefly, the total CS activity in the whole skeletal muscle homogenate was initially calculated multiplying the CS activity per mg of protein by the total amount of protein in the whole skeletal muscle homogenate, being further normalized to muscle wet weight (g). In order to estimate the mitochondrial density, the obtained value was divided by the CS specific activity assessed in isolated mitochondrial suspension. The recovery of mitochondria was measured as citrate synthase (CS) activity in the mitochondrial suspension relative to that in the skeletal muscle tissue homogenate.

Mitochondrial preparation for transmission electron microscopy (TEM)

Mitochondrial preparation for TEM analyses and further morphometric characterization has been previously described (38). Briefly, 100 µL of the mitochondrial suspension was centrifuged at 7,000*g* during 10 minutes and the resulting pellet was fixed with 2.5% glutaraldehyde, post-fixed with 2% osmiumtetroxide, deyhdrated in graded alcohol, and embedded in LR White.

Ultra-thin sections mounted on copper grids (300 Mesh) were contrasted with uranyl acetate and lead citrate for transmission electron microscopy (Zeiss EM 10A) analysis. In order to obtain a global characterization of the pellet several grids were prepared (5-8 grids per animal each containing 3 to 4 sections) from different zones ranging through the whole pellet.

Morphometric analysis of the mitochondrial pellet

Morphometric analysis was performed as described in detail elsewhere (38) in at least 50 photos *per* mitochondrial pellet using a morphometric processing program (ImageJ, NIH Image). For each pellet the mean number of mitochondria *per* µm and μm^2 was calculated from all analyzed micrographs and their product was used to calculate the mitochondrial concentration in the pellet (number *per* μm^3 with further adjustment to the number *per* mL).

The mitochondrial concentration in each mitochondrial suspension was assessed as previously described (38) and used to normalize all functional and biochemical data.

Measurement of mitochondrial respiratory activity

Traditional tests. After the determination of the total protein concentration in the mitochondrial suspension (estimated spectrophotometrically with the biuret method using bovine serum albumin as standard), the mitochondrial respiratory function was polarographically measured using a Clark-type oxygen electrode (Hansatech DW 1, Norfork, UK). All assays were conducted in a 0.75 ml closed, magnetically stirred, and temperature controlled (25°C) glass chamber. Mitochondria were added to the chamber (0.5 mg of protein) in a reaction buffer of 225 mM mannitol, 75 mM sucrose, 10 mM Tris, 10 mM KCl, 10 mM K_2HPO_4, and 0.1 mM EDTA, pH 7.5 (39). After a 1-min equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) for Complex I-linked substrate assay, or succinate (10 mM) plus rotenone (4 µM) for Complex II-linked substrate assay. State 3 respiration was determined after adding ADP to a final concentration of 200 µM; state 4 respiration was measured as the rate of oxygen consumption in the absence of

ADP phosphorylation. The RCR, i.e., the ratio between state 3 and state 4 respiration, and ADP/O were calculated according to Estabrook (40), using 235 nmol O_2/ml as the value for the solubility of oxygen at 25°C. To quantify mitochondrial inner membrane permeability and the maximal rate of uncoupled oxidative phosphorylation, oligomycin (final concentration of 1.5 µg/ml) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; 2 µM), respectively, were sequentially added during ADP-stimulated (final concentration of 1 mM) state 3 respiration. The utilization of oligomycin avoids the permeability to protons through the ATP synthase creating an oligomycin-inhibited state 3 respiration; the comparison of oligomycin-inhibited state 3 respiration with a normal state 4 allows establishing the degree of mitochondrial inner membrane integrity. After obtaining the oligomycin-inhibited state 3 respiration, the addition of CCCP induces an uncoupling of the respiratory chain enabling to assess its maximal activity, since the permeability in the presence of CCCP is always maximal (41).

Consecutive ADP-stimulation test. In order to mimic the *in vivo* metabolic stress imposed to mitochondria during muscular exercise we performed a series of repeated and consecutive bouts of ADP-stimulated mitochondrial respiration using in a Clark-type oxygen electrode to determine oxygen consumption (Hansatech DW 1, Norfork, UK) following a previously described protocol (42). Briefly, this *in vitro* test consisted of four assays performed on each of the mitochondrial suspensions (figure 1); assay I: after a 60 second (s) equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30s stabilization; assay II: after a 60s equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30s stabilization and the addition of one pulse of ADP followed by 90s; assay III: after a 60s equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2 mM) followed by 30s stabilization and the addition of 3 consecutive pulses of ADP interspersed by 90s each; assay IV: after a 60s equilibration period, mitochondrial respiration was initiated by adding pyruvate (5 mM) plus malate (2

mM) followed by 30s stabilization and the addition of 6 consecutive pulses of ADP interspersed by 90s each.

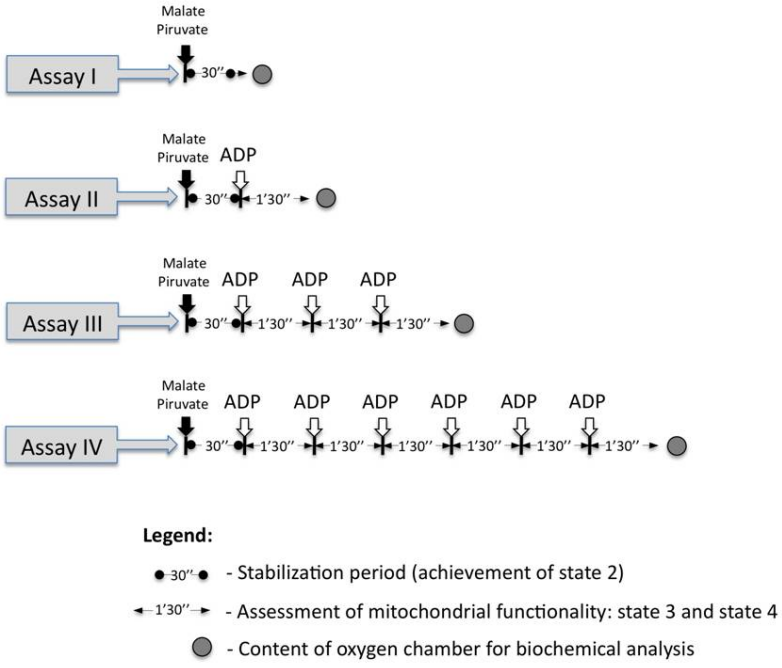

Figure 1- Consecutive ADP-stimulation test. See text for detailed description.

In assays II, III and IV, mitochondrial respiratory function was assessed by analyzing several functional parameters (i.e., state 3 and state 4 respiratory rates, the respiratory control ratio (RCR) and ADP/O) in each of the ADP cycles. At the end of each of the four assays, the content of the oxygen chamber was collected and used for biochemical analysis (figure 1). The content of the oxygen chamber at the end of assay I represent the mitochondrial basal condition (pre-stimulation); the content of the oxygen chamber after assays II, III and IV correspond to different degrees of ADP-stimulation. In all assays a certain quantity of the oxygen chamber (500 µL) was used for the determination of protein carbonyl derivatives and total mitochondrial SH groups. Furthermore, a portion of the oxygen chamber (250 µL) was centrifuged for 15 minutes at 10,000*g* in order to determine the content of extramitochondrial cytochrome *c*. Aconitase activity was also quantified in assay I in order to indirectly assess the age-related superoxide radical generation at basal conditions.

Aconitase activity. The activity of aconitase (ACON) was assayed because ACON activity is redox sensitive, and diminished ACON activity has been interpreted as an index of superoxide radical generation and mitochondrial oxidative damage (43). Aconitase activity was measured spectrophotometrically by monitoring the formation of *cis*-aconitate at 240 nm after the addition of 20 mM isocitrate at 25°C, according to Krebs and Holzach (44). One unit of activity was defined as the amount of enzyme necessary to produce 1 μM *cis*-aconitate/min [molar extinction coefficient (ε) at 240nm (ε_{240}) = 3.6 $mM^{-1} \bullet cm^{-1}$].

Protein carbonyl derivatives. For the protein carbonyl derivatives assay, a certain volume (vol) of the oxygen chamber containing 20 μg of protein was derivatized with dinitrophenylhydrazine. Briefly, the sample was mixed with 1 vol of 12% SDS plus 2 vol of 20 mM dinitrophenylhydrazine prepared in 10% trifluoroacetic acid, followed by 30 min of dark incubation, after which 1.5 vol of 2 M Tris/18.3% β-mercaptoethanol was added. A negative control was simultaneously prepared for each sample. After the derivatized proteins were diluted in TBS to obtain a final concentration of 0.001 μg/μl, a 100 μl volume was slot blotted into a Hybond-polyvinylidene difluoride membrane. Immunodetection of carbonyls was then performed using rabbit polyclonal anti-dinitrophenyl (1:2000; V0401 DakoCytomation) as the primary antibody and anti-rabbit IgG peroxidase (Amersham Pharmacia) as the second antibody (1:2,000 dilution). The bands were visualized by treating the immunoblots with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, MO). The films were analyzed with QuantityOne Software version 4.3.1 (Bio-Rad).

Total mitochondrial sulfhydryl (SH) groups. The oxidatively modified SH groups, including GSH and other SH-containing proteins, were quantified by spectrophotometric measurement according to the method proposed by Hu (45). Briefly, after 1 min centrifugation at 1,000*g*, 50 μl of supernatant was added to a medium containing 150 μl of 0.25 M Tris, 790 μl of methanol, and 10

µl of 10 mM 5,5'-dithio-bis (2-nitrobenzoic acid). The colorimetric assay was performed at 414 nm against a blank test. Total SH content was calculated using the following molar extinction coefficient: ε_{414}= 13.6 mM^{-1} • cm^{-1}.

Extramitochondrial cytochrome c. The content of extramitochondrial cytochrome *c* was determined in the supernatant after centrifugation and isolation of the mitochondrial pellet. Equal amounts of supernatant proteins were electrophoresed on a 15% SDS-PAGE gel, followed by blotting on a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech). After blotting, non-specific binding was blocked with 5% nonfat dry milk in TTBS (Tris-buffered saline (TBS) with Tween 20) and the membrane was incubated with anti-cytochrome *c* (1:1000; cat. number 556433; Pharmingen) antibody for 2 hours at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse (Amersham Pharmacia Biotech) for 2 hours. The membrane was then washed and developed with Western blotting chemiluminescence reagents (Amersham Pharmacia Biotech) according to manufacturer's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (Bio-Rad).

Statistical analysis

For all dependent variables, a one-way analysis of variance was used to analyze the differences between the three groups. When appropriate *post-hoc* comparisons were determined with Scheffe test. The Statistical Package for the Social Sciences (SPSS version 10.0) was used for all analyses. Significance was taken as $p< 0.05$.

Results

Lifelong voluntary physical activity of OA animals are shown in figure 2. Voluntary physical activity levels of the OA animals peaked at the 4th and 5th week of the experiment. Note that activity declined during the subsequent 10

weeks and reached a new plateau of activity at ~ week 16 with animals running approximately 5km per day until the end of the experiment (Figure 2).

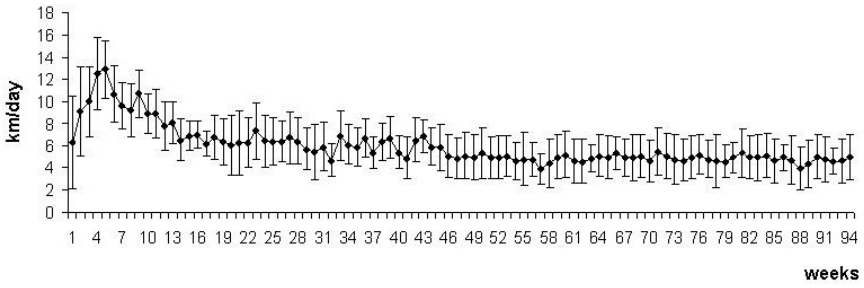

Figure 2- Lifelong voluntary physical activity. Data points represent the daily average distance per day (km) (mean±standard deviation).

Animal body weights and skeletal muscle CS activity are reported in table 1. As indicated from the skeletal muscle wet weight and muscle weight/body weight ratio, aging and inactivity was associated with a decline in skeletal muscle mass in OS animals. This age-related decline in skeletal muscle mass was attenuated by physical activity. Compared to both, the young and sedentary old animals, skeletal muscle CS activity was significantly higher in the old active animals. Interestingly, this CS activity in the old sedentary animals did not differ from the younger animals.

Table 1. Body weights, skeletal muscle wet weights, and CS activity in muscle homogenate and in isolated mitochondria from young, old sedentary, and old active animals.

Measured parameters	Young	Old Sedentary	Old Active
Body weights (g)	27.16±0.90	36.14±3.36*	32.09±1.99**
Pooled skeletal muscle weight (*Soleus*, *Gastrocnemius*, *Tibialis anterior*, and *Quadriceps*) (g wet wt)	1.90±0.11	1.76±0.14*	1.97±0.16**
Ratio muscle weight/body weight	0.07±0.002	0.04±0.007*	0.06±0.005***
Skeletal muscle CS activity (nmol/min/mg)	46.68±7.16	47.71±5.59	58.24±8.43**
Skeletal muscle mitochondrial density (mg/g muscle wet wt)	25.95±9.86	17.05±4.57	37.78±9.22**
Isolated mitochondria CS activity (nmol/min/mg)	504.94±55.33	664.63±57.22*	512.14±99.40***
Recovered mitochondria (%)	14.88±4.80	20.81±4.55*	13.34±4.28***

Notes: Data are mean ± standard deviation of ten separate experiments in young and old active animals and eight separate experiments in old sedentary animals. Skeletal muscle mitochondrial density was estimated by the division of CS activity of skeletal muscle homogenate (U/g wet wt) by the CS specific activity in isolated mitochondrial suspension (U/mg mitochondrial protein). Recovered mitochondria was calculated from the fraction of muscle CS activity recovered in the isolated mitochondria.

* Significantly different from young animals ($p < .05$);

** Significantly different from young and old sedentary animals ($p < .05$);

*** Significantly different from old sedentary animals ($p < .05$).

Quantification of the number of mitochondria revealed a significantly higher number of mitochondria in the old sedentary animals compared to both the young and old active animals (table 2). This finding is corroborated by the measurement of CS activity within the mitochondrial suspension (table 1); however, the protein content in the mitochondrial suspensions was not significantly different among groups.

Table 2. Characterization of the mitochondrial suspensions obtained from young, old sedentary, and old active animals.

Measured parameters	Young	Old Sedentary	Old Active
Protein content (mg/mL)	17.08±2.72	18.38±3.02	22.28±4.61
CS activity (nmol/min/mL)	8624.4±1388.4	12215.9±1151.3 *	11410.5±2339.0 *
Mitochondria (nx10^{11}/mL)	1.99±0.27	3.47±0.67 *	2.36±1.05 ***

Notes: Data are mean ± standard deviation. Biochemical data represents ten experiments in young and old active animals and eight separate experiments in old sedentary animals. The morphometric data represents the counts in at least 50 micrographs of each mitochondrial pellet. After the counts the correction factor of each sample was applied in order to estimate the concentration of mitochondria in the suspension (number of mitochondria/mL).

* Significantly different from young animals ($p < .05$);

*** Significantly different from old sedentary animals ($p < .05$).

Previously we have reported (38) that varying degrees of contamination exist in skeletal muscle mitochondrial isolation across animals of differing age groups. Therefore, to avoid this important confounding variable we determined the number of mitochondria in the mitochondrial suspension of each animal in order to normalize all functional and biochemical data to the number of mitochondria in the assay sample.

Lifelong Inactivity and Mitochondrial Functional Alterations

Traditional measurements of mitochondrial respiration. Skeletal muscle mitochondria were isolated with high integrity in all groups under study as documented by the RCR values (table 3) and by their morphological appearance in the TEM analysis (data not shown).

Table 3. Functional data obtained from skeletal muscle mitochondria isolated from young (Y), old sedentary (OS), and old active (OA) animals with complex I-linked substrates.

Measured parameters		Young	Old Sedentary	Old Active
State 3	(nmol O_2/min/mg)	241.8 ± 22.5	114.5 ± 10.9 *	133.4 ± 11.0 *
	(nmol O_2/CS)	0.47 ± 0.05	0.17 ± 0.005 *	0.25 ± 0.02 **
	(nmol O_2/min/mit)	2.0 $x10^{-8}$ ± 0.40 $x10^{-8}$	0.6$x10^{-8}$ ± 0.13 $x10^{-8}$ *	1.2$x10^{-8}$± 0.29 $x10^{-8}$ **
State 4	(nmol O_2/min/mg)	28.9 ± 3.2	30.7 ± 3.7	23.1 ± 2.4
	(nmol O_2/CS)	0.05 ± 0.004	0.04 ± 0.004	0.04 ± 0.004
	(nmol O_2/min/mit)	2.4$x10^{-9}$± 0.66 $x10^{-9}$	1.6$x10^{-9}$± 0.59 $x10^{-9}$	2.2$x10^{-9}$± 0.49 $x10^{-9}$
RCR		8.3 ± 1.9	3.7 ± 1.1 *	5.7 ± 1.6 *
ADP/O		1.59 ± 0.5	1.78 ± 0.5	1.8 ± 0.5
ADP/O per mit		2.6 x 10^{-10} ± 1.1 x 10^{-10}	1.8 x 10^{-10} ± 0.3 x 10^{-10} *	3.4 x 10^{-10} ± 1.0 x 10^{-10} **

Notes: Data are mean ± standard deviation of ten separate experiments in young and old active animals and eight separate experiments in old sedentary animals. State 3 and state 4 respiratory rates are expressed as nmol O_2 consumed/min/mg of protein, as nmol O_2 consumed/citrate synthase (CS) activity, and as nmol O_2 consumed/min/mitochondria (mit). RCR, respiratory control ratio; ADP/O data is expressed as number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed (ADP/O) and number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondria (ADP/O per mit.).

* Significantly different from young animals ($p < .05$);

** Significantly different from young and old sedentary animals ($p < .05$);

*** Significantly different from old sedentary animals ($p < .05$).

Functional data obtained from skeletal muscle mitochondria of all groups is shown in tables 3 and 4. Compared to young animals, OA and OS animals evidenced a significant decline in state 3 respiration and RCR with both complex I and II-linked substrates, being this reduction more pronounced in OS animals. To assess the mitochondrial phosphorylation efficiency we calculated the ADP/O ratio normalized to the number of mitochondria (table 3 and 4). Our results reveal that when expressed per mitochondria, ADP/O values are significantly diminished in the old sedentary animals compared to both the young and old active animals. Interestingly, compared to both the young and old

sedentary animals, ADP/O ratios (complex I substrates) were significantly higher in the old active animals; this finding suggests that aging *per se* did not influence on mitochondrial phosphorylation efficiency.

Table 4. Functional data obtained from skeletal muscle mitochondria isolated from young (Y), old sedentary (OS), and old active (OA) animals with complex II-linked substrates (succinate (10mM) and rotenone (4μM)).

Measured parameters		Young	Old Sedentary	Old Active
State 3	(nmol O_2/min/mg)	285.7 ± 36.5	112.6 ± 9.0 *	133.4 ± 16.8 *
	(nmol O_2/CS)	0.56 ± 0.1	0.16 ± 0.01 *	0.25 ± 0.03 **
	(nmol O_2/min/mit)	$2.41 \times 10^{-8} \pm 0.5 \times 10^{-8}$	$0.6 \times 10^{-8} \pm 0.2 \times 10^{-8}$ *	$1.2 \times 10^{-8} \pm 0.4 \times 10^{-8}$ **
State 4	(nmol O_2/min/mg)	81.9 ± 2.9	81.2 ± 4.1	51.1 ± 1.8 **
	(nmol O_2/CS)	0.16 ± 0.01	0.12 ± 0.005	0.09 ± 0.003 *
	(nmol O_2/min/mit)	$6.9 \times 10^{-9} \pm 2.2 \times 10^{-9}$	$4.3 \times 10^{-9} \pm 0.9 \times 10^{-9}$	$4.8 \times 10^{-9} \pm 1.4 \times 10^{-9}$
RCR		3.48 ± 1.0	1.38 ± 0.4 *	2.62 ± 0.4
ADP/O		1.10 ± 0.1	0.92 ± 0.2	0.98 ± 0.1
ADP/O per mit		$1.8 \times 10^{-10} \pm 0.1 \times 10^{-10}$	$0.9 \times 10^{-10} \pm 0.1 \times 10^{-10}$ *	$1.8 \times 10^{-10} \pm 0.1 \times 10^{-10}$ ***

Notes: Data are mean ± standard deviation of ten separate experiments in young and old active animals and eight separate experiments in old sedentary animals. State 3 and state 4 respiratory rates are expressed as nmol O_2 consumed/min/mg of protein, as nmol O_2 consumed/citrate synthase (CS) activity, and as nmol O_2 consumed/min/mitochondria (mit). RCR, respiratory control ratio; ADP/O data is expressed as number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed (ADP/O) and number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondria (ADP/O per mit) .

* Significantly different from young animals ($p < .05$);

** Significantly different from young and old sedentary animals ($p < .05$);

*** Significantly different from old sedentary animals ($p < .05$).

Our results collectively indicate that the age-related functional impairment of skeletal muscle mitochondria is targeted at the state 3 respiratory rates and that a lifelong sedentary behavior exacerbates this age-related mitochondrial dysfunction. In order to confirm this data we have performed an additional

assay where we analyzed the oligomycin-inhibited state 3 respiration and CCCP-induced uncoupled respiration (table 5).

Table 5. Effect of age and lifelong physical activity on oligomycin-inhibited state 3 respiration and CCCP-induced uncoupled respiration in skeletal muscle mitochondria isolated from young, old sedentary, and old active animals.

Measured parameters		Young	Old Sedentary	Old Active
State Oligomycin	(nmol O_2/min/mg)	32.0 ± 3.4	39.4 ± 5.0	23.1 ± 2.5
	(nmol O_2/CS)	0.06 ± 0.008	0.05 ± 0.004	0.04 ± 0.004
	(nmol O_2/min/mit)	$2.7x10^{-9}$±$1.1x10^{-9}$	$2.1x10^{-9}$±$0.9x10^{-9}$	$2.2x10^{-9}$±$1.1x10^{-9}$
State CCCP	(nmol O_2/min/mg)	218.1 ± 19.9	95.7± 9.3 *	124.0 ± 11.5 **
	(nmol O_2/CS)	0.43 ± 0.061	0.14 ± 0.012 *	0.24 ± 0.019 **
	(nmol O_2/min/mit)	$18.4x10^{-9}$±$8.9x10^{-9}$	$5.1x10^{-9}$±$2.9x10^{-9}$ *	$11.8x10^{-9}$±$5.3x10^{-9}$ **

Notes: Data are mean ± standard deviation and represents ten experiments in young and old active animals a eight separate experiments in old sedentary animals. Results are expressed as nmol O_2 consumed/min/mg protein, as nmol O_2 consumed/citrate synthase (CS) activity, and as nmol of O_2 consumed/min/mitochond (mit). Respiration was induced with pyruvate (5mM) and malate (2mM) as energizing substrates and saturat ADP concentration (1mM) to initiate state 3 respiration. State 3 was inhibited after the addition of oligomycin (fi concentration 1,5 µg/ml) and CCCP (final concentration 2 µM) to uncouple mitochondrial respiration.

* Significantly different from young animals ($p < .05$);

** Significantly different from young and old sedentary animals ($p < .05$).

No significant differences existed between the experimental groups in the oligomycin-inhibitied state 3 respiration. In contrast, uncoupled respiration with CCCP was significantly diminished with age although the decrease in state CCCP respiration was greater in the old sedentary animals.

Consecutive ADP-stimulation test. Similar to the age-related changes in mitochondrial function observed with the traditional tests, the age and sedentary-related alterations in mitochondrial function were also present in the consecutive ADP-stimulation tests (Figure 3). Specifically, state 3 respiratory

rate and RCR were acutely diminished in all groups with increasing number of trials. In contrast, state 4 respiration rate was significantly higher in the 6th ADP-stimulation, when compared with data from the 1st and 2nd ADP challenge, in all groups under investigation. As to the ADP/O ratio normalized to the number of mitochondria, no change occurred across the successive ADP-stimulations.

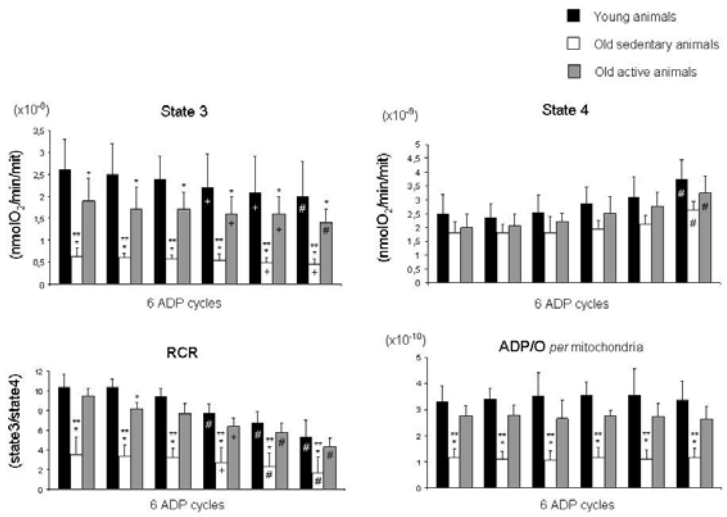

Figure 3- Functional data obtained from skeletal muscle mitochondria isolated from young, old sedentary, and old active animals with complex I linked substrates, pyruvate (5mM) and malate (2mM), in the consecutive ADP-stimulation test. Data are mean ± standard deviation of ten separate experiments in young and old active animals and eight separate experiments in old sedentary animals. State 3 and state 4 respiratory rates are expressed as nmol of O_2 consumed per minute per mitochondria (mit). RCR, respiratory control ratio; ADP/O, number of nanomoles of ADP phosphorylated by nanomoles of O_2 consumed per mitochondria. * Significantly different from young animals ($p < .05$); ** Significantly different from old active animals ($p < .05$); + Significantly different from 1st cycle ($p<0.05$); # Significantly different from 1st and 2nd cycle ($p < .05$).

A schematic representation of the mitochondrial oxygen consumption in the consecutive ADP-stimulation test (assay IV) is shown in figure 4 for young, old sedentary and old active animals. Note that isolated mitochondria from old sedentary animals were unable to re-establish baseline state 4 conditions after the 4th, 5th and 6th ADP stimulation. In contrast, this functional impairment seems to be not that much apparent in young and in old active animals.

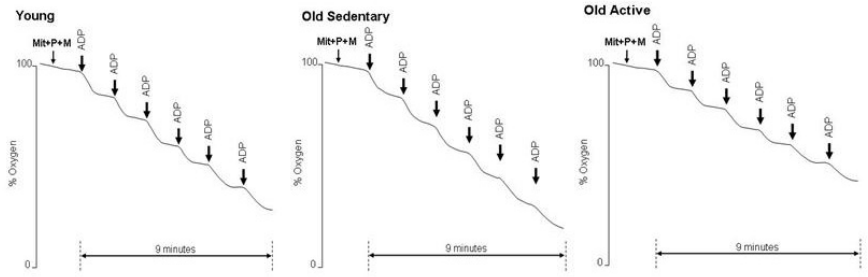

Figure 4- Schematic representation of the assay IV (six ADP “cycles”) from the consecutive ADP-stimulation test in examples of one young, one old sedentary, and one old active animal.

Consecutive ADP-stimulation test. When stimulated with consecutive ADP additions, mitochondria from all groups exhibited a significant increase in protein carbonyls and extramitochondrial cytochrome *c* content. In contrast, consecutive ADP additions resulted in a decrease of the SH group levels in all groups under study (Figure 5). Note that these oxidative modifications of mitochondria were not present after a single ADP stimulation (assay II) in all groups under study (data not shown). This observation suggests that the repeated ADP stimulations resulted in increased ROS production as the test proceeded.

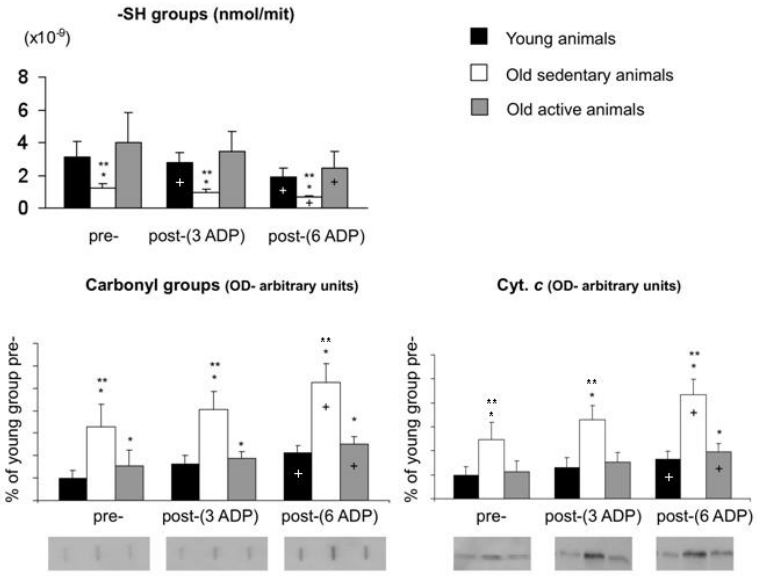

Figure 5- Acute biochemical alterations induced by the consecutive ADP-stimulation test in skeletal muscle mitochondria isolated from young, old sedentary and old active animals. Pre-stimulation (pre-) represents basal conditions; post- (3 ADP) and post- (6 ADP) represent the acute alterations induced by 3 and 6 ADP additions, respectively. Data are mean ± standard deviation. Biochemical data represents ten experiments in young and old active animals and eight separate experiments in old sedentary animals. Data from SH groups is expressed as nmol *per* mitochondria. Protein carbonyls and cytochrome *c* data is expressed as optical density arbitrary units. Protein carbonyl formation (blots) shows a representative pattern of anti-dinitrophenol (DNP)-specific interaction with DNP for each group. Immediately below the cytochrome *c* histogram the panel shows representative western blots from each group and ADP-stimulation test condition. * Significantly different from young animals ($p < .05$); ** Significantly different from old active animals ($p < .05$); $^{+}$ Significantly different from pre-stimulation ($p < .05$).

Impact of age and physical inactivity on mitochondrial oxidative damage

To evaluate the impact of lifelong inactivity on the level of mitochondrial oxidative damage we determined the activity and content of several oxidative stress biomarkers in mitochondria of young, old sedentary and old active animals in basal conditions (assay I) (table 6 and figure 5 "*pre-stimulation"*).

Table 6. Biochemical data obtained from skeletal muscle mitochondria isolated from young, old sedentary, and old active animals.

Measured parameters	Young	Old Sedentary	Old Active
Aconitase (U/mit)	$6.0x10^{-9} \pm 2.9x10^{-9}$	$2.1x10^{-9} \pm 0.8x10^{-9}$ *	$15.0x10^{-9} \pm 4.9x10^{-9}$ **
Carbonyls (OD/mit)	$2.3x10^{-7} \pm 0.8x10^{-7}$	$4.4x10^{-7} \pm 1.9x10^{-7}$ *	$3.3x10^{-7} \pm 1.7x10^{-7}$ **
SH groups (nmol/mit)	$3.1x10^{-9} \pm 1.0x10^{-9}$	$1.2x10^{-9} \pm 0.3x10^{-9}$ *	$4.0x10^{-9} \pm 1.8x10^{-9}$ ***

Data are mean ± standard deviation. Biochemical data represents ten experiments in young and old active animals and eight separate experiments in old sedentary animals. All data is expressed per mitochondria. Protein carbonyl derivatives are expressed as optical density arbitrary units *per* mitochondria.

* Significantly different from young animals ($p < .05$);

** Significantly different from young and old sedentary animals ($p < .05$);

*** Significantly different from old sedentary animals ($p < .05$).

Compared to both young and old active animals, aconitase activity was significantly lower in the old sedentary animals. Moreover, compared to both young and old sedentary animals, mitochondrial from old active animals contained significantly higher aconitase activity. We also observed an age-related increase in mitochondrial protein oxidation as indicated by the increased content of protein carbonyls; note that the age-related increase in protein oxidation was exacerbated in the old sedentary group. Similarly, mitochondria from sedentary animals contained a diminished level of SH groups when compared with both young and active old animals.

Discussion

Overview of the principal findings

The main message of this study is that lifelong sedentary behavior accelerates both age-related sarcopenia and skeletal muscle mitochondrial dysfunction in mice. Moreover, our findings reveal that lifelong sedentary behavior increases the level of oxidative damage in skeletal muscle mitochondria. A detailed

discussion of these major findings and a discussion of the experimental model follows.

Rationale behind the experimental model

The goal of these experiments was to determine whether a lifetime sedentary behavior would exacerbate aged-related sarcopenia and skeletal muscle mitochondrial dysfunction. To achieve this objective, we studied two groups of animals; one group was confined to a small cage for 23 months (i.e. old sedentary) and the second group was housed with free access to a running wheel (i.e., old active). We reason that the activity pattern of the old active animals resembles the normal activity of animals in the wild and therefore these animals served as our control group (i.e., normal activity). In contrast, our sedentary old animals were housed in an environment that fostered an abnormal activity pattern of sedentary behavior and therefore, we consider these animals to be the experimental group (i.e., forced sedentary activity). In this context, we believe that the findings obtained from the normal activity group represents the aging process *per se*, whereas the results obtained from the sedentary animals represent the impact of sedentary behavior on the aging process.

In addition, consistent with previous findings (38), we also observed varying levels of impurity among the mitochondrial pellets (data not shown) indicating contamination of the mitochondrial suspensions. Specifically, we performed morphological analyses of the isolated mitochondria (data not shown) and observed varying degrees of mitochondria-like membranous debris in samples with large variability between both experimental groups and among animals within the same group. Further, our mitochondrial suspensions reveal a significantly higher yield of intact mitochondria in the old sedentary animals (table 2) which agrees with the CS activity measured within the mitochondrial fraction and with the mitochondrial recovery data (table 1). Note, however, the protein content in the mitochondrial suspensions did not differ between groups. Therefore, normalizing our mitochondrial functional measures to total protein content, as typically done in the literature, could bias our experimental results.

In this context, although CS activity is a better marker of mitochondrial mass than the total protein content (38) the normalization of our results to the CS activity also has limitations since the mitochondrial suspensions can be contaminated with extra-mitochondrial CS resulting from damage to mitochondria during the isolation procedure. Therefore, given these important methodological considerations, our discussion will focus on the mitochondrial respiratory data normalized to the number of mitochondria.

Lifelong sedentariness has a major impact on sarcopenia

To compare skeletal muscle mass among our three experimental groups that differed in body mass, we used the ratio of skeletal muscle mass to body mass. Our findings reveal that sedentary behavior exacerbates age-related sarcopenia in mice as indicated by the observation that the skeletal muscle/body mass ratio was significantly greater in the old active animals compared to that of old sedentary animals (Table 1). This conclusion is further supported by the finding that no differences existed in the skeletal muscle mass/body mass ratio between old active animals and the young animals. Collectively, these data support the concept that sedentary behavior plays a major role in the etiology of sarcopenia and that long-term prevention strategies to retard age-induced sarcopenia should include a lifelong program of physical activity (46).

Lifelong sedentary behavior exacerbates age-related functional impairments of mitochondria

Traditionally, mitochondrial function has been evaluated by the assessment of oxygen consumption during a single measurement of both state 3 and state 4 respiration. Using this approach, several studies have reported that aging is associated with diminished mitochondrial function (38, 47, 48). Similarly, compared to young animals, our results demonstrate an age-related impairment in mitochondrial function in both the old active and sedentary animals as documented by a decrease in state 3 respiratory rate with both complex I and complex II-linked substrates. When comparing the state 3 respiratory rates between complex I and complex II-linked substrate assays, we observed a

similar functional impairment for both the OS and OA animals. These results indicate that both complex I and complex II supported respiration were similarly influenced by increasing age and further exacerbated by sedentary lifestyles, as documented by the higher mitochondrial functional impairment in OS animals in both complex I and complex II-linked substrates. These results agree with previous findings suggesting that age-related mitochondrial functional impairment might be explained by decreased activities of complex I, III and IV of mitochondrial ETC, caused by oxidative lesions of mitochondrial DNA that encode these subunits (49). Our finding that the mitochondrial functional impairments are greatest in the old sedentary group (OS) compared to the old active animals (OA) suggests that lifelong inactivity has a negative impact on mitochondrial function. Notably, we postulate that the age-related decline in state 3 mitochondrial respiration is age- and inactivity-related and not due to confounding variables (e.g., membrane damage during isolation) because no differences in state 4 respiration existed between the OA and OS groups. This argument is also supported by the finding that the oligomycin-inhibited state 3 respiration does not differ between groups indicating that the inner mitochondrial membrane integrity was not affected by age, inactivity, or by the mitochondrial isolation procedures. In support of this position, Navarro and Boveris (50) have recently concluded that there is no clear evidence that inner mitochondrial membrane permeability is altered with age.

As an extension beyond traditional mitochondrial function testing, our experiments included a new and innovative approach for testing mitochondrial function using a series of consecutive ADP-stimulation tests. This *in vitro* test was an attempt to assess the capacity of mitochondria to re-establish their homeostatic balance between consecutive ADP-stimulations (e.g. simulated bouts of exercise). We hypothesized that, compared to young animals, mitochondria from old animals would have a lower capacity to maintain homeostasis when challenged with repeated ADP-stimulations. Moreover, we predicted that lifelong inactivity would exacerbate this mitochondrial functional impairment in old animals. Our results support this postulate as we observed an age-associated decline in the maximal mitochondrial respiratory capacity as

documented by the lower state 3 respiratory rates and RCR in both the old sedentary group and old active group, when compared with the young animals. Moreover, old sedentary animals demonstrated a greater impairment of mitochondrial function when compared to old active animals. Although the pattern of functional decline across ADP stimulation trials was similar in all groups (Figure 3), mitochondria from old sedentary animals exhibited significant functional impairments during each ADP stimulation cycle (i.e., lower state 3 respiratory rate, RCR and ADP/O ratios). Finally, state 4 respiratory rates were increased significantly in all experimental groups during the repetitive ADP trials, indicating an increase in mitochondrial respiratory uncoupling.

Our results suggest that mitochondria from younger and old active animals were able to phosphorylate all the ADP added in each of the repetitive ADP "cycles" (Figure 4). In contrast, mitochondria isolated from old sedentary animals were unable to respond similarly; in fact, it was evident in all the experiments (data not shown) that from the 4^{th} ADP "cycle" onwards, mitochondria from the old sedentary animals were unable to phosphorylate all the ADP added, suggesting that ATP homeostasis could not be maintained in response to this energetic challenge. This finding could be due to a decreased ADP phosphorylation capacity at the level of electron transport and/or proton pumping, thereby diminishing the proton electrochemical gradient and compromising the generation of ATP. Ultimately, the ADP/O, normalized to the number of mitochondria was not altered between the ADP "cycles" in all groups studied, suggesting that the efficiency of ADP phosphorylation was not acutely changed.

Inactivity increases mitochondrial oxidative damage in skeletal muscle

Another important finding of this study is that age-related increases in mitochondrial oxidative damage are exacerbated by a lifelong of sedentary behavior. Indeed, our data reveal increased levels of oxidative damage in skeletal muscle mitochondria isolated from old sedentary animals, documented by the diminished aconitase activity, significantly lower sulfhydryl groups and higher levels of protein carbonyls. Mitochondria isolated from old active animals also contained increased levels of oxidative damage, but, the levels were lower

than the old sedentary animals. Collectively this data suggests that aging *per se* does contribute to increased mitochondrial oxidative damage and that sedentary behavior exacerbates this problem.

It is feasible that oxidative damage to mitochondria may account for, at least in part, the age and inactivity-related decline in mitochondria function. For example, our findings indicate that lifelong inactivity results in diminished aconitase activity, which is likely linked to an oxidative-induced down-regulation of enzyme activity. Indeed, this enzyme is particularly sensitive to a reaction with superoxide which damages the iron-sulfur clusters [4Fe-4S] in its active site (51). This increased oxidative damage to this mitochondrial protein could diminish TCA cycle flux leading to decreased electron flow within the respiratory chain and therefore, decreased oxidative phosphorylation (52).

Previous studies have reported an age-related decline in aconitase activity in skeletal muscle mitochondria (52). Interestingly, our results reveal that this age-related decline in aconitase activity is eliminated by a lifetime voluntary activity. Our data do not disclose the mechanism behind the ability of physical exercise to prevent an age-related decline in aconitase activity. However, a reduction in mitochondrial H_2O_2 production after exercise training has been reported in skeletal muscle (53) and Judge et al. (29) found that H_2O_2 production in cardiac subsarcolemmal and interfibrillar mitochondria is reduced in rats subjected to lifelong physical activity. Therefore, the commonly observed age-related decline in aconitase activity in skeletal muscle may not due to aging *per se*, but may result from a lifetime of inactivity. A potential explanation for this postulate is that skeletal muscle inactivity is associated with higher levels of mitochondrial ROS production and therefore, increased oxidative damage to mitochondrial proteins such as aconitase (54).

When assessing the basal levels of mitochondrial oxidative damage, our results confirm the existence of an age-related increase in mitochondrial oxidative damage (table 6) and these findings agree with published reports (51, 52, 55-57). In fact, the present study clearly demonstrates an age-related increase in mitochondrial protein carbonyls and this increase is exacerbated in the old

sedentary group. Moreover, old sedentary animals exhibit a diminished content of SH groups when compared to both the younger and old active animals.

Note that the repetitive ADP-stimulation test (six ADP-stimulations) resulted in a significant increase in the release of cytochrome *c* from the mitochondria in all groups. This finding suggests that this rigorous challenge increased mitochondrial outer membrane permeability. Importantly, the magnitude of cytochrome *c* release following our repetitive ADP challenges was consistently higher in mitochondria obtained from the old animals compared to the young. Furthermore, when comparing the old active animals versus the old sedentary at resting conditions, cytochrome *c* release was already significantly higher in mitochondria from the old sedentary animals. Also, note that an increase in mitochondrial protein oxidation occurred with the repetitive ADP-stimulations in all experimental groups. This increase in protein oxidation is particularly evident in the older animals and further exacerbated in the sedentary animals, and could be due to a (i) higher production of ROS resulting from the increase in mitochondrial respiration, or (ii) an impaired antioxidant capacity. Similar conclusions have been reached by McArdle et al. (58).

Summary and future directions

In summary, our study demonstrates that lifelong sedentary behavior has a major negative impact on the age-related changes of mice skeletal muscle mitochondrial respiration, namely at the level of the complex I and complex II and perhaps additional impairment in the function of the ATP synthase complex. We postulate that this mitochondrial dysfunction may be associated with an age- and sedentary behavior-dependent increased oxidative damage to the mitochondrial biomolecules. Note, however, that our results only provide evidence of an age- and sedentary-related dysfunction of isolated mitochondria obtained from mixed skeletal muscle fiber types of mice, as our final mitochondrial suspensions were obtained from differing muscle phenotypes containing different mitochondrial characteristics (9, 59-61). Therefore, given the well described variability of age-related alterations among skeletal muscles and fiber types (5, 62, 63) the extrapolation of our results to a specific muscle or

fiber type must be done with caution. Additionally, despite being potentially applied to other species, the extrapolation of our results to other type of animals should be done with prudence due to the variability of the aging process between species.

Future experiments should address the biochemical mechanisms responsible for the potential of regular physical exercise to prevent age-related impairments in skeletal muscle mitochondrial function. Finally, based upon our results, it can be argued that many of the age-related events that occur in skeletal muscle mitochondria of rodents housed in small cages without assess to running wheels may be due to inactivity and not to the aging process *per se*.

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General Discussion

General Discussion

Overview of the principal findings

The main findings of the present document support the hypothesis that aging is associated with a diminished respiratory function of skeletal muscle mitochondria of C57BL/6 strain male mice. Additionally, lifelong sedentariness exacerbates the reduction in skeletal muscle mitochondrial respiratory function with age. Moreover, our data demonstrates that the age- and sedentary-related decline in mitochondrial function is associated with higher levels of mitochondrial oxidative damage. A general discussion of these findings and of the selected experimental models follows.

Rationale behind the experimental models

One of the main goals of this work was to estimate the amount of mitochondria in mitochondrial suspensions obtained from skeletal muscle of young and mature mice (paper III). The rationale for this was based on the fact that the process of tissue preparation and mitochondrial extraction for *in vitro* studies, namely when concerning one of the most important steps in the process of mitochondrial isolation (Rasmussen et al., 2003a), the homogenization procedure, may leave some non-mitochondrial proteins in the mitochondrial suspensions which might bias the results (Rasmussen et al., 2003b). Conventionally, the normalization of mitochondrial functional and biochemical data is reported *per* total protein content in suspension, and a major reason for the lack of consensus regarding the age-related mitochondrial dysfunction (paper II and III) could be the assumption that the mitochondrial suspensions obtained from young and aged muscles contained equivalent amounts of non-mitochondrial material and thereby similar degrees of impurities. Our data strongly supports that different degrees of contamination exists in the mitochondrial suspensions obtained from skeletal muscle of mice with different ages (paper III). In fact, when analyzing the transmission electron microscopy (TEM) micrographs obtained from skeletal muscle of young and mature animals we found clear evidences of non-mitochondrial material in both groups, which

was more pronounced in the younger animals. Additionally, despite the inexistence of significant differences in the total protein content of the mitochondrial suspensions between young and aged animals the morphometric data clearly indicate a higher number of mitochondria in the suspensions of the older animals (papers III, IV and V). Furthermore, when concerning the analyses of mitochondrial areas we did not find significant differences between the young and the mature animals (paper III). Collectively, this data supports that mitochondrial suspensions with the same amount of protein do not necessarily have the same content of integral mitochondria. In an attempt to surpass this methodological limitation imposed by non-mitochondrial protein contamination, several mitochondrial enzymatic reference activities have been occasionally used in the literature to normalize mitochondrial functional and biochemical data (Hood, 2001; Wibom, Hagenfeldt, & von Dobeln, 2002). Citrate synthase activity has been used as a marker of mitochondrial mass (Rasmussen et al., 2003a), and in fact our results drawn from TEM analysis of the mitochondrial suspensions supports its utilization (paper III). However, we may argue that despite it is claimed that this enzyme is little affected by the homogenization procedure (Rasmussen et al., 2003a), some degree of contamination of the mitochondrial suspensions with extra-mitochondrial CS might exist, resulting from the rupture of mitochondrial membranes caused by mechanical stress during the isolation procedure. Indeed, the qualitative analysis of the TEM micrographs revealed in all the performed studies (papers III, IV and V) and in all groups under study several degrees of mitochondria-like membranous debris that were not taken into account for the final mitochondrial counts. In this context, despite supporting the notion that CS activity is a better marker of mitochondrial mass than protein content, our data also show that the utilization of this enzyme to establish a reference activity could as well present some limitations. Bearing this in mind, in the context of the experimental work of this thesis (papers III, IV and V) we have normalized all the mitochondrial functional and biochemical data to the number of mitochondria drawn from TEM analysis of the mitochondrial suspensions.

A further methodological concern that has arisen during the preparation of this work was related with the lifelong interventions in aging studies. This raises an interesting discussion about the used terminology, namely the definition of sedentary and active animals (normal voluntarily active and not imposed activity) and the posterior definition of experimental and control groups in aging studies that uses rodents as the animal model. Conventionally, in aging studies, laboratory animals are confined all their lives to a rodent cage. We argue that the results obtained from this experimental approach do not solely represent the impact of the aging process on the selected variables but are also representative of the influence of sedentary behavior that was "imposed" to these animals during all their lives. In fact, in aging studies one of the most difficult tasks is to differentiate the influence of aging *per se* from the repercussions of sedentary lifestyle that is commonly associated with increasing age. Considering this we have used an experimental design that incorporated lifelong sedentariness versus lifelong voluntary activity (paper V). We argue that the activity pattern of animals that have free access to a running wheel during all their lives resembles the normal activity of animals in the wilderness. In contrast, the animals who were confined to a rodent cage during all their lives can not be representative of a normal behavior; instead they are housed in an environment that created an abnormal activity pattern of sedentary behavior. Considering all of the above, for the purpose of this thesis we considered that the animals confined all their lives to a rodent cage without any additional means to spontaneously perform physical activity represents not only the aging process but also the influence of lifelong sedentariness (paper III, IV and OS animals in paper V). In contrast, animals that have free access to a running wheel throughout all their lives, fulfilling their physiological voluntary activity needs, represents the aging process *per se* (OA animals in paper V).

Age- and lifelong sedentary-related sarcopenia

Aging and inactivity are the two main conditions leading to skeletal muscle atrophy in animals (Clarke, 2004; D'Antona et al., 2003; Dirks, Hofer, Marzetti, Pahor, & Leeuwenburgh, 2006; Morley, Baumgartner, Roubenoff, Mayer, &

Nair, 2001; Welle, 2002). Increasing age is associated with progressive and deleterious alterations at the level of skeletal muscle (reviewed in paper I) with enormous repercussions in the functional capacity of the elderly (Clarke, 2004).
In order to study the alterations in skeletal muscle mass with age and lifelong sedentariness we used the ratio of skeletal muscle mass to body mass. Our results demonstrate clearly that lifelong sedentariness plays a major role in the etiology of sarcopenia. Data from paper III, IV and V shows a marked decrease in the ratio skeletal muscle mass/body mass of the older and sedentary animals when compared with the younger animals, supporting evidences already demonstrated in the literature about the impact of physical inactivity in sarcopenia (Marcell, 2003; Vandervoort, 2002). Additionally, the findings from paper V also reveal that sedentary behaviors exacerbate age-related sarcopenia as indicated by the observation that skeletal muscle mass/body mass ratio was significantly lower in the aged-sedentary animals when compared with their age-matched active counterparts. Moreover, no significant differences existed in the skeletal muscle mass/body mass ratio between old active animals and the younger ones (paper V) suggesting that a normal and lifelong voluntary pattern of physical activity has the capacity to attenuate the sedentary-related sarcopenia, supporting that lifelong interventions based on improvements in physical activity levels are one important public health intervention for this condition.

Lifelong sedentary behavior exacerbates the age-related skeletal muscle mitochondrial respiratory dysfunction

One of the main purposes of the present document was to study skeletal muscle mitochondrial function in animals of different ages and the impact of different lifelong interventions on this organelle functionality. The assessment of isolated skeletal muscle mitochondrial respiratory capacity has been widely studied by means of *in vitro* respiratory polarographic assays which involve the measurement of several respiratory parameters in the presence of various substrates (reviewed in paper II). In our experimental work several traditional and standardized *in vitro* tests were used in order to evaluate the maximal

functionality of isolated skeletal muscle mitochondria (paper III, IV and V). Additionally, in paper IV and V we have designed a standardized *in vitro* test characterized by consecutive ADP additions in an attempt to investigate the acute mitochondrial functional and biochemical responses induced by successive variations between a state of high metabolic activity with rapid oxygen consumption (state 3 respiration) and a state of reduced metabolic activity with low rates of oxygen consumption (state 4 respiration), thereby studying their capacity to withstand several successive ADP-stimulations and re-establish their respiratory homeostatic balance.

Taken together our data from paper III, IV and V clearly supports the existence of an age-related impairment of isolated skeletal muscle mitochondrial respiratory capacity, further exacerbated by lifelong inactivity. In fact, our results revealed that the maximal rate of mitochondrial oxygen consumption is diminished both in aged sedentary and aged voluntary active animals as demonstrated by the impaired state 3 respiratory rate both with complex I and complex II-linked substrates. These results were further confirmed by the maximal rate of uncoupled respiration that was reduced in aged animals and further exacerbated when sedentariness was taken into account, and collectively supports previously reported findings of diminished mitochondrial function with increasing age (Desai, Weindruch, Hart, & Feuers, 1996; Drew et al., 2003; Faist, Koenig, Hoeger, & Elmadfa, 1998; Mansouri et al., 2006; Sastre, Pallardo, & Vina, 2003; Short et al., 2005; Tonkonogi et al., 2003). Data from our studies also demonstrates that the functional impairment observed during state 3 respiration could be similar or even greater in complex I comparatively to complex II-linked substrates, suggesting that both complex I and succinate-supported respiration were affected with increasing age. These results supports several published data who demonstrates that the activity of the electron transport chain complexes, namely complex I, III and IV, is diminished with age (Van Remmen & Richardson, 2001). Overall, the results from paper III, IV and V are consistent with an age-related and sedentary-exacerbated reduction in mitochondrial respiration with both complex I and complex II-linked substrates. In contrast to the above mentioned results we

have failed to demonstrate any age- or sedentary-related alterations in state 4 respiratory rate, suggesting that the permeability of the inner mitochondrial membrane was not apparently affected by age or physical inactivity. This statement was further confirmed by an additional assay in which mitochondrial respiration in the presence of olygomycin was assessed. In fact, in all our experimental work (paper III, IV and V) we have not found any significant alterations in the olygomycin-inhibited state 3 respiration between groups or when considering the different lifelong interventions, which supports our argument that the integrity of the inner mitochondrial membrane was unaffected by age or sedentariness. Moreover, considering the functional integrity of mitochondria and bearing in mind that RCR could be interpreted as a measure of functional and structural integrity of this cellular organelle (Magalhaes et al., 2005) our results demonstrate a decreased RCR with age which was further exacerbated by sedentary lifestyles. This reduction is mainly a consequence of the decrease in state 3 respiratory rate as state 4 was unchanged, and is consistent with a diminished functionality of mitochondria of aged animals when compared with the younger ones.

When exposed to successive ADP-stimulations (paper IV and V) our results support our initial postulate, specifically that mitochondria from older animals have a lower capacity to withstand the imposed stimuli and that physical inactivity intensify the observed functional impairment. In fact, data from paper IV and V showed that mitochondrial function declined following each successive ADP-stimulation being the pattern of functional decline similar across the groups under study. Actually, we observed lower state 3 respiratory rates and RCR in both old sedentary and old active groups, when compared with the younger animals, suggesting that skeletal muscle mitochondria from young animals exhibited superior functional responses during each ADP stimulation. Accordingly, our results demonstrated a greater impairment of mitochondrial function in the sedentary animals when compared with the old active animals (paper V). A further observation drawn from the *in vitro* ADP-stimulation test was that state 4 respiratory rate increased in all groups under study at the completion of the test. Interestingly, this increase in state 4 respiration was

greater in younger animals both when compared with the aged sedentary and active animals; the mechanism to explain this observation in unclear as state 4 respiration is governed by several factors including the integrity of the inner membrane and the content of uncoupling proteins along with ATP synthase complexes (Kerner et al., 2001). In the context of this *in vitro* ADP-stimulation test we may argue that the acute challenge imposed by the successive ADP additions have induced an increase in mitochondrial respiratory uncoupling, demonstrated by the increased state 4 respiratory rates and decreased RCR in all groups under study. We must emphasize that although mitochondrial RCR decreased during the alternating state 3/state 4 conditions, in the young and in the old active animals' RCR's remained relatively high and were indicative of functional mitochondria (paper IV and V). In contrast, mitochondria from skeletal muscle of old sedentary animals were unable to phosphorylate all the ADP added from the 4^{th} and 5^{th} ADP addition onwards suggesting that ATP homeostasis could not be maintained in response to this successive variations between states characterized by high metabolic activity with rapid oxygen consumption (state 3 respiration) and states of reduced metabolic activity with low rates of oxygen consumption (state 4 respiration). This observation could be explained by a decreased ADP phosphorylation capacity due to a decreased functionality at the level of the electron transport and hydrogen pumping thereby diminishing the proton electrochemical gradient and limiting the ATP production. Consistent with the abovementioned results supporting an age-associated skeletal muscle mitochondrial dysfunction and bearing in mind its potential role as a mechanism behind the reduction of skeletal muscle fiber function with increasing age, mitochondrial respiratory chain proteins might be seen as cellular redundant components, being the ceasing of its functionality assumed as a loss of redundancy within the skeletal muscle fiber with consequent reduction in the reserve capacity and therefore maximal functionality (reviewed in paper II). Additionally, the age-related loss of oxidative capacity of skeletal muscle fibers reflects not only the reduced capacity of mitochondria themselves but also a diminished mitochondrial density (Conley et al., 2000), and in attempt to counteract the age-related skeletal muscle fiber dysfunction several cellular

mechanisms of mitochondrial biogenesis exist (reviewed in paper II). As shown by the indirect biochemical assessment of skeletal muscle mitochondrial density (paper III and V) our results demonstrate that lifelong sedentariness has a major negative impact on skeletal muscle mitochondrial density and that lifelong voluntary physical activity is able to counteract this reduction. These findings might be explained by voluntary activity-dependent up-regulation of genes originating from nuclear and mitochondrial genomes inducing an increase in mitochondrial protein synthesis (reviewed in paper II) and supports several previous reported findings that physical activity is able to influence skeletal muscle mitochondrial content even in the aged skeletal muscle (Conley et al., 2000; Menshikova et al., 2006). In this context, a lifelong voluntary active lifestyle may have a potential positive effect on the skeletal muscle fiber oxidative capacity not only by attenuating the deleterious sedentary-related effects on the specific activity of individual mitochondria but also contributing to the expansion of mitochondrial mass.

Age and lifelong sedentariness increases skeletal muscle mitochondrial oxidative damage

One study hypothesis of the present thesis was that age-related skeletal muscle mitochondrial dysfunction is associated with increased levels of mitochondrial oxidative damage. Moreover, we further hypothesized that lifelong sedentariness exacerbate the age-induced loss of skeletal muscle mitochondrial function which would also be associated with increased sedentary-related levels of mitochondrial oxidative damage.

Our results confirm the enunciated hypothesis since we have found that mitochondria isolated from skeletal muscle of aged sedentary animals possessed significant fewer sulfhydryl groups, lower aconitase activity and higher levels of protein carbonyls when compared with age-matched voluntary active animals and young animals. Additionally, aged voluntarily active animals also demonstrated significantly fewer sulfhydryl groups and higher levels of protein carbonyls, when compared with the younger animals, supporting several observations described in the literature about the age-related increase in the

levels of mitochondrial oxidative damage (reviewed in paper II). In this context, it has been suggested that oxidative damage to mitochondrial biomolecules with increasing age can have negative functional consequences and it has been hypothesized to constitute an important mechanism linking mitochondria, oxidative stress and aging (Barja, 2004; Dirks et al., 2006; Sastre et al., 2003; Short et al., 2005; Sohal, 2002). Our findings indicated that lifelong inactivity results in diminished aconitase activity which is likely linked to an oxidative-induced down-regulation of enzyme activity. In reality, this enzyme is particularly sensitive to reaction with superoxide and consequently to oxidative damage due to their iron-sulfur clusters [4Fe-4S] in its active site (Floyd, West, & Hensley, 2001). Aconitase oxidative inactivation could diminish TCA cycle flux leading eventually to a decreased hydrogen supply to the respiratory chain and therefore to a diminished oxidative phosphorylation (Yan, Levine, & Sohal, 1997). Despite previous studies have reported an age-related decline in aconitase activity in skeletal muscle mitochondria (Sastre et al., 2003; Yan et al., 1997), our results reveal that a pattern of lifelong voluntary activity is able to eliminate this reduction (paper V). In fact, the results from paper V enable us to observe an increase in aconitase activity in the animals that were voluntarily active throughout all their lives, both when compared with data drawn from the young and age-matched sedentary animals. Nonetheless, our results do not allow us to establish the mechanisms behind this age-related increase; however, we may speculate that it may be related with an augmented redundancy of enzymes of this metabolic pathway. The enhanced CS activity in old active animals, determined in whole skeletal muscle (table 1 of paper V) supports this assumption. Therefore, on the basis of our results we may speculate that the age-related decline in skeletal muscle aconitase activity, usually reported in the literature, may not be due to aging *per se* but may result from a lifetime of sedentary behaviors. Further studies are needed in order to fully clarify this topic.

Independently of the underlying causes (age and/or sedentariness) it has been suggested that mitochondrial protein oxidation might be protein-specific and appears to be linked to ACON and adenine nucleotide translocase (ANT) (Yan

et al., 1997; Yan & Sohal, 1998). In this context, in addition to ACON oxidative inactivation, oxidative damage to ANT leading to its functional inactivation could promote a decrease in state 3 respiration and increased mitochondrial H_2O_2 production due to enhanced reduction of the ETC mitochondrial complexes (Sohal, 2002). In agreement with previous reports (reviewed in paper II) the results from paper IV and V reveal an age-related increase in mitochondrial oxidative damage further exacerbated by lifelong inactivity, demonstrated by increased levels of protein carbonyls and diminished content of SH groups. This age- and sedentary-related increased oxidative damage is functionally significant because oxidative damage to mitochondrial complexes could impair electron transport and compromise mitochondrial respiration; this may actually explain the lower mitochondrial respiratory capacity in aged and aged sedentary animals reported in our experimental work. In fact, we hypothesize that the exacerbation of mitochondrial dysfunction in the old sedentary animals may be explained by higher levels of mitochondrial ROS production and therefore by the increased oxidative damage that is usually associated with skeletal muscle inactivity (Kondo, Nakagaki, Sasaki, Hori, & Itokawa, 1993).

As already mentioned we have not found any age- or lifelong sedentary-related alteration at the level of the functional integrity of the inner mitochondrial membrane. Interestingly, several published reports indicate that phospholipid membrane environment may became less functional with age (Kwong & Sohal, 2000) due to increased levels of lipid peroxidation (Faist et al., 1998; Paradies, Petrosillo, Pistolese, & Ruggiero, 2002; Spiteller, 2001). However, in paper IV we failed to observe any significant alterations in mitochondrial levels of MDA with increasing age.

When mitochondria were exposed to successive ADP-stimulations, our results demonstrated an acute increase in the levels of oxidative damage biomarkers in all groups under study (paper IV and V). Additionally, we observed that the acute oxidative damage induced by the ADP-stimulations was particularly evident in the aged animals and further exacerbated by lifelong inactivity. The results from paper IV showed an increase in the lipid peroxidation levels at the completion of the test, both for young and older animals which may be

representative of an enhanced phospholipid membrane dysfunction with alterations in membrane fluidity. This result may explain the acute functional observations drawn from this *in vitro* test, namely the increased state 4 respiration and decreased RCR at the end of the ADP-stimulations; actually, it is described that alterations in inner membrane functionality can have a significant functional impact on the activity of the respiratory chain as well as in the generation of the electrochemical proton gradient resulting in increased state 4 and decreased RCR (Kwong & Sohal, 2000). Moreover, an increase in mitochondrial protein oxidation occurred with the repetitive ADP-stimulations in all groups under study, being particularly evident in mitochondria from the older animals and further exacerbated in the sedentary ones. This may be explained partly by a (i) higher production of ROS resulting from the repetitive acute stimulation, or (ii) an impaired antioxidant capacity in these groups. During the consecutive ADP-stimulation test it was also observed an extra-mitochondrial increased of cytochrome *c* content (paper IV and V), which is suggestive of an enhanced outer mitochondrial membrane permeability in all groups under study (Kroemer, Galluzzi, & Brenner, 2007). Note that the content of extra-mitochondrial cytochrome *c* was significantly higher in the old sedentary animals, not only after stimulation (3 and 6 ADP stimulations) but also at basal conditions. In this context, we may speculate that the enhanced permeability of the outer mitochondrial membrane at basal conditions and after stimulation will favor the occurrence of apoptotic phenomenon in aged sedentary animals (Kroemer, Dallaporta, & Resche-Rigon, 1998; Kroemer et al., 2007). Furthermore, and in addition to its important role in the apoptotic processes, it is suggested that cytochrome *c* extrusion might depend on its interaction with cardiolipin (Kroemer et al., 2007) presumably through cardiolipin oxidation (Orrenius & Zhivotovsky, 2005), which reinforces not only an increase in the outer membrane permeabilization but also a possible increase in the cardiolipin oxidation, a phospholipid of the inner mitochondrial membrane that plays an important role in the function of a number of major integral inner membrane proteins (Hoch, 1992), namely complex I activity (Paradies et al., 2002). Globally, these findings and assumptions may partly explain the greater

functional impairment imposed by the successive ADP-stimulations in the older sedentary animals when compared with their age-matched controls and the younger animals as well as in the older voluntary active animals when compared with the young group.

Conclusions and Future Directions

Conclusions and Future Directions

Based on the analysis of the results drawn from the different studies presented in this document, it is possible to consider the following conclusions and considerations:

-Mitochondrial suspensions obtained from skeletal muscle of animals from different age groups exhibited several degrees of contamination, which supports our initial hypothesis; this finding reveals that the widely accepted assumption that mitochondrial suspensions obtained from animals of different ages contains equivalent amounts of non-mitochondrial material, and thereby similar degrees of impurity, is erroneous;

-Mitochondrial suspensions with similar amounts of total protein, obtained from skeletal muscle of animals with different ages, had significantly different mitochondrial mass, indicating that the normalization of the mitochondrial functional and biochemical data to the protein content will bias the results of the investigations;

-Lifelong sedentariness had a major impact on the reduction of skeletal muscle mass supporting that, more than aging *per se*, lifelong physical inactivity has a major impact in the etiology of sarcopenia;

-In our animals, aging was associated with a loss of skeletal muscle mitochondrial function even when considering a lifelong voluntary active aging behavior, supporting clearly the notion that increasing age by itself has a negative impact on the mitochondrial respiratory capacity;

-Lifelong sedentariness was associated with an altered status of isolated mitochondria from mice skeletal muscle, supporting our initial hypothesis that lifelong sedentary behaviors exacerbates the age-related changes in mitochondrial respiratory capacity;

-The reported mitochondrial dysfunction was associated with an age- and sedentary behavior-dependent increased oxidative damage to mitochondrial biomolecules, which supports our enunciated hypothesis that oxidative damage to mitochondrial biomolecules has negative

functional consequences at the level of mitochondrial respiratory capacity and, moreover, this damage might constitutes an important mechanism linking aging, loss of function and individual behavior.

-Based on the results of our work it may be argued that many of the age-related alterations reported in the literature at the level of skeletal muscle mitochondria of rodents are not only due to the aging process *per se* but also represents the impact of physical inactivity on the selected variables.

In the context of this thesis it is important to highlight that the final mitochondrial suspensions were obtained from different muscle phenotypes containing different mitochondrial characteristics. Bearing this in mind we must be aware that our results only provide evidence of an age- and sedentary-related dysfunction in isolated mitochondria from mixed muscle types in mice and caution must be done when attempting to extrapolate our results to a specific muscle, fiber type or even mitochondrial populations. Additionally, despite the fact that our results indicate that aging and lifelong inactivity were associated with a loss of muscle mass and with an impairment in skeletal muscle mitochondrial function, our studies have not identified the mechanism(s) behind these observations. Future cause-effect experiments using mitochondrial targeted antioxidants could prove useful in determining some of the underlying mechanisms of age- and sedentary-related mitochondrial dysfunction. Attention must be done also to the chronic adaptations to lifelong voluntary physical activity or forced exercise modalities (exercise training protocols) and the regulatory mechanisms responsible for age-related sarcopenia, skeletal muscle mitochondrial biogenesis and mitochondrial functionality. In fact, in the context of our work lifelong voluntary active behaviors might have had a potential positive effect on the skeletal muscle fiber oxidative capacity not only by attenuating the deleterious sedentary-related effects on the specific activity of individual mitochondria but also contributing to the expansion of mitochondrial mass. Further studies are needed in order to confirm these assumptions.

Finally, it would be also interesting to study mitochondria isolated from other tissues in order to correlate the age- and lifestyle-related alterations in mitochondria from organs with different metabolic profiles.

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