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Skeletal muscle disuse atrophy: implications on intracellular signaling pathways and mitochondrial permeability transition pore function

by

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Université de Montréal
Faculté des études supérieures

This thesis entitled :

Skeletal muscle disuse atrophy: implications on intracellular signaling pathways and mitochondrial permeability transition pore function

(Étude de l'inactivité musculaire sur les voies de signalisation intracellulaire et les fonctions du pore de transition de perméabilité mitochondriale)

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SUMMARY

In all biological systems, a balance between cell growth and death is required for normal development as well as for adaptation to a changing environment. Skeletal muscle is considered a mechanosensitive cell type since mechanical forces such as stretch, tension, and loading have been shown to play a critical role in regulating the dynamic processes of protein synthesis, degradation, cellular proliferation and apoptosis. Despite intensive investigation, the intracellular mechanisms by which muscle disuse is perceived and converted to biochemical responses leading to muscle remodeling and atrophy have yet to be completely understood. Whereas it was appreciated a long time ago that muscle atrophy comes about via an increase in protein degradation concomitant with suppression of protein synthesis, only recently have specific signaling pathways and cellular processes been identified. The identification of a growing number of apoptosis-associated factors and events in disused muscle is providing increasing evidence that apoptotic cell-death plays a role in muscle fiber atrophy due to a variety of causes.

This thesis is composed of three research studies developed with the objective of contributing to the present understanding of the intracellular processes regulating disuse muscle atrophy. The first study explores the intracellular signaling potential in disused muscle in response to a mechanical stimulus. The results of this study show that c-jun NH_2-terminal kinase (JNK) phosphorylation response to mechanical stimulation is decreased following hindlimb suspension indicating that atrophic muscle may lose the ability to transduce mechanical signals to the mitogen-activated protein (MAP) kinase pathways. However, we also show that basal JNK activity is increased in disused muscle. Since the contribution of cellular apoptosis has been proposed as a possible mechanism regulating the loss of myofibers as a result of reduced mechanical loading, increased JNK-mediated regulation of cellular apoptosis may explain the increased basal phosphorylation levels measured in muscle following hindlimb suspension. The second study investigates the effect of loss of neural input to muscle fibers on changes in the sensitivity and modulation of the mitochondrial permeability transition pore (PTP), a structure responsible for initiating the release of pro-apoptotic factors from the mitochondrial intermembrane space and thought to be implicated in a form of

programmed cell death, or apoptosis. We show that muscle atrophy caused by denervation (for 21 days) is associated with heightened sensitivity of the PTP to opening in response to progressive Ca^{2+} loading. These findings are substantiated by studies with cyclosporin A (CsA), a pharmacological agent which inhibits PTP opening by binding to cyclophilin D (CypD). We report that the inhibitory effects of CsA are significantly more potent in mitochondria from denervated muscle. The major finding that the susceptibility to PTP opening is dramatically favored following denervation provides evidence for a previously unreported mechanism that could at least partly account for the activation of the mitochondrial death pathway in denervation disorders in animal models and humans. The third study is a characterization of the properties and function of the mitochondrial PTP in different skeletal muscles characterized by different fiber types. Considering the well-established metabolic differences between oxidative and glycolytic muscle fiber phenotypes, there exist surprisingly few studies which have investigated intrinsic mitochondrial properties in relation to fiber type. We demonstrate that basic PTP function and sensitivities are different depending on the type of muscle fiber from which mitochondria are isolated. Our findings support the emerging view that mitochondria display distinct properties that differ qualitatively according to muscle fiber type.

SOMMAIRE (FRANÇAIS)

Dans tous les systèmes biologiques, l'équilibre entre la croissance et la mort cellulaire est nécessaire au développement normal ainsi qu'aux adaptations face aux changements environnementaux. Les fibres du muscle squelettique sont considérées comme des cellules mécanosensitives puisque les forces mécaniques comme l'étirement, la tension et la charge jouent un rôle important dans la régulation des processus dynamiques comme la synthèse et la dégradation de protéines ainsi que la prolifération cellulaire et l'apoptose. Malgré l'existence de plusieurs études sur le sujet, les mécanismes intracellulaires par lesquelles l'inactivité musculaire est convertie en réponse biochimique entraînant le remodelage et l'atrophie musculaire ne sont pas encore bien compris. Bien qu'il est accepté depuis longtemps que l'atrophie musculaire résulte d'une concomitance entre l'augmentation de la dégradation et la suppression de la synthèse protéique, ce n'est que récemment que des voies de signalisation et des processus cellulaires spécifiques ont été identifiés. L'identification de plus en plus de facteurs et d'événements associés à l'apoptose prenant place dans l'inactivité musculaire suggèrent que la mort cellulaire par apoptose joue un rôle dans l'atrophie des fibres musculaires induite par différentes causes.

La présente thèse comprend trois projets de recherche qui ont pour objectif de contribuer à l'avancement de la compréhension actuelle des processus intracellulaires régulant l'atrophie musculaire. La première étude explore le potentiel de la signalisation intracellulaire dans l'inactivité musculaire en réponse à un stimulus mécanique. Les résultats de cette étude montrent que la phosphorylation de c-jun NH_2-terminal kinase (JNK) en réponse à un stimulus mécanique est réduite lors de la suspension de l'arrière-train, indiquant que le muscle atrophié peut perdre l'habilité à traduire un signal mécanique à la voie des mitogen-activated protein (MAP) kinases. Nous démontrons également que l'activité basal de JNK est augmentée dans l'inactivité musculaire. Puisqu'il a été proposé que l'apoptose soit un mécanisme possible dans la régulation de la perte de myofibres résultant d'une diminution de charge mécanique, il est possible que l'augmentation de la régulation de l'apoptose par JNK explique la phosphorylation

de base plus élevée mesurée dans le muscle suivant une période de suspension de l'arrière-train. La deuxième étude examine l'effet de la perte d'innervation des fibres musculaires sur les changements dans la sensibilité et dans la modulation du pore de perméabilité transitionnelle (PTP), une structure responsable pour l'initiation de la relâche de facteurs pro-apoptotiques résidants normalement dans l'espace intermembranaire mitochondrial et impliqué dans la mort cellulaire programmé, ou apoptose. Nous démontrons que l'atrophie musculaire induite par dénervation (21 jours) est associée à une sensibilité accrue de l'ouverture du PTP en réponse à un chargement progressif de Ca^{2+}. Ces résultats sont appuyés par des expériences effectuées avec la cyclosporine A (CsA), un agent pharmacologique qui inhibe l'ouverture du PTP en se liant à la cyclophilin D (CypD). Nous rapportons que l'effet inhibiteur de la CsA est significativement plus élevé dans les mitochondries provenant de muscles dénervés. La découverte importante que la susceptibilité de l'ouverture du PTP est considérablement augmentée suivant la dénervation suggère l'existence d'un mécanisme non identifié jusqu'à présent et qui pourrait, du moins en partie, être responsable de l'activation de la voie mitochondriale de mort cellulaire dans les désordres neurologiques observés dans le modèle animal et humain. La troisième étude porte sur la caractérisation des propriétés et des fonctions du PTP mitochondrial dans différents muscles squelettiques se distinguant par leurs types de fibres. Considérant les différences métaboliques déjà bien établies entre les phénotypes des fibres musculaires oxydatives et glycolytique, il existe étonnamment peu d'études qui ont investigué les propriétés intrinsèques mitochondriales en relation avec le type de fibre. La troisième étude présentée dans cette thèse examine certaines propriétés fonctionnelles du PTP dans des mitochondries isolées de muscles provenant des pattes postérieures de rat, qui sont caractérisés par différents types de fibres. Nous démontrons que les fonctions de bases et la sensibilité du PTP sont différentes selon le type de fibres musculaires d'où proviennent les mitochondries. Nos résultats supportent l'idée grandissante que les mitochondries ont des propriétés distinctes, qui diffèrent qualitativement selon le type de fibre musculaire.

KEYWORDS

skeletal muscle
atrophy
disuse
intracellular signaling
mitochondrial permeability transition pore
mitogen-activated protein kinase
cyclophilin D
denervation
hindlimb suspension

MOTS CLÉS

muscle squelettique
atrophie
inactivité
signalisation intracellulaire
pore de transition de perméabilité mitochondrial
mitogen-activated protéine kinase
cyclophilin D
dénervation
suspension des membres arrières

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ABBREVIATIONS

ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
ANOVA	analysis of variance
ANT	adenine nucleotide translocator
Apaf-1	apoptosis protease activating factor-1
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium
COX	cyclooxygenase
CRC	calcium retention capacity
CsA	cyclosporin A
CTL	control
CypD	cyclophilin D
DISC	death-inducing signaling complex
DEN	denervated
EDL	extensor digitorum longus
EGTA	ethylene glycol-bis(ß-aminoethyl ether) tetraacetic acid
EndoG	endonuclease G
FOXO	forkhead box O
HLS	hindlimb suspension
IAP	inhibitor of apoptosis protein
JNK	c-jun NH_2-terminal kinase
kDa	kilo Dalton
MAPK	mitogen-activated protein kinase
MiCK	mitochondrial isoform of creatine kinase
MG	medial gastrocnemius
MHC	myosin heavy chain
PAGE	polyacrylamide gel electrophoresis
PLN	plantaris
PPIase	pepidyl propyl-*cis, trans*-isomerase
PTP	permeability transition pore
ROS	reactive oxygen species
SOL	soleus
TUNEL	terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick-end labeling
VDAC	voltage-dependent anion channel

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To my families: old, new, and future…..

Chapter 1: Introduction

1 INTRODUCTION

1.1 Presentation of the experimental studies

The purpose of the work presented in this thesis was to broaden our understanding of the cellular processes involved in mediating the adaptations of skeletal muscle to disuse. Thus, this thesis investigates two major axes of research relating to the disuse atrophy of skeletal muscle.

The first area of investigation extends on the research interests of Dr. Phil Gardiner and pertains to the effects of reduced weight-bearing on the sensitivity of mechanically-responsive intracellular signaling pathways in muscle. Mechanical forces play an important role in the regulation of muscle size (Vandenburgh, 1987). Since there appears to exist a relationship between muscle unloading and atrophy, the first study presented in this thesis investigates the sensitivity of mechanically-responsive intracellular signaling pathways in order to establish the extent to which a given mechanical stimulus can influence the trophic response of muscle following atrophy. We focused on c-jun NH_2-terminal kinase (JNK), a member of the mitogen-activated protein (MAP) kinase family that is activated by phosphorylation in skeletal muscle in response to a number of cellular stresses including changes in loading conditions. JNK activation was also reported to activate programmed cell death (*i.e.* apoptosis) (Davis, 2000; Papadakis *et al.*, 2006), a process that is activated in the disused muscle (Kandarian *et al.*, 2006) and may account for the loss of entire myofibers and/or of some nuclei within remaining myofibers. The results of this study showed that basal JNK activation state (*i.e.* phosphorylation) is increased in response to muscle atrophy, which may reflect activation of cellular apoptosis. On the other hand, we reported that the capacity to activate JNK by phosphorylation in response to an acute mechanical challenge is reduced following hindlimb unloading. These results led us to propose that atrophic muscle may lose the ability to transduce mechanical signals to the MAP kinase pathways.

The second axis of research of this thesis extends on the general interests of Dr.

Yan Burelle pertaining to the role of mitochondrial plasticity in heart and skeletal muscle under physiological and pathological conditions. A particular focus of this second research area relates to the investigation of the mitochondrial permeability transition pore (PTP), a structure identified as a key player in signaling necrosis and apoptosis in several tissues and cell types (Zoratti & Szabo, 1995; Hengartner, 2000). Despite a large number of experimental studies providing evidence implicating the PTP as a trigger for cell death, little is known regarding the regulation and behavior of the PTP in skeletal muscle and whether or not it is affected in the process of severe muscle atrophy.

In the second study presented in this thesis we determined whether the sensitivity or occurrence of PTP opening is altered in response to muscle denervation, a model of severe muscle disuse that mimics several denervation disorders observed in humans (Tews, 2002). The results from this study showed that a loss of innervation for 21 days led to a dramatic increase in the vulnerability of isolated mitochondria to opening of the PTP. This phenomenon was at least partly caused by a significant increase in the endogenous Ca^{2+} content of both myofibers and mitochondria in response to denervation. In addition, we made the novel observation that cyclophilin D (CypD), a matrix protein that was recently shown to act as a regulator by sensitizing the PTP to Ca^{2+}-induced opening (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005) was upregulated compared to several other mitochondrial proteins including other putative PTP component, namely the adenine nucleotide translocator (ANT) protein and the voltage-dependent anion channel (VDAC), and enzymes of the respiratory chain (*i.e.* cytochrome oxidase). To our knowledge, this study provides the first evidence that changes in the expression of CypD could play a role in a non-genetic model of disease and suggests that opening of the PTP could be involved in the activation of apoptosis generally observed in response to disuse atrophy.

Another question that remains unanswered with regards to the PTP is whether its regulation varies across muscle fiber types. This question is particularly relevant as the appearance and progression of several neuromuscular disorders is very heterogeneous in muscles with various phenotypes (Tews, 2002), which may in part reflect a different

vulnerability of mitochondria to PTP opening and activation of mitochondrial death pathways across fiber types. In the third study presented in this thesis, we isolated mitochondria from muscles displaying different fiber type compositions and determined their sensitivity to PTP opening *in vitro* and examined a selected number of physiological regulators of pore opening at the mitochondrial level including endogenous Ca^{2+} levels, production of reactive oxygen species (ROS) and content of putative PTP components including CypD. The results from this study indicated that mitochondria from the slow-twitch soleus composed predominantly of type I fibers, displayed a significantly greater vulnerability to PTP opening compared to mitochondria isolated from the plantaris and white gastrocnemius muscles, composed primarily of type II fibers. ROS production, which is a well-known PTP inducer, was lower in mitochondria from soleus compared to the other muscles implying that factors other than ROS were involved. One of these factors appeared to be the greater endogenous Ca^{2+} content within mitochondria from the soleus as compared to plantaris and white gastrocnemius. However, this could not entirely account for the differences in vulnerability to pore opening among muscles. We observed that the expression of ANT and VDAC, two putative PTP components, was significantly greater in mitochondria from soleus compared to mitochondria from the other muscles, which may increase the likelihood that these proteins undergo conformational changes under conditions that favor PTP opening. On the other hand, pharmacological and molecular evidence indicated that CypD expression was similar across muscle types suggesting that this protein was not involved. This led us to speculate that the existence of a fiber type-specificity in the regulation of PTP opening could at least partly account for the heterogeneous progression of neuromuscular disorders across muscles.

1.2 Introduction to the review of literature

The review of literature is divided into four main sections. The objective of the first section is to provide the reader with an overview of skeletal muscle fiber types and some of the particular aspects that are pertinent to the understanding of disuse atrophy. In particular, the literature available on the differences in mitochondrial profile that exist between muscles with different metabolic and contractile phenotypes will be reviewed.

The second section of the review will focus on muscle disuse. First, an overview of the experimental models from which much of our understanding with regards to how muscles react to disuse was obtained will be presented and some of the classic changes that occur with disuse will be reviewed. As one of the important mechanisms responsible for the loss of muscle mass involves an increase in protein degradation and a reduction in protein synthesis, some of the recent advances in the identification of signaling pathways that could mediate these effects, including the Akt/PkB, nuclear-factor-kB (NFkB) and MAP kinase pathways and highlight the role for these pathways in apoptotic signaling will then be presented.

In the third section, an overview of the apoptotic machinery, including the intrinsic pathway of cell death, where mitochondria are believed to play a pivotal role, is presented. Given the nature of the experimental work presented in this thesis, the consequences of PTP opening as well as the structure of this pore and its regulation are covered in more detail. Finally, the fourth section of the review will focus particularly on cell death in models of muscle disuse and on the evidence supporting a role for mitochondria in this process.

2 SKELETAL MUSCLE FIBER TYPES

2.1 Contractile proteins

Skeletal muscle tissue is composed of heterogeneous muscle fibers with different contractile and metabolic profiles. This fiber diversity confers, to a certain extent, the property of adaptability to skeletal muscles. Mammalian skeletal muscle is a multinucleated, highly specialized tissue made up of fibers with a diverse range of properties (Schiaffino & Reggiani, 1996; Bottinelli & Reggiani, 2000). The general properties of a given muscle typically result from the distinct properties of the represented fiber types combined with their proportions. The classification of muscle fibers into slow and fast 'types' is based primarily on the kind of myosin heavy chain (MHC) isoform predominantly expressed (Schiaffino & Reggiani, 1996). Myosin is considered the main structural and regulatory protein in skeletal muscle and plays a prominent role in dictating skeletal muscle functional and contractile properties. In the

limb muscles of the rat, the MHC protein exists as four main isoforms: the slow type I and the fast types IIa, IIx, and IIb. Table I summarizes the proportions of MHC isoforms in selected muscles of humans and rats. Interestingly, protein expression of the IIb MHC isoform is lacking in humans (Pereira Sant'Ana *et al.*, 1997).

Table 1-1: Comparison of MHC isoforms in selected muscles of the rat and human

	Rat				**Human**		
Muscle	I	IIa	IIx	IIb	I	IIa	IIx
Soleus	89	11	0	0	67	27	6
MedialGastrocnemius	7	10	25	58	58	27	15
Vastus Lateralis	0	0	2	98	39	43	18

Values are in percent (%). Table adapted from Talmadge, 2000.

In addition to the four major MHC isoforms already mentioned, other isoforms have been identified including embryonic, neonatal, extraocular, and laryngeal-specific isoforms (Pette *et al.*, 2000), but their expression levels in limb muscles are negligible and therefore, will not be further discussed. Since the contractile properties of a muscle fiber are in large part determined by the MHC composition (Bottinelli & Reggiani, 2000; Reiser *et al.*, 1985; Schiaffino & Reggiani, 1996; Fitts *et al.*, 1998; Widrick *et al.*, 1999), the nomenclature for the four major fiber types identified in limb muscles suitably follows the type of MHC isoform present (*i.e.* fiber types are designated as type I, IIA, IIX, or IIB) (Rivero *et al.*, 1998). Presently, there is some debate as to whether or not 'discrete' fiber types even exist as it is becoming increasingly evident that fiber types might actually exist on a continuum of contractile velocities and metabolic properties rather than representing discrete entities (Talmadge *et al.*, 1993; Pette *et al.*, 2000).

2.2 Proteins involved in excitation-contraction coupling and Ca^{2+} handling

Although slow and fast-twitch skeletal muscles are used to perform distinct functions, all vertebrate skeletal muscles have the same basic structure and use the same basic contractile system (Rome & Lindstedt, 1998). It is the specific modifications of the proteins involved in the contractile system that allow the muscles to perform these

diverse tasks. Both qualitative and quantitative modifications of several muscle proteins seem to underlie the physiological differences between slow and fast twitch fibers. For instance, slow-twitch fibers, compared with fast-twitch fibers, contain slower MHC isoforms, which have a reduced maximal velocity of shortening (Reiser *et al.*, 1985), a decreased content of sarcoplasmic reticulum (SR) (Appelt *et al.*, 1989), with a slower isoform of the Ca^{2+} pump (SERCA2 in slow-twitch vs. SERCA1 in fast-twitch) (Lytton *et al.*, 1992), and a lower concentration of the cytoplasmic Ca^{2+} buffering protein parvalbumin (Heizmann *et al.*, 1982). Calsequestrin, a Ca^{2+} binding protein located in the lumen of the SR, also has a unique isoform expression pattern in slow-twitch and fast-twitch muscles of rabbit and rat (Damiani & Margreth, 1994). Finally, the ryanodine receptors are also considered a part of this molecular excitation-contraction-associated machinery that varies across fiber types and molecular evidence indicates that distinct isoforms of ryanodine proteins are expressed in tuna slow- and fast-twitch skeletal muscle (Franck *et al.*, 1998). During transitions in muscle fiber type, these proteins are co-regulated with the expression of specific MHC isoforms.

2.3 The myonuclear domain

A special feature of skeletal muscle fibers compared to most other cells is that they are multinucleated and successive muscle fiber segments are controlled by individual nuclei. Mammalian skeletal muscle fibers have been shown to maintain a relatively finite, fiber type-specific relationship between myofiber size and myonuclear number (Hikida *et al.*, 1997). In fact, the relationship between myonuclear number, cell size, succinate dehydrogenase activity (a marker of mitochondrial density), and MHC type has previously been examined in single fiber segments mechanically dissected from soleus and plantaris muscles of rats (Tseng *et al.*, 1994). The results from this study show that cytoplasmic volume per myonucleus is higher in fast and slow plantaris fibers (112 vs. 34 x 10^3 microns^3) than fast and slow soleus fibers (40 vs. 30 x 10^3 microns^3), respectively. The authors report that slow fibers always had small cytoplasmic volumes per myonucleus, regardless of fiber diameter, succinate dehydrogenase activity, or muscle of origin. Slow soleus fibers had significantly greater numbers of myonuclei/mm than did either fast soleus or fast plantaris fibers (116 vs. 55 and 44, respectively). These data suggest that the size of the myonuclear domain is

more limited in slow than fast fibers, and in the fibers with a high, compared to a low, oxidative metabolic capacity (Tseng *et al.*, 1994).

Interestingly, the myonuclear domain maintains a degree of plasticity by adapting to changes in muscle fiber size, and likely phenotype. The atrophic response due to unloading on the number of myonuclei, and size of the myonuclear domain in fibers of rat hindlimb muscles has been studied. It would appear that as a muscle undergoes a disuse-induced phenotypic transition from slow-to-fast or from fast-to-faster, existing myonuclei are eliminated to support not only the reduction in fiber size, but also the inherent change in phenotype. Although intuitively sound, this rationale implies that it is fiber phenotype and size that dictate myonuclear number. The question has been raised whether the loss of myonuclei in response to muscle disuse is the early event which causes muscle atrophy and slow-to-fast directional transitions in fiber type. Most studies have shown that the loss of muscle mass becomes significant between 3 and 7 days following the onset of disuse (Allen *et al.*, 1997; Krawiec *et al.*, 2005) even though protein synthesis is suppressed as promptly as 6 hours after muscle disuse (Watson *et al.*, 1984; Thomason *et al.*, 1989). However, a very recent study shows that in response to unloading of the rat hindlimb muscle, loss of myonuclei is already increased at a time when no measurable loss of muscle mass or cross sectional area has occurred, suggesting that myonuclear loss is an early, and possibly the causative event in the process of muscle atrophy (Dupont-Versteegden *et al.*, 2006). The significance of this observation needs further investigation since it is still unclear whether loss of myonuclei initiates, or occurs as a consequence of the loss of muscle mass during atrophy.

2.4 Mitochondrial profiles

Beside substantial differences in contractile proteins, components involved in excitation-contraction coupling, and size of myonuclear domains, muscle fibers also display dramatic differences in their metabolic profile. Given that in the present thesis a main focus is on mitochondria, this section examines differences in the mitochondrial profile that occurs across muscle fiber types including quantitative differences in mitochondrial volume density as well as qualitative differences that may exist in terms of ultra-structure, function, and regulation of respiration.

2.4.1 Mitochondrial volume density

The most important difference in mitochondrial profile that can be observed across fiber types is the volume of muscle cells occupied by mitochondria, which has been termed mitochondrial volume density. Morphometric analysis of electron micrographs obtained from muscles with different fiber type compositions indicate that mitochondrial volume density is approximately 6 % in soleus, a muscle composed predominantly of type I oxidative fibers (Schwerzmann *et al.*, 1989). In type IIa fibers, which have a high capacity for oxidative phosphorylation as well as for glycolysis, mitochondrial volume density is similar and in rat specifically, may even reach higher values than type I fibers (this is not the case in humans where type I ≥ IIa (Gardiner, 2001)). Of note however, mitochondria in IIa fibers appear smaller and more numerous compared to type I fibers in which mitochondria are less numerous but larger (Shah & Sahgal, 1991). Finally, type IIb fibers have the lowest mitochondrial density at 2-3 % of fiber volume (Schwerzmann *et al.*, 1989), which reflects their high dependence on glycolysis for ATP production.

2.4.2 Intrinsic properties of mitochondria

Although it is clear that skeletal muscle exhibits considerable variation in mitochondrial volume density among fiber types, it is less clear whether the mitochondria also present specific functional characteristics and/or distinct regulatory properties that allow fine adjustments of mitochondrial performance to the conditions and needs encountered in various fibers. The few studies available on this topic have used mainly mitochondria (or less frequently saponin-permeabilized fibers) isolated from fast and slow muscles from rabbits (Jackman & Willis, 1996; Howlett & Willis, 1998; Gueguen *et al.*, 2005a; Gueguen *et al.*, 2005b), cats (Schwerzmann *et al.*, 1989), pigs (Gueguen *et al.*, 2005c), and rats (Pande & Blanchaer, 1971; Yajid *et al.*, 1998; Capel *et al.*, 2004; Mogensen & Sahlin, 2005; Anderson & Neufer, 2006), which express predominantly type IIb and I fibers, respectively. Fish white and red muscles have also been used because they offer the unique advantage of having muscle compartments with very homogeneous fiber types (Leary *et al.*, 2003). As discussed

below, properties that have been compared include mitochondrial enzyme content, respiratory capacities, coupling efficiency between oxidation and phosphorylation, proton conductance of the inner membrane (the so-called proton leak), membrane fluidity, and production of ROS.

2.4.2.1 Enzymology and respiratory capacity

The measurement of maximal respiratory capacity in the presence of various respiratory substrates has been measured in several studies. The group of Weibel in Switzerland (Schwerzmann *et al.*, 1989) reported that maximal ADP-stimulated respiration was similar in mitochondria isolated from the cat soleus and gracilis muscles when energized with a combination of substrates feeding the respiratory chain at the level of complex I (pyruvate-malate, glutamate-malate), complex II (succinate), as well as complex IV (reduced cytochrome c after rupture of the outer membrane) (Figure 1-1A). Similarly, Yajid et al. (Yajid *et al.*, 1998) observed no significant difference in maximal state 3 respiration of mitochondria isolated from several rat muscles (soleus, extensor digitorum longus (EDL), tibialis anterior (TA), gastrocnemius) neither in the presence of glutamate-malate nor succinate (Figure 1-1B). This lack of difference also appeared in mitochondria isolated from red and white muscles in fish (Figure 1-1C), which displayed nearly identical rates of state 3 respiration in the presence of pyruvate-malate (Figure 1-1D). Finally, in mitochondria from rabbit muscle, state 3 respiration in the presence of pyruvate-malate was reported to be significantly (25 %) higher in mitochondria from the soleus compared to the fast gracilis muscle (Jackman & Willis, 1996), while no difference was observed with the respiratory substrate 2-oxoglutarate. In general, the available data suggest that the respiratory capacity per milligram of mitochondrial protein is fairly constant across fiber types and that upregulation of mitochondrial volume density is probably the main mechanism by which the oxidative potential of a fiber can be increased.

This relative similarity in respiratory capacity in mitochondria across fiber types is in general agreement with the available data regarding enzyme content. Indeed, Leary et al. (Leary *et al.*, 2003) reported that the activity of the respiratory chain complexes (I, II, I+III and IV) as well as that of the ATP synthase (complex V) and citrate synthase

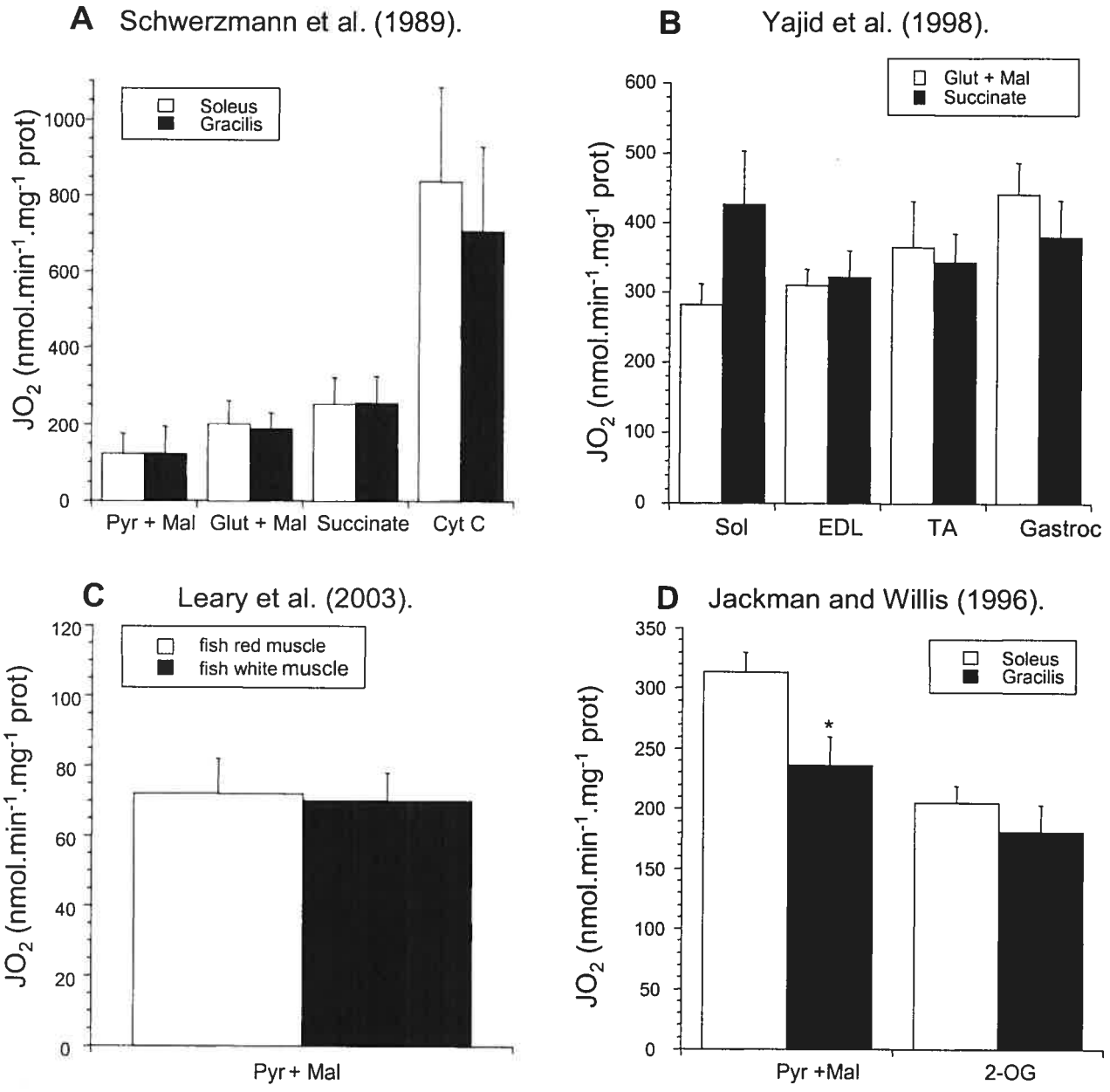

Figure 1-1:

Respiratory properties measured in mitochondria isolated from skeletal muscles with oxidative and glycolytic phenotypes in rabbit (panels A and D), rat (panel B), and fish (panel C). Respiratory rates are expressed per mg of mitochondrial protein and were determined in the presence of various substrates: substrates for complex I (pyruvate (Pyr) + malate (Mal), glutamate (Glut) + Mal, 2-oxoglutarate (2-OG)); complex II (succinate); and exogenous cytochrome c (Cyt C) (following rupture of the outer membrane). Abbreviations for panel B: Sol: soleus; EDL: extensor digitorum longus; TA: tibialis anterior; Gastroc: gastrocnemius.

was similar overall in mitochondria isolated from the white and red muscles of rainbow trout (Figure 1-2A). However, the activity of aconitase and 2-oxoglutarate dehydrogenase, two other enzymes of the TCA cycle, was significantly lower in mitochondria from white compared to red muscle, which led this group to suggest that subtle differences in the stoichiometry of TCA cycle enzymes could occur across fiber types (Figure 1-2A). Schwerzmann et al. (Schwerzmann *et al.*, 1989) also reported that the activity of complex IV of the respiratory chain was similar in mitochondria isolated from cat soleus and gracilis. Similarly, Philippi and Sillau (Philippi & Sillau, 1994) reported that the content of cytochromes $c+c_1$ and $a+a_3$, which provide an indication of the content of cytochrome c as well as complexes III and IV, was similar in mitochondria isolated from the white gastrocnemius and soleus muscles in rats. Of note, this study analyzed the subsarcolemmal (SSM) and intermyofibrillar (IMF) mitochondria separately and found no difference between the two populations of mitochondria neither within nor between muscles (Philippi & Sillau, 1994) (Figure 1-2B and C).

In stark contrast with these studies, Jackman and Willis (Jackman & Willis, 1996) reported that in rabbit muscle, the activities of several complexes of the respiratory chain were 1.6 to 2.0 fold greater in mitochondria from the soleus compared to the gracilis (Figure 1-3A). Although the differences were more modest, they also observed higher activities for citrate synthase and malate dehydrogenase. On the other hand, isocitrate dehydrogenase activity was two-fold higher in the gracilis compared to soleus (Figure 1-3B). These data are somewhat surprising given that in this study, maximal state 3 respiration was unchanged (in the presence of 2-oxoglutarate) or only 25 % higher (in the presence of pyruvate + malate) in the mitochondria from the soleus compared to the gracilis (Figure 1-4). Except for this apparent discrepancy, the data available in the literature would suggest that mitochondria from different fiber types do not substantially differ with respect to the maximal capacity of the respiratory chain.

However, under some conditions, differences do appear between mitochondria of slow and fast muscles. This is the case when mitochondria are energized with lipid

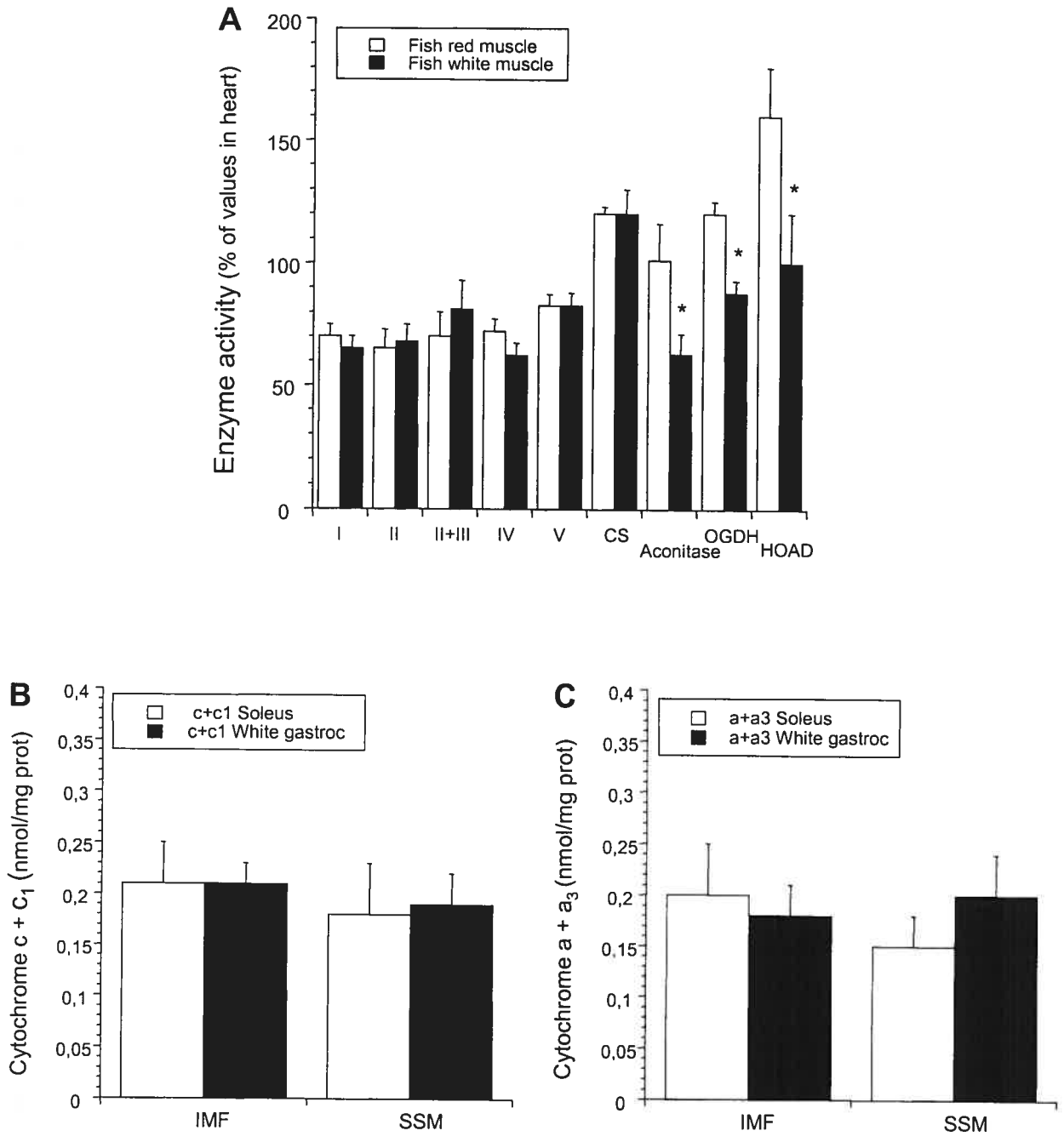

Figure 1-2:

Activity or content of enzymes of the respiratory chain, TCA cycle and β -oxidation pathway in mitochondria isolated from fish and rat muscles. Abbreviations in panel A are : HOAD: hydroxyacyl-CoA dehydrogenase; OGDH: 2-oxoglutarate dehydrogenase, CS: citrate synthase, I-IV respiratory chain complexes; V: ATP synthase. Abbreviations in panels B and C: SSM: subsarcolemmal mitochondria; IMF: intermyofibrillar mitochondria.

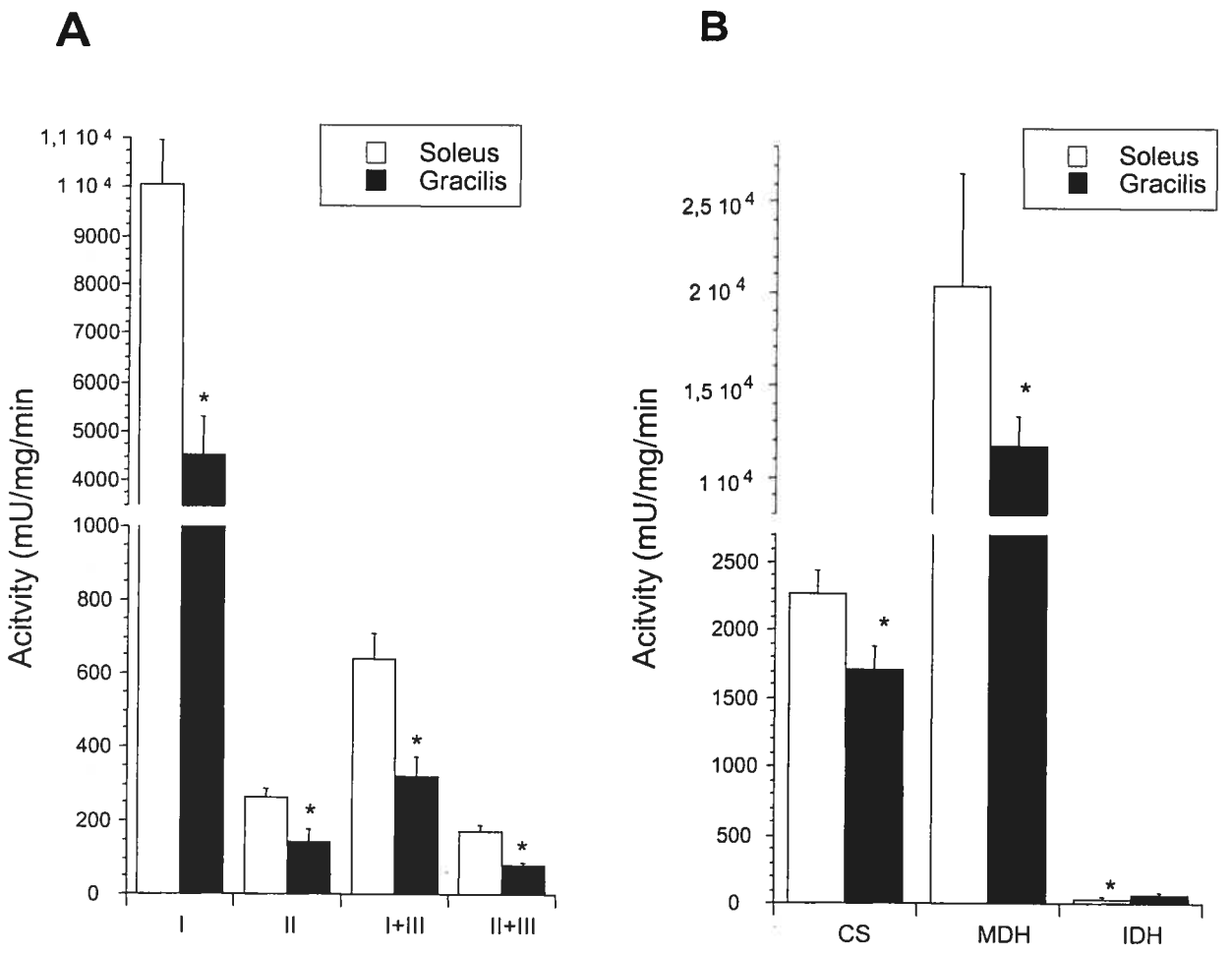

Figure 1-3:

Activity or content of enzymes of the respiratory chain, TCA cycle and β -oxidation pathway in mitochondria isolated from rabbit muscles. Data are from Jackman and Willis (1996). In panel A, numbers on the x-axis refer to the respiratory chain complexes. In panel B, abbreviations are the following: CS: citrate synthase; MDH: malate dehydrogenase; IDH: isocitrate dehydrogenase.

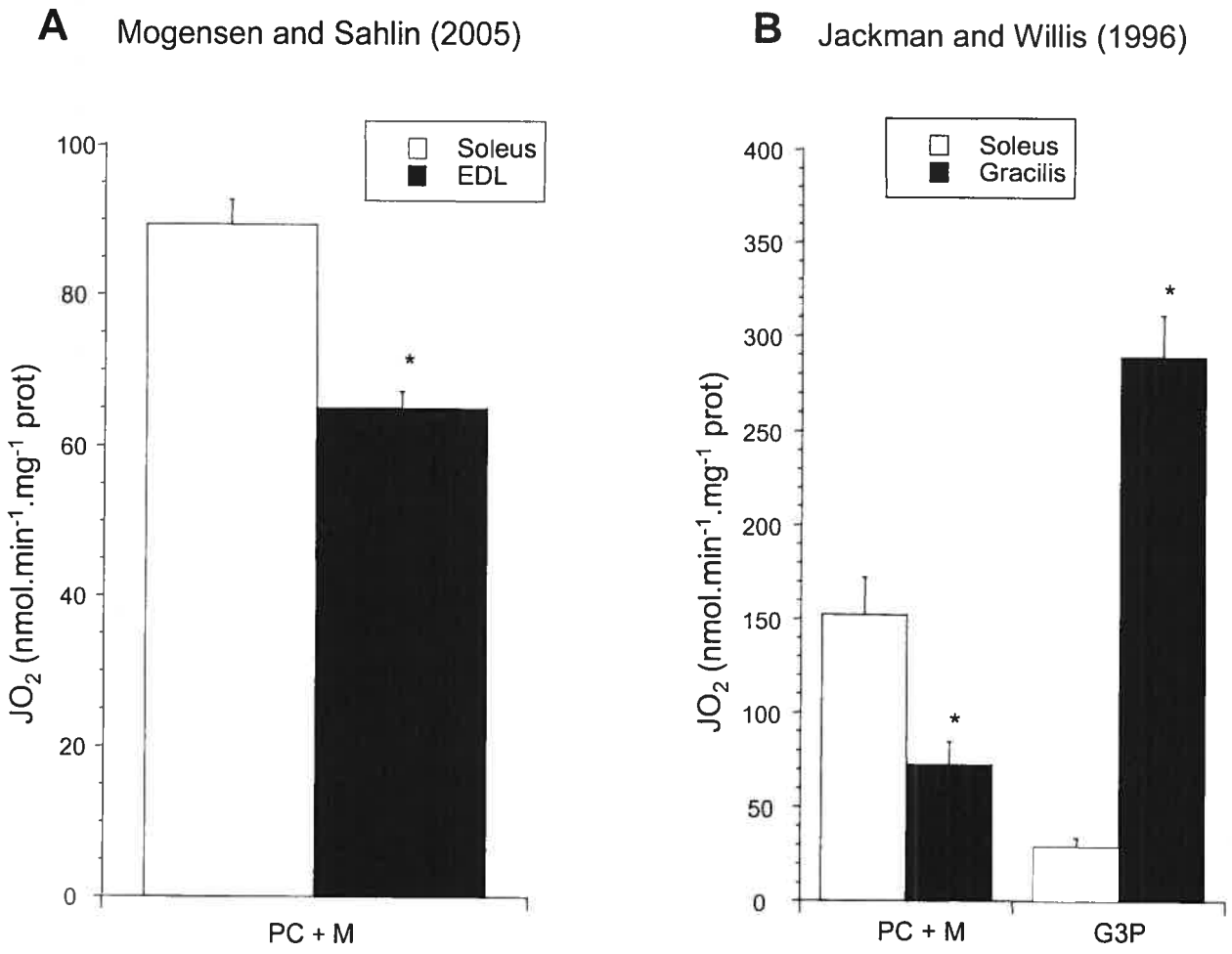

Figure 1-4:

Respiratory properties measured in mitochondria isolated from skeletal muscles with oxidative and glycolytic phenotypes in rat (panel A) and rabbit (panel B). Respiratory rates are expressed per mg of mitochondrial protein and were determined in the presence of lipid substrates palmitoyl carnitine + malate (PC+M) or glycerol-3-phosphate (G3P).

substrates or with glycerol-3-phosphate, a glycolytic intermediate involved in the shuttling of cytosolic reduced equivalent to mitochondria. Indeed, both in rat and rabbit, mitochondria from slow oxidative muscles (soleus) display a higher state 3 respiration in the presence of palmitoylcarnitine compared to mitochondria from glycolytic muscles (EDL or gracilis) (Figure 1-5A and B). These data are consistent with the fact that the activities of enzymes of the β-oxidation pathway such as hydroxyacyl-CoA dehydrogenase are 1.6 to 1.9-fold higher in mitochondria from oxidative *vs* glycolytic muscles (Figure 1-4A and B). This difference also appears consistent with the fact that oxidative muscles derive a significant portion of their energy from the oxidation of circulating fatty acids and express much higher levels of proteins involved in sarcolemmal fatty acid transport (FAT/CD36, FATP, $FABP_{pm}$) and fatty acid intracellular binding (FABP) (Bonen *et al.*, 2002).

Conversely, because in type IIb fibers the high glycolytic rates achieved during short burst contractions are likely to result in the accumulation of cytosolic reducing equivalent and glycolytic intermediates, mitochondria in these fibers may have an increased capacity to shuttle cytosolic reducing equivalent into mitochondria for oxidation. The only available evidence to support this possibility comes from the study by Jackman and Willis (Jackman & Willis, 1996) which showed that compared to mitochondria from the soleus, mitochondria from the rabbit gracilis muscle had a ten-fold higher state 3 respiration when glycerol-3-phosphate was the respiratory substrate (Figure 4B). They showed that this was probably due to the fact that mitochondria from the gracilis muscle expressed more glycerol-3-phosphate dehydrogenase and were thus able to produce mitochondrial $FADH_2$ at a much higher rate. Therefore, at least in the rabbit, glycolytic muscles may depend more on the α-glycerolphosphate shuttle compared to other muscles in which the malate-aspartate shuttle may predominate (Jackman & Willis, 1996).

Finally, it should be mentioned that although several mitochondrial properties appear similar in mitochondria across muscle fibers at steady state, oxidative capacity and the stoichiometric relationships among the various mitochondrial proteins can

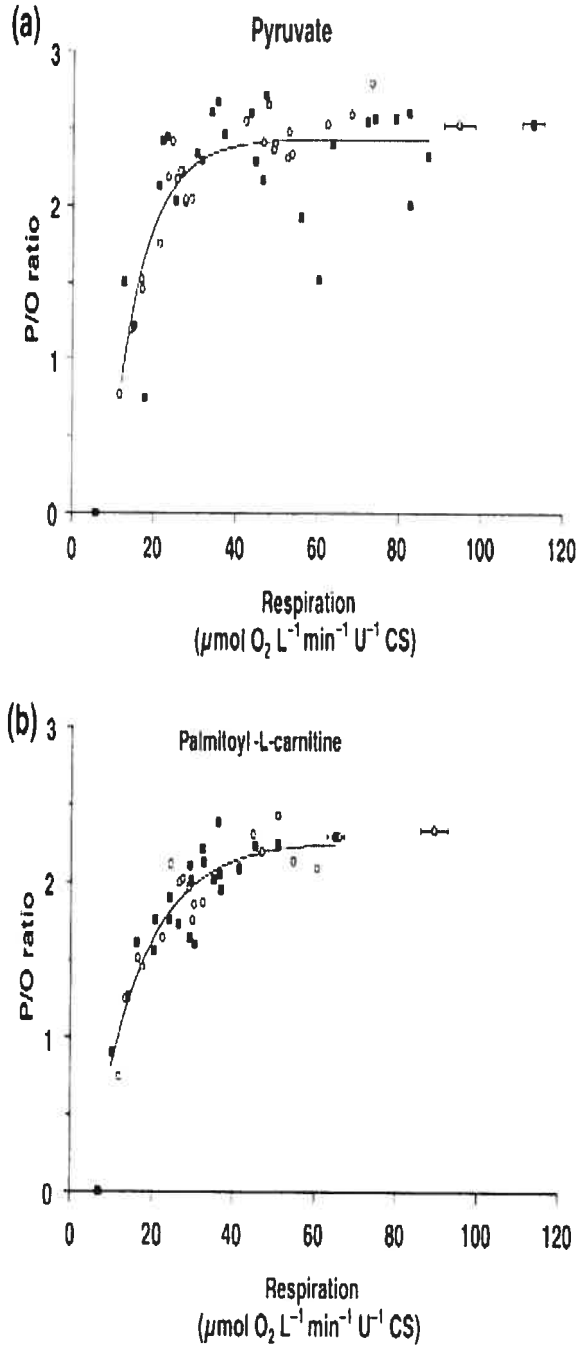

Figure 1-5:

P/O ratio at different respiration rates with pyruvate (a) and palmitoylcarnitine (b) in soleus (open circles) and extensor digitorum longus (dark squares). P/O ration at Vmax is shown as the mean +/- SEM. The mean state 4 respiration is shown on the x-axis when P/O equals 0. Adapted from Mogensen and Sahlin (2005).

change acutely in response to signals that alter mitochondrial biogenesis. For example, mitochondrial enzymes increase significantly during myogenesis (Moyes *et al.*, 1997) and during electrical stimulation of skeletal muscle (Henriksson *et al.*, 1986; Henriksson *et al.*, 1989; Reichmann *et al.*, 1991), but individual respiratory chain complexes and TCA cycle enzymes do not change in parallel. This phenomenon is probably related to the extreme complexity of the mitochondrial biogenesis program, which involves several stimuli and transcription factors, the coordination of transcription of genes located in two separate genomes (nuclear and mitochondrial), importation of pre-proteins into mitochondria, and assembly of multi-complex enzymes and holoenzymes (Hood, 2001; Moyes & Hood, 2003). It is thus possible that at least transitorily, mitochondria undergo changes in their normal composition.

2.4.2.2 Coupling efficiency, proton conductance and membrane properties

Very few studies have investigated whether variations across fiber types exist with respect to the coupling efficiency of oxidative phosphorylation. This parameter, known as the P/O ratio, is conventionally measured by adding a quantity of ADP to isolated mitochondria and measuring the amount of oxygen consumed to phosphorylate ADP into ATP. It is well established that the P/O ratio decreases as respiration is progressively decreased from maximal ADP-stimulated respiration to submaximal respiration rates (Gnaiger *et al.*, 2000). The reasons for which mitochondrial efficiency is progressively lowered as respiration approaches resting values are complex. However, one of the main factors appears to be that the proton leak, *i.e.* the passive re-entry of protons into the mitochondrial matrix, accounts for an increasing fraction of respiration as it approaches resting values (Brand *et al.*, 1994), thereby increasing O_2 consumption that is not devoted to ATP synthesis.

In one of the earliest studies comparing P/O ratios in mitochondria isolated from different skeletal muscle fibers in the rat, Pande & Blanchaer (1971) were unable to find differences when using pyruvate or palmitoyl-carnitine as substrate. Of note, in this study P/O values were only measured at maximal respiration rates in the presence of saturating amounts of ADP, which does not allow one to exclude the existence of differences when mitochondria are respiring at submaximal rates (as is usually the case

in vivo). However, Mogensen and Sahlin (Mogensen & Sahlin, 2005) recently compared the P/O ratios over the entire range of respiratory capacity in mitochondria isolated from the rat soleus and the fast EDL. These authors reported no significant difference in the P/O ratios between mitochondria from the two muscles, both in the presence of pyruvate or palmitoylcarnitine (Figure 1-5). Direct measurement of proton leak also revealed an absence of significant difference in mitochondria isolated from red and white fish muscle (Leary *et al.*, 1998). However, these authors noted that when proton leak was expressed per unit of complex IV instead of per mg of total mitochondrial proteins, the leak appeared greater in mitochondria from white compared to the heart or red muscle. The authors argued that under certain circumstances, normalization against a marker of the respiratory chain capacity could be more appropriate than total protein to express mitochondrial parameters. Leary et al. (Leary *et al.*, 2003) also determined the fluidity of mitochondrial membranes since local lipid environment can affect structure and function of mitochondrial proteins. These authors observed that the membrane fluidity of mitochondria in red muscles was significantly greater than in white muscles. This phenomenon could be due to many factors including variations in phospholipid profiles (*i.e.* chain length, saturation, or cardiolipin content). However no information is available regarding the phospholipid profile in mitochondria across fiber types and it also remains unclear how this could affect the *in vivo* activity of membrane-bound proteins. Taken as a whole, these data suggest that although some membrane properties and the proton leak may slightly differ, the coupling efficiency of mitochondria appears to be relatively constant across fiber types.

2.4.2.3 Sensitivity of oxidative phosphorylation to ADP and creatine

One of the most striking and systematically reported differences between mitochondria from oxidative and glycolytic muscles relates to the mechanisms by which changes in the concentration of cellular adenylates regulate the rate of oxidative phosphorylation. The majority of the experimental evidence in favor of such differences has been obtained using saponin permeabilized fibres, which allows for the investigation of mitochondrial function within a relatively preserved cyto-architectural environment. Several studies have shown that the apparent K_m of mitochondrial respiration for ADP is several-fold higher in slow oxidative muscles predominantly

composed of type I fibers (200-300 μM for the heart and soleus) compared to muscles expressing mainly type II fibres (10-30 μM in EDL and white gastrocnemius) (Kay *et al.*, 1997; Saks *et al.*, 1998; Braun *et al.*, 2001; Saks *et al.*, 2001; Seppet *et al.*, 2001) (Figure 1-6). This apparently low sensitivity of oxidative fibers to ADP was shown to be at least partly due to the fact that the porin pore VDAC, which is responsible for the transport of ADP across the outer membrane, has a low conductance for ADP in slow compared to fast muscles (Saks *et al.*, 1998). Indeed, if the mitochondrial outer membrane is disrupted by a carefully controlled osmotic shock, the K_m for ADP becomes similar in both types of mitochondria (20-30 μM) (Saks *et al.*, 1993).

Another important difference is that in mitochondria from oxidative muscles, the phosphocreatine shuttle appears to play a predominant role in the control of respiration compared to that observed in mitochondria from glycolytic muscles (Kay *et al.*, 1997; Saks *et al.*, 1998; Braun *et al.*, 2001; Saks *et al.*, 2001; Seppet *et al.*, 2001). Indeed, mitochondria from oxidative muscles express high levels of the mitochondrial isoform of creatine kinase (MiCK). MiCK is located in the intermembrane space where it associates with VDAC in the outer mitochondrial membrane and the ATP/ADP exchanger (*i.e.* ANT) of the inner membrane (Brdiczka *et al.*, 1998). In contrast to ADP, the conductance of VDAC for creatine coming from the cytosol is relatively high in all muscles. Therefore in oxidative fibers, the presence of creatine allows MiCK to preferentially use the ATP exiting the ANT exchanger to directly regenerate ADP in the mitochondria thus acting as a powerful stimulator of oxidative phosphorylation (Figure 1-7; scheme of the CK shuttle).

Finally, recent evidence also suggests that subtle differences in the regulation of respiration by the ATP/ADP ratio exist between mitochondria isolated from oxidative and glycolytic muscles, independent of the phosphocreatine shuttle. Indeed, Gueguen et al. (Gueguen *et al.*, 2005c) showed that in mitochondria from glycolytic muscle, respiration was more sensitive to inhibition by ATP compared to mitochondria from oxidative muscle. Given that the ANT exchanger is known to exert a significant amount of control over respiration (Groen *et al.*, 1982), the authors postulated that the content

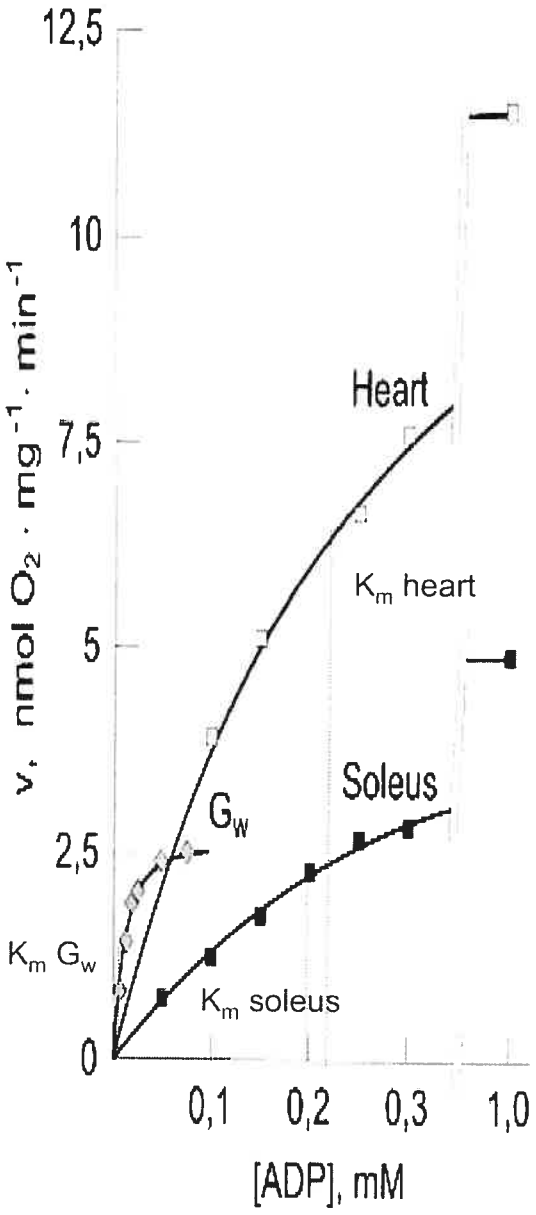

Figure 1-6:

Respiration rates of saponin permeabilized fibers from rat heart, soleus, and white superficial portion of the gastrocnemius (Gw) as a function of ADP concentration in the incubation medium. Dotted lines indicate the [ADP] required to elicit half of the maximal respiratory rate (K_m). Respiration is expressed in nmol/min/mg dry weight. Adapted from Kay et al. (1997).

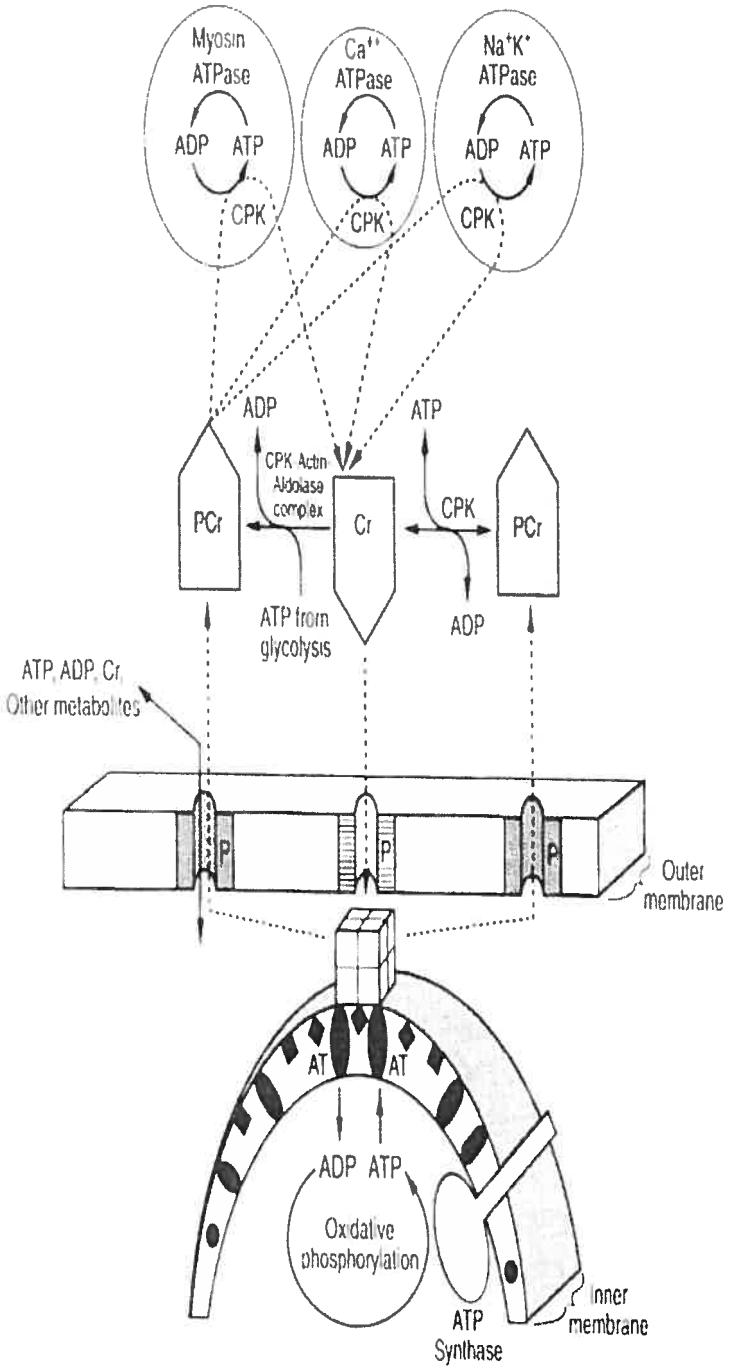

Figure 1-7:

Schematic representation of the phosphocreatine shuttle network in muscle cells. In mitochondria, the specific isoform of creatine kinase (MiCK) form an octamer that is structurally and functionally coupled to porin pores (P) in the outer membrane and the ATP/ADP exchanger (AT) of the inner membrane. Cytosolic creatine generated by CK isozymes located at sites of ATP consumption enter mitochondria through porin pores and stimulates MiCK. MiCK through a preferential access to mitochondrial ATP will generate ADP at the vicinity of AT exchangers resulting in a powerful stimulation of respiration. This system is predominant in oxidative muscles such as the heart and soleus. On the other hand, it is virtually absent in mitochondria from fast muscle. Instead, in these muscles mitochodondrial respiration is directly regulated by changes in cytosolic ADP. Diagram originally published by Wallimann et al. (1992).

or the regulation of ANT could be different in both types of mitochondria.

In general, the literature available on the regulation of respiration indicates that in glycolytic muscles, respiration is predominantly regulated by changes in cytosolic [ADP] and ATP/ADP ratio, while in oxidative muscles, respiration is predominantly regulated by the phosphocreatine circuit which couples sites of ATP consumption to sites of ATP production.

2.4.2.4 Production of reactive oxygen species (ROS)

It is well established that the mitochondrial respiratory chain is one of the main source of ROS in cells. ROS production arises mainly from the leak of electrons at complex I and III of the respiratory chain, which then react with O_2 to produce the superoxide anion (Brand *et al.*, 2004; Muller *et al.*, 2004; Andreyev *et al.*, 2005). Electron leaks at these sites are favored by conditions such as high membrane potential, high levels of reduction of respiratory chain complexes, or physical damage to the respiratory chain (Nicholls, 2004; Brand, 2005). Not surprisingly, mitochondria have an elaborate antioxidant system composed of enzymatic and non-enzymatic mechanisms. The main enzymatic system involves the mitochondrial isoform of superoxide dismutase (MnSOD) which converts the highly toxic superoxide anion into the somewhat less reactive H_2O_2 (Fridovich, 1995). Other enzymatic systems discovered more recently involve specific mitochondrial isoforms of thioredoxin (TRX-2) and peroxyredoxin (Prx-3) which work in concert to scavenge H_2O_2 (Tanaka *et al.*, 2002; Yamawaki & Berk, 2005; Matsushima *et al.*, 2006). Finally, the main non-enzymatic system is the mitochondrial reduced gluthatione pool and the enzymes associated glutathione metabolism (glutathione peroxidase and reductase) which are also involved in H_2O_2 scavenging.

Only three studies have determined whether mitochondrial ROS production varies across muscle fiber types. Recently, Anderson et al. (Anderson & Neufer, 2006) measured rates of H_2O_2 production by mitochondria *in situ* in saponin permeabilized fiber bundles from muscles with distinctive fibers types including the soleus (type I), the red gastrocnemius (type IIA) and the white gastrocnemius (type IIB). If H_2O_2

production was solely a function of mitochondrial content, then fiber bundles from soleus and red gastrocnemius muscles would be expected to generate the highest levels of H_2O_2 production. Surprisingly, these authors observed that mitochondrial free radical leak (H_2O_2 produced/O_2 consumed) was two- to three-fold higher in white gastrocnemius (type IIB) than in red gastrocnemius (type IIA) or soleus (type I) muscle fibers during basal respiration supported by complex I or complex II substrates (Figure 1-8) despite the fact that the number of mitochondria in white gastrocnemius was 50 % less. When normalized for mitochondrial content, total H_2O_2 scavenging capacity was lower in red gastrocnemius and white gastrocnemius fibers, whereas glutathione peroxidase activity, which is largely responsible for H_2O_2 removal in mitochondria, was similar in all three muscle types indicating that factors other than the activity of this enzyme were responsible for the lower scavenging capacity in white gastrocnemius. The fact that mitochondrial H_2O_2 production observed among the three types of muscle fibers did not mirror differences in respiratory capacity or mitochondrial content suggest that mitochondria possess distinct features that affect their ROS production and/or removal. It would appear that type II muscle fibers, particularly type IIB, possess unique properties that potentiate mitochondrial H_2O_2 production and/or release (Anderson & Neufer, 2006). Similarly, the results of another study measuring mitochondrial ROS production in various muscles of the rat show that in soleus muscle, glutamate/malate (complex I) supported mitochondrial H_2O_2 release was lower than in tibialis anterior muscle (Capel *et al.*, 2004), consistent with the observations reported by Anderson et al. (Anderson & Neufer, 2006). Leary et al. (Leary *et al.*, 2003) made a similar observation in fish when mitochondria isolated from red and white muscles were compared. Of note, in this study, Leary et al. reported that the activity of the TCA cycle enzyme aconitase was significantly lower in mitochondria from white muscle. This phenomenon could be at least partly due to the greater amounts of ROS produced in these mitochondria as it is well known that aconitase is very sensitive to inactivation in the presence of oxidative stress (Benderdour *et al.*, 2004).

As a general conclusion to this section, it appears that despite the fact that mitochondria from different fiber types may not differ substantially in terms of maximal respiratory capacity, they do have distinct properties at least in terms of regulation of

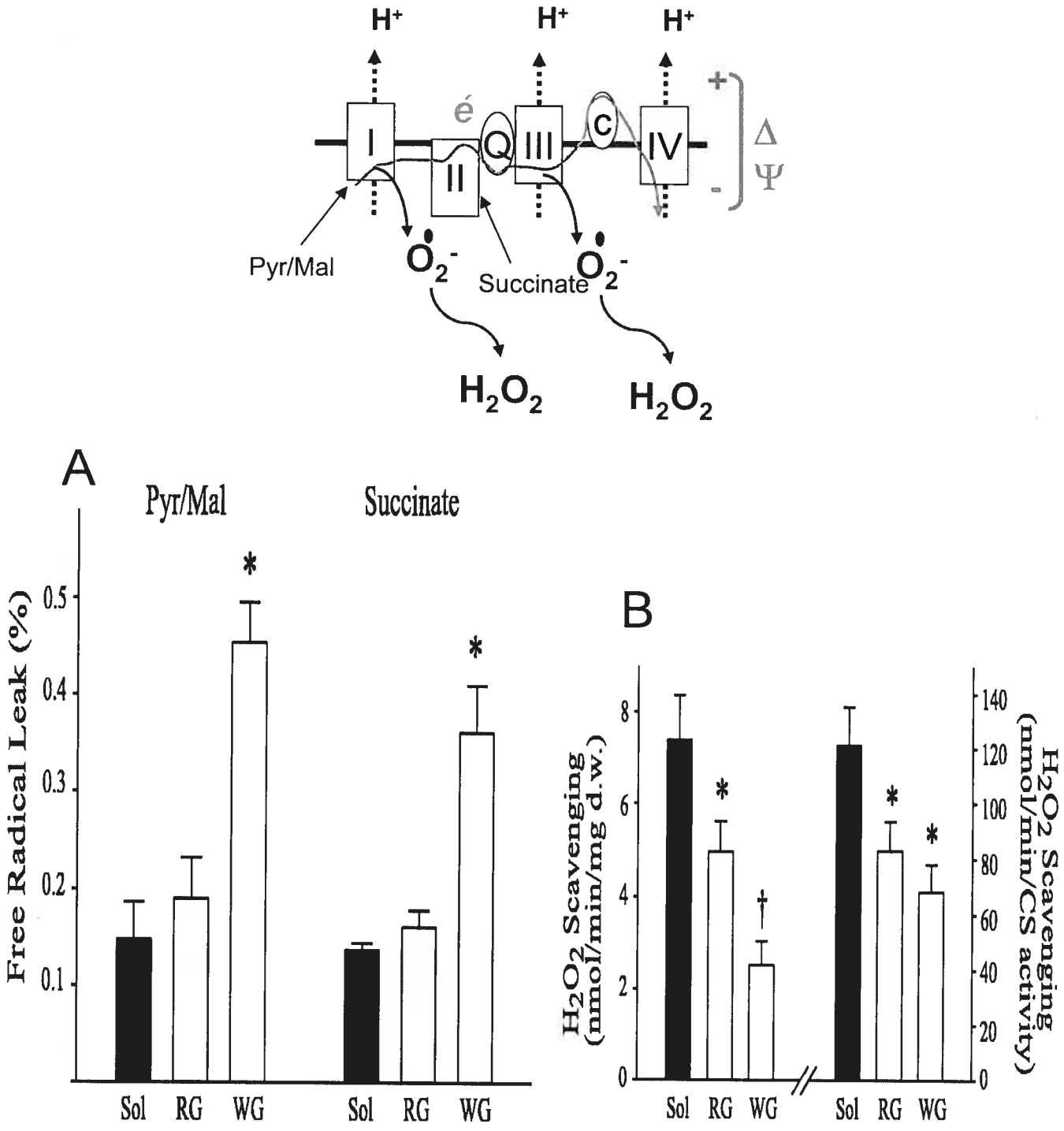

Figure 1-8:

Panel A shows the production of reactive oxygen species (expressed as a % of O_2 consumed) from complex I and III of the respiratory chain is significantly higher in saponin permeabilized fiber bundles from the white gastrocnemius (WG) compared to more oxidative muscles such as the soleus (Sol) or the red portion of the gastrocnemius (RG). This phenomenon is observed both in the presence of substrates feeding complex I (pyruvate + malate (Pyr/Mal)) and II (succinate) of the respiratory chain. Panel B shows total ROS scavenging capacity in fiber bundles from the three muscles expressed per mg of dry fiber weight or per unit of citrate synthase (CS) to take into account differences in mitochondrial content. Data from Andersen and Neufer (2006).

respiration and production of ROS. It is entirely possible that other differences exist as well, however, further studies are needed to explore this possibility. Of note, there is apparently no study available regarding possible variations across fiber types in the mitochondrial properties that pertain to their role in the initiation of cell death. For example, the question of whether differences exist in the mitochondrial content of several proteins involved in the regulation of apoptosis remains to be established. In addition, there are currently very few studies available on the mitochondrial PTP in skeletal muscle (Fontaine *et al.*, 1998a; Irwin *et al.*, 2003) and none have investigated whether its regulation varies across fiber types. Clearly, these questions need to be addressed especially in the context of understanding the response of muscle fibers to disuse.

3 SKELETAL MUSCLE ADAPTATIONS TO DISUSE

In this section of the literature review, the response of muscle fibers to disuse will be discussed. First, an overview of the experimental models from which much of our understanding of how muscles react to disuse was obtained will be presented, followed by an overview of some of the classic changes that occur in muscle in response to disuse. The molecular etiology of muscle disuse will then be discussed. As one of the important mechanisms responsible for the loss of muscle mass involves an increase in protein degradation and a reduction in protein synthesis, this section will present some of the recent advances in the identification of factors that could mediate these effects, including stimuli such as mechanical stretch and signaling pathways such as the Akt/PkB, nuclear-factor-kB (NFkB) and MAP kinase pathways. The role that these pathways play in apoptotic signaling will also be highlighted.

3.1 Models of disuse and overview of muscle response to disuse

Since it is difficult to investigate the mechanisms responsible for disuse muscle atrophy in humans, most investigations have used laboratory animals (namely mice, rats, rabbits, cats, and guinea pigs) to understand the underlying causes and cellular processes implicated in muscle remodeling following disuse. These models vary in their degree of invasiveness and extent of muscle inactivation ranging from essentially

complete muscle inactivity (nerve blockade, denervation, and spinal cord isolation) to decreased weight bearing and mechanical activity (hindlimb suspension, limb casting, spaceflight). Although comparisons of available muscle disuse atrophy data are confounded by differences in species, type of muscles studied, duration of disuse, and the use of different immobilization techniques, some of the important effects on muscle morphology, function and expression of proteins that dictate the contractile phenotype are summarized below.

3.1.1 Morphological and functional changes associated with muscular inactivity

It has been repeatedly shown that immobilization of the hindlimb of the rat results in preferential atrophy of slow twitch fibers compared to fast twitch fibers (Booth & Kelso, 1973; Fitts *et al.*, 1986; Thomason & Booth, 1990; Oishi, 1993; Talmadge *et al.*, 1996a; Baldwin & Haddad, 2001). Although the underlying cause for this fiber type disparity is unclear, one hypothesis used to explain this widely observed phenomenon is that wasting may be greater in those muscles that are used more regularly. This is based on the rationale that in frequently used muscles, the disuse stimulus is perceived to a greater magnitude, as there is a larger difference between the 'normal' usage patterns and disuse (Musacchia *et al.*, 1988; Hudson & Franklin, 2002). Since fast twitch fibers are typically recruited only intermittently or during high force-requiring movements, the relative size of the disuse stimulus is proportionately smaller than that observed by slow twitch fibers. Thus, the elicited atrophic response would be comparably larger in slow, relative to fast twitch fibers.

Although muscle atrophy is one of the most predictable consequences of disuse, this adaptation is also associated with significant alterations in the functional properties of muscle, which again can vary according to the type of muscle. The debilitating effects of disuse on muscle strength and susceptibility to fatigue have been documented (Fell *et al.*, 1985; McDonald *et al.*, 1992; Falempin *et al.*, 1997). Indeed, in a study by Witzmann et al. (Witzmann *et al.*, 1983), soleus and EDL muscles were stimulated to contract *in situ* following hindlimb immobilization for a 6 week period. These investigators observed significant fatigue, as measured by a loss of muscle contractile tension over a stimulation period lasting 30 minutes. Compared to control muscles,

atrophied muscles exhibited a greater reliance on anaerobic energy production, as suggested by a greater rate of glycogen disappearance. Thus, disused muscles show increased fatigability and this may be partly attributable changes in the muscle's substrate utilization (Baldwin *et al.*, 1993; Grichko *et al.*, 2000). In response to disuse, the decrease in muscle strength or twitch force appears to be commensurate with the degree of muscle atrophy, with the effect being generally more significant in soleus muscle. A reduction in force production (twitch and tetanic tensions), and decreases in the time to peak tension during the twitch have been reported in soleus muscle in response to unloading by hindlimb suspension for two weeks (Falempin *et al.*, 1997). Similarly, Witzmann et al. (Witzmann *et al.*, 1982) cast immobilized the hindlimbs of rats for a period of 1-42 days and noted significant reductions in the time to peak tension development and ½ relaxation times in soleus muscle whereas in EDL muscle, both speed-related contractile parameters became prolonged by immobilization. This effect occurred early in the disuse period and was suggested to be related to alterations in function of the SR. Similar studies by Templeton et al. (Templeton *et al.*, 1984) confirm these findings in soleus muscle reporting a decrease of 25% in the time to peak tension development and ½ relaxation times following suspension for 2 weeks. The precise mechanisms for these changes in functional parameters remain unclear however. It seems reasonable to suggest that a combination of decreased muscle strength, shifts in fiber phenotype, and altered metabolic capacities may all contribute.

3.1.2 Changes in proteins that dictate phenotype in response to muscle inactivity

MHC gene regulation is a highly adaptable process that is modulated by a variety of factors through finely orchestrated cellular processes. Changes in the mechanical load, contractile activity, and the electrical activity typically experienced by a muscle can result in modifications in the MHC composition of its fibers. In studies using the model of hindlimb unloading in rodents, a shift in MHC isoform from slow-to-fast accompanied by a transition from lipid towards carbohydrate metabolism is commonly reported and appears more pronounced in tonically-contracting postural muscles (Fitts *et al.*, 1986; Oishi, 1993; Talmadge *et al.*, 1996a; Baldwin & Haddad, 2001). Although the effects of unloading in fast muscles are not as pronounced, the changes can nevertheless be described as a transition in profile from fast-to-faster (Adams *et al.*,

2000; Stevens *et al.*, 2000). This phenomenon is consistent with the notion that atrophy in fast muscles, that are not frequently recruited, is less than in tonic postural muscles as previously mentioned (section 3.1.1). Similar transitions in MHC profile have also been reported following neuromuscular transmission blockade (Cormery *et al.*, 2000) and it is likely to occur in other animal models of disuse such as tenotomy, joint immobilization and prolonged bed rest.

In humans, muscle disuse due to limb immobilization has been shown to induce a shift in muscle fiber type. It was found that leg immobilization for 3 weeks caused a 9% decrease in type I muscle fibers, no change in type IIA fibers, and an increase of 7% in type IIX fibers. These shifts in fiber type were associated with a 28% decrease in type I MHC and an increase of 200% in type IIx MHC (Hortobagyi *et al.*, 2000). However, it appears that the effects of disuse in humans are less apparent than in rodents, a phenomenon that may be linked to the much higher metabolic rates of rodents compared to humans. Indeed, Hudson et al. (Hudson & Franklin, 2002) observed that in general, the extent of muscle disuse atrophy observed among species is highly correlated with mass-specific metabolic rate, although a mechanistic explanation for this observation has not been proposed.

Recent findings show that under specific conditions, individual muscle fibers may simultaneously express several MHC isoforms (di Maso *et al.*, 2000). Under steady state conditions, the prominence of 'hybrid' fibers containing multiple isoforms of MHC protein is typically low but the prevalence of such fibers is known to increase significantly in muscles undergoing a change in fiber type (Talmadge *et al.*, 1999) such as occur during disuse (Talmadge *et al.*, 1996b; Caiozzo *et al.*, 1998; Cormery *et al.*, 2000). For instance, using specific antibodies to identify MHC isoforms in soleus and EDL muscle fibers, Michel et al. (Michel *et al.*, 1996) reported that after two weeks of pharmacological nerve blockade, fibers from predominantly fast or slow muscles display an increase in the proportion of fibers containing multiple MHC isoforms. Similarly, in our laboratory, previous studies performed by Cormery et al. (Cormery *et al.*, 2000) using immunohistochemical techniques have shown that the proportion of fibers displaying pure MHC isoforms in the rat soleus and gastrocnemius muscle after

two and four weeks of TTX paralysis is decreased. After four weeks of paralysis, only 14% of fibers in the soleus contained pure type I MHC with the remaining fibers also containing developmental (76%), IIa (26%), or IIx (18%) MHC isoforms.

Curiously, the fast MHC isoform that increases the most in the unloaded rat soleus is the IIx isoform (Talmadge *et al.*, 1996a). This is surprising considering that the soleus muscle normally expresses type I and IIa isoforms. It has been proposed that the new expression of primarily the type IIx MHC isoform is occurring at the expense of type I, with little or no increase in the IIa isoform, suggesting that previously slow type I fibers might 'bypass' the expression of type IIa MHC and express type IIx MHC directly (Talmadge *et al.*, 1996a; Caiozzo *et al.*, 1998). The authors note that these observations shed some doubt on the established idea that muscle fibers are obligated to follow a prescribed directional transition of their MHC isoform from I → IIa → IIx → IIb (Andersen *et al.*, 1999). However, prior to making broad assumptions of this nature, it is important to consider the possibility that the authors' observation of little or no increase in IIa MHC isoform does not obligatorily imply a bypass of the latter isoform in the transition from type I to IIx. That the proportion of type IIa remained unchanged in this study does not necessarily mean that these are the same IIa as previously identified. Thus, it seems that the sequential 'nearest-neighbour' transitional scheme proposed in 1992 by Pette and Vrbova (Pette & Vrbova, 1992) for transforming fibers remains a well-established hypothesis.

3.1.3 The case of muscle denervation

3.1.3.1 Muscle mass and functional properties

It is well known that neural activity is an important factor that determines muscle phenotype (Pette & Vrbova, 1992; Fluck & Hoppeler, 2003). Thus, when neural control is removed, as occurs in various muscle denervation disorders in humans and experimental models of muscle denervation in laboratory animals, muscle atrophy and changes in fiber phenotype occur in both slow and fast muscles. The changes observed in these situations are not only caused by lack of contractile activity but also by the absence of nerve-derived trophic factors that are normally delivered to the muscle via

the neuromuscular junction and appear essential for the maintenance of normal muscle morphology and function. These factors are not removed in other models of disuse where the integrity of the motoneurons is preserved.

Loss of muscle innervation in rodents results in a loss of muscle mass that can be detected after three days and is rapid in the ensuing two months. At the end of this period, only 20 to 40 % of the original muscle mass remains and a significant portion becomes occupied by connective tissue. The magnitude of muscle atrophy observed following denervation is similar to that observed in models of muscle disuse in which the motoneuron is not damaged which suggests that most, if not all, of the loss in muscle mass is caused by a lack of contractile activity. On the other hand, it is not totally clear whether the atrophy varies across fiber types as conflicting results have been reported in the literature. Indeed, early studies by Stonnington and Engel (Stonnington & Engel, 1973) indicated similar levels of atrophy in red and white fibers. In contrast, a more recent study has shown that in the rat EDL, atrophy is only observed in the fast fibers whereas slow fibers seem relatively unaffected (Borisov *et al.*, 2001).

As expected, denervation causes changes in muscle functional properties. There is a profound reduction in single twitch and tetanic tension. However, in contrast to what is observed in other models of disuse (see section 3.1.1), twitches become significantly slower both in slow and fast muscles. The mechanisms responsible for this phenomenon are not entirely clear but appear to be related to a prolongation of the muscle fiber action potential (MacIntosh *et al.*, 2005).

3.1.3.2 Changes in protein expression dictating metabolic and contractile phenotype

Several changes in the expression of proteins of the contractile and E-C coupling apparatus occur following the loss of neural imput. It appears that the expression of MHC I and IIb are decreased at the expense of an increase in the expression of type IIa and IIx (Huey & Bodine, 1998; Jakubiec-Puka *et al.*, 1999). In the IIB fibers of the rat EDL muscle, several changes in E-C coupling proteins have also been reported including a decrease in the expression of SERCA 1 protein isoform, parvalbumin, and calsequestrin (Germinario *et al.*, 2002). These changes, which are suggestive of a fast-

to-slow phenotype transition, have also been confirmed at the transcriptional level in mouse EDL muscle 7 days after denervation. Results from microarray analysis indeed revealed a significantly reduced expression of genes for SERCA1 and parvalbumin, and a downregulation of the gene coding for Myh4, the gene that codes for the IIb MHC protein isoform (Raffaello *et al.*, 2006). In this study, *in vitro* experiments were also performed on single fibers and showed that these changes in expression were associated with a significant reduction in twitch time parameters, confirming the transition towards a slow phenotype. It therefore appears that the increase in action potential duration as well as alterations in Ca^{2+} handling (Raffaello *et al.*, 2006), rather than modifications of MHC profile, are the main factors responsible for the alterations in contraction velocity.

3.1.3.3 Other changes within muscle fibers

At this point, it is important to remark that muscle denervation is associated with changes in fiber morphology and subcellular architecture that are either absent or attenuated in other models of disuse and that are likely attributable to the lack of trophic influence from motoneurons.

Beside simple atrophy, a fraction of the fibers within denervated muscles will display signs of further degeneration after several months. This includes centralization of myonuclei within muscle fibers, nuclear swelling and fragmentation, disorganization of sarcomeres, formation of cytosolic vacuoles, thickening followed by disintegration of the sarcolemma, and invasion by monocytes (MacIntosh *et al.*, 2005). These phenomena constitute clear evidence that apoptotic and necrotic cell death occurs in this situation and may lead to degeneration of segments within some fibers or to the complete loss of fibers.

3.2 Molecular etiology of disuse muscle atrophy

One of the most important mechanisms responsible for the loss of muscle mass in response to disuse involves a dramatic reduction in protein content. This is achieved by a down regulation of protein synthesis and by a simultaneous acceleration in the degradation of a number of proteins. Although elucidation of the signal transduction

pathways mediating these effects remains incomplete in part due to their complexity, it appears clear that atrophy is not the result of a simple reversal of hypertrophic signaling pathways and that distinct mechanisms are involved (Jagoe *et al.*, 2002; Haddad *et al.*, 2003; Stevenson *et al.*, 2003; Lecker *et al.*, 2004). This section will focus on some of the identified factors that could impinge upon protein synthesis and degradation in the disused state including stimuli such as mechanical stretch and signaling pathways such as the Akt/PkB, NFkB, and MAP kinase pathways. The role that these pathways play in apoptotic signaling will also be highlighted.

3.2.1 The Akt/PkB pathway

Akt is a serine-threonine kinase, also called protein kinase B (PkB) that is activated by PI3 kinase and has been shown to have growth-promoting effects in skeletal muscle (Rommel *et al.*, 2001). Bodine and co-workers demonstrated the importance of the PI3 kinase/Akt pathway for inducing muscle hypertrophy by regulating factors essential to the protein synthesis process (Bodine *et al.*, 2001b). Once phosphorylated, Akt can promote muscle growth by influencing protein synthesis through subsequent phosphorylation of mTOR, p70s6k, and the translation initiation factor 4E-BP-1 pathway.

Recent evidence suggests that chronically deactivated Akt plays an important physiological role in the progression of muscle atrophy by decreasing rates of protein synthesis (Stitt *et al.*, 2004; Song *et al.*, 2005). For example, it has been shown that suspension-induced atrophy of hindlimb muscles in the rat is associated with decreased protein expression and phosphorylation of Akt, decreased activation of p70s6k, and increased binding of 4E-BP1 to the transcriptional elongation factor eIF4E, suggesting that the pathology of disuse muscle atrophy may involve a signaling defect in the mTOR/ p70s6k/ 4E-BP1 pathway (Bodine *et al.*, 2001b; Hornberger *et al.*, 2001). Consistent with this line of evidence, it was shown that a knockout strain of mice devoid of Akt experience significant muscle atrophy (Peng *et al.*, 2003) whereas overexpression of activated Akt partially inhibited atrophy in denervated muscle (Bodine *et al.*, 2001b). However, it presently remains unclear whether this latter observation reflects the activation of a muscle growth pathway or inhibition of a

potential muscle atrophy pathway.

Interestingly, Akt has also been described as a protein with anti-apoptotic function. Although the mechanism by which signaling through the Akt pathway prevents cell death remains unclear, it was shown that Akt can directly phosphorylate and inactivate caspase-9 (Cardone *et al.*, 1998). It has also been shown that activation of Akt is sufficient to inhibit the release of cytochrome c from mitochondria and inhibits apoptosis and cytochrome c release induced by several proapoptotic Bcl-2 family members (Kennedy *et al.*, 1999). Taken together, these results show that Akt promotes cell survival by intervening in the apoptosis cascade prior to cytochrome c release and caspase activation (Kennedy *et al.*, 1999).

Recently, Bodine and coworkers reported that activation of the Akt pathway suppresses the activation of the E3 ligating enzyme atrogin-1 and MuRF-1 through activation of the forkhead box O (FOXO) family of transcription factors (Stitt *et al.*, 2004). FOXO is a class of transcription factors that shuttle between the nucleus and the cytoplasm and are implicated in the regulation of genes involved in the atrophy process. Upon phosphorylation, FOXO becomes sequestered in the cytoplasm and is thus inactive. However, dephosphorylation of FOXO has been shown to cause its translocation to the nucleus where it activates cellular apoptotic processes (Sandri *et al.*, 2004). Thus, reduced levels of activated Akt would be expected to lead to decreased phosphorylation of the FOXO which would increase its nuclear translocation where it may promote apoptotic activity.

Taken together these data suggest that the reduced expression and activity of Akt observed in disused muscles could have a double effect. The first effect would be to suppress growth while the second effect would be the activation of apoptosis. In light of these observations, strategies that stimulate Akt signaling might prove effective in preventing muscle atrophy.

3.2.2 The NFkB pathway

NFkB is a nuclear transcription factor complex expressed in several tissues

including skeletal muscle and is formed by the association of its molecular members into a heterodimer formation. The five nuclear transcription factors belonging to the NFkB family include p60, p52, p50, c-Rel, and Rel-B, and all family members are present in skeletal muscle tissue (Hunter *et al.*, 2002). In its inactive form, NFkB is sequestered in the cytoplasm, bound by members of the IkB family of inhibitor proteins. The various stimuli that activate NFkB cause phosphorylation of IkB, which is followed by its ubiquitination and subsequent degradation. This results in the heterodimer association of NFkB subunits and the subsequent translocation of the activated complex to the nucleus where it activates the transcription of various genes.

Among the activators of NFkB, elevations in intracellular Ca^{2+} (Hughes *et al.*, 1998) and increased cellular levels of ROS (Schreck *et al.*, 1991; Li *et al.*, 1998) have been identified, both of which occur in atrophic muscle. Activated NFkB has been shown to regulate a large number of cellular processes including apoptosis, inflammation, and cellular differentiation (Baldwin, 1996), and has been implicated in muscle atrophy attributable to muscle disuse (Hunter *et al.*, 2002). Furthermore, sustained activation of NFkB in muscle has been shown to lead to increased expression of MuRF-1, a sarcomere-associated protein that is a component of the ubiquitin–proteasome system of protein degradation and has been shown to be up-regulated by conditions that provoke atrophy (Bodine *et al.*, 2001a; Glass, 2003).

In a recent study by Hunter and coworkers (Hunter *et al.*, 2004), it was reported that 7 days of mechanical unloading increases NFkB activity in the soleus muscle of the mouse. Interestingly, the results of gene expression analyses suggest that the proposed heterodimer formation that is operative during disuse atrophy is different from the heterodimer formation reported during cachexia-induced muscle atrophy (Hunter *et al.*, 2002). This indicates that differences exist in the molecular signaling for these two types of atrophy and suggests that the NFkB pathway activated by muscle unloading is distinct.

3.2.3 Role of mechanical stretch and the MAP kinase pathway

The importance of mechanical loading for the maintenance of skeletal muscle

mass was first presented by Allan Goldberg. In 1975, Goldberg made the seminal observation that when mechanical loading on a skeletal muscle is increased by tenotomy of a synergistic muscle, its weight may increase by 30-50% within a 6-day period (Goldberg *et al.*, 1975). On the basis of these, and numerous other studies in which the amount of loading has been manipulated in an attempt to understand the effects of tension on skeletal muscle, it has become widely accepted that chronically unloading a muscle results in a decrease in mass (Goldberg *et al.*, 1975; Goldspink, 1977; Vandenburgh, 1987).

More recently, knowledge at the cellular and molecular levels as to how mechanical forces regulate muscle size and function has depended largely on *in vitro* models using modern tissue engineering techniques (Vandenburgh, 1987; Vandenburgh *et al.*, 1991). In a cell culture model using avian myotubes, Vandenburgh (Vandenburgh, 1987) demonstrated that mechanical stretch applied intermittently produced a large increase in protein synthesis resulting in myotube growth. Consistent with the important role of mechanical tension and force in the regulation of muscle mass, several studies have demonstrated the importance of passive muscle stretch to prevent muscle fiber atrophy, and the loss of sarcomeres in immobilized muscle (Yang *et al.*, 1997; Fowles *et al.*, 2000; Gomes *et al.*, 2006) and passive stretch is now recognized as a powerful stimulant of muscle growth and protein synthesis (Loughna *et al.*, 1986; Goldspink, 1999; Goldspink *et al.*, 2002). For example, it was shown that as little as one session of passive stretching per week applied to soleus muscle of rats immobilized in the shortened position was sufficient to provide significant protection against muscle fiber atrophy (Gomes *et al.*, 2004). Similarly, passive muscle stretch, achieved by brief sessions of daily muscle lengthening in the absence of contractile activity, was shown to maintain the structural integrity of muscle fibers by significantly reducing the formation of central core-like lesions in the soleus muscle of hindlimb-suspended rats (Baewer *et al.*, 2004). While it is not entirely understood how mechanical stretch counteracts the formation of central core-like lesions and protein breakdown, results by Hornberger and coworkers (Hornberger & Esser, 2004) demonstrate that intermittent passive stretch can activate mTOR-dependent signaling events, but through an Akt-independent mechanism. On the other hand, Siu and Alway

(Siu & Alway, 2005a) suggest that stretch provides its atrophy-preventing effects on muscle through modulation of apoptotic cell death processes. Specifically, these authors show that following stretch of the anterior latissimus dorsi muscle in an avian model, the incidence of DNA fragmentation, and pro-apoptotic factors (discussed later in more detail) are reduced, whereas activation of inhibitors of apoptosis proteins (IAP) and the content of chaperone heat shock proteins are elevated. The authors propose that apoptotic components are responsive to stretch and the presence of mechanical stretch may prevent muscle atrophy by promoting anti-apoptotic processes (Siu & Alway, 2005a). Therefore, while the importance of mechanical forces and stretch for the promotion of muscle growth through protein synthetic pathways is well-recognized, an apparent new role for mechanical stimuli in the protection against atrophy by promoting anti-apoptotic processes is becoming unveiled.

Proteins of the MAP kinases constitute a family of intracellular signaling proteins associated with membrane-linked cytoskeletal proteins involved in the transduction of mechanical forces from the cell-surface to the cell nucleus (Davis, 2000; Sakamoto & Goodyear, 2002). These kinases are activated by a variety of signals, including mechanical forces (Boppart *et al.*, 2001; Martineau & Gardiner, 2001; Kumar *et al.*, 2003; Wang *et al.*, 2005). JNK is an identified member of the MAP kinase family. In skeletal muscle, JNK is highly expressed and muscle contraction associated with exercise has been shown to robustly increase JNK activity in both rats and humans (Aronson *et al.*, 1997; Aronson *et al.*, 1998; Boppart *et al.*, 1999; Boppart *et al.*, 2000; Boppart *et al.*, 2001; Widegren *et al.*, 2001). Furthermore, the involvement of JNK in the transduction of mechanical forces from the cell surface to the nucleus in skeletal muscle has been demonstrated (Martineau & Gardiner, 2001; Csukly *et al.*, 2002; Hornberger *et al.*, 2005). Considering the importance of mechanical stimuli in the regulation of muscle size and function, it is feasible to suppose that a period of unloading, or absence of mechanical loading, might cause modifications in the muscle's capacity for transmission and/or detection of mechanical signals. Indeed, recent studies have reported an increase in the basal JNK MAP kinase phosphorylation status in muscle immediately following a period of immobilization (Childs *et al.*, 2003; Hilder *et al.*, 2003), although the significance of this observation is not entirely clear.

The first study presented in this thesis was designed to investigate the sensitivity of a mechanically-responsive signaling pathway in atrophic muscle following an acute mechanical challenge. Specifically, we examined the phosphorylation of the mechanically sensitive JNK MAP kinase signaling protein in atrophied rat soleus muscle in response to a mechanical challenge *in situ*. The results of this study are presented in the second chapter of this thesis.

4 CELL DEATH AND ITS ROLE IN MUSCLE DISUSE

As already mentioned, there is an increasing body of experimental evidence supporting the notion that in addition to large-scale changes in protein synthesis and degradation, apoptosis (and in a lesser manner necrosis) contributes to muscle remodeling in response to disuse. Over the recent years, it has become universally recognized that mitochondria play a critical role in cell death in several tissues and cell types (Kroemer *et al.*, 1998; Desagher & Martinou, 2000; Hengartner, 2000; Bernardi *et al.*, 2001; Lemasters *et al.*, 2002; Mattson & Kroemer, 2003; Mohamad *et al.*, 2005; Siu & Alway, 2005b). However, despite this knowledge, limited information is available on the potential role that these organelles might play in cell death signaling in disused muscles. This section of the thesis provides a general description of the main pathway involved in apoptotic signaling with a particular emphasis on the role played by mitochondria and PTP opening.

4.1 General distinctions between apoptosis and necrosis

The term apoptosis, which refers to programmed cell death, was introduced in 1965 following the observation that cell death that occurs during normal development is not of accidental nature but follows a sequence of controlled steps leading to cellular self destruction (Lockshin & Williams, 1965). Apoptosis can be distinguished from necrosis in several ways. In apoptotic cells, lysosomes are generally intact, as indicated by staining for acid phosphatase and shrinkage of the cytoplasm, and the condensation of chromatin and the appearance of furrowed plasma membrane are typically observed.

Cells dying from apoptosis finally convert into small, fragmented, spherical bodies that undergo phagocytosis by macrophages. Therefore, apoptosis does not typically induce death of neighboring cells and takes place in the absence of inflammation. By contrast, cell death by necrosis is relatively uncontrolled and is characterized by cellular swelling and lysis of the cell membrane causing the release of intracellular contents followed by inflammation, and damage to the surrounding cells.

4.2 Overview of apoptotic processes and known pathways

Within the last decade, considerable progress has been made towards the identification of stimuli that trigger apoptosis and the understanding of the molecular pathways involved. It is a common belief that in most cells, two main apoptosis signaling pathways, the extrinsic and the intrinsic pathways, exist (Sprick & Walczak, 2004). Both pathways differ with respect to the initiating stimuli and molecular signals involved as discussed below. However, they both lead to common mechanisms of cellular dismantlement.

4.2.1 The caspase family

The central apoptotic mechanism involves the activation of caspases, a family of cysteine proteases that cleave substrates after aspartic acid residues (Li *et al.*, 1997; Desagher & Martinou, 2000; Mayer & Oberbauer, 2003; Crow *et al.*, 2004; Danial & Korsmeyer, 2004). Caspases, which are synthesized primarily as inactive zymogens (pro-caspases), are divided into two categories: 1) upstream or activating caspases; 2) effector caspases. Upstream caspases, which include caspases 2, 8, 9, 10 and 12 are activated by dimerization upon specific stimulation and are responsible for the proteolytic cleavage and activation of the downstream effector caspases including caspases 3, 6 and 7. These caspases achieve the dismantlement of cells by proteolytic cleavage of a number of cellular proteins and by activation of nuclear DNA fragmentation, the latter phenomenon being readily detectable by terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and measurement of histone complexed DNA fragments.

4.2.2 Extrinsic pathway

Extrinsic death signaling is initiated by the binding of death ligands to their cognate death receptor at the cell surface (Baker & Reddy, 1998; Crow *et al.*, 2004). The ligand may be an integral membrane protein on the surface of a second cell (*i.e.* Fas [CD95/Apo-1] ligand), or a soluble extracellular protein (*i.e.* Tumor necrosis factor-α: TNF-α). Ligand binding induces the formation of a multiprotein complex called the death-inducing signaling complex (DISC). Activation of DISC leads to the recruitment of procaspase-8 and its activation by dimerization. Once activated, caspase-8 can cleave and activate procaspase-3 and Bid, a pro-apoptotic member of the Bcl-2 family of proteins. As discussed later, cleavage of Bid into truncated Bid (tBid) targets the mitochondria and therefore links the intrinsic and the extrinsic pathways of apoptosis (Crow *et al.*, 2004).

4.2.3 Intrinsic pathway

It is now widely accepted that the mitochondrial release of factors contained within their intermembrane and matrix space initiates apoptotic events (Green & Reed, 1998; Kroemer *et al.*, 1998; Bernardi, 1999a; Desagher & Martinou, 2000; Mayer & Oberbauer, 2003; Newmeyer & Ferguson-Miller, 2003). Thus, the barrier to apoptosis induction via the intrinsic pathway is related to the integrity of the mitochondrial membrane such that when the integrity of the mitochondrial membrane is breached, pro-apoptotic factors, which are usually confined to the inter membrane space, are released into the cytosol. These pro-apoptotic factors include the mobile electron carrier cytochrome c, a flavoprotein called apoptosis inducing factor (AIF), endonuclease G (EndoG), the second mitochondria-derived activator of caspases-direct inhibitor of apoptosis binding protein with low Pi (Smac/Diablo), and the serine protease Omi/HtrA (Desagher & Martinou, 2000; Hengartner, 2000). These proteins act through different mechanisms and at different sites to mediate the sequential process of cellular dismantlement, a process that is highly regulated.

4.2.3.1 Caspase-dependent mechanisms

The first protein shown to be released from mitochondria upon apoptotic stimuli

is cytochrome c, an essential component of the respiratory chain (Hengartner, 2000). Upon release into the cytoplasm, cytochrome c binds to the protease activating factor-1 (Apaf-1) and recruits and activates procaspase-9 to form a complex called the apoptosome (Desagher & Martinou, 2000). Activated caspase-9, can then cleave pro caspase-3 and in so doing, initiates the proteolytic cascade leading to cell death. It is important to note that this process requires ATP and thus the maintenance of minimal cellular capacities for energy production. Therefore, cells that have suffered extensive damage will probably initiate the apoptotic cascade but will ultimately become necrotic.

The catalytic function of cytochrome c in the formation of the apoptosome is regulated by members of the inhibitor of apoptosis proteins (IAP), which includes XIAP. XIAP normally acts as an inhibitor that prevents accidental formation of apoptosome complexes. However, some mitochondrial pro-apoptotic proteins have the ability to alter the activity of IAP family members. This is the case for Smac/Diablo which acts to promote apoptosis by neutralizing or negatively regulating proteins of the IAP family (Du *et al.*, 2000). Omi/HtrA2 also exert similar effects on XIAP but in addition is able to amplify caspase activity directly by virtue of its protease activity (Cain, 2003)

4.2.3.2 Caspase-independent mechanisms

Other key proteins released from mitochondria are AIF and EndoG. AIF and EndoG also provoke cell death, but in a caspase-independent manner (Susin *et al.*, 2000). AIF and EndoG become active cell killers since upon release into the cytosol, they translocate to the nucleus where they are involved in nuclear destruction through DNA fragmentation and chromatin condensation (Daugas *et al.*, 2000). Exactly how AIF and EndoG exert their destructive nuclear functions remains elusive. However, they could exert their effects by facilitating the cleavage and inactivation of Poly(ADP-ribose)polymerase-1 (PARP-1), a nuclear enzyme activated by DNA breaks which serves a role in DNA repair through the formation of polymers (poly(ADP)ribosylation) at sites of DNA damage.

Paradoxically, there is some evidence that AIF may exert a protective function

in some cell types. Apparently, the results of studies using both *in vitro* cell models and *in vivo* AIF-deficient mouse models have established that AIF is required for the correct assembly or function of complex I of the respiratory chain (Vahsen *et al.*, 2004; Urbano *et al.*, 2005). Interestingly, AIF deficiency compromises oxidative phosphorylation by inhibiting respiratory chain complex I *in vitro* and *in vivo*, revealing a 'life' function for AIF (Vahsen *et al.*, 2004). However, the mechanistic connections between the redox activity of AIF, oxidative phosphorylation and cell survival remain unclear.

4.2.4 Mechanisms of release of mitochondrial pro-apoptotic proteins

As we have seen, the loss of mitochondrial membrane integrity and the consequent release of apoptogenic factors into the cytosol is evidently an event of primary importance to the progression of cell death. But how are such proteins released and how do they pass through the mitochondrial membranes? Although the precise mechanism by which apoptogenic proteins are released from the mitochondrial intermembrane space is still debated, two prevailing models exist: (1) the formation of autonomous channels by members of the Bcl-2 subfamily; (2) the opening of the non-selective permeability transition pore (PTP). The next section reviews the current state of knowledge regarding the role of each of these proposed processes in mediating the release of apoptogenic proteins from the mitochondrial intermembrane space.

4.2.4.1 *The role of the Bcl-2 family of proteins*

The Bcl-2 proteins have multifunctional capabilities that enable them to serve as potent regulators of cellular apoptosis by either promoting or preventing permeabilization of the outer mitochondrial membrane (Reed, 1997; Wang, 2001). As such, the Bcl-2 family of proteins plays a pivotal role in determining whether a cell will live or die (Gross *et al.*, 1999). All Bcl-2 family members possess at least one of four motifs known as Bcl-2 homology (BH) domains (BH1−BH4). Most anti-apoptotic members, including Bcl-2 and Bcl-$_{xL}$, contain all four domains, whereas the pro-apoptotic members such as Bax and Bak lack the BH4 domain. Other pro-apoptotic members, the so-called BH3 domain-only proteins, include Bid, Bim, and Bad and, as their name implies, contain *only* the BH3 domain (Gross *et al.*, 1999).

In normal, healthy cells, Bax is localized mostly in the cytoplasm. But in response to stressful stimuli, Bax undergoes conformational changes and redistributes to the outer mitochondrial membrane (Martinou & Green, 2001). Following mitochondrial translocation, it is believed that Bax exerts its pro-apoptotic action by facilitating the release of cytochrome c from the mitochondrial intermembrane space into the cytosol by either forming a pore, through oligomerization, in the outer mitochondrial membrane, or by promoting the opening of other channels (Shimizu *et al.*, 1999). In this regard, Bax was shown to directly induce opening of the mitochondrial PTP (Narita *et al.*, 1998; Pastorino *et al.*, 1999; Brenner *et al.*, 2000), which suggests that Bax can cause membrane permeation at least by two mechanisms.

As for Bak, these proteins are present in the mitochondrial membrane even in healthy cells, but undergo a conformational change during apoptosis causing the formation of aggregates that increase the permeabilization of the outer mitochondrial membrane resulting in the mitochondrial release of apoptogenic factors (Danial & Korsmeyer, 2004).

Bad is regulated by its phosphorylation, and sequestration by its cytosolic anchoring protein 14-3-3. For instance, deprivation from growth factors has been shown to result in the inactivation of Akt which induces the dephosphorylation and activation of Bad (Datta *et al.*, 1997). Bad activation causes its translocation to the mitochodnria where it was shown to facilitate the activation of Bak (Cheng *et al.*, 2003).

As for Bid, this protein remains inactive in the cytosol until cleaved to tBid in response to death stimuli. tBid then translocates to the mitochondrial membrane where it is believed to exert its pro-apoptotic function by facilitating the mitochondrial release of cytochrome c (Luo *et al.*, 1998). As previously mentioned, caspase-8 has the capability to cleave Bid, which allows cross talk between the death receptor-mediated (extrinsic) and the mitochondrial-mediated (intrinsic) pathways of apoptosis. The importance of this crosstalk between the cell surface and the mitochondria is demonstrated by studies performed in Bid-knockout mice. The hepatocytes from Bid-deficient mice are resistant to TNF-induced apoptosis, although cells of these mice

retain their susceptibility to apoptosis following exposure to other known apoptosis-inducing agents that do not act via the death receptor pathway (Yin *et al.*, 1999). Bid can also be cleaved by calpain, a Ca^{2+} sensitive protease. The importance of this process in mediating ischemic damage in the heart has recently been demonstrated (Chen *et al.*, 2001).

The activities of the pro-apoptotic proteins such as Bax, Bak, Bad, and Bid can be neutralized by the anti-apoptotic members of the family such as Bcl-2 and Bcl-$_{xL}$. To this effect, it is well established that the balance of interaction between pro- and anti-apoptotic members of the Bcl-2 family (*i.e.* Bax:Bcl-2 ratio) can constitute an important element in the determination of whether a cell is destined to survive or die. In fact, the ratio of anti- to pro-apoptotic molecules has been described as a rheostat that sets the threshold of cellular susceptibility to apoptosis via the intrinsic pathway (Danial & Korsmeyer, 2004).

Bcl-2 and Bcl-$_{xL}$ exert their anti-apoptotic function, at least in part, by blocking the oligomerization of Bax and Bak, thereby disrupting their ability to form pore-like structures which compromise the barrier function of the outer mitochondrial membrane (Newmeyer & Ferguson-Miller, 2003). In fact, the evidence from a study performed in artificial membranes composed of mitochondrial lipids demonstrates that the channel-forming ability of Bax is directly inhibited in the presence of Bcl-$_{xL}$ (Kuwana *et al.*, 2002). Bcl-2 and Bcl-$_{xL}$ do not seem to interrupt, however, the translocation of Bax, Bid or Bad proteins to the mitochondria (Wei *et al.*, 2001). There has also been some suggestion that Bcl-2 and Bcl-$_{xL}$ may exert their anti-apoptotic effects by binding to Apaf-1 thereby inhibiting its association with caspase-9 to form the apoptosome (Haraguchi *et al.*, 2000).

Another way Bcl-2 and Bcl-$_{xL}$ proteins block the mitochondrial release of pro-apoptotic factors is by inhibiting the opening of the PTP. In fact, Kroemer's group has proposed that PTP opening is the fundamental mechanism for the release of pro-apoptotic proteins of the intermembrane space (Susin *et al.*, 1996). Although the effects of Bcl-2 family members on the probability of PTP opening still await a mechanistic

explanation, both pro- and anti-apoptotic proteins of the Bcl-2 family interact with components of the PTP, and they do so in a manner that is consistent with their effects on apoptosis (Bax, stimulatory; Bcl-2, inhibitory). A detailed description of the PTP is presented in the next section.

4.2.5 The permeability transition pore

4.2.5.1 The phenomenon of permeability transition

The mitochondrial permeability transition was initially described in isolated mitochondria as a sudden increase of the inner membrane permeability to solutes in the presence of a high Ca^{2+} concentration (Haworth & Hunter, 1979). Although initially thought to be due to unspecific membrane damage, it is now widely accepted that this phenomenon is actually caused by the opening of the PTP, a non-specific high conductance channel of the inner membrane (see (Zoratti & Szabo, 1995) for an extensive review). Prolonged and widespread opening of the PTP leads to the equilibration of ions and solutes of < 1500 Da between the matrix and the intermembrane space resulting in the collapse of the proton motive force, uncoupling of oxidation from phosphorylation, and massive ATP hydrolysis caused by the reversal of F_0F_1 ATPsynthase (Di Lisa & Bernardi, 1998; Bernardi, 1999b; Bernardi *et al.*, 1999; Crompton, 1999; Suleiman *et al.*, 2001). At least *in vitro*, the increased permeability to solutes also results in swelling of the mitochondrial matrix. Because the inner membrane has a large surface and presents several folds, it can accommodate important changes in matrix volume. However, because the outer membrane has a much smaller surface, swelling eventually results in its rupture, causing the release of mitochondrial pro-apoptotic factors.

Depending on cell type, mitochondria supply almost 95% of the ATP used by cells. Because of the dramatic effects of PTP opening, one would therefore expect that over evolutionary time such a process would have been eliminated by natural selection. While there is no consensus regarding the precise physiological role of PTP, a number of hypotheses have been proposed. Among these are that PTP provides a way of clearing the matrix of unwanted or damaged molecules (Gunter & Pfeiffer, 1990), or

that PTP opening is a way of getting rid of damaged mitochondria (Lemasters *et al.*, 2002) or to reduce the number of mitochondria during physiological fiber type transition (Moyes & Hood, 2003). Opening of the PTP *in vivo* or *in situ* in intact cells has been demonstrated in several organs (heart, brain, skeletal muscle, liver) in response to stress such as acute graft rejection, ischemia-reperfusion, oxidative stress, glutamate excytotoxicity, and Ca^{2+} overload.

4.2.5.2 Regulation of the permeability transition pore

The open-closed transitions of the PTP are highly regulated by numerous physiological factors, but matrix Ca^{2+} has been identified as the single most important factor for pore opening (Hunter & Haworth, 1979). Physiological factors such as increased inorganic phosphate (Pi), oxidation of pyridine nucleotides, matrix alkalinization (pH >7.6), increased matrix volume or swelling, and decreased membrane potential promote PTP opening (Connern & Halestrap, 1994, 1996; Halestrap *et al.*, 1997). Molecules such as Mg^{2+} and other divalent cations are considered to compete directly with Ca^{2+} and thus strongly inhibit the open pore conformation (Hunter & Haworth, 1979; Bernardi *et al.*, 1992).

Table 1-2: Modulators of the mitochondrial permeability transition pore

LEVEL OF REGULATION	OPEN PROBABILITY		REFERENCE
	Increase	Decrease	
Voltage	Depolarization	Hyperpolarization	(Bernardi *et al.*, 1992)
Matrix pH	Alkalinization	Acidification	(Szabo *et al.*, 1992)
Surface potential	More negative	More positive	(Broekemeier & Pfeiffer, 1995)
Cyclophilin D	-	CsA	(Crompton *et al.*, 1988)
Pyridine nucleotides	Oxidation	Reduction	(Duchen *et al.*, 1993)
Quinones	-	Decylubiquinone	(Fontaine *et al.*, 1998b)
Matrix Me^{2+} site	Ca^{2+}	Mn^{2+}, Mg^{2+}	(Hunter & Haworth, 1979)
External Me^{2+} site	-	Ca^{2+}, Mg^{2+}	(Hunter & Haworth, 1979)

The PTP can also be modulated by a variety of drugs classified as inhibitors or activators depending on whether they desensitize or sensitize, pore opening in response

to a given concentration of cellular Ca^{2+} (Zoratti & Szabo, 1995). In this regard, a major advancement in the study of the PTP was achieved in 1988 when Crompton and colleagues (Crompton *et al.*, 1998) discovered that cyclosporine A (CsA), a potent immunosuppressant used to decrease organ rejection after transplant surgery, could specifically inhibit pore opening at micromolar concentrations. The inhibition of PTP by CsA in isolated mitochondria was the trigger for studies designed to identify the molecular target of CsA. The 18 kDa protein cyclophilin D (CypD), found exclusively in mitochondria was later identified as the molecular target of CsA (Connern & Halestrap, 1992).

However, it is important to note that CsA is not a true pore inhibitor since inhibition can be overcome by large increases in matrix Ca^{2+} (Bernardi *et al.*, 1993). This suggests that CypD likely acts as a key regulator of PTP function, rather than a structural protein component of the complex. Indeed, some studies have shown that CypD may actually translocate from the matrix to the inner mitochondrial membrane where it can interact with the PTP under conditions of Ca^{2+} overload (Connern & Halestrap, 1994), supporting a regulatory role for CypD in PTP function.

4.2.5.3 Molecular identity of the PTP

Despite its relatively detailed functional characterization, the molecular identity of the PTP is not clearly established and remains a subject of intense debate (Bernardi, 1999b; Forte & Bernardi, 2005; Zoratti *et al.*, 2005). However, it appears relatively clear that it is composed of several specific mitochondrial proteins, which under pathological conditions undergo conformational changes that transform them into non-specific, high conductance channels.

The ANT-VDAC hypothesis

The most popular and well documented hypothesis is that the PTP includes (although not necessarily exclusively) three basic units, the ANT (Halestrap & Davidson, 1990; Halestrap *et al.*, 1997; Woodfield *et al.*, 1998) the porin pore VDAC (Beutner *et al.*, 1998; Brdiczka *et al.*, 1998; Crompton *et al.*, 1998; Ruck *et al.*, 1998)

and the matrix enzyme CypD (Halestrap & Davidson, 1990; Andreeva *et al.*, 1995). These proteins form a complex at the contact sites between the inner and outer mitochondrial membranes together with other kinases including mitochondrial creatine kinase and hexokinase (Beutner *et al.*, 1998; Ruck *et al.*, 1998).

Recent controversy has, however been raised following a publication by Kokoszka et al. (Kokoszka *et al.*, 2004) demonstrating the existence of a functional PTP in liver mitochondria lacking the two isoforms of ANT derived from a genetically modified, double-mutant ANT-knockout mouse model. Mitochondria isolated from ANT-deficient livers were able to undergo Ca^{2+}-dependent permeability transition suggesting that in the absence of ANT, other proteins can substitute to form the PTP. However, the PTP from these ANT-deficient mitochondria required significantly higher levels of Ca^{2+}, on the order of three-fold, for activation compared to control mitochondria. Moreover, the absence of ANT resulted in the loss of PTP regulation by the ANT ligand atractyloside. Considered together, these data suggest that ANT might control PTP function by sensitizing PTP opening in response to Ca^{2+}. Thus, while the results of Kokoszka (Kokoszka *et al.*, 2004) would seem to unequivocally demonstrate that the ANT is not obligatory for PTP formation, this conclusion has not been universally accepted among experts in the area (Halestrap, 2004). Although not published yet, there is emerging evidence suggesting that genetic ablation of VDAC is also unable to fully prevent PTP opening (C.P. Baines, unpublished observations).

Role of cyclophilin D

Recently, two articles have appeared in *Nature*, in which the gene for CypD was inactivated in mice (Baines *et al.*, 2005; Nakagawa *et al.*, 2005). An examination of mitochondria lacking CypD revealed that PTP can assume the open conformation in the absence of CypD, confirming its role as a regulator but not necessarily a core component of the protein complex. Indeed, both Baines et al. (2005) and Nakagawa et al. (2005) found that mice lacking CypD, as assessed by Western blot and PCR analysis, developed normally, without any detectable phenotypic abnormalities, but the mitochondria of these mice were resistant to Ca^{2+}-induced opening of the PTP. In

addition to the ability of these CypD-null mitochondria to accumulate higher levels of Ca^{2+} prior to PTP opening, the presence of CsA was totally ineffective at providing inhibition thus proving that CypD is the molecular target of CsA. However, the PTP response to other known modulators that do not depend on CypD such as depolarization and pH was not different between CypD-null and wild-type mice. Similarly, although loss of CypD expression protected against Ca^{2+} and oxidative stress-induced mitochondrial-mediated cell death, it offered no protection against non-PTP-mediated cell death such as that mediated by TNF (Baines *et al.*, 2005).

Since CypD appears to be an activator of the PTP, it might be expected that overexpression of this protein would lead to increased sensitivity to factors promoting cell death. Indeed, heart mitochondria overexpressing CypD displayed changes in mitochondrial architecture, swelling, an overall increased TUNEL staining, increased cytochrome c release, increased caspase-9 activation and a reduction in cardiac function over time (Baines *et al.*, 2005). Each of these phenomena is consistent with activation of the PTP-mediated cell death pathway. Thus, considered together, these studies confirm that PTP opening can still occur in mitochondria devoid of CypD when the concentrations of Ca^{2+} are greatly increased.

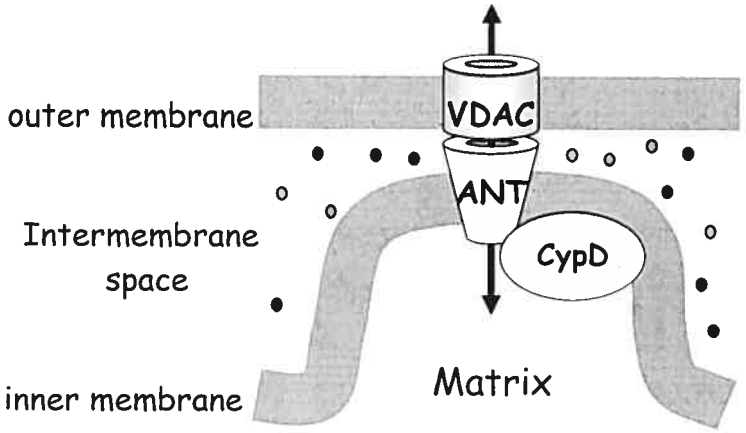

Figure 1-9:

The proposed molecular structure of the mitochondrial permeability transition pore (PTP). In the open configuration, adenine nucleotide translocator (ANT), voltage-dependent anion chanel (VDAC) and cyclophilin D (CypD) form a pore-like structure at the contact site between the inner and outer membranes allowing the release of proteins normally confined to the intermembrane space. Figure adapted from Desagher & Martinou (2000).

5 EVIDENCE FOR THE ROLE OF APOPTOSIS IN MUSCLE DISUSE ATROPHY

The last part of this review focuses particularly on cell death in models of muscle disuse and on evidence that supports a role for mitochondria in this process.

5.1 Regulation of myonuclear domain size

In most cell types, apoptosis of the nucleus implies the death of the cell. However, since skeletal muscle is a multinucleated cell type, apoptosis of a nucleus may not necessarily equate to apoptosis of the whole muscle fiber. Indeed, when muscle fibers atrophy, the size of the nuclear domain remains the same or changes slightly in function of the phenotypic remodeling of the fibers. Allen et al. (Allen *et al.*, 1999) put forth the hypothesis that apoptotic-like mechanisms may be responsible for the elimination of myonuclei from a muscle fiber in an attempt to maintain the relative size of the myonuclear domain during situations of reductive remodeling. These same authors also demonstrated that the number of nuclei showing double-stranded DNA fragmentation seen by TUNEL histochemical staining, an indicator of apoptosis, was significantly increased after 14 days of hindlimb unloading and the number of fibers containing morphologically abnormal nuclei was also significantly greater in suspended compared with control rats (Allen *et al.*, 1997). Furthermore, the authors report that treatment with growth hormone and insulin-like growth factor I and resistance exercise attenuated the increase in TUNEL-positive nuclei by approximately 26%, and significantly decreased the number of fibers with abnormal nuclei. At the time of publication, the authors interpreted this data to suggest that "programmed nuclear death" contributes to the elimination of myonuclei from atrophying fibers and that insulin-like growth factor I administration plus muscle loading ameliorates the apoptosis associated with hindlimb unloading.

Of interest, is whether mitochondria play a role in this apoptotic process. Indeed, recent studies by Siu et al. (Siu *et al.*, 2005) investigated the hypothesis that apoptosis is associated with hindlimb suspension-induced muscle loss. In the suspended animals, muscles had a 73% greater Bax mRNA content compared with the control muscle. The

Bax protein content increased by 43 % after suspension and the Bcl-2 protein content in the muscle after suspension was 55 % higher than in the control muscle. The authors speculate that the elevation of Bcl-2 may be an adaptive or compensatory change that is invoked in response to suspension in an attempt to reduce the apoptotic loss of myonuclei and, consequently, muscle mass. As for mitochondria-mediated apoptosis factors, ELISA analysis on the mitochondria-free cytosolic fraction indicated that the protein content of cytosolic cytochrome c in the suspended muscle was elevated by 41% compared with control muscle. Given that cytochrome c mainly resides in the mitochondria under normal conditions, this finding indicates that cytochrome c was relocated or released into the cytosol in response to suspension. The authors were unable to find any difference in the mRNA content of Apaf-1 between the suspended and control muscles. Furthermore, the release/accumulation of mitochondrial Smac/Diablo was estimated by using immunoblot analyses on the mitochondria-free cytosolic fraction, but no change with suspension was reported (Siu *et al.*, 2005). Although these data suggest the involvement of the mitochondrial death pathway in the muscle atrophy process induced by hindlimb suspension, they provide no indication that this process is directly responsible for the elimination of nuclei that occurs in disused muscle.

5.2 Loss of muscle fibers in various models of disuse

5.2.1 Ageing

Sarcopenia is the term used to define the progressive loss of muscle mass and strength associated with the ageing process. Although the cause of the loss of muscle mass with age likely involves multiple factors, sarcopenia results in a decrease in the number of muscle fibers and atrophy of the remaining fibers. Although the mechanisms through which ageing skeletal muscles atrophy are largely unidentified, proposed mechanisms have included the activation of proteolytic pathways, loss of innervation, hormonal adaptations, and processes associated with mitochondrial dysfunction and mitochondrial-mediated apoptosis (Dirks & Leeuwenburgh, 2002).

Determining whether mitochondrial dysfunction is the cause or consequence of

ageing has been difficult to clarify. A large body of evidence has led to a theory now recognized as the *mitochondrial theory of ageing* (Harman, 1972). According to this theory, mitochondrial damage and respiratory chain dysfunction leading to enhanced ROS production (oxidative damage) are linked in a downward spiral that culminate in progressive mitochondrial dysfunction leading to impaired cell function and ultimately, compromised cell viability. Indeed, a decline in mitochondrial oxidative function and an increase in the incidence of mitochondrial DNA damage have been shown to occur in various tissues with age and there is support that ROS play an important role in these processes (Wallace *et al.*, 1995; Cadenas & Davies, 2000). Ironically, the very feature that makes mitochondria unique among the various cell organelles - having their own DNA - gives rise to a major problem. Whereas nuclear DNA is protected by histone proteins and various repair enzymes, which minimizes damage to nuclear DNA from free radicals/oxidants, mitochondrial DNA has no histone protection or enzyme repair systems to offer protection against free radicals (Richter, 1995). As such, mitochondrial DNA is much more subject to free radical damage. The accumulation of mitochondrial DNA mutations with age can interfere with the synthesis of proteins and enzymatic pathways involved in the transfer of electrons along the respiratory chain and ATP production (Wallace *et al.*, 1995; Papa & Skulachev, 1997).

An expanding body of evidence now shows that apoptosis may play a role in regulating the process of muscle loss with ageing (Pollack *et al.*, 2002; Alway *et al.*, 2003; Dirks & Leeuwenburgh, 2004; Dirks *et al.*, 2005; Leeuwenburgh *et al.*, 2005). Indeed, studies suggest that the rate of apoptosis in skeletal muscle increases with age, and the specific apoptotic pathways which are responsible for the loss of muscle fibers that occurs in sarcopenic muscle are becoming clearer (Dirks & Leeuwenburgh, 2002; Alway *et al.*, 2003; Dirks *et al.*, 2005). For instance, the protein levels of the caspase-3 enzyme, in both its activated and inactivated forms, was reported to be significantly increased in ageing skeletal muscle and the cellular levels of the proapoptotic protein AIF has been shown to increase by approximately 50% in aged compared to young muscle (Dirks & Leeuwenburgh, 2004). These results suggest an increased apoptotic potential in aged muscle. Furthermore, Alway et al. (Alway *et al.*, 2003) have shown that Bax levels increase and Bcl-2 levels decrease with age in the plantaris muscle of

rats, providing a mechanism to explain the elevated apoptotic potential in aged muscle. In fact, there is even some evidence to suggest that activation of the mitochondrial PTP is enhanced with ageing (Mather & Rottenberg, 2000). This evidence comes from a study showing that mitochondria isolated from brain and liver of 20 month-old mice show increased susceptibility to PTP activation by Ca^{2+} overload suggesting that ageing-induced enhanced activation of the PTP may be a general phenomenon that contributes to the increased susceptibility of several tissues to cell damage that is associated with ageing (Mather & Rottenberg, 2000). Although more research is required to determine the precise role of apoptosis in the loss of muscle fibers and the loss of fiber cross sectional area with ageing, the available evidence tends to suggest that apoptotic mechanisms involving the mitochondria are indeed implicated.

5.2.2 Denervation

Muscle denervation is an exemplary cause for progressive loss of muscle fiber mass and size. In fact, there has been some speculation as to whether denervated muscle fibers solely become increasingly atrophic or whether they degenerate completely. It has been suggested that a lack of innervation, or defective innervation may incite muscle fibers to activate an intrinsic suicide program, the implication being that muscle fibers that are not properly/adequately innervated may be programmed to die (Tews *et al.*, 1997a; Tews *et al.*, 1997b; Tews, 2002). The activation of mitochondria-associated apoptosis has been shown in skeletal muscle during denervation (Siu & Alway, 2005b). Specifically, rat skeletal muscle denervated for 14 days demonstrated augmentation of apoptotic DNA fragmentation, increases in the Bax:Bcl-2 ratio, mitochondrial release of cytochrome c into the cytoplasm, increases in Smac/Diablo and AIF, increases in the protein content and activity of caspase-3 and caspase-9, and decreases in XIAP (an inhibitor of apoptosis) (Siu & Alway, 2005b).

In fact, these findings are corroborated by the results of another recent study by these same authors using a mouse model deficient for the Bax gene (Siu & Alway, 2006). Compared to denervated muscles from wild-type mice, denervated muscles taken from the mice that were deficient for the Bax gene had significantly decreased levels of DNA fragmentation, decreased caspase-3 and caspase-9 activities, and suppressed

mitochondrial cytochrome c release (Siu & Alway, 2006). These results indicate that in response to the removal of nerve supply in skeletal muscle deficient for the Bax gene, the extent of muscle loss is attenuated and is accompanied by the suppression of pro-apoptotic signaling, provide further evidence that apoptotic signaling has a significant role in denervated muscle and may be involved in mediating muscle wasting.

Further evidence supporting the notion that events associated with apoptosis are upregulated in denervated muscle has been provided by experiments where rat facial muscle was denervated and subsequently reinnervated (Tews *et al.*, 1997b). The results of this study show that apoptotic degradation of denervated muscle fibers appears to be mediated by intrinsic, mitochondria-associated mechanisms that involve proteins of the Bcl-2 family, increased levels of caspase-9, TUNEL-positive DNA fragmentation, and features of apoptotic nuclear degradation that could be observed at the ultrastructural level (Tews & Goebel, 1996; Tews *et al.*, 2005). These findings provide additional strong evidence supporting the speculation that apoptotic mechanisms are active and contribute to muscle fiber loss with denervation.

The results presented in the second study presented in the present thesis, provide supporting evidence that PTP opening could be one of the mechanisms by which mitochondria activate cell death pathways.

6 PRESENTATION OF THE MANUSCRIPTS

The objective of the work presented in this thesis is to provide a contribution to the current understanding of the cellular processes regulating disuse muscle atrophy. This thesis consists of three original research articles that contribute novel information to: 1) the understanding of intracellular signaling potential in disused muscle in response to a mechanical stimulus; 2) the effect of loss of neural input to muscle fibers on changes in the sensitivity and modulation of PTP opening; 3) a characterization of the properties and function of the mitochondrial PTP in different skeletal muscles characterized by different fiber types.

6.1 Summary of study I:

"SENSITIVITY OF RAT SOLEUS MUSCLE TO A MECHANICAL STIMULUS IS DECREASED FOLLOWING HINDLIMB UNWEIGHTING"

As discussed, mechanical forces play an important role in the regulation of muscle size and phenotype. Since there exists a relationship between muscle unloading and atrophy, the aim of this study was to investigate the sensitivity of mechanically-responsive intracellular signaling pathways in order to establish the extent to which a given mechanical stimulus can influence the trophic response of muscle following atrophy. The results of this study show that basal JNK activation state changes in response to muscle atrophy. The JNK pathway has been shown to regulate apoptotic signaling events in various cell types. Since the contribution of cellular apoptosis has been proposed as a possible mechanism regulating the loss of myofibers which occurs as a result of reduced mechanical loading, increased JNK-mediated regulation of cellular apoptosis may explain the increased basal phosphorylation levels measured in muscle following hindlimb suspension. Following a mechanical challenge, we report that JNK phosphorylation response is significantly reduced in muscle atrophied by unloading. We propose that this decreased JNK phosphorylation response to mechanical stimulation indicates that atrophic muscle may lose the ability to transduce mechanical signals to the MAP kinase pathways.

6.2 Summary of study II:

"MUSCLE DENERVATION INCREASES THE EXPRESSION OF MITOCHONDRIAL CYCLOPHILIN D AND PROMOTES OPENING OF THE PERMEABILITY TRANSITION PORE"

Given recent evidence implicating a role for mitochondrial PTP opening in cell death, and since there are currently very few data available on the PTP in skeletal muscle, this study investigated PTP sensitivity in skeletal muscle atrophied by a loss of neural input. The model of denervation was used because of the severity of the atrophic

response associated with loss of neural input to the muscle.

In this study, we show for the first time that muscle atrophy caused by denervation was associated with heightened sensitivity of the PTP to opening in response to progressive Ca^{2+} loading. This response is partly due to a progressive muscle and mitochondrial Ca^{2+} overload. Ca^{2+} retention capacity was found to be dramatically reduced in muscle following denervation but surprisingly, this could be significantly improved in the presence of inhibitors of PTP opening, namely CsA. This result prompted us to measure protein expression of CypD, a matrix peptidyl-prolyl-cis-isomerase believed to be an integral molecular component of the PTP complex and the protein target of CsA. We found CypD expression to be significantly increased in mitochondria of muscle atrophied by denervation leading to over-representation of this protein relative to ANT and VDAC, other structural components of the PTP. Our explanation linking changes in CypD expression to PTP dysregulation is as follows: by virtue of its peptidyl-prolyl-cis-isomerase activity, CypD is believed to act as a protein foldase that presumably interacts with chaperone proteins to limit mitochondrial protein misfolding that is known to occur following various perturbations. However, in the presence of elevated Ca^{2+}, CypD assumes a secondary role by promoting the formation of non-specific channels, possibly the PTP. In summary, we report that CypD expression is increased in muscle atrophied by denervation as an initial defense or protective mechanism against protein misfolding. However, as mitochondria become progressively overloaded with Ca^{2+}, this would evolve into a maladaptive response by promoting PTP formation and opening.

6.3 Summary of study III:

"MITOCHONDRIA FROM SOLEUS MUSCLE EXHIBIT INCREASED SENSITIVITY TO CA^{2+}-INDUCED OPENING OF THE PERMEABILITY TRANSITION PORE: EVIDENCE FOR PHENOTYPE-SPECIFIC PORE REGULATION"

The mitochondria under examination in the former study were isolated from mixed groups of plantar hindlimb muscles including soleus, plantaris, and whole

gastrocnemius. Since the contractile, biochemical, and morphological characteristics of these muscle groups are largely different (i.e. soleus is composed mainly of slow-twitch type I fibers with a large volume of mitochondria having a high oxidative enzyme level, whereas gastrocnemius is largely dominated by fast-twitch type II fibers with relatively less mitochondria), it is possible that these muscle groups, as well as their isolated mitochondria, might have dissimilar responses to the denervation stimulus. To date, the characterization of PTP properties has been based primarily on studies in mitochondria isolated from cardiac or hepatic tissue, due in part to the rich mitochondrial content and the relative ease of mitochondrial isolation from these tissues compared to skeletal muscle tissue. Consequently, relatively little information is available regarding PTP function in mitochondria isolated from skeletal muscle tissue. In light of the transitions in fiber phenotype known to accompany muscle disuse atrophy, a characterization of basic parameters of PTP function in different muscle fibers is necessary. In this third study, we demonstrate that indeed, basic PTP function and sensitivities are different depending on the type of muscle fiber from which mitochondria are isolated.

Chapter 2: Original Research Article I

Title:

Sensitivity of rat soleus muscle to a mechanical stimulus is decreased following hindlimb unweighting

Authors:

Kristina Csukly, Tanguy Marqueste, Phillip Gardiner

Journal publication reference:

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ABSTRACT

Mechanical loading is thought to be an important stimulus regulating muscle mass. However, the responsiveness of a muscle atrophied by a period of mechanical unloading to a subsequently imposed mechanical challenge is not well understood. This study examined the phosphorylation of the mechanically sensitive p54 c-jun NH_2-terminal kinase (JNK) signaling protein in atrophied rat soleus muscle in response to a mechanical challenge in-situ (isometric contractions; 100 Hz, 150 ms, once every 1 s for 5 min). Rats underwent either 7 or 14 days of hindlimb suspension (HLS) following which phosphorylation of JNK was measured biochemically. Immunofluorescence analysis revealed that phosphorylated JNK was localized in myonuclei. Baseline JNK phosphorylation measured in non-stimulated soleus muscles of 7 and 14-day HLS groups was 3.0 and 2.8 fold, respectively, the baseline phosphorylation measured in muscle of weight-bearing control animals (CTL). Following a mechanical challenge, JNK phosphorylation in stimulated CTL and 7-day HLS groups was significantly increased by 3.2 and 1.8-fold the non stimulated baseline levels, respectively. In stimulated muscle of 14-day HLS, JNK phosphorylation levels did not significantly differ from the baseline levels suggesting that the ability to elicit a mechanically-induced phosphorylation of the JNK signaling protein gradually decreases with unweighting and is attenuated after 14-day HLS. Changes in the responsiveness of mechanically sensitive intracellular signaling pathways in atrophic muscle may contribute to the functional impairment experienced by muscle in the absence of weight bearing for prolonged periods.

INTRODUCTION

The regulation of skeletal muscle mass is particularly sensitive to mechanical loading such that overload causes fiber hypertrophy whereas unloading causes fiber atrophy (Fitts et al. 2000; Goldberg et al. 1975; Goldspink et al. 1986; Roy et al. 1985; Vandenburgh 1987). In addition to the control of fiber size, mechanical forces contribute to the regulation of numerous other cellular processes in skeletal muscle fibers, including the control of muscle phenotype (Thomason et al. 1987), metabolism (Ihlemann et al. 1999), morphology and architecture (Ihlemann et al. 1999; Vandenburgh et al. 1991; Vandenburgh and Karlisch 1989). Detailed reviews on disuse atrophy have shown that an absence of mechanical loading results in a rapid and substantial atrophy, particularly of the slow-twitch soleus muscle, which acquires properties typical of fast-twitch muscles (Booth and Baldwin 1996; Fitts et al. 2000; Thomason and Booth 1990). Thus, myocytes are capable of detecting and responding to the presence and/or absence of mechanical stimuli by activating signal transduction pathways, resulting in changes in gene expression. Despite the growing understanding of the cellular processes regulating muscle adaptations to unweighting, the activation of mechanically-responsive signal-transduction pathways to an acute stimulus in muscle subjected to a period of unloading is not well defined.

The mitogen-activated protein kinases (MAPKs) constitute a family of intracellular signaling proteins associated with membrane-linked cytoskeletal proteins involved in the transduction of mechanical forces from the cell-surface to the cell nucleus (Labrador et al. 2003). The mechanical responsiveness of the MAPKs in skeletal muscle (Goodyear et al. 1996; Hayashi et al. 1999; Martineau and Gardiner 2001; Ryder et al. 2000; Wretman et al. 2000) and in various other cell types (Komuro et al. 1991; Sadoshima and Izumo 1993; Schmidt et al. 1998; Seko et al. 1999; Zou et al. 1998) is well documented. Considering the importance of mechanical loading in the maintenance and control of

skeletal muscle homeostasis, it is presumed that a period of unweighting will cause modifications in the muscle's capacity for transmission and/or detection of mechanical signals. Indeed, recent studies have reported increased basal p54 c-jun NH_2-terminal (JNK) MAPK phosphorylation status in muscle immediately following a period of immobilization (Childs et al. 2003; Hilder et al. 2003; Morris et al. 2004). However, information regarding JNK signaling in atrophied muscle following acute contractile activity, a mechanical stimulus known to favor the restoration of muscle mass, is lacking. Accordingly, in the present study we sought to investigate the sensitivity of this mechanically-responsive signaling pathway in atrophic muscle following an acute mechanical challenge. For this purpose, the activation (i.e. phosphorylation) of JNK was assessed in rat soleus muscles atrophied by HLS for either 7 or 14 days in both the non-stimulated basal condition and following a 5-min isometric mechanical challenge applied in situ. Although the influence of an acute mechanical challenge on the JNK phosphorylation response in atrophic muscle is unknown, muscle contraction performed immediately after immobilization has been shown to rapidly instigate profound changes in gene expression associated with muscle hypertrophy and remodeling (Jones et al. 2004). Therefore, we proposed that the responsiveness of unloaded muscle to an acute mechanical stimulus would be increased as part of a compensatory effort to reestablish the muscle's pre-atrophy state.

MATERIALS AND METHODS

Hindlimb Suspension Protocol

Female Sprague-Dawley rats (Charles River, Saint-Constant, Quebec), weighing 197±5 g, were housed in an environmentally controlled room maintained at 23 °C and kept on a 12:12-h light-dark cycle. The rats were provided water and food ad libitum. Animals were randomly assigned to one of three groups; weight-bearing control (CTL); n = 10, 7-day HLS; n = 10, or 14-day HLS; n = 10. Animals belonging to a HLS group were suspended in a head-tilt position at an angle of approximately 30 degrees from the horizontal plane via a non-invasive apparatus affixed to the proximal end of the tail (Morey-Holton and Globus 2002). Briefly, the animal's tail was washed, dried and wrapped in breathable adhesive tape with a paper clip attached to the end. The paper clip acted as a hook by which the animal could be secured onto an elevated swivel system build into the top of the cage. The hindlimbs were prevented from touching any supportive surfaces of the cage while the forelimbs maintained full contact with the cage floor allowing free movement and access to food and water. Daily inspection of the animals' tail was performed, verifying for discoloration or lesions. Body mass was evaluated every 48 h as an indication of tolerance to the suspension condition. Any animal demonstrating signs of distress or intolerance was immediately excluded from the experimental protocol. All procedures were approved by the animal ethics committee of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care.

In-situ Nerve-Muscle Preparation

The in situ mechanical stimulation experiments were performed as described elsewhere (Martineau and Gardiner 2001). Briefly, animals were anesthetized by intraperitoneal injection of ketamine and xylazine (61.5, 7.7 mg/kg, respectively). An incision was made in the distal posterior segment of the

left hindlimb and the soleus muscle and its tendon were exposed. Following isolation of the muscle from the other extensors, the calcaneous was clipped, leaving a bone chip attached to the tendon and a silk ligature was tied securely around the bone-tendon interface. The animal was placed in the prone position within a stereotaxic frame with the left knee securely pinned in a slightly flexed position and the left foot clamped. The silk thread was attached to the lever arm of a muscle servomotor (305-LR; Aurora Scientific). Care was taken to ensure no tension was placed upon the isolated muscle during the preparation. The skin of the hindlimb was pulled to form a bath filled with heated mineral oil. Body and muscle bath temperatures were maintained at 36-37 °C for the duration of the experiment.

Mechanical Stimulation Protocol

The optimal muscle length (L_o) for producing maximum twitch force was determined by twitch stimulation achieved by indirect stimulation through a bipolar electrode placed under the sciatic nerve. Single square pulses (5 V, 0.05 ms) were delivered once every 3 s while the length of the muscle was slowly increased from a relatively slack length at ~ 1 mm intervals. Electrical stimulation in the form of supramaximal single square pulses (0.05 ms in duration) was delivered to the sciatic nerve at a frequency of 100 Hz once every second for a duration of 150 ms, for 5 min. Baseline values were determined following determination of L_o, by ceasing electrical stimulation and holding the muscle at this length for 5 min without any further stimulation. For the isometric contractile protocol, L_o was determined and maintained constant as the muscle was indirectly stimulated to contract for 5 min. Immediately-following the 5-min experimental period, the isolated muscle was excised and rapidly frozen in liquid nitrogen. The animal was euthanised by anesthetic overdose.

Biochemical Tissue Analysis

Tissue processing, electrophoretic separation of proteins, and measurements of phosphorylated p54 JNK and total JNK2 contents by immunoblotting using phospho-specific antibodies against phospho-p54 JNK and an anti-JNK2 antibody (Santa Cruz Biotechnology) were performed as previously described in detail (Martineau and Gardiner 2001). Briefly, frozen muscles were weighed and powdered in liquid nitrogen. Approximately 100 mg of powder was solubilized in 10 volumes of ice-cold modified RIPA buffer containing protease and phosphatase inhibitors. Muscle lysates containing 180 µg of protein were prepared for SDS-PAGE by dilutions with reducing sample buffer followed by a 10-min immersion in near-boiling water. Samples were resolved on 9% gels and simultaneously transferred to a PVDF membrane (Millipore). Equal sample loading was confirmed by Ponceau S stain. The membrane was blocked and incubated overnight at 4 °C with primary antibody solution followed by a 90-min incubation at room temperature in secondary antibody solution (Jackson Immunoresearch). Revelation was performed by enhanced chemiluminescence (Amersham) with film exposure times ranging from 5 to 45 min. Films were scanned and bands quantified by NIH Image software. Data were analysed using one-way ANOVA followed by Fisher's post-hoc test. Significance was assumed at 0.05.

Immunohistochemistry

Frozen soleus muscles in liquid nitrogen were cut (30 µm) at -20 °C using a cryostat. Forty-eight slices were analyzed from 3 soleus muscles (16 from one control soleus, 32 from two stimulated solei). Tissues were fixed with acetone for 3 min, permeated by incubating in TBS containing 0.1% triton X-100 for 10 min, then washed 3 x 5 min in TBS. Blocking was performed by pre-incubating in TBS containing 10% donkey serum for 1 h at room temperature, then slides were incubated with primary antibody for p54 JNK (anti-Active JNK pAb, Promega) raised in rabbit (diluted 1:200 in TBS + 0.2%

triton X-100 + 0.1% Na azide) for 24 h in a humidified chamber at room temperature. After 3 x 10-min washes with TBS, the fluorescent secondary antibody (fluorescein-FITC conjugated donkey anti rabbit IgG, Jackson Immuno Research) raised in donkey (diluted in 1:200 in TBS) was placed on the section and incubated in a humidified chamber for 2 h at room temperature. After a final wash 3 x 10 min in TBS, coverslides were mounted on with antifade medium and sealed.

Localization of phosphorylated p54 JNK was performed using a Nikon E600 microscope mounted with a Nikon Y-FL fluorescent device, using an epifluorescent XF22 cube. Pictures were digitalized with a monochrome Cool Snap Pro camera and Image Pro Plus 4.1 Software (Media Cybernetics, Inc). Nuclei of the same slices were double stained with Mayer's Hematoxylin Solution 0.1% (Sigma) during 1 min, then washed in TBS.

RESULTS

Body and muscle weights

Significant reductions in the soleus wet weight occurred as a result of HLS for 7 and 14 days (Table 1). Consistent with the literature (Jiang et al. 1993; Roy et al. 1991), the greatest decrease in muscle mass occurred during the first 7 days of HLS. Compared to CTL, after 7 days HLS, the soleus wet weight decreased by 27% relative to CTL whereas the soleus muscle-to-body weight ratio decreased by 23% relative to CTL. After 14 days HLS, the soleus wet weight decreased by 33% relative to CTL and the muscle-to-body weight ratio decreased by 31% relative to CTL (Table 1).

Contractile studies

Compared to muscle twitch force in rats from the CTL group, soleus twitch force was decreased to 75% and 64% in the 7 and 14-day HLS groups, respectively (Table 1). Tetanic force was influenced more by HLS than twitch force; by 7 and 14-days HLS, tetanic force was 62% and 39%, respectively, of the CTL value. Consequently, while normalized twitch force (i.e., twitch force divided by muscle wet weight) was not significantly influenced by HLS, normalized tetanic force decreased significantly by 14 days to 58% of CTL values (Table 1).

p54 JNK phosphorylation

Amount of total p54 JNK protein, independent of phosphorylation state, was not different among groups (Fig. 1). Basal JNK phosphorylation levels were clearly increased relative to CTL in 7 and 14-day HLS groups by 3.0 and 2.8-fold, respectively (Fig. 2). In response to mechanical stimulation, the JNK phosphorylation response in the 14-day HLS group did not significantly differ from its baseline value whereas in the CTL and 7-day HLS groups, significant increases in JNK phosphorylation of 3.2-fold and 1.8-fold baseline, respectively, were observed (Fig. 3). In order to illustrate the effect of

tetanic force production on the JNK phosphorylation response, we also normalized JNK phosphorylation as a function of the tetanic tension capability of the muscle (Fig. 4).

Phosphorylated p54 JNK localization

Double staining of nuclei with Mayer's Hematoxylin solution indicates that phosphorylated JNK signal is localized in the cell nuclei (Fig. 5 a and b). Double omission of primary and secondary antibodies gave no fluorescent signal in control and stimulated muscles. Single omission of primary antibodies in both control and activated muscles (Fig. 6 a and b) gave a very weak signal and few non-specific fixations of the secondary FITC antibodies. The dilution of 1:200 of primary antibodies showed a bright signal located in the nuclei of each cell. Four or more nuclei can be easily identified per cell. These images (Fig. 6 c and d) were observed in both control and stimulated muscle slices.

DISCUSSION

The main finding of this study is that JNK signaling is altered in soleus muscle atrophied by HLS and that this signaling changes dramatically during the initial 2 weeks of muscle weight loss. The finding of increased basal JNK phosphorylation levels measured in non-stimulated soleus muscle of HLS groups is in accordance with the findings of other recent reports (Childs et al. 2003; Hilder et al. 2003; Morris et al. 2004). In this study, we show that phosphorylation of JNK is restricted to myonuclei. The changes in basal JNK phosphorylation following HLS are particularly interesting given the increase in myonuclei per mg of muscle which is known to occur after this condition (Allen et al. 1997). After two weeks of HLS, soleus cytoplasmic volume per myonucleus changes from 13,000 to 7,000 cubic micrometers, which signifies almost a doubling of myonuclear number per unit weight of muscle. This could signify that, while the increased basal phosphorylation of JNK after HLS might reflect the increased concentration of myonuclei (ie., basal phosphorylation per nucleus remains unchanged), the mechanical stimulation response of JNK would be even less per myonucleus than the significantly attenuated response seen when expressed per mg of wet muscle weight.

The JNK pathway has been shown to regulate apoptotic signaling events in various cell types (Aoki et al. 2002; Davis 2000). The contribution of the cellular process of apoptosis in situations of cellular remodeling has been discussed as a possible explanation for the loss of myofibers which eventually occurs as a result of reduced mechanical loading (Allen et al. 1997). Thus, increased JNK-mediated regulation of cellular apoptotic events may partially explain the increased JNK phosphorylation observed in atrophic muscles of HLS groups at rest. On the other hand, atrophying muscles may come under increased mechanical stress in spite of lack of weight-bearing, this resulting in increased JNK phosphorylation at "rest".

The JNK signaling protein is highly expressed in skeletal muscle whereby muscle contraction and passive stress are known to induce a robust phosphorylation that is both rapid and transient in nature (Boppart et al. 2000; Carlson et al. 2001; Martineau and Gardiner 2001). However, in the present study, it appears that JNK phosphorylation in response to mechanical stimulation by isometric contraction gradually becomes considerably reduced in soleus muscle of rats of the HLS groups. To our knowledge, this is the first study to measure the phosphorylation of a mechanically responsive signaling molecule in skeletal muscle atrophied by HLS, in response to an acute mechanical challenge in the form of evoked contractile activity. These findings suggest that while basal JNK phosphorylation levels are increased in atrophied soleus muscle, phosphorylation of this mechanically sensitive signaling molecule following isometric contractile activity gradually attenuates as the duration of muscle unloading progresses to 14 days.

One possible interpretation for the absence of a significant stimulation-induced JNK phosphorylation response in atrophied muscles of HLS animals is that mechanical sensitivity might already be approaching maximal levels at baseline in these groups. To address this possibility, we performed a mechanical stimulation protocol previously shown to maximally activate JNK phosphorylation in skeletal muscle (Martineau and Gardiner 2001). This stimulation protocol, consisting of eccentric contractile activity for 5 min, produced a JNK phosphorylation response that was 8-fold baseline ($n = 6$, data not shown) in HLS groups, compared to the 3.2 and 1.8–fold increase that occurred with isometric contractions for CTL and 7-day HLS, respectively (Figure 3), negating the possibility that maximal JNK activation may have been attained at baseline levels in the HLS groups.

To verify whether the decreased JNK phosphorylation in stimulated soleus of HLS groups was a reflection of the muscle's force generating capacity, we also normalized phosphorylated JNK results to the tetanic force capacity of the muscle. If the JNK phosphorylation response were uniquely a reflection of the force produced by the muscle, we would expect to observe the greatest values in the CTL group since the capacity for tetanic force production in this group was significantly greater relative to 7 and 14-day HLS. However, the significantly increased JNK phosphorylation observed in 7-day HLS suggests that unloaded muscle might be more sensitive in response to a given mechanical stimulus at early time periods following the onset of unweighting. Consistent with the reports from previous studies, the loss of soleus mass resulting from unloading is greatest during the first 7 days of unweighting (Jiang et al. 1993; Roy et al. 1991) and stabilizes with continued unloading. It is also suggested that much of the slow-to-fast transitions in myosin heavy chain occurs within the first week of unweighting (Staron et al. 1998). Therefore, there may exist important differences in the signaling responses to a mechanical stimulus in atrophying versus atrophied soleus muscle.

CONCLUSION

In conclusion, the results of this study suggest that while there is a marked increase in basal levels of JNK phosphorylation in HLS groups, the phosphorylation response of this protein to a mechanical stimulus is gradually attenuated in soleus muscles as the duration of HLS progresses. This decrease in JNK phosphorylation response to mechanical stimulation with increasing duration of HLS indicates that atrophic soleus may lose the ability to transduce mechanical signals to the MAPK pathways. Thus, in light of the muscle's declining responsiveness to mechanical stimuli with continued unweighting, countermeasures designed to preserve muscle mass in situations of mechanical unloading such as bed rest, immobilization, or spaceflight, should be implemented promptly following initiation of

mechanical unloading. Further investigations on the effects of mechanical stimuli on the phosphorylation response of the JNK substrate c-jun might provide further insight into the changes in JNK signaling resulting from mechanical interventions.

ACKNOWLEDGEMENTS

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Table 1- Effect of HLS on soleus muscle mass, isometric twitch force, and tetanic force

	CTL	7-day HLS	14-day HLS
Muscle mass (g)	0.132 ± 0.004	0.097 ± 0.003 *	0.089 ± 0.003 *
Muscle mass/ body mass ratio (mg/g)	0.65 ± 0.018	0.50 ± 0.013 *	0.45 ± 0.010 *, ⊥
Twitch force (N)	0.303 ± 0.015	0.229 ± 0.018 *	0.195 ± 0.021 *
Normalized twitch force (N/g)	2.35 ± 0.11	2.43 ± 0.17	2.12 ± 0.19
Tetanic force (N)	1.34 ± 0.073	0.832 ± 0.033 *	0.520 ± 0.060 *, ⊥
Normalized tetanic force (N/g)	10.32 ± 0.49	8.89 ± 0.73	5.97 ± 0.60 *, ⊥

Values are expressed as means ± SE where n = 10 for each group. Significantly different ($p \leq 0.05$) from * CTL group; ⊥ 7-day HLS group. Significance determined by Fisher post-hoc.

FIGURE LEGENDS

Figure 1: Total muscle p54 JNK content in soleus muscle, irrespective of phosphorylation state, expressed in arbitrary densitometry units (a.d.u.). Data expressed as means ± SE where n = 6 for the CTL group and n = 4 for the 7-day and 14-day HLS groups. The same amount of muscle protein was added to each gel.

Figure 2: Baseline p54 JNK phosphorylation per mg wet muscle weight measured in non-stimulated soleus muscles. Data presented as fold-CTL JNK phosphorylation level and expressed as means ± SE where n = 6 for CTL group and n = 4 for the 7-day and 14-day HLS groups. * denotes a significant difference ($p \leq 0.05$) from CTL condition. Significance determined by Fisher post-hoc.

Figure 3: p54 JNK phosphorylation measured at basal levels in non-stimulated soleus muscles (solid bars) and in response to 5 min of isometric contractions (100 Hz, 150 ms/s, repeated once per second; hatched bars). Data are presented in arbitrary densitometry units (a.d.u.) and expressed as means ± SE where n = 6 for CTL baseline group and n = 4 for the 7-day and 14-day HLS baseline groups. For the isometrically stimulated groups, n = 4 for the CTL group and n = 6 for the 7-day and 14-day HLS groups. * denotes a significant difference ($p \leq 0.05$) in the phosphorylation response of isometrically stimulated muscle from its respective baseline condition. Significance determined by Fisher post-hoc.

Figure 4: p54 JNK phosphorylation measured in soleus muscles in response to 5 min of isometric contractions (100 Hz, 150 ms/s, repeated once per second). Data presented per unit tetanic force capacity of the muscle and expressed as means ± SE where n = 4 for the CTL group and n = 6 for the

7-day and 14-day HLS groups. * denotes a significant difference ($p \leq 0.05$) from CTL group. Significance determined by Fisher post-hoc.

Figure 5: Soleus muscle section after 5 min of sciatic stimulation (a). Dilution at 1:200 for primary antibody showed a bright signal located in the nuclei. Slice is 30 μm thick, stained with primary anti-Active JNK and secondary fluorescein-FITC conjugated anti IgG antibody. Picture of the same slice in bright light condition (b), shows nuclei stained in black by Mayer's Hematoxylin.

Figure 6: Single omission of primary antibody in control soleus muscles (a) and after 5 min of sciatic stimulation (b). Dilution at 1:200 of primary antibody showed a bright signal located in the nuclei, in control (c) and after muscle activation (d). Slices are 30 μm thick, stained with primary anti-Active JNK and secondary fluorescein-FITC conjugated anti IgG antibody.

Figure 1

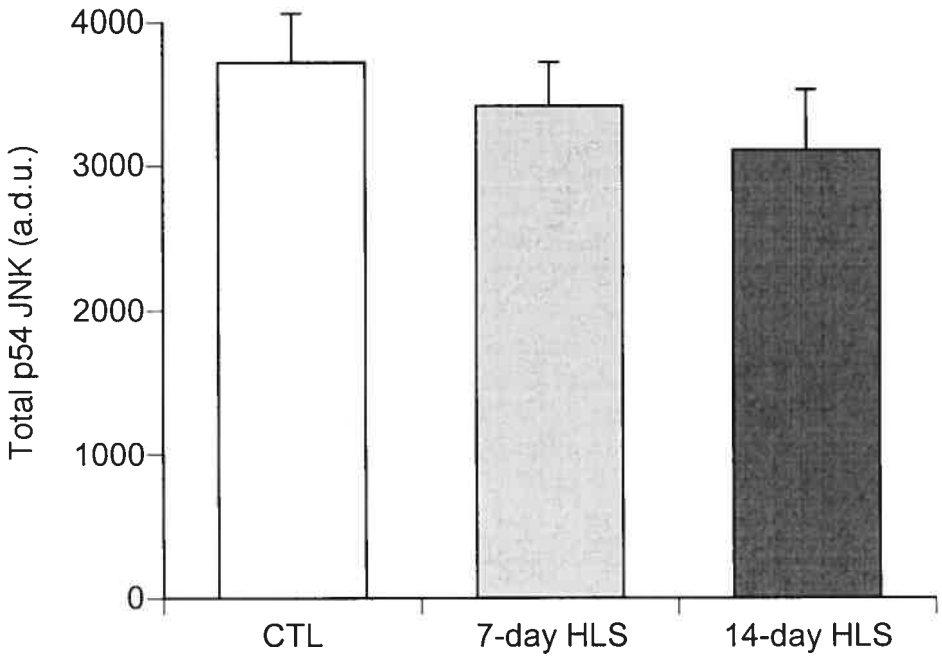
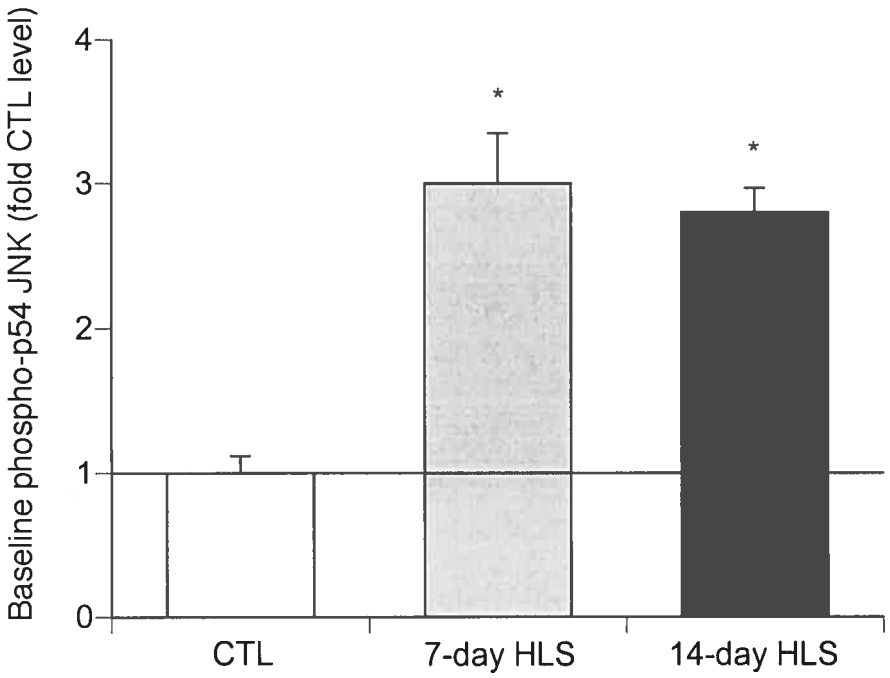


Figure 2


Baseline phospho-p54 JNK (fold CTL level)
4
3
2
1
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CTL
7-day HLS
14-day HLS

Figure 3

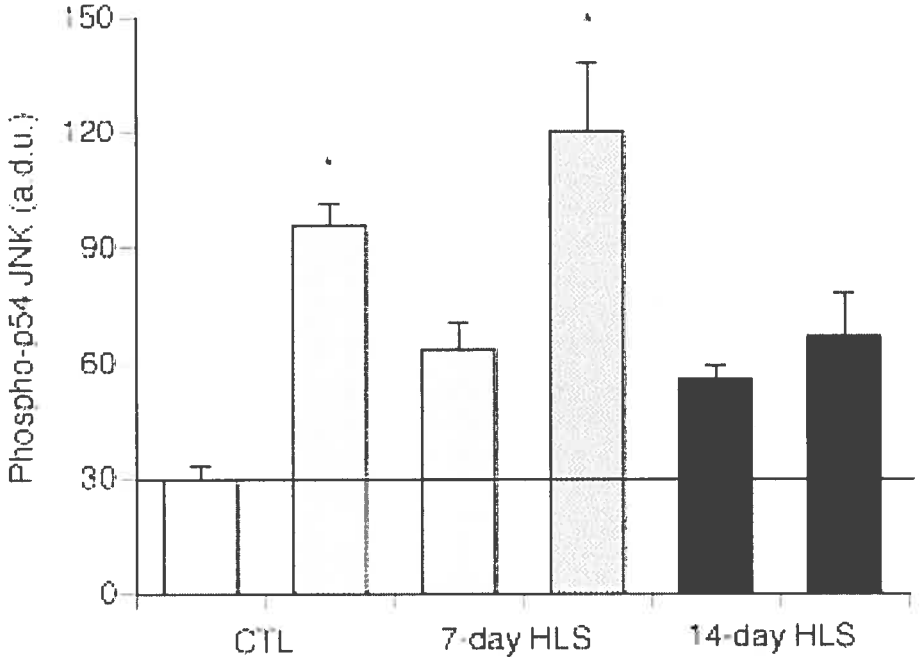
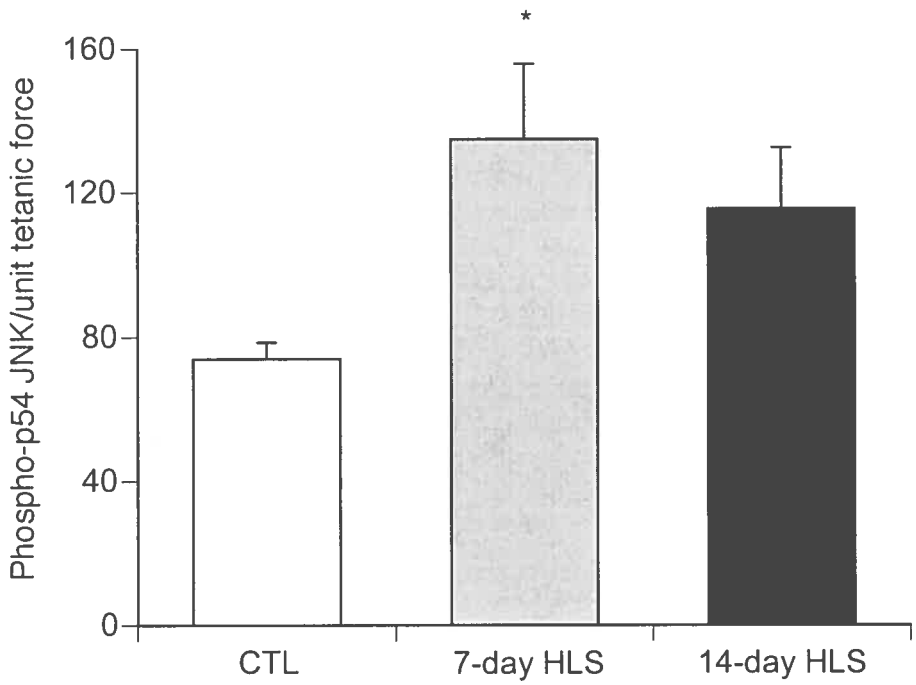


Figure 4


*
160
120
80
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Phospho-p54 JNK/unit tetanic force
CTL
7-day HLS
14-day HLS

Figure 5

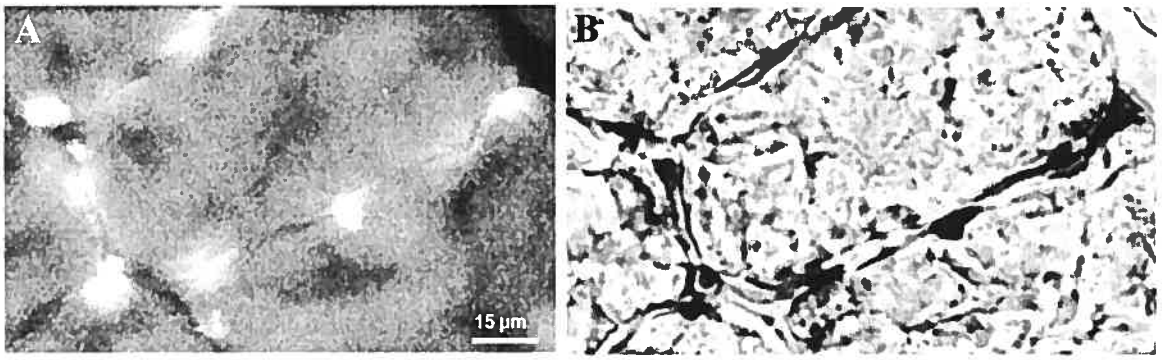
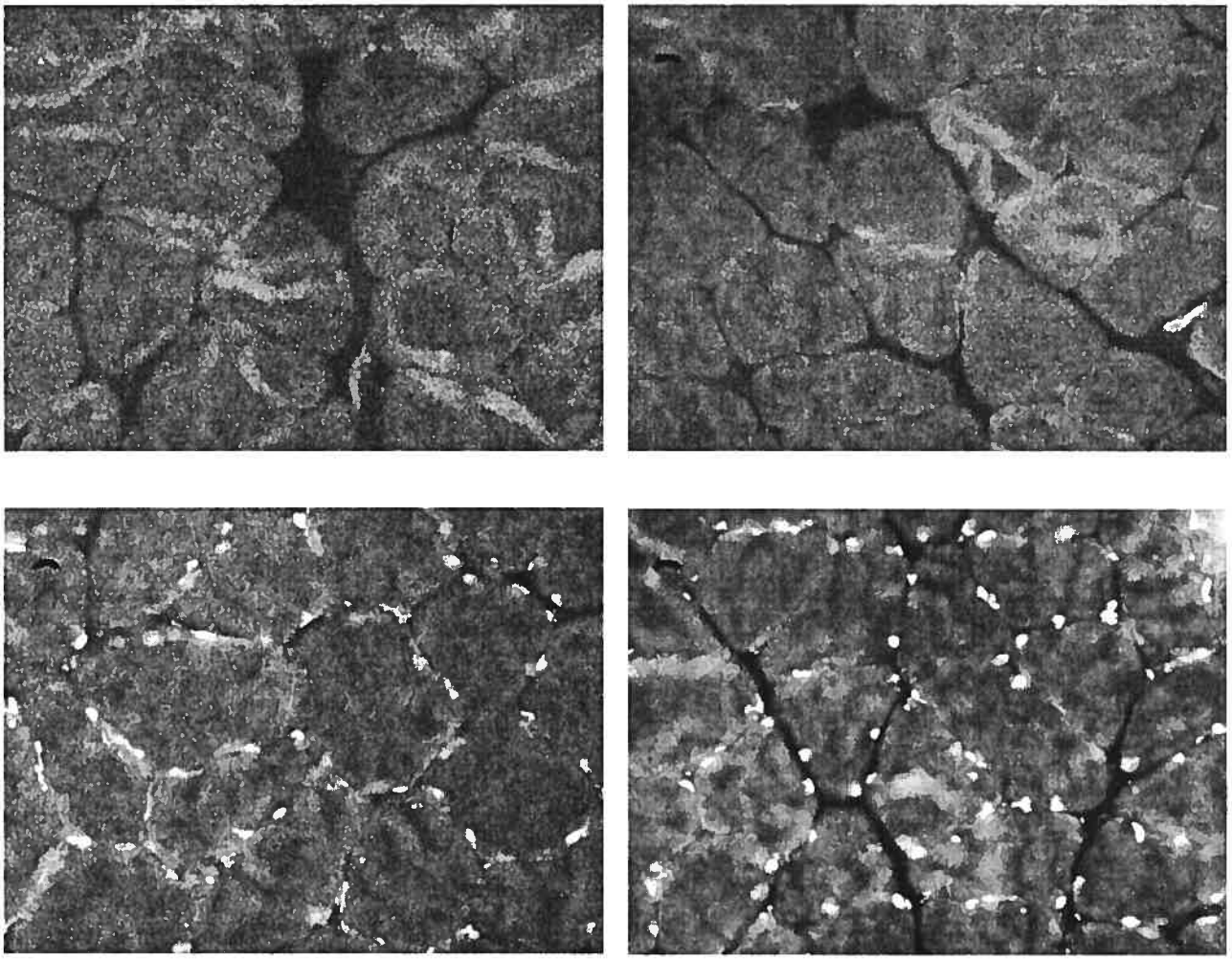


Figure 6



Chapter 3: Original Research Article II

Title:

Muscle denervation promotes opening of the permeability transition pore and increases the expression of mitochondrial cyclophilin D

Authors:

Kristina Csukly, Alexis Ascah, Jimmy Matas, Phillip Gardiner, Éric Fontaine, Yan Burelle

Journal publication reference:

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ABSTRACT

Loss of neural input to skeletal muscle fibres induces atrophy and degeneration with evidence of mitochondria-mediated cell death. However, the effect of denervation on the permeability transition pore (PTP), a mitochondrial protein complex implicated in cell death, is uncertain. In the present study, the impact of 21 days of denervation on the sensitivity of the PTP to Ca^{2+}-induced opening was studied in isolated muscle mitochondria. Muscle denervation increased the sensitivity to Ca^{2+}-induced opening of the PTP, as indicated by a significant decrease in calcium retention capacity (CRC: 111 ± 12 vs. 475 ± 33 nmol/mg protein for denervated and sham, respectively). This phenomenon was partly attributable to in vivo mitochondrial and whole muscle Ca^{2+} overload. Cyclosporin A, which inhibits PTP opening by binding to cyclophilin D (CypD), was significantly more potent in mitochondria from denervated muscle and restored CRC to the level observed in mitochondria from sham-operated muscles. In contrast, the CypD independent inhibitor trifluoperazine was equally effective at inhibiting PTP opening in sham and denervated animals and did not correct the difference in CRC between groups. This phenomenon was associated with a significant increase in the content of the PTP regulating protein CypD relative to several mitochondrial marker proteins. Together, these results indicate that Ca^{2+} overload in vivo and an altered expression of CypD could predispose mitochondria to permeability transition in denervated muscles.

INTRODUCTION

Several progressive neuromuscular diseases are associated with severe atrophy and loss of muscle fibres resulting in muscle wasting as the main clinical presentation. There is growing evidence that activation of cell death through apoptosis, and in a lesser manner necrosis, is involved in the loss of muscle fibres (Lu et al., 1997; Borisov & Carlson, 2000; Tews, 2002; Siu & Alway, 2005). In addition, apoptosis also occurs in localized segments of the remaining myofibres and may play a role in atrophic remodelling (Allen et al., 1997; Allen et al., 1999; Leeuwenburgh et al., 2005). These phenomena have been described in denervation disorders (Tews & Goebel, 1997b; Yoshimura & Harii, 1999; Borisov & Carlson, 2000; Siu & Alway, 2005), muscular dystrophy (Sandri et al., 1995; Sandri et al., 2001), inactivity (Leeuwenburgh et al., 2005; Siu et al., 2005a) and ageing-induced sarcopenia (Leeuwenburgh et al., 2005; Siu et al., 2005a; Siu et al., 2005b).

Although the mechanisms responsible for the activation of cell death pathways in muscle are not well defined, there is evidence that at least in denervation atrophy, mitochondria may play a role. Indeed, several studies have reported an increase in the bax/bcl-2 ratio in various denervation disorders (Tews & Goebel, 1996, 1997b; 1997a,Tews, 2002; Siu & Alway, 2005), which was suggested to induce permeation pathways through mitochondrial membranes. In addition, recent studies have shown that two weeks of denervation in rat muscle leads to the release of several mitochondrial pro-apoptotic proteins (e.g. cytochrome c, AIF, smac/Diablo) and to the activation of downstream events including the activation of caspase 9 and 3 and DNA fragmentation (Siu & Alway, 2005).

One of the well-described mechanisms of membrane permeation in mitochondria involves the opening of the permeability transition pore (PTP) (see (Zoratti & Szabo, 1995) for extensive review). The PTP

is a high conductance, non-specific pore presumably formed by a supramolecular complex spanning the double membrane system of the mitochondria mainly at contact sites. Although it is increasingly recognized that the molecular composition of the PTP is probably variable (He & Lemasters, 2002; Kim et al., 2003; Zoratti et al., 2005), the prevailing hypothesis is that the adenylate translocator (ANT), the porin pore (VDAC) and the regulatory matrix protein cyclophilin-D (CypD) are the major proteins forming the complex (Crompton, 1999; Halestrap et al., 2000). Opening of the PTP results in matrix swelling, loss of membrane potential (ψ), uncoupling of oxidative phosphorylation, and ATP hydrolysis and was also shown to cause the release of several mitochondrial pro-apoptotic proteins including cytochrome c, AIF, Smac/Diablo, endonuclease G and Omi/HtrA2 (Di Lisa & Bernardi, 1998; Bernardi, 1999; Bernardi et al., 1999; Crompton, 1999; Suleiman et al., 2001). Although the regulation of PTP gating varies depending on the tissue studied, accumulation of Ca^{2+} in the matrix is the obligatory and most important trigger for its opening (Zoratti & Szabo, 1995; Bernardi, 1999). Several factors, including the type of substrate used, membrane potential, redox state of pyridine nucleotides, reactive oxygen species, [adenylates] and [proton] can however act as co-regulators by affecting the sensitivity of the pore to Ca^{2+} (Zoratti & Szabo, 1995; Bernardi, 1999).

There are currently very few data available on the PTP in skeletal muscle. Opening of the PTP was shown to mediate the myotoxic effect of the anaesthetic bupivacaine, which could be prevented by inhibiting the pore with cyclosporin-A (CsA) (Irwin et al., 2002). More recently, mitochondrial dysfunction caused by an increased vulnerability to PTP opening was observed in mice harbouring a knockout of the collagen VI gene, which is involved in Bethlem myopathy and Ullrich congenital muscular dystrophy (Irwin et al., 2003). However, whether the vulnerability of mitochondria to PTP opening is altered in denervated muscle remains largely unknown. In the present study, surgical

denervation was used to determine whether loss of neural input and/or its functional sequellae alters the sensitivity of isolated mitochondria to PTP opening and if this could be associated with changes in some factors favouring permeability transition.

METHODS

Muscle Denervation Procedure

Female Sprague-Dawley rats (Charles River, Saint-Constant, Quebec), weighing 225-250 g at the beginning of the experiment, were housed in an environmentally controlled room (23 °C, 12:12-h light-dark cycle) and provided water and food ad libitum. Animals were randomly assigned to one of two groups; muscle denervation or sham-operated control where n = 24 animals per group. Following anesthesia (ketamine / xylazine : 61.5 / 7.7 mg/kg, i.p.) muscle denervation was performed bilaterally by extracting a 10 mm segment of the sciatic nerve through an incision in the mid-posterolateral area of the thigh. The incision was closed with silk sutures and the animals were administered buprenorphine (0.05 mg/kg, i.p.) as post-operative analgesia. The incision site was washed with antibacterial solution to prevent infection. Daily inspection of the animals' hindlimbs was performed, verifying for absence of the toe-spread reflex. Body mass was evaluated every 48 h as an indication of tolerance to the denervation procedure. Any animal demonstrating signs of distress or automutilation of the distal ends of the feet and/or toes was immediately excluded from the experimental protocol. All procedures were approved by the animal ethics committee of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care.

Materials

All chemicals were purchased from Sigma (St-Louis, MO, USA), with the exception of Cyclosporin A (Tocris, Ellisville, MO, USA), and CaGreen-5N (Molecular Probes, Eugene, OR, USA).

Mitochondrial Isolation Procedure

Isolation of mitochondria was performed as previously described (Fontaine et al., 1998) with minor modifications. Twenty one days following surgical denervation, animals were anesthetized (ketamine / xylazine : 61.5 / 7.7 mg/kg, i.p.) and the plantar group of muscles (soleus, plantaris, gastrocnemius) was dissected from the surrounding connective tissue, rapidly removed, trimmed clean of visible connective tissue, weighed, and placed in 10 ml of ice-cold mitochondrial isolation buffer (in mM : 150 sucrose, 75 KCl, 50 Tris Base, 1 KH_2PO_4, 5 $MgCl_2$, 1 EGTA, 0.2% BSA, pH 7.4). Animals were subsequently euthanized by lethal overdose of ketamine/xylazine. All steps were performed at 4°C. Muscles were minced with scissors, incubated for 1 min with Nagarse protease (0.2 mg/ml) and homogenized using a motor-driven Teflon pestle homogenizer. The homogenate volume was completed to 40 ml with cold isolation buffer and centrifuged at 700 g for 10 min. The supernatant was decanted and centrifuged at 10 000 g for 10 min. The pellet was resuspended in 40 ml of suspension buffer (in mM : 250 sucrose, 10 Tris-Base, 0.1 EGTA, pH 7.4) and centrifuged at 8 000 g for 10 min. The final mitochondrial pellet was resuspended in 0.5 ml of suspension buffer and protein concentrations were determined by bicinchoninic acid method.

Respirometry and enzyme activity

Mitochondrial oxygen consumption was measured polarographically at 25 °C, using Clark-type electrodes (Oxygraph, Hansatech Instruments, Kings Lynn, England). Experiments were started with the addition of 0.3 mg mitochondria in 1ml of respiration buffer (in mM:125 KCl, 10 Pi-Tris, 0.05 EGTA, 10 MOPS, 2.5 $MgCl_2$) supplemented with glutamate-malate (5: 1 mM). The medium was then supplemented with 0.25 mM ADP to measure maximal rate of oxidative phosphorylation (V_{ADP}).

For the measurement of the activity of the respiratory chain complex IV cytochrome oxidase (COX), aliquots of the mitochondrial suspension were treated with 0.1 % Triton X-100 for 60 min on ice. Following centrifugation for 5 min at 10 000 g, COX activity in the supernatant was determined spectrophotometrically as previously described (Burelle & Hochachka, 2002) and reported in mU/mg prot.

Ca^{2+} challenge

Mitochondria (0.15 mg.ml-1) were incubated at 25 °C in 1.5 ml of buffer (in mM: 250 sucrose, 10 MOPS, 0.05 EGTA, 10 Pi-Tris, pH 7.3) containing glutamate-malate (5:2.5 mM) or succinate-rotenone (5 mM - 1 µM). All substrates were free acids buffered to pH 7.3 with Tris. Changes in extra-mitochondrial calcium concentration were monitored fluorimetrically (Hitachi, F4500 or Ocean Optics SD2000 spectrofluorometer) using Calcium-green 5N (1 µM, excitation-emission: 505-535 nm) as described by Ichas et al. (Ichas, 1997). Residual calcium concentration was adjusted to the same level at the beginning of every experiment by adding a small amount of EGTA. Unless stated otherwise, Ca^{2+} pulses (83 nmol/mg prot.) were then added at 2 min intervals until a Ca^{2+}-induced mitochondrial Ca^{2+} release was observed. We have previously showed that this Ca^{2+} release is invariably accompanied by loss of ψ and high amplitude swelling of the mitochondrial matrix (Marcil et al., 2005). Calcium retention capacity (CRC) was taken as the total amount of Ca^{2+} accumulated by mitochondria prior to the Ca^{2+} pulse triggering Ca^{2+} release. This value represents a reliable index of the threshold [Ca^{2+}] required to open the PTP in the whole mitochondrial population studied.

Endogenous Ca^{2+} content

Muscles were sampled from one hindlimb and freeze-clamped in liquid N_2 for determination of whole muscle Ca^{2+} content. Muscles from the contralateral limb were used for the isolation of mitochondria as described above except that all buffers were free of EGTA in order to avoid chelating Ca^{2+}. Ground muscle samples and isolated mitochondrial pellets were diluted in 0.6 N HCl (1/10 w/v), homogenized with a polytron (2x10 sec at a setting of 3) and sonicated (2x10 sec at 40% of maximal power output). Following 30 min of incubation in boiling water, samples were centrifuged 5 min at 10 000 g and the supernatant was recovered. Ca^{2+} content in the supernatant was determined spectrophotometrically (VERSAmax, Molecular Devices) using an O-Cresolphthalein Complexone assay according the manufacturer's instructions (TECO Diagnostics, Anaheim, CA). Results were expressed in nmoles Ca^{2+}/mg prot.

Immunoblot Analysis

The protein expression of CypD, ANT and VDAC was determined in the isolated mitochondrial fraction. Samples were prepared for SDS-PAGE by dilutions with reducing sample buffer followed by a 10-min immersion in near-boiling water. Twenty micrograms of protein were loaded in each lane and resolved on 12% polyacrylamide mini-gels at room temperature. The gels were transferred to a PVDF membrane (Millipore). Equal sample loading was confirmed by Ponceau S stain (Sigma-Aldrich). The membrane was fixed for 10 min with 0.05% glutaraldehyde in phosphate-buffered saline with 0.1 % Tween 20 (PBS-T) then blocked in 5 % non fat milk (CypD, VDAC) or 5% BSA (ANT) in PBS-T at room temperature for 90 min, and incubated overnight at 4 °C with the following primary antibodies diluted in PBS-T with 5 % BSA: anti-CypD (1:2000 dilution, Affinity Bioreagents), anti-VDAC (1:2000 dilution, Alexis Biochemicals) and anti-ANT (1:2000 dilution, Calbiochem). Membranes were

then incubated for 90 min at room temperature in secondary antibody solution (1: 50000 dilution, Jackson Immunoresearch). Bands were visualized by enhanced chemiluminescence with film exposure times ranging from 5 to 45 min. Films were scanned and bands quantified by ImagePro software.

Statistical analysis

Data were analyzed using ANOVA followed by Fischer's Post hoc tests. Where appropriate (i.e. in Ca^{2+} challenge experiments), the Bonferonni correction was applied to the P value obtained to correct for multiple comparisons. A corrected P value < 0.05 was considered significant.

RESULTS

Morphometric parameters and mitochondrial protein isolation yield

Denervation for 21 days caused an atrophy of the hindlimb muscles as evidenced by a significant reduction in absolute muscle mass and muscle mass to body mass ratio (Table 1). The amount of protein recovered in the mitochondrial fraction per gram of muscle was not significantly different between the two experimental groups. However, because of muscle atrophy, the total amount of protein recovered was significantly lower in denervated compared to sham-operated animals.

Respiratory parameters

Figure 1 shows the results of respirometry experiments aimed to determine oxidative capacity. In mitochondria from denervated muscles, basal ADP restricted (V_0) and maximal rate of ADP-stimulated (V_{ADP}) respiration were significantly decreased relative to sham when expressed per mg of protein. This reduction of respiratory activity was similar in magnitude to that of the respiratory chain marker enzyme COX, which was 1.8 fold lower in denervated compared to sham-operated animals. Consequently, when respiration rates were normalized per unit of COX, V_0 and V_{ADP} were similar in the two experimental groups.

Vulnerability to Ca^{2+}-induced PTP opening

Figure 2 shows representative Ca^{2+} uptake traces in mitochondria from sham and denervated muscles submitted to progressive Ca^{2+} loading. When energized with the complex II substrate succinate, mitochondria from sham muscle were able to accumulate 475 ± 33 nmol Ca^{2+}/mg prot before Ca^{2+} was released as a result of PTP opening. In contrast, under the same conditions 4.3 fold less Ca^{2+} was required to trigger pore opening in mitochondria from denervated muscle (111 ± 12 nmol Ca^{2+}/mg

prot) and the Ca^{2+} uptake kinetics were substantially slower compared to sham (time to 50% uptake: 48 ± 13 vs 14 ± 1 sec, $P<0.05$).

In energized mitochondria, the regulation of PTP opening is known to vary according to the type of substrate oxidized, with substrates feeding complex I acting as sensitizers compared to substrates for complex II (Fontaine et al., 1998; Bernardi, 1999; Leverve & Fontaine, 2001; Marcil et al., 2005). Therefore, experiments were also performed in the presence of the complex I donors glutamate-malate (Figure 2). In line with previous results, CRC in the presence of glutamate-malate was significantly lower than in the presence of the complex II substrate succinate in the two experimental groups. However, CRC was still reduced in denervated compared to sham-operated animals.

In order to determine whether changes in CRC could be due to changes in Ca^{2+} present in the mitochondrial matrix at baseline, endogenous Ca^{2+} levels were determined in the mitochondria and whole muscle. Figure 3 indicates that mitochondria from denervated muscles had a greater Ca^{2+} content compared to sham, which was accompanied by a substantial muscle Ca^{2+} overload.

As indicated by the lower (~ 2 fold) content of both inner (COX, ANT) and outer membrane (VDAC) markers (Figure 1 and 5), denervation appeared to reduce the number of mitochondria present in the mitochondrial fraction. Therefore, CRC was also determined with a fixed amount of COX (Figure 4). Under these conditions, the difference in CRC observed between the two experimental groups was significantly less (2.4 fold) than the results obtained with a fixed amount of protein (4.3 fold). In addition, if endogenous Ca^{2+} present in mitochondria at baseline was taken into account, the difference in CRC between sham and denervated muscles decreased further to 1.5 fold, but remained significant.

Figure 4 also shows the results of experiments performed in the presence of inhibitors of the PTP. Incubation in the presence of 1 μM CsA, which inhibits pore opening by binding to the putative regulatory protein CypD (Davidson & Halestrap, 1990; Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005), resulted in a 1.6 ± 0.1 fold increase in CRC in mitochondria from sham muscles. In contrast, in the denervated group the same treatment had a significantly more potent effect and increased CRC by 2.4 ± 0.2 fold. As a consequence, CsA was able to abolish the difference in CRC observed between the two experimental groups. In order to determine if this phenomenon was unique to CsA, experiments were also performed in the presence of trifluoperazine, which inhibits PTP opening through a CypD-independent mechanism (Bernardi et al., 1993). Incubation with trifluoperazine increased CRC in both experimental groups but did not correct for the difference in CRC observed between sham and denervated at baseline in the absence of inhibitors.

Immunoblot analysis of the mitochondrial fraction

Since the increased potency to pharmacological PTP inhibition in the denervated group appeared to be specific to CsA the mitochondrial content of CypD, the putative receptor for CsA (Davidson & Halestrap, 1990; Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005) was determined (Figure 5). No significant difference in the absolute content of CypD was observed between groups. However similar to what was observed with COX activity (Figure 1) the content of VDAC and ANT, two abundant mitochondrial proteins was significantly lower in the mitochondrial fraction of denervated compared to sham-operated muscles. Therefore, following denervation, CypD appeared to be over expressed by 2-3 fold relative to each protein marker evaluated (Figure 5).

DISCUSSION

Recent studies have shown that mitochondria, through their capacity to signal cell death, may be involved in the atrophic remodelling of muscles following denervation (Tews & Goebel, 1996, 1997a, 1997b; Tews, 2002; Siu & Alway, 2005) but the mechanisms remain largely undefined. In the present study we show that mitochondria isolated from denervated muscles display some respiratory dysfunction and a greater susceptibility to opening of the PTP, one of the mechanisms involved in mitochondria-mediated cell death. This phenomenon is due in part to the existence in vivo of a muscle and mitochondrial Ca^{2+} overload, which favours permeability transition. In addition, we provide evidence that mitochondria from denervated muscle have an increased CypD content relative to several marker proteins and display a selective increase in the sensitivity to CsA, suggesting that CypD further facilitates pore opening in the denervated state.

Effect of denervation on respiratory capacity

Similar to what has been previously reported (Joffe et al., 1981a), we observed that mitochondria isolated from denervated muscle display a reduction in V_0 and V_{ADP} per mg of protein, which would indicate that denervation caused a significant reduction in oxidative capacity. However, the amplitude of this reduction should be interpreted with caution. Indeed, while milligram protein is generally a good denominator to express respiratory parameters because the majority of mitochondrial proteins consist of respiratory chain enzymes, in some cases its use can introduce distortions in the results (Leary et al., 2003). For instance, in the present study denervation caused minimal changes in the protein concentration of the mitochondrial fraction (Table 1), whereas COX and other mitochondrial marker proteins were significantly reduced. Because the relationship between total protein and specific mitochondrial markers was altered by denervation, respiration was also expressed per unit of COX

(Leary et al., 2003). When expressed this way, the effect of denervation on oxidative capacity was much less pronounced, which suggests that the negative impact of denervation on the metabolic potential of mitochondria could be less important than previously thought (Joffe et al., 1981a).

Effect of denervation on the susceptibility to Ca^{2+}-induced PTP opening

Previous studies in rats denervated for 21 days have reported that respiratory uncoupling occurred prematurely in mitochondria when incubated in the presence of Ca^{2+}, although the mechanisms were not investigated (Joffe et al., 1981c, 1981b). The results obtained in the present study clearly establish a role for the PTP in Ca^{2+}-induced dysfunction. Indeed, CRC was substantially lower in denervated compared to sham-operated muscles when expressed per mg of protein or per unit of COX and the CRC displayed sensitivity to classic PTP inhibitors, particularly CsA.

Ca^{2+}-induced permeability transition is known to be modulated by a variety of physiological effectors, including the flow of electrons through complex I, which increases the sensitivity of the PTP to Ca^{2+}-induced opening (Fontaine et al., 1998; Bernardi, 1999; Leverve & Fontaine, 2001; Marcil et al., 2005). However, in the present study, bypassing complex I using succinate did not abolish the difference in CRC between experimental groups, suggesting that the greater sensitivity of mitochondria to pore opening in denervated muscle was not related to a mechanism involving electron flow through complex I.

In accordance with a previous study (Joffe et al., 1981c), we observed a significant elevation in the endogenous level of Ca^{2+} in mitochondria from denervated muscles. This phenomenon has been reported in certain myopathies (Joffe et al., 1981c; Marchand et al., 2001) and is probably due to a

progressive increase in intramuscular Ca^{2+} levels ((Joffe et al., 1981c) and Figure 3) which appears to occur as a result of an increase in the leakiness of the sarcolemma and the abnormal morphology and functioning of the sarcoplasmic reticulum (Joffe et al., 1981c; Takekura et al., 2003). Since the accumulation of Ca^{2+} in the matrix is the main trigger for PTP opening (Zoratti & Szabo, 1995) this increase in endogenous Ca^{2+} clearly contributed to the increased mitochondrial vulnerability to pore opening in the denervated group. However, the fact that CRC still remained 1.5 fold lower in the denervated group after taking endogenous Ca^{2+} into account indicated that other factors further predisposed mitochondria to permeability transition.

In this regard CypD, a matrix peptidyl-prolyl cis-trans isomerase, was recently shown to play an important role in sensitizing the pore to Ca^{2+} (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Indeed, in mitochondria from Ppif $^{-/-}$ mice, which are devoid of CypD, the amount of Ca^{2+} required to trigger PTP opening is increased several fold (Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). In addition, the inhibitory effect of CsA is completely abolished in these mice, which indicates that CypD expression is the key factor that confers sensitivity to CsA (Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Consistent with these data, Baines et al. (Baines et al., 2005) also reported that in mice over-expressing CypD in a cardiac-restricted manner, PTP opening occurred more readily and that CsA could prevent this effect. Our observations that CsA was selectively more potent in mitochondria from denervated muscle and was able to restore CRC to the level observed in sham-operated muscle therefore suggest a role for CypD in increasing the vulnerability to PTP opening following denervation. The fact that CypD was over expressed by 2-3 fold relative to all mitochondrial protein markers measured following denervation is consistent with

this idea. To our knowledge, this is the first study to suggest a link between changes in CypD expression and PTP dysregulation in a non-transgenic model of disease.

CONCLUSION

In conclusion, the results reported in the present study indicate that conditions that substantially increase the vulnerability of mitochondria to undergo permeability transition are observed 21 days after denervation. This includes a significant increase in mitochondrial and whole muscle Ca^{2+} content and an increase in the expression of CypD, which further predispose the Ca^{2+} overloaded mitochondria to permeability transition. This observation is of significance as the occurrence of PTP opening in some mitochondria in vivo may partly account for the activation of mitochondria-mediated cell death that is known to occur in the process of denervation atrophy (Tews & Goebel, 1996, 1997a, 1997b; Tews, 2002; Siu & Alway, 2005).

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Table 1: Morphometric data and mitochondrial yield in muscle from sham and denervated animals

	Sham	Denervated
Body weight (g)	262.3 ± 4.3	262.0 ± 3.7
Hindlimb muscle weight (mg)	1679 ± 27	649 ± 15 [a]
Muscle mass to body mass ratio (mg/g)	6.42 ± 0.009	2.49 ± 0.05 [a]
Mitochondrial isolation yield (mg prot./g)	3.926 ± 0.286	3.207 ± 0.388
Mitochondrial protein yield (mg)	6.610 ± 0.449	3.332 ± 0.322 [a]

[a]: Significantly different from sham, $P<0.05$

FIGURE LEGENDS

Figure 1: Respiratory function in mitochondria from sham and denervated animals. Panel A shows basal ADP-restricted (V_0), and maximal rate of oxidative phosphorylation following addition of 1 mM ADP (V_{ADP}) expressed per mg of protein. Panel B shows the activity of the terminal respiratory chain enzyme COX in the mitochondrial fraction of sham-operated and denervated animals. Panel C shows V_0 and V_{ADP} expressed per unit of COX present in the mitochondrial fraction of both experimental groups. For respirometry experiments mitochondria were energized with glutamate-malate (5, 2.5 mM). Data are presented as means ± SE, for n = 6 and n = 5 in the denervated and sham groups, respectively. a : Significantly different from sham ($P < 0.05$).

Figure 2: Response to Ca^{2+} challenge in mitochondria from sham and denervated animals. The figure shows typical Calcium-green 5 N tracing of muscle mitochondria (0.15 mg/mL) energized with succinate-rotenone (5 mM, 1 µM) and glutamate-malate (5, 2.5 mM) in sham operated (panel A and C) and denervated animals (panel B and D). Tracings show progressive Ca2+ accumulation followed by release of accumulated Ca^{2+} secondary to PTP opening. Each spike indicates the addition of a calcium pulse of 83 nmol/mg. Panel E shows the average Ca2+ retention capacity of mitochondria in the various experimental conditions. Means ± SE are presented for n = 8 and 9 in the sham and denervated groups respectively. a : Significantly different from sham ($P < 0.05$), b: Significantly different from succinate + rotenone within the same experimental group ($P < 0.05$).

Figure 3: Effect of denervation on endogenous mitochondrial and whole muscle Ca^{2+} content in sham and denervated animals. The figure presents endogenous Ca^{2+} content measured in the whole muscle homogenate and the mitochondrial fraction of sham and denervated animals. Data are presented as means ± SE, for n = 8 and n = 9 in the denervated and sham groups, respectively. a : Significantly different from sham ($P < 0.05$).

Figure 4: Total Ca^{2+} retention capacity and effect of PTP inhibitors in mitochondria from sham and denervated animals. The figure shows total Ca^{2+} retention capacity in absence and presence of the PTP inhibitors cyclosporin-A (1 μM) and trifluoperazine (10 μM). Values are expressed as the sum of endogenous Ca^{2+} (black bars) and of Ca^{2+} added during the in vitro challenge and are expressed per unit of the marker enzyme cytochrome oxidase (COX). Means ± SE are presented for n = 8 and 9 in the sham and denervated groups respectively. a : Significantly different from sham ($P < 0.05$), b: Significantly different from baseline ($P < 0.05$).

Figure 5: Cyclophilin D, VDAC and ANT content of mitochondrial fraction from sham and denervated animals. Representative immunoblots of cyclophilin D (CypD: 21 kDa), voltage dependent anion channel (VDAC: 31 kDa) and adenine nucleotide translocase (ANT: 31 kDa) in muscle mitochondrial fraction isolated from sham and denervated animals. Each line represents a single mitochondrial preparation in each group. Densitometric analysis for each protein represents the mean ± SE of 4 preparations in each experimental group. Histograms A and B show CypD content expressed relative to that of VDAC and ANT respectively. Histogram C shows CypD content relative to the activity of COX measured in the same samples. a : Significantly different from sham ($P < 0.05$).

Figure 1

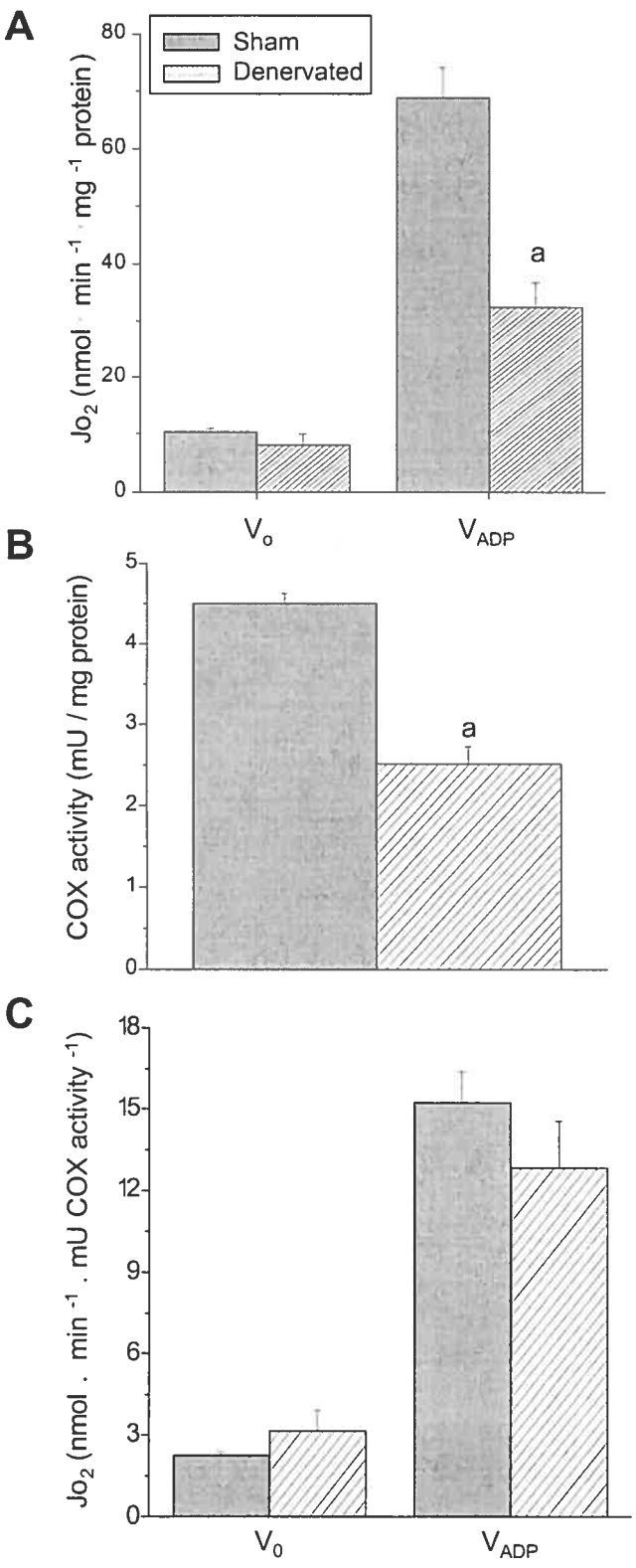

A
Sham
Denervated
Jo_2 (nmol · min^{-1} · mg^{-1} protein)
80
60
40
20
0
a
V_o
V_{ADP}
B
COX activity (mU / mg protein)
5
4
3
2
1
0
a
C
Jo_2 (nmol . min^{-1} . mU COX activity^{-1})
18
15
12
9
6
3
0
V_0
V_{ADP}

Figure 2

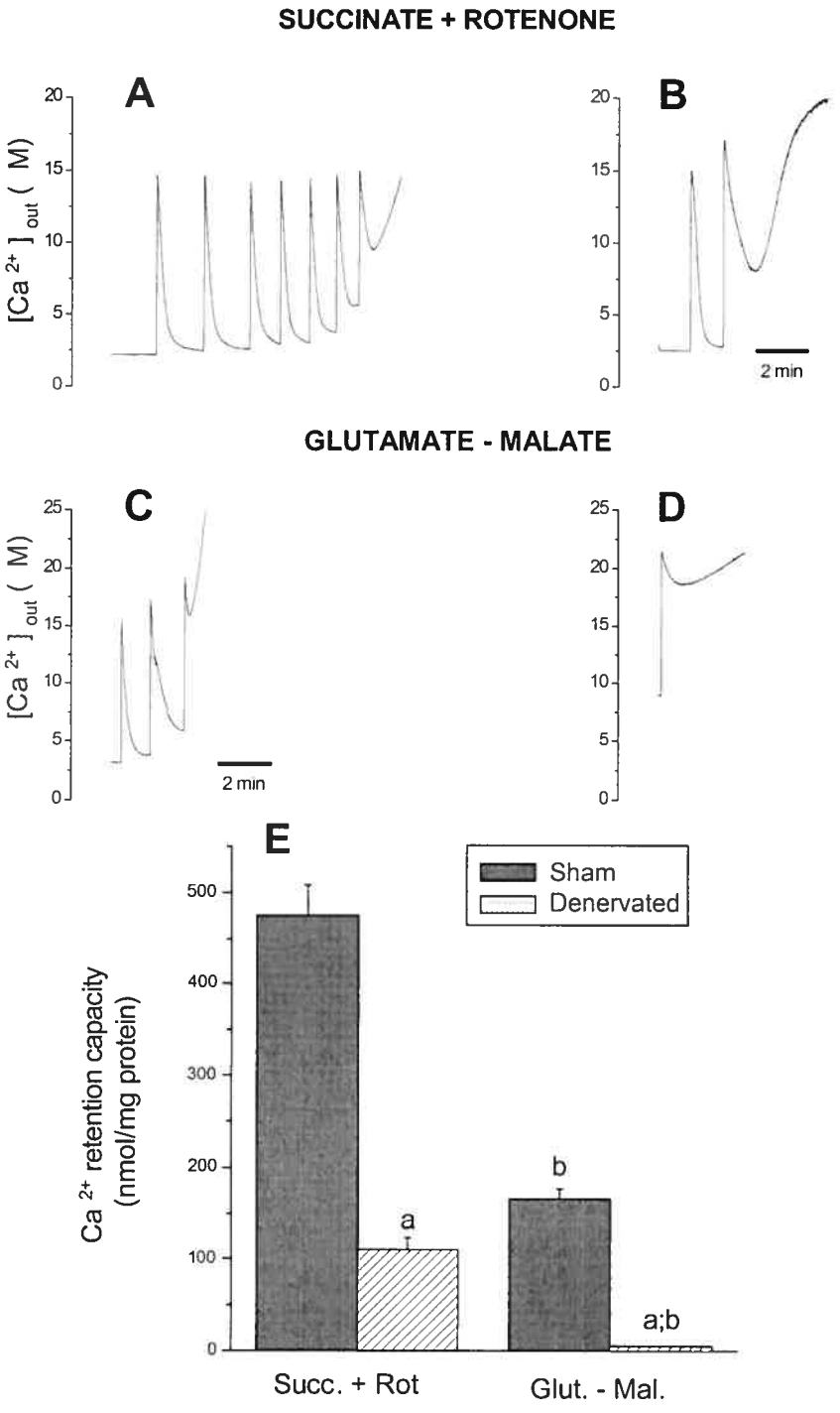

SUCCINATE + ROTENONE
A
B
[Ca 2+] out (M)
20
15
10
5
0
2 min
GLUTAMATE - MALATE
C
D
25
E
Sham
Denervated
Ca 2+ retention capacity (nmol/mg protein)
500
400
300
200
100
0
a
b
a;b
Succ. + Rot
Glut. - Mal.

Figure 3

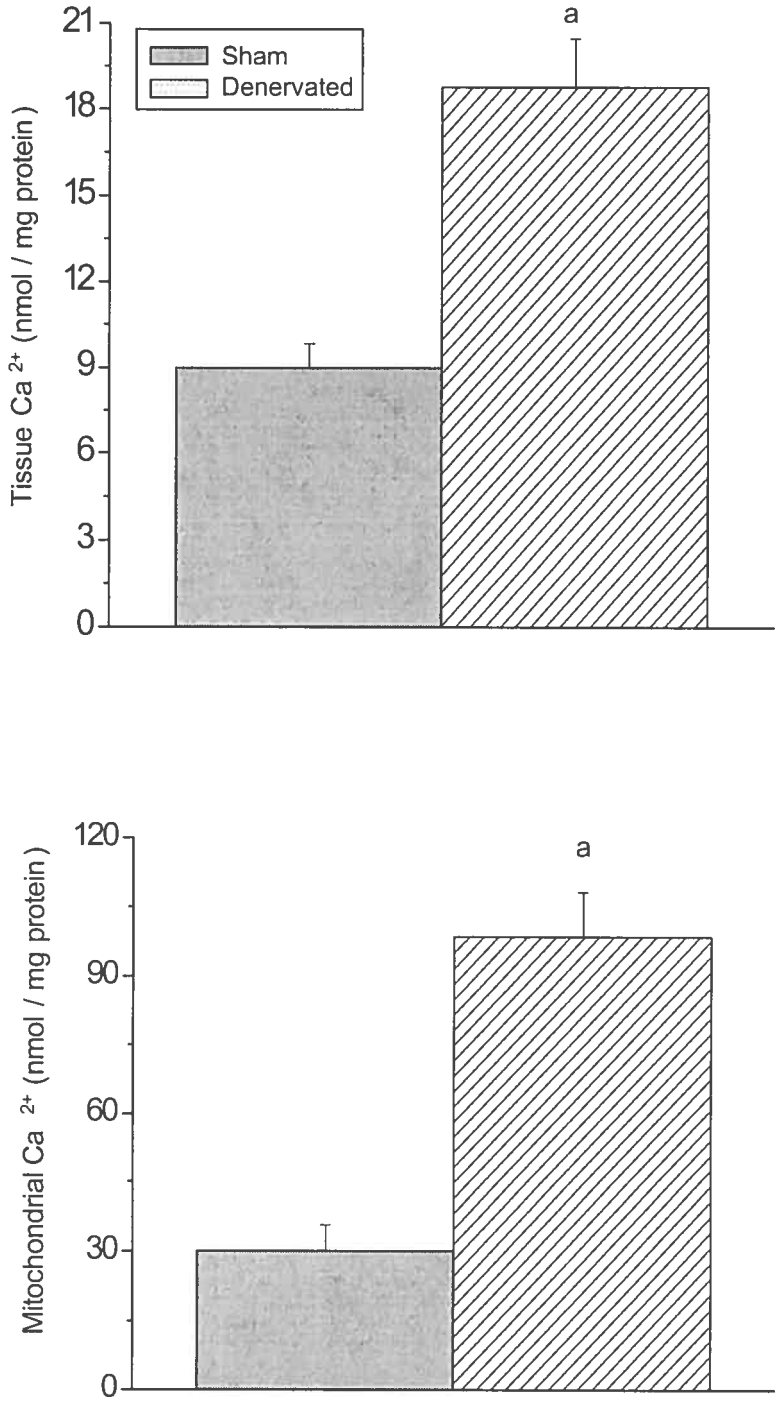
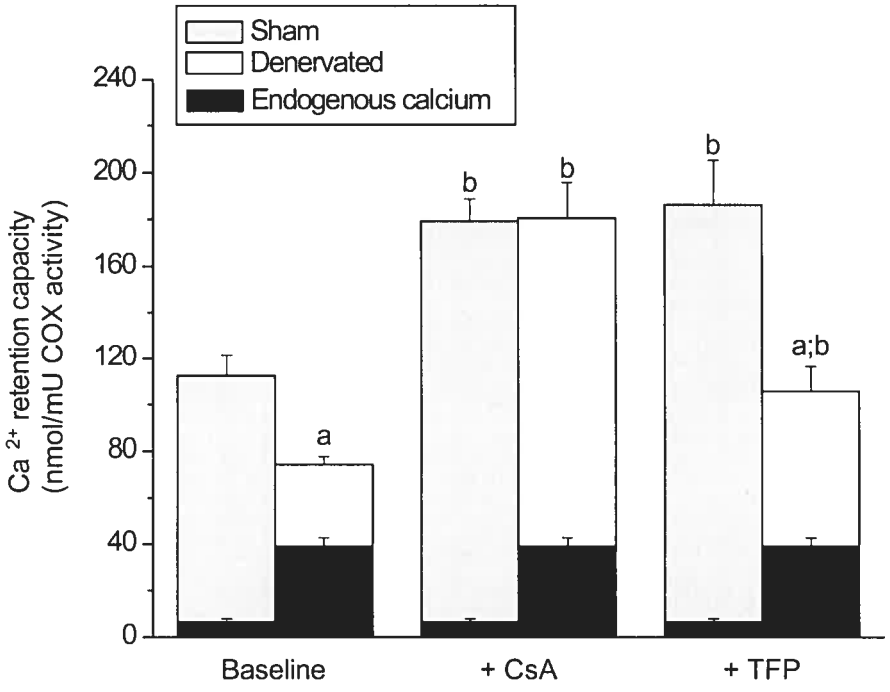

21
18
15
12
9
6
3
0
Tissue Ca 2+ (nmol / mg protein)
Sham
Denervated
a
120
90
60
30
0
Mitochondrial Ca 2+ (nmol / mg protein)
a

Figure 4


Sham
Denervated
Endogenous calcium
Ca 2+ retention capacity (nmol/mU COX activity)
240
200
160
120
80
40
0
a
b
b
b
a;b
Baseline
+ CsA
+ TFP

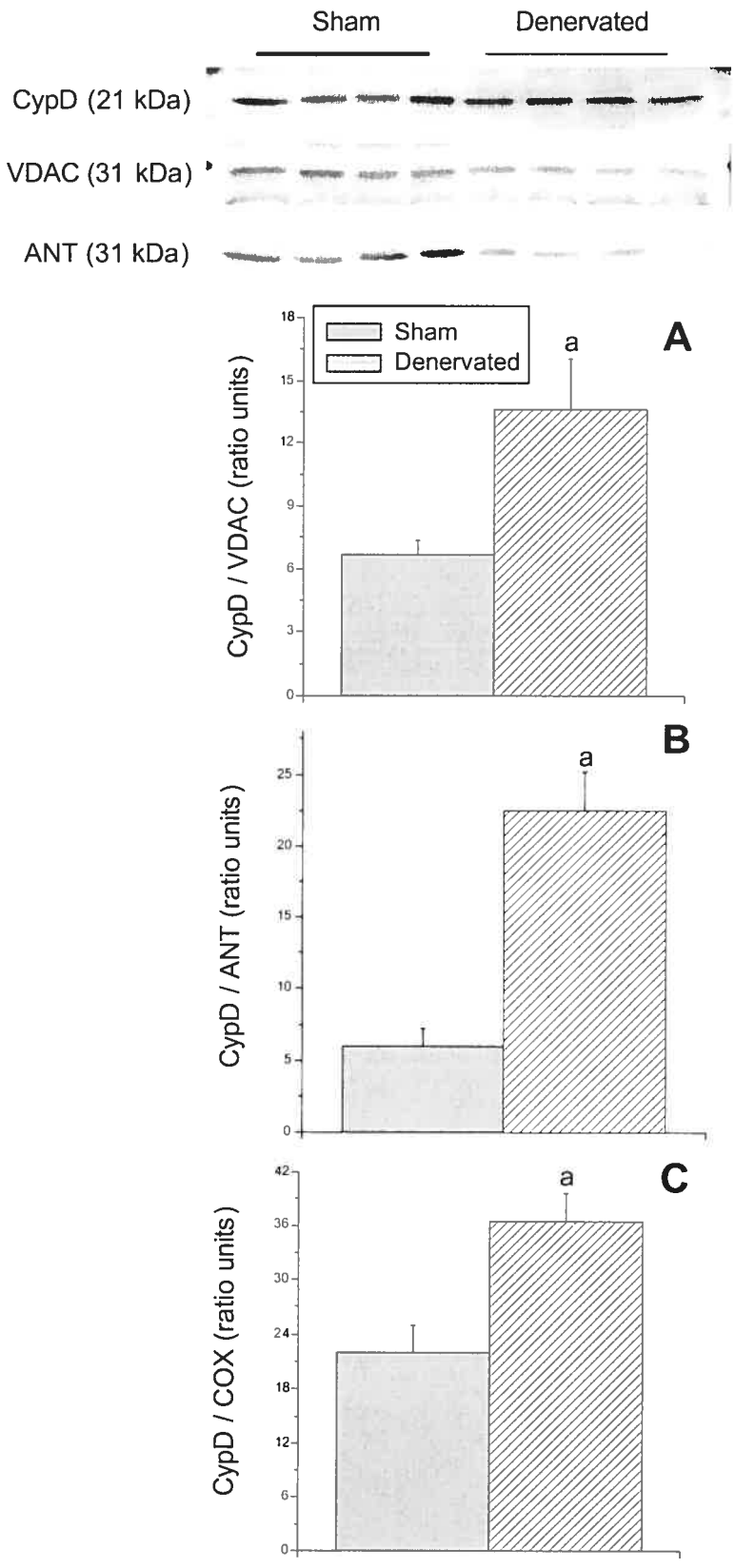

Sham
Denervated
CypD (21 kDa)
VDAC (31 kDa)
ANT (31 kDa)
Sham
Denervated
A
a
CypD / VDAC (ratio units)
B
a
CypD / ANT (ratio units)
C
a
CypD / COX (ratio units)

Figure 5

Chapter 4: Original Research Article III

Title:

Mitochondria from soleus muscle exhibit increased sensitivity to Ca2+-induced opening of the permeability transition pore: evidence for phenotype-specific pore regulation

Authors:

Kristina Csukly, Alexis Ascah, Phillip Gardiner, Yan Burelle

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ABSTRACT

In the present study, mitochondria were isolated from rat soleus (SOL), plantaris (PLN) and white gastrocnemius (WG), three muscles characterized by different fiber type composition, and the regulation of pore opening was investigated *in vitro*. Mitochondria isolated from these three muscles did not differ significantly with respect to their content of the marker enzymes (CS and COX) and their respiratory capacity. On the other hand, significantly less Ca^{2+} was required to trigger PTP opening in mitochondria from SOL compared to PLN and WG when energized with complex II (Ca^{2+} retention capacity (CRC): 343 ± 16 vs. 585 ± 62 and 513 ± 79 nmol/mg prot, respectively) or complex I (CRC: <83 vs 120 ± 60 and 120 ± 60 nmol/mg prot, respectively) substrates. This phenomenon was partly caused by a 2-fold increase in endogenous Ca^{2+} levels in mitochondria from SOL compared to PLN and WG, but was not due to an increased production of reactive oxygen species (ROS) since ROS production was lower in SOL compared to PLN and WG. Incubation in presence of cyclosporin A increased CRC by a similar magnitude irrespective of muscle type consistent with the absence of difference in the content of cyclophilin D, its molecular target and putative pore component. However, the levels of ANT and VDAC, two proteins involved in pore formation, were significantly higher in SOL compared to PLN and WG. The present study provides the first evidence that the regulation of permeability transition varies according to the phenotype of muscle fibers, which may contribute to explain the heterogeneous progression of neuromuscular disorders across muscles.

Keywords: mitochondria, permeability transition pore, skeletal muscle, fiber phenotype

INTRODUCTION

Besides their well-established importance for ATP production, it is now well known that mitochondria play a crucial role in cellular dysfunction and death by their capacity to trigger necrosis and apoptosis (Hengartner, 2000; Kim *et al.*, 2003; Mayer & Oberbauer, 2003; Green & Kroemer, 2004). A key event in these phenomena is the appearance of permeation pathways across mitochondrial membranes through various mechanisms, one of the best described being the opening of the permeability transition pore (PTP). The PTP is a high conductance, non-specific channel formed by a supramolecular complex spanning the double membrane system of mitochondria (Zoratti & Szabo, 1995; Crompton, 1999; Zoratti *et al.*, 2005). Although it is increasingly recognized that the PTP is probably not composed of a unique set of proteins, the adenylate translocator (ANT) and the porin pore (VDAC), which are among the most highly expressed mitochondrial proteins, are known for their ability to form the PTP (Crompton *et al.*, 1999; Halestrap *et al.*, 2002) together with the matrix regulatory protein cyclophilin D (CypD) (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Opening of the PTP results in the loss of membrane potential, matrix swelling, uncoupling of oxidative phosphorylation, ATP hydrolysis and the release of several pro-apoptotic factors including cytochrome c, AIF, Smac/Diablo, endonuclease G and Omi/HtrA2 (Di Lisa & Bernardi, 1998; Bernardi, 1999; Crompton, 1999; Suleiman *et al.*, 2001). The accumulation of Ca^{2+} in the matrix is the obligatory and most important trigger for PTP opening (Zoratti & Szabo, 1995; Bernardi, 1999). However, other factors, including the type of substrate used, membrane potential, redox state, matrix pH and adenylate content as well as ROS production, act as modulators by affecting the sensitivity of the pore to Ca^{2+}-induced opening (Zoratti & Szabo, 1995; Bernardi, 1999).

Recent studies have shown that an increased vulnerability to opening of the PTP occurs in skeletal muscle under various pathological conditions including denervation atrophy (Csukly *et al.*, 2006), myopathies related to collagen VI deficiencies (Irwin *et al.*, 2003) as well as bipuvacaine-induced myotoxicity (Irwin *et al.*, 2002). However, despite this knowledge very few data are available on the regulation of the PTP in skeletal muscle (Fontaine *et al.*, 1998) and the question of whether the vulnerability of mitochondria to pore opening varies across fiber type remains unanswered. This question is particularly relevant as the appearance and progression of several neuromuscular disorders is very heterogeneous in muscles with various phenotypes (Tews, 2002), which may in part reflect a different vulnerability of mitochondria to PTP opening and activation of mitochondrial death pathways across fiber types. In the present study we isolated mitochondria from three muscles displaying divergent fiber type composition ranging from predominantly slow-twitch type I to predominantly fast-twitch type IIb fibers and determined their vulnerability to PTP opening under various conditions. We also determined whether these changes were associated with muscle type-specific alterations in selected PTP effectors and putative regulatory and structural pore components.

METHODS

Mitochondrial Isolation Procedure

Female Sprague-Dawley rats (Charles River, Saint-Constant, Quebec), weighing 225-250 g were housed in an environmentally controlled room (23 °C, 12:12-h light-dark cycle) and provided water and food ad libitum. Isolation of mitochondria was performed as previously described (Madsen *et al.*, 1996) with minor modifications. Animals were anesthetized (ketamine / xylazine : 61.5 / 7.7 mg/kg, i.p.) and the plantar group of muscles was dissected from the surrounding connective tissue, rapidly removed, trimmed clean of visible connective tissue, weighed, and placed in 10 ml of ice-cold mitochondrial isolation buffer (in mM : 150 sucrose, 75 KCl, 50 Tris Base, 1 KH_2PO_4, 5 $MgCl_2$, 1 EGTA, 0.2% BSA, pH 7.4). Animals were subsequently euthanized by cervical dislocation. All procedures were approved by the animal ethics committee of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care.

All steps were performed at 4°C. The soleus (SOL), plantaris (PLN) and white portion of the gastrocnemius (WG) were minced separately with scissors, incubated for 1 min with Nagarse protease (0.2 mg/ml) and homogenized using a motor-driven Teflon pestle. The homogenate volume was completed to 40 ml with cold isolation buffer and centrifuged at 700 g for 10 min. The supernatant was decanted and centrifuged at 10 000 g for 10 min. The pellet was resuspended in 40 ml of suspension buffer (in mM : 250 sucrose, 10 Tris-Base, 0.1 EGTA, pH 7.4) and centrifuged at 7 000 g for 10 min. The final mitochondrial pellets were resuspended in 0.3 ml of suspension buffer for PLN and WG and 0.2 ml for SOL and protein concentrations were determined by the bicinchoninic acid method (Sigma, St-Louis Missouri, USA).

Materials

All chemicals were purchased from Sigma (St-Louis, MO, USA), with the exception of Cyclosporin A (Tocris, Ellisville, MO, USA), and CaGreen-5N (Molecular Probes, Eugene, OR, USA).

Respirometry and enzyme activity

Mitochondrial oxygen consumption was measured polarographically at 25 °C, using Clark-type electrodes (Oxygraph, Hansatech Instruments). Experiments were started with the addition of 0.3 mg mitochondria in 1ml of respiration buffer (in mM:125 KCl, 10 Pi-Tris, 0.05 EGTA, 10 MOPS, 2.5 $MgCl_2$) supplemented with glutamate-malate (5:2.5 mM) or succinate-rotenone (5 mM - 1 μM). The medium was then supplemented with 0.25 mM ADP to measure maximal rate of oxidative phosphorylation (V_{ADP}). The respiratory control ratio (RCR) was calculated as the ratio V_{ADP}/V_0 where V_0 represents baseline respiration in the absence of ADP.

For the measurement of citrate synthase (CS) and cytochrome oxidase (COX) activity, aliquots of the mitochondrial suspension were treated with 0.1 % Triton X-100 for 60 min on ice. Following centrifugation for 5 min at 10 000 g, the activites of CS and COX in the supernatant were determined spectrophotometrically as previously described (Csukly *et al.*, 2006) and reported in mU/mg prot.

Ca^{2+} challenge

Mitochondria (0.15 mg/ml) were incubated at 25 °C in 1.5 ml of experimental buffer (in mM: 250 sucrose, 10 MOPS, 0.05 EGTA, 10 Pi-Tris, pH 7.3) containing glutamate-malate (5:2.5 mM) or

succinate-rotenone (5 mM - 1 μM). All substrates were free acids buffered to pH 7.3. Changes in extra-mitochondrial calcium concentration were monitored fluorimetrically (Hitachi, F4500 or Ocean Optics SD2000 spectrofluorometer) using Calcium-green 5N (1 μM, excitation-emission: 505-535 nm) as described by Ichas et al. (Ichas *et al.*, 1997). Residual calcium concentration was adjusted to the same level at the beginning of every experiment by adding a small amount of EGTA. Ca^{2+} pulses (83 nmol/mg prot.) were then added at 2 min intervals until a Ca^{2+}-induced mitochondrial Ca^{2+} release was observed. CRC was taken as the total amount of Ca^{2+} accumulated by mitochondria prior to the Ca^{2+} pulse triggering Ca^{2+} release. This value represents a reliable index of the threshold [Ca^{2+}] required to open the PTP in the whole mitochondrial population studied (Zoratti & Szabo, 1995; Bernardi, 1999).

Endogenous Ca^{2+} content

Mitochondria from the different muscles were isolated as described above except that all buffers were free of EGTA in order to avoid chelating Ca^{2+}. Isolated mitochondrial pellets were diluted in 0.6 N HCl (1/10 w/v), homogenized with a polytron (2x10 sec at a setting of 3) and sonicated (2x10 sec at 40% of maximal power output). Following 30 min of incubation in boiling water, samples were centrifuged 5 min at 10 000 g and the supernatant was recovered. Ca^{2+} content in the supernatant was determined spectrophotometrically (VERSAmax, Molecular Devices) using an O-Cresolphthalein Complexone assay according the manufacturer's instructions (TECO Diagnostics). Results were expressed in nmoles Ca^{2+}/mg prot.

Production of reactive oxygen species (ROS)

Mitochondrial H_2O_2 production was measured fluorimetrically with amplex red (20 µM: excitation-emission: 560-584). Mitochondria (0.1 mg/ml) were incubated at 25 °C in 2 ml of experimental buffer (in mM: 250 sucrose, 10 MOPS, 0.05 EGTA, 10 Pi-Tris, pH 7.3) containing glutamate-malate (5:2.5 mM) or succinate (5 mM) and supplemented with 1.2 U/ml of horseradish peroxidase. At the conclusion of each test, 176 pM of H_2O_2 was added to allow for the calculation of endogenous ROS production.

Western immunoblot analysis

The protein contents of cyclophilin D (CypD), Adenylate translocator (ANT) and Porin (VDAC) were determined in the isolated mitochondrial fraction. Samples were prepared for SDS-PAGE by dilutions with reducing sample buffer followed by a 10-min immersion in near-boiling water. Twenty micrograms of protein were loaded in each lane and resolved on 12% polyacrylamide mini-gels at room temperature. The gels were transferred to a PVDF membrane (Millipore). Equal sample loading was confirmed by Ponceau S stain (Sigma-Aldrich). The membrane was fixed for 10 min with 0.05% glutaraldehyde in phosphate-buffered saline with 0.1 % Tween 20 (PBS-T) then blocked in 5 % non-fat milk (CypD, VDAC) or 5% BSA (ANT) in PBS-T at room temperature for 90 min, and incubated overnight at 4 °C with the following primary antibodies diluted in PBS-T with 5 % BSA: anti-CypD (1:2000 dilution, Affinity Bioreagents), anti-VDAC (1:2000 dilution, Alexis Biochemicals) and anti-ANT (1:2000 dilution, Calbiochem). Membranes were then incubated for 90 min at room temperature in secondary antibody solution (1: 50000 dilution, Jackson Immunoresearch). Revelation was performed by enhanced chemiluminescence

with film exposure times ranging from 5 to 45 min. Films were scanned and bands quantified using ImagePro software.

Statistical analysis

Data were analyzed using one-way ANOVA followed by Fisher's post-hoc test. Significance was assumed at $P < 0.05$.

RESULTS

Isolation of mitochondria from SOL and PLN yielded significantly more mitochondrial protein per gram of muscle compared to WG (Table 1). This is consistent with the predominant phenotype of these muscles, with the SOL containing a high proportion of slow-oxidative type I fibers (87 % type I, 15 % type IIA-IIX, 0% type IIB), PLN containing a mixture of predominantly fast-oxidative and fast-glycolytic fibers (6 % type I; 45 % type IIA – IIX and 47 % type IIB), whereas the WG contains more than 90 % fast-glycolytic fibers (0% type I, 8 % type IIA- IIX, 92 % type IIB) (Delp & Duan, 1996). As for the activity of the marker enzymes CS and COX present in the mitochondrial fraction, no significant differences were observed among muscles.

Table 2 shows the results of respirometry experiments aimed to determine basic parameters of oxidative phosporylation in presence of substrates for complex I and II. Values for V_{ADP} were not significantly different among muscles, regardless of the substrate oxidized. Basal ADP-restricted respiration tended to be higher in PLN and WG compared to SOL, but this difference reached statistical significance only when mitochondria were energized with the complex II donor succinate. RCR values ranged from 9.4 to 11 in presence of glutamate-malate and from 4.9 to 7.4 in presence of succinate, indicating that mitochondria from the three muscles were well-coupled. RCR values tended to be higher in SOL compared to PLN and WG, but this difference was not statistically significant. Overall, these results indicated no substantial variations in respiratory properties across muscle type under our conditions.

Figure 1 shows the results of experiments in which the sensitivity of mitochondria to Ca^{2+}-induced PTP opening was determined. Figure 1 A-C shows typical Ca^{2+} uptake traces during Ca^{2+} challenge experiments in mitochondria from the three muscles when energized with the complex II donor succinate in presence of rotenone. Under this condition, mitochondria from PLN and WG were able to accumulate 531 ± 62 and 456 ± 79 nmol Ca^{2+}/mg prot respectively before PTP opening was triggered (Figure 1D). In contrast, under the same conditions, ~ 55 % less Ca^{2+} was required to trigger pore opening in mitochondria from SOL muscle (232 ± 16 nmol/mg prot), indicating an enhanced vulnerability to permeability transition. In addition, the kinetics of Ca^{2+} uptake was significantly slower in SOL compared to PLN and WG (time to 50% uptake: 39 ± 1, 24 ± 3 and 20 ± 1 sec respectively).

In energized mitochondria, the type of substrate oxidized is known to influence Ca^{2+}-induced PTP opening, with substrates feeding complex I acting as sensitizers compared to substrates for complex II (Fontaine *et al.*, 1998; Csukly *et al.*, 2006). In order to determine if the difference in CRC across muscle types was specific to the substrate oxidized, Ca^{2+} challenge experiments were also performed in presence of the complex I substrates glutamate-malate. In line with previous results (Fontaine *et al.*, 1998; Csukly *et al.*, 2006), CRC measured in presence of glutamate-malate was significantly lower than with succinate in the three muscles (Figure 1D). In addition, CRC remained lower in mitochondria from SOL compared to PLN and WG. Indeed, in presence of glutamate-malate, mitochondria from PLN and WG were able to accumulate 121 ± 60 nmol Ca^{2+}/mg prot whereas mitochondria isolated from SOL were unable to accumulate the first Ca^{2+} pulse of 83 nmol/mg prot, implying that CRC was below this value.

In presence of Ca^{2+}, reactive oxygen species are known to favor PTP opening (Halestrap *et al.*, 1997; Halestrap *et al.*, 2002). ROS production by the respiratory chain was therefore measured in mitochondria in order to determine whether this could account for differences in CRC across muscle types (Figure 2). In presence of the complex II donor succinate, ROS production was significantly lower in mitochondria from SOL compared to WG, and mitochondria from PLN displayed intermediate values. In presence of substrates for complex I, ROS production in mitochondria from the three muscles displayed a similar profile, although the differences between muscles did not reach statistical significance. In accordance with previous studies (Capel *et al.*, 2004; Kudin *et al.*, 2005; Anderson & Neufer, 2006), ROS production in mitochondria energized with complex II substrates was significantly higher than that observed in presence of complex I substrates, irrespective of the muscle type.

Since Ca^{2+} accumulation in the matrix is a key factor triggering pore opening, the endogenous Ca^{2+} levels present in mitochondrial extracts prior to the Ca^{2+} challenge was determined. Figure 3 shows that endogenous Ca^{2+} levels in mitochondria from SOL were 111 ± 11 nmol/mg prot, ~2-fold higher than in mitochondria from PLN and WG (54 ± 7 and 56 ± 4 nmol/mg prot respectively). Figure 4 shows Ca^{2+} retention capacity of mitochondria normalized for endogenous Ca^{2+} present at baseline before addition of exogenous Ca^{2+}. Differences in endogenous Ca^{2+} levels across muscle type significantly accounted for the lower CRC observed in mitochondria from SOL when energized with glutamate-malate. However, this was not the case when mitochondria were energized with succinate, since CRC values normalized for endogenous Ca^{2+} remained significantly ~ 40 % lower in SOL compared to WG and PLN. Figure 4 also shows the results of experiments performed in presence of CsA, a drug that reduces vulnerability to Ca^{2+}-

induced PTP opening by binding to CypD (Crompton *et al.*, 1988). CsA significantly increased CRC in mitochondria from all muscles but did not abolish the differences among muscle groups. Immunoblot analysis performed on mitochondrial fractions showed no significant differences in the content of the regulatory protein CypD (Figure 5). However, the levels of ANT and VDAC were significantly higher in SOL compared to PLN and WG.

DISCUSSION

In the present study, we report that mitochondria isolated from skeletal muscles with different fiber type compositions are heterogeneous with respect to their vulnerability to Ca^{2+}-induced PTP opening, with mitochondria from the SOL being more vulnerable compared to PLN and WG. This greater sensitivity appears in the absence of major differences in respiratory capacities and despite the fact that ROS production, a factor known to favor PTP opening, was the lowest in the SOL. Our results also indicate that the increased sensitivity to PTP opening in SOL is observed irrespective of the type of substrate oxidized and is at least partly due to the greater endogenous Ca^{2+} levels.

Respiratory function

The lack of substantial variations in the content of the marker enzymes CS and COX and in respiratory properties across muscle type reported in the present study is in general agreement with previous reports that have characterized fiber type differences in mitochondrial function. Indeed, the activities of the TCA cycle enzyme CS (Yajid *et al.*, 1998; Leary *et al.*, 2003), several respiratory chain complexes including COX (Schwerzmann *et al.*, 1989; Leary *et al.*, 2003) and ATP synthase (Leary *et al.*, 2003) were found to be relatively similar in mitochondria from oxidative and glycolytic muscles. In addition, mitochondria from slow-oxidative muscles were reported to have similar (Pande & Blanchaer, 1971; Schwerzmann *et al.*, 1989; Yajid *et al.*, 1998; Leary *et al.*, 2003; Mogensen & Sahlin, 2005) or slightly higher (Jackman & Willis, 1996) capacities for oxidative phosphorylation and similar values of ADP-restricted respiration (Jackman & Willis, 1996; Yajid *et al.*, 1998; Leary *et al.*, 2003; Mogensen & Sahlin, 2005) compared to mitochondria from fast-glycolytic muscles when energized with TCA cycle

substrates feeding complex I or complex II. Of note, fiber type specificity in the capacity to oxidize lipid substrates (Baldwin *et al.*, 1972; Jackman & Willis, 1996; Mogensen & Sahlin, 2005) as well as in the relative stoichiometries of some TCA cycle enzymes (Jackman & Willis, 1996; Leary *et al.*, 2003) have been reported but these differences, if present in our preparations, had no significant impact on respiratory function under our experimental conditions.

In muscle, it is well known that ANT exerts a major control on oxidative phosphorylation under physiological conditions (Vignais, 1976; Bohnensack, 1981; Balaban, 1990). In the present study, we observed that the mitochondrial content of ANT was significantly lower in PLN and WG compared to SOL despite the absence of difference in V_{ADP} among muscles. These results clearly indicate that the degree of control exerted by ANT on maximal oxidative phosphorylation varies across muscle types, being more important in predominantly fast-twitch muscles compared to slow-twitch muscles. Variations in ANT content across muscle fiber type could thus contribute to explain why mitochondrial respiration in fast muscles is mainly controlled by changes in [ADP] and [ATP] while in slow-oxidative muscle, changes in the concentration of adenylates play a less important role (Hochachka & McClelland, 1997).

Vulnerability to permeability transition

Very little information is currently available on the regulation of the PTP in skeletal muscle and the question of whether fiber type specific differences in its regulatory properties exist has not been addressed previously. Fontaine et al. (Fontaine *et al.*, 1998) have shown that in mitochondria isolated from pooled hindlimb muscles, CRC is 3-4 fold lower in mitochondria energized with complex I (glutamate-malate) compared to complex II (succinate + rotenone)

substrates. This phenomenon was found to be due to the fact that electron flow through complex I acted as a potent sensitizer to Ca^{2+}-induced PTP opening (Fontaine *et al.*, 1998). Results from the present study confirm and extend these data by showing that CRC was substantially lower in presence of glutamate-malate compared to succinate in all muscles, which indicates that this regulatory feature is conserved across muscle fiber types.

However our results clearly indicate that irrespective of the respiratory substrate oxidized, mitochondria isolated from muscles composed predominantly of slow type I fibers (SOL) are significantly more vulnerable to PTP opening in response to Ca^{2+} loading compared to mitochondria from muscle expressing mainly fast type II fibers (PLN and WG). Among the factors known to increase the sensitivity to permeability transition is ROS production, which favors pore opening by oxidizing critical SH residues of pore forming proteins (Zoratti & Szabo, 1995). However, in the present study, we observed that the rate of ROS production in presence of succinate was lower in SOL compared to WG while mitochondria from PLN displayed intermediate values. A similar trend was observed in presence of glutamate-malate, although no significant difference between muscle types was noted. These results are in accordance with recent studies showing that mitochondrial ROS production is higher in isolated mitochondria and saponin-skinned fibers from muscles expressing mainly type II fibers (Capel *et al.*, 2004; Anderson & Neufer, 2006). Overall, these data argue against a role for ROS production in explaining the fiber type difference in PTP regulation under our experimental conditions.

Endogenous Ca^{2+} was two-fold higher in mitochondria from SOL compared to PLN and WG muscles. This observation is consistent with the fact that steady state $[Ca^{2+}]$ in the cytosol is

significantly higher in slow twitch compared to fast twitch fibers likely because of a lower capacity of the sarcoplasmic reticulum for Ca^{2+} storage and to the absence of the cytosolic Ca^{2+}-binding protein parvalbumin (Fryer & Stephenson, 1996; Carroll *et al.*, 1997; Carroll *et al.*, 1999). Since the accumulation of Ca^{2+} in the matrix is the main trigger for PTP opening (Zoratti & Szabo, 1995), this difference in endogenous Ca^{2+} at least partly contributed to the greater vulnerability to PTP opening in mitochondria from SOL compared to PLN and WG. This was particularly evident when mitochondria were incubated in presence of glutamate-malate since under this condition the Ca^{2+} threshold for PTP opening (between 111-170 nmol/mg prot) was equal to or only slightly higher than the endogenous Ca^{2+} levels (54-111 nmol/mg prot) due to the sensitizing effect of complex I substrate on pore opening (Fontaine *et al.*, 1998). In contrast, when complex I was bypassed with succinate, CRC was substantially higher than endogenous Ca^{2+} levels in all muscles and therefore differences in endogenous Ca^{2+} had a proportionately smaller role in determining the sensitivity to external Ca^{2+}. However, under this condition CRC was still ~ 40 % lower in SOL, implying that in this muscle additional factors were responsible for the increased vulnerability to pore opening.

In this regard, the matrix protein foldase CypD was recently shown to play an important role in sensitizing the PTP to Ca^{2+} (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Recent studies have indeed shown that CypD-knockout mice are more resistant to Ca^{2+}-induced PTP opening (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005) while overexpression of CypD leads to enhanced mitochondrial swelling (Baines *et al.*, 2005). Moreover, the loss of sensitivity to CsA was shown to parallel the loss of CypD expression indicating that CypD is the molecular target of CsA and is responsible

for its inhibitory effect on the PTP. In the present study, the potency of CsA at inhibiting PTP opening was similar in mitochondria, irrespective of muscle type. In addition, the expression of CypD was similar in the three muscles, suggesting that CypD does not play a role in the fibre type difference in the sensitivity to PTP opening (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). On the other hand, in mitochondria from SOL we observed a significantly greater expression of ANT, an abundant protein known for its capacity to form the PTP together with VDAC (Beutner *et al.*, 1998; Crompton *et al.*, 1999; Halestrap *et al.*, 2002). Recent studies have shown that in mice harboring a liver-specific knockout of both isoforms of ANT, PTP opening can still occur indicating that ANT is not absolutely required. However, significantly greater amounts of Ca^{2+} are required to induce PTP opening in the absence of ANT suggesting that ANT confers greater Ca^{2+} sensitivity (Kokoszka *et al.*, 2004). Therefore one possibility could be that in presence of conditions favoring permeability transition the greater abundance of ANT and its binding partner VDAC in the SOL may increase the likelihood that some of these proteins loose their role of specific exchangers to participate in the formation of the PTP.

An important consideration regards the possibility that the so-called white portion of the gastrocnemius also includes portions of intermediate gastrocnemius, which has a fiber type composition similar to that of the PLN (Delp & Duan, 1996). This possibility could partially account for the general lack of difference reported in this study between PLN and WG with respect to parameters such as endogenous Ca^{2+} content, vulnerability to Ca^{2+}-induced PTP opening, Ca^{2+} retention capacity, and protein content of ANT, VDAC, and CypD. In light of this possible caveat, it may be more suitable to compare only slow and fast muscle (including two

different fast muscles), rather than proposing distinctions according to predominant fiber types. In future studies, it would be valuable to include actual measurements of the fiber type composition of the representative samples.

In conclusion, the present study provides the first evidence that in predominantly slow twitch oxidative muscles, mitochondria are more sensitive to PTP opening compared to mitochondria from predominantly fast-twitch muscles and display greater levels of ANT and VDAC, two proteins that can be involved in pore formation (Crompton *et al.*, 1999; Halestrap *et al.*, 2002). This greater vulnerability to permeability transition is at least partly due to the greater mitochondrial Ca^{2+} levels present in mitochondria from slow-twitch oxidative muscles but not to differences in the production of ROS or to the expression of the regulatory protein CypD. Thus, increased susceptibility of PTP opening might provide a mechanism that acts to predispose SOL muscle to significant atrophy and slow-to-fast phenotypic transitions with loss of mitochondrial mass under various conditions where normal activity patterns are modified (unloading, spaceflight, immobilization) (Marshall *et al.*, 1989).

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Table 1: Muscle mass and mitochondrial isolation yields and enzymatic activities

	SOL	PLN	WG
Muscle mass (mg)	274 ± 8.78 [b,c]	589 ± 9.55 [a,c]	889 ± 24.7 [a,b]
Mitochondrial yield (mg)	1.19 ± 0.119	2.37 ± 0.195	2.40 ± 0.212
Mitochondrial yield (mg/g)	4.34 ± 0.421	4.04 ± 0.346	2.68 ± 0.194 [a,b]
COX activity (mU/mg)	7.39 ± 0.431	7.37 ± 1.00	7.77 ± 0.780
CS activity (mU/mg)	8.83 ± 0.852	8.05 ± 0.365	6.32 ± 0.593

[a]: Significantly different from SOL; [b]: significantly different from PLN; [c]: significantly different from WG, $P<0.05$, where $n = 5$ for each muscle.

Table 2: Respiratory function in mitochondria from SOL, PLN, and WG muscle. Presented are values for basal ADP-restricted (V_0), maximal rate of oxidative phosphorylation following addition of 1 mM ADP (V_{ADP}), and respiratory control ratio (RCR) in mitochondria energized with glutamate-malate (5, 2.5 mM) or with succinate-rotenone (5mM- 1μM). Data are presented as means ± SE, where n = 7 for each muscle. a : Significantly different from SOL ($P < 0.05$).

Table 2: Values for mitochondrial respiration V_o, V_{ADP} and calculated respiratory control ratio for mitochondria isolated from SOL, PLN, and WG muscles

	Succinate/ Rotenone			Glutamate/ Malate		
	V_o	**V_{ADP}**	**RCR**	**V_o**	**V_{ADP}**	**RCR**
SOL	16.9 ± 1.3 [b]	122.0 ± 9.4	7.4 ± 0.8	11.9 ± 2.1	127.2 ± 19.9	11.1 ± 1.3 [a]
PLN	31.3 ± 3.2	160.4 ± 13.1	5.3 ± 0.4	16.5 ± 1.2 [a]	159.2 ± 18.6	9.4 ± 0.9 [a]
WG	32.8 ± 4.3	156.6 ± 15.8	4.9 ± 0.3	14.8 ± 1.3 [a]	146.8 ± 14.6	9.9 ± 0.8 [a]

[a] significant difference between substrates
[b] significantly different from PLN and WG

FIGURE LEGENDS

Figure 1: Calcium uptake kinetics during Ca^{2+} challenge experiments in mitochondria from SOL, PLN, and WG muscle. The figure shows typical Calcium-green 5N tracing of muscle mitochondria (0.15 mg/mL) energized with succinate-rotenone in SOL (panel A), PLN (panel B) and WG (panel C). Tracings show progressive Ca^{2+} accumulation followed by release of accumulated Ca^{2+} secondary to PTP opening. Each spike indicates the addition of a Ca^{2+} pulse of 83 nmol/mg. Calcium retention capacity measured in several experiments in presence of succinate + rotenone (SR) or glutamate-malate (GM) is shown in Panel D for each muscle. Data are presented as means ± SE, where n = 5 for SOL and PLN, and n= 6 for WG. a: significantly different from PLN and WG.

Figure 2: Mitochondrial H_2O_2 production. The figure shows H_2O_2 production (picoM· mg prot $^{-1}$· min $^{-1}$) in mitochondria isolated from SOL, PLN, and WG muscles energized with substrates for complex II or complex I (hatched bars). Data are presented as means ± SE, where n = 9 for each muscle. a: significantly different from SOL and PLN; b: significantly different from condition with complex II substrate, ($P < 0.05$).

Figure 3: Endogenous Ca^{2+} content of mitochondria isolated from SOL, PLN, and WG muscles. The figure presents endogenous Ca^{2+} content measured in the mitochondrial fraction of SOL, PLN, and WG muscles. Data are presented as means ± SE, where n = 5 for SOL, and n = 6 for PLN and WG muscles. a : significantly different from PLN and WG ($P < 0.05$).

Figure 4: Effect of substrate and the PTP inhibitor CsA on calcium retention capacity in mitochondria from SOL, PLN, and WG muscles. The figure shows the effect of incubation with glutamate-malate and succinate-rotenone with or without cyclosporin-A (1μM) on calcium retention capacity. Values are expressed as the sum of the contribution of endogenous mitochondrial Ca^{2+} (black bars) and the Ca^{2+} added during the in vitro challenge. Data are presented as means ± SE, where n = 5 for SOL and PLN, and n= 6 for WG. a: significantly different from PLN and WG; b: significantly different from condition with glutamate-malate; c: significantly different from condition with succinate-rotenone.

Figure 5: Cyclophilin D, VDAC and ANT content of mitochondrial fraction from SOL, PLN, and WG muscles. Representative immunoblots of cyclophilin D (CypD: 21 kDa), voltage dependent anion channel (VDAC: 31 kDa) and adenine nucleotide translocase (ANT: 31 kDa) in muscle mitochondrial fraction isolated from SOL, PLN, and WG muscle. Densitometric analysis for each protein represents the mean ± SE, where n = 6 for SOL and PLN, and n = 5 for WG. a : Significantly different from PLN and WG, ($P < 0.05$).

Figure 1 A

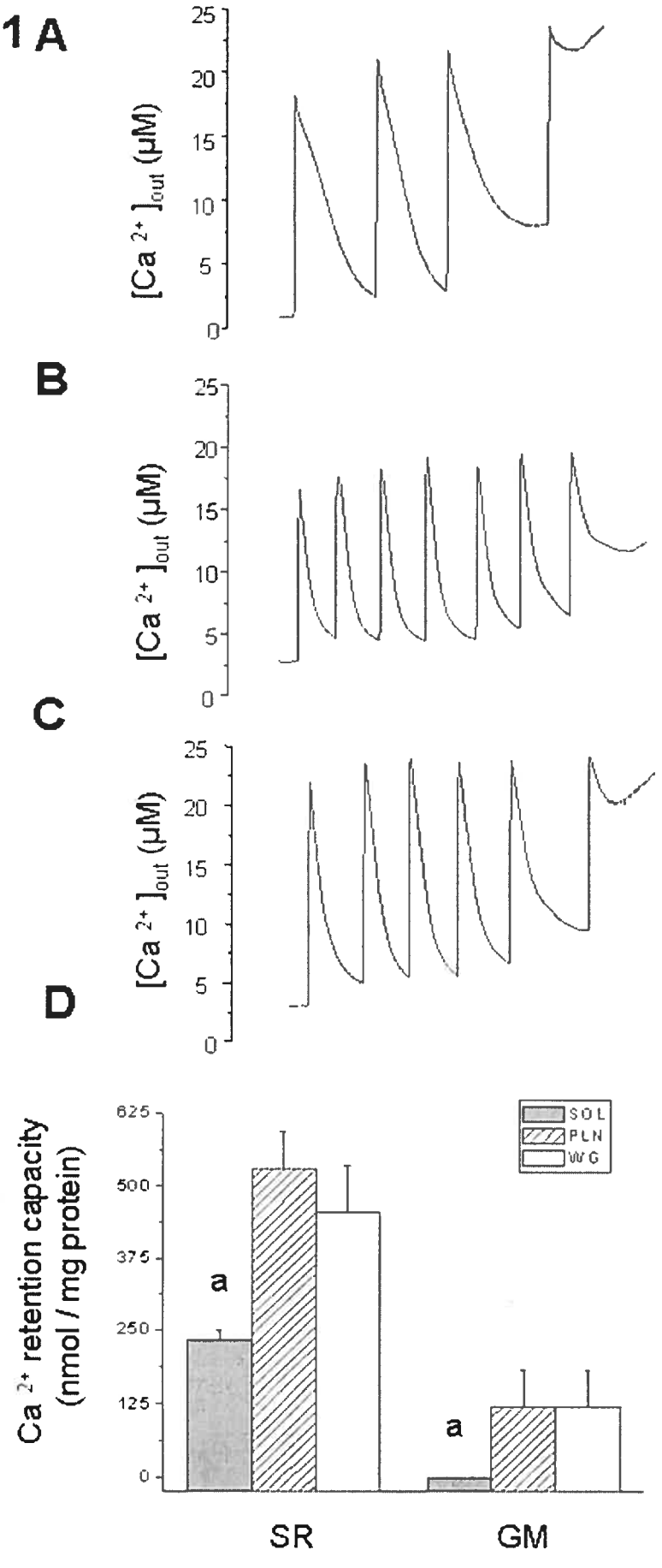

25
20
15
10
5
0
[Ca 2+]out (μM)
B
C
D
625
500
375
250
125
0
Ca 2+ retention capacity
(nmol / mg protein)
a
a
SOL
PLN
WG
SR
GM

Figure 2

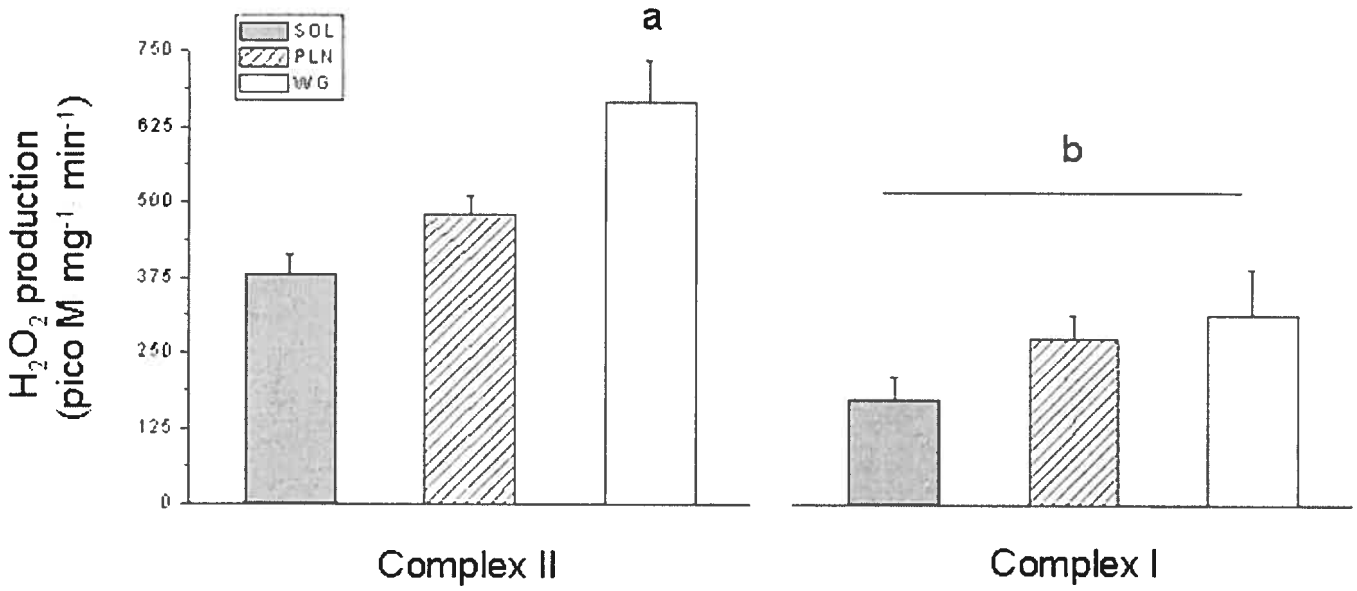

SOL
PLN
WG
750
625
500
375
250
125
0
H_2O_2 production
(pico M mg^{-1} min^{-1})
a
b
Complex II
Complex I

Figure 3

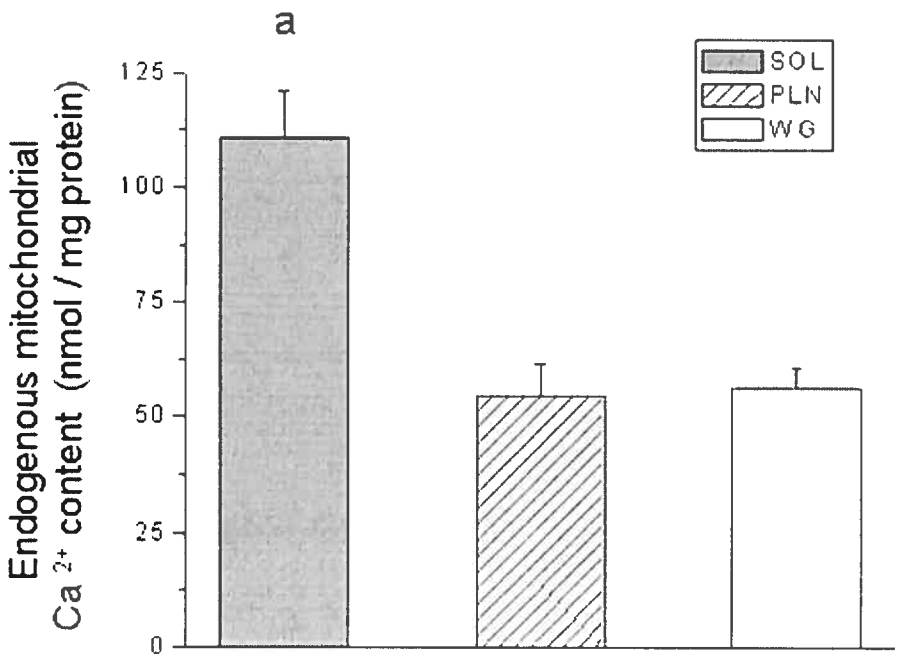
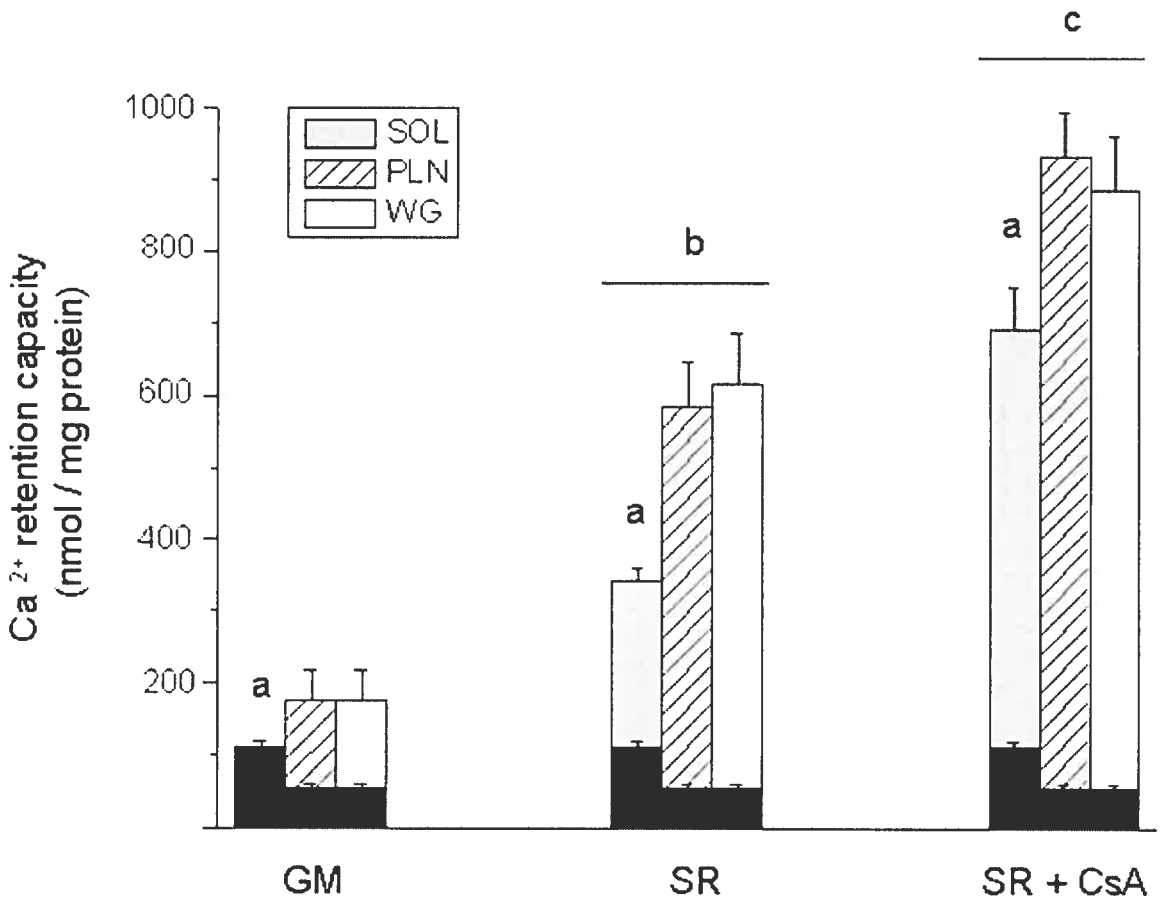

a
SOL
PLN
WG
Endogenous mitochondrial
Ca 2+ content (nmol / mg protein)
125
100
75
50
25
0

Figure 4


c
1000
800
600
400
200
SOL
PLN
WG
b
a
a
a
Ca 2+ retention capacity
(nmol / mg protein)
GM
SR
SR + CsA

CypD

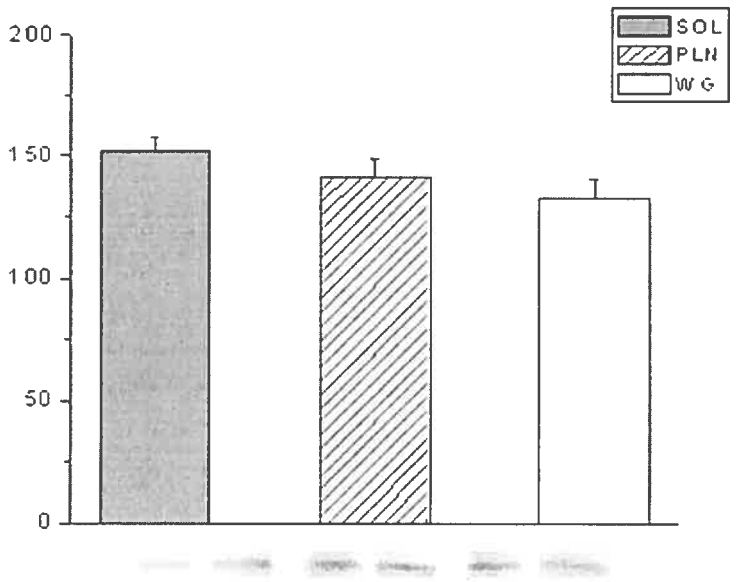
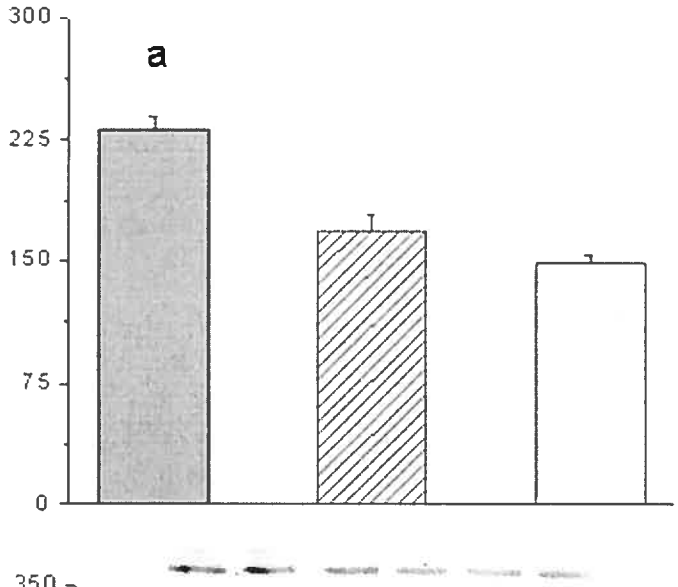
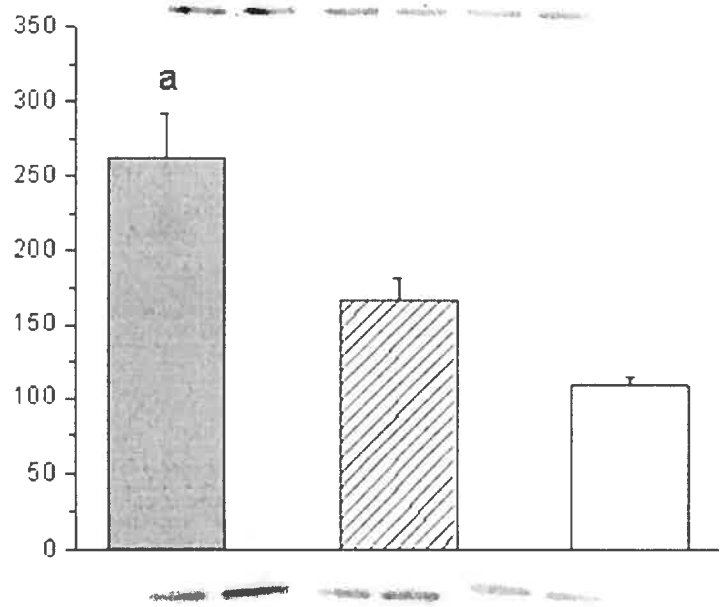


Figure 5

VDAC



ANT



Chapter 5: Conclusion

5. CONCLUSIONS AND GENERAL DISCUSSION

The three original studies presented in this thesis have provided information regarding the cellular processes regulating disuse muscle atrophy with respect to: 1) the understanding of intracellular signaling potential in disused muscle in response to a mechanical stimulus; 2) the effect of loss of neural input to muscle fibers on changes in the sensitivity and modulation of PTP opening; 3) a characterization of the properties and function of the mitochondrial PTP in different skeletal muscles characterized by different fiber types. Based on the observations and results of these studies, we propose the following conclusions:

1. decreased JNK phosphorylation response to mechanical stimulation indicates that atrophic muscle may lose the ability to transduce mechanical signals to the MAPK pathways. In light of this decline in muscle responsiveness to mechanical stimuli with continued unweighting, we propose that countermeasures designed to preserve muscle mass in situations of unloading (bed rest, spaceflight, immobilization) should be implemented promptly following the onset of mechanical unloading;

2. since the contribution of cellular apoptosis has been proposed as a possible mechanism regulating the loss of myofibers which occurs as a result of reduced mechanical loading, increased JNK-mediated regulation of cellular apoptosis may explain the increased basal phosphorylation levels measured in muscle following hindlimb suspension;

3. we suggest that the expression of CypD, by virtue of its PPIase activity, is increased in muscle atrophied by denervation as an initial defense or protective mechanism against protein misfolding. But as mitochondria become progressively overloaded with Ca^{2+}, this evolves into a maladaptive response by promoting PTP formation and opening;

4. we contribute important information regarding the characterization of PTP

properties in mitochondria isolated from skeletal muscle tissue. We demonstrate that basic PTP function and sensitivities are different depending on the type of muscle fiber from which mitochondria are isolated. Mitochondria isolated from SOL display increased sensitivity to Ca^{2+}-induced PTP opening as evidenced by a significant decrease in Ca^{2+}-retention capacity and significantly slower Ca^{2+}-uptake kinetics. This greater sensitivity appears in the absence of major differences in respiratory capacities and despite the fact that ROS production, a factor known to favor PTP opening, is the lowest in the SOL. This phenomenon may be partly caused by an increase in endogenous mitochondrial Ca^{2+} levels in this muscle but, unlike in the denervation study, is not associated with differences in the protein expression of CypD. This is corroborated by the finding that PTP inhibition by CsA, an inhibitor whose molecular target is CypD, is equally effective in all muscle types studied. However, the levels of ANT and VDAC proteins are significantly higher in SOL compared to PLN and WG. Thus, in presence of conditions favoring permeability transition, the greater abundance of ANT and its binding partner VDAC in the SOL muscle may increase the likelihood that some of these proteins loose their role of specific exchangers to participate in the formation of the PTP. Increased susceptibility of PTP opening might provide a mechanism that acts to predispose SOL muscle to significant atrophy and slow-to-fast phenotypic transitions with loss of mitochondrial mass under various conditions where normal activity patterns are modified (unloading, spaceflight, immobilization). These findings support the emerging view that mitochondria display distinct properties that differ qualitatively according to muscle phenotype.

5.1 General Discussion

A loss of skeletal muscle mass and functional capacity is an undesirable, yet inherent consequence of muscle disuse. Inactivity-mediated protein breakdown occurs in many circumstances ranging from events such as illness or injury, to unique environments such as microgravity, and to less blatant causes such as the progression of aging in the elderly. Regardless of the underlying cause, the consequences of inactivity are readily

observable and debilitating.

The functional adaptations to disuse in the muscles of humans are often difficult to investigate because adaptations occur over a period of weeks, months, or even years and there is a large variability in response to muscle disuse among humans. To circumvent these issues, various animal models have been developed which quickly result in extensive muscle atrophy. Such models allow precise control of the loading, innervation, and can provide accounts of muscle activation throughout the disuse intervention.

Mechanistically, the loss of lean muscle mass during inactivity is the result of a chronic imbalance between muscle protein synthesis and breakdown. The results of numerous studies have demonstrated that these processes are controlled by multiple signals originating both inside and outside the muscle, with downstream intracellular signal transduction pathways interacting in complex ways (Hunter *et al.*, 2002; Glass, 2003; Hornberger & Esser, 2004; Jackman & Kandarian, 2004; Glass, 2005; Kandarian & Jackman, 2006). There is accumulating new evidence suggesting that apoptosis is a key process during muscle atrophy resulting from diverse causes. Although apoptosis can occur through several mechanisms, mitochondria have been implicated as major regulatory centers for apoptosis. The discovery that mitochondria operate not only as providers of cellular energy necessary for life, but also as sources of signals for cell death has prompted great interest in the investigation of mitochondria-associated signaling of apoptosis.

As we have seen, mitochondria contain a structure that, *in vitro* forms a large unspecific channel called the PTP under conditions of high Ca^{2+}, P_i, and oxidative stress (Crompton *et al.*, 1988). Opening of this so-called PTP channel has mostly been studied for its involvement in apoptosis. It was believed that opening of the PTP would cause two important changes to mitochondria: 1) it would interfere with mitochondrial electron transport, and therefore ATP production; 2) it would cause mitochondrial swelling due to the disequilibrium of water and ions between the cytosol and matrix resulting in bursting of the mitochondrial membranes and the release of their pro-

apoptotic contents (Kroemer *et al.*, 1998; Green & Kroemer, 2004). Presently, it is not entirely clear whether the mitochondrial PTP complex has a role in normal cell physiology. It has been suggested by some that the pore may represent a route for Ca^{2+} to escape from overloaded mitochondria under physiological conditions (Ichas *et al.*, 1997). However, it is well known that pore opening destroys the protonmotive force so it has been argued that it is unlikely that mitochondrial Ca^{2+} levels are regulated by a mechanism that causes enzymes of the electron transport chain to be lost to the cytosol and ATP to be hydrolyzed (Crompton & Costi, 1990).

An interesting and important task that remains to be completed is to determine the molecular composition of the mitochondrial PTP. Although several hypotheses are being considered, there is still no clear resolution of this issue (Green & Kroemer, 2004). One of the curious aspects of the PTP complex is that it is assembled from components that have other well-established roles in cells. For instance, the function of VDAC is to allow specific solutes of low molecular weight access to the transport systems of the inner membrane. As for ANT, the physiological role for this protein is to mediate the exchange of ADP for ATP, a process that is essential for the primary bioenergetic function of the mitochondria.

5.2 Possible alternate functions of PTP components

The function of CypD is not as well established, but a likely role is the control of protein misfolding. In our second study, we show that denervation induces an increase in the relative expression of CypD and an over-representation of this protein compared to other putative PTP components, establishing a condition which may predispose mitochondria to Ca^{2+}-induced permeability transition. Recently, CypD was shown to play an important role in sensitizing the pore to Ca^{2+} (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Indeed, in mitochondria from *Ppif*$^{-/-}$ mice, which lack CypD, the amount of Ca^{2+} required to trigger PTP opening was increased several fold compared to that observed in mitochondria from strain-matched wild type mice (Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). In addition, in the absence of CypD, PTP opening in response to Ca^{2+} loading was

absolutely insensitive to CsA indicating that CypD is the molecular target responsible for the inhibitory effect of CsA on pore opening (Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). A striking parallel can be made between the P*pif*$^{-/-}$ mice data and the results obtained in our study. Indeed, we observed that CsA was significantly more potent at inhibiting Ca^{2+}-induced PTP opening in mitochondria from denervated muscles, while trifluoperazine, which inhibits pore opening through mechanisms that are independent from CypD (Bernardi *et al.*, 1993), was equally effective in the sham and the denervated groups. Immunoblot analysis on mitochondrial fractions confirmed the hypothesis that CypD expression may be increased following denervation, leading to an over-representation of this protein relative to ANT and VDAC, two other putative structural components of the PTP (Zoratti & Szabo, 1995; Crompton, 1999; Halestrap *et al.*, 2000; Kim *et al.*, 2003b; Mayer & Oberbauer, 2003; Zoratti *et al.*, 2005). Taken together, these results indicate that an increased expression of CypD significantly contributed to the sensitization of mitochondria to Ca^{2+}-induced pore opening in denervated muscle mitochondria. To our knowledge, this is the first study to demonstrate a link between changes in CypD expression and PTP dysregulation in a non-transgenic model of disease.

The basis for this apparent increase in CypD expression in response to loss of innervation is currently unclear. CypD by virtue of its PPIase activity is thought to act as a protein foldase that presumably interacts with one or more chaperonins to limit mitochondrial protein misfolding that occurs after oxidative stress or other various perturbations (He & Lemasters, 2002; Kim *et al.*, 2003a). Lemasters et al. (He & Lemasters, 2002; Kim *et al.*, 2003b) recently proposed a model in which the PTP is formed by clusters of misfolded proteins. In this model, CypD initially binds these clusters to refold proteins to their native states. However, in presence of Ca^{2+}, CypD turns these clusters into non-specific channels, an effect that is antagonized by CsA (He & Lemasters, 2002; Kim *et al.*, 2003b). Therefore, one possibility is that CypD expression is increased in denervated muscles as an initial defense mechanism against potential protein misfolding in denervated muscle. Further studies will be necessary to fully understand this potential alternate role for CypD.

In our study on the vulnerability of different fiber types to PTP opening, we reported that mitochondria isolated from skeletal muscles with different fiber type compositions are heterogeneous in their response to Ca^{2+}-induced PTP opening, with mitochondria isolated from muscle predominantly composed of slow-twitch fibers (SOL) being more vulnerable than mitochondria from muscle expressing mainly fast-twitch fibers (PLN and WG). We measured the protein content of the putative PTP structural proteins and observed that the mitochondrial content of CypD was similar across fiber types, but that the contents of VDAC and ANT were significantly lower in PLN and WG than in SOL muscle. Recent studies in ANT knockout mice have shown that despite the absence of ANT, PTP opening can still occur, but significantly greater amounts of Ca^{2+} are required to induce opening (Kokoszka *et al.*, 2004). Given this observation, we suggest that in slow-twitch muscle fibers under conditions that favor opening of the mitochondrial PTP, a greater ANT abundance may increase the likelihood of PTP formation, a phenomenon which could account for the greater sensitivity of SOL muscle to PTP opening observed in our study. Again, further studies will be required to clarify this alternate role for ANT.

5.3 Study limitations

In order to understand the ways in which mitochondria behave in relation to other aspects of cell physiology, and to understand how other cellular functions respond to changes in mitochondrial function, then it is necessary to have experimental models in which these processes can be studied in intact cells. This approach also has the advantage that tissues where the sample size might be too small for the preparation of isolated mitochondria for conventional biochemical studies, may become accessible for study. In fact, one of the major challenges we encountered during our studies of the properties of mitochondria from skeletal muscle tissue was the small size of the protein isolation yields, especially in the muscle denervation study. The weight of tissue harvested from SOL muscle denervated for 21 days was, in some cases, inferior to 100 ug. This sample size proved to be too small to isolate a sufficient quantity of viable mitochondria. Since there was no way to increase sample size, other than by pooling together numerous SOL muscles, we needed to modify the isolation procedure such that

viable mitochondrial samples could be isolated from smaller tissue volumes, a task that unexpectedly proved to be more difficult than originally anticipated.

Mitochondrial studies performed on preparations of mitochondria isolated from major tissues (heart, liver, and brain) have provided a large portion of our understanding of the ways in which mitochondria function in their control of the processes of life and death. Properties of the PTP have been studied most extensively in isolated mitochondria. Because of the signal averaging over a large population of mitochondria in these types of experiments, PTP opening is evident as a gradual process. The permeability transition of each individual mitochondria, however, is believed to be a very fast phenomenon and the gradual response recorded from mitochondrial populations is caused by the successive recruitment of organelles undergoing pore transitions (Gunter *et al.*, 1994). In vivo, mitochondria are organelles that are highly organized within structural compartments. For example, structural contacts between the sarcoplasmic reticulum and the mitochondria are involved in the control of Ca^{2+} homeostasis and ATP production (Szalai *et al.*, 2000). In 1988, it was found that the kinetics of respiration by ADP measured in mitochondria *in situ* in carefully prepared permeabilized muscle fibers are significantly different from those observed *in vitro* in preparations of isolated mitochondria (Kummel, 1988). In the studies presented here, parameters of PTP function were investigated in isolated mitochondrial preparations only. In order to extrapolate our results to parameters of integrated physiological function, it would be imperative to investigate these parameters in intact, permeabilized muscle fibers. Ideally, parameters of mitochondrial PTP function should be monitored in intact muscles, however, this procedure is not yet feasible.

Since apoptosis is an ATP-dependent event, should cellular ATP levels decrease beyond a critical threshold, the cell would inevitably die by necrosis. Thus, if PTP opening is prolonged rather than transient, or if numerous mitochondria experience PTP opening at the same time, ATP levels would not be maintained and cellular necrosis would result. Therefore, it would appear that the balance between the number of "closed" and "open" PTPs within a mitochondrial population is critical in determining whether a cell will die or recover. It has been suggested that depending on the severity of the apoptosis-

initiating stimulus, only a small population of the mitochondria might adopt the open pore conformation (Zoratti & Szabo, 1995). Alternatively, it has been proposed that perhaps PTP opening is a transient event causing a "flickering" effect (Duchen *et al.*, 1998). Unaffected mitochondria remain capable of either producing sufficient ATP to support formation of the apoptosome or are able to uptake the Ca^{2+} released by mitochondria whose PTPs have opened. This is currently a topic of debate among experts in the area. Considering the *in vitro* nature of the experiments presented in this thesis, it is not possible to determine whether our observations reflect the activity of the mitochondrial population as a whole, or whether our measurements reflect the behavior of a select subpopulation of the mitochondria within the sample. Thus, although the *in vitro* methods commonly used to investigate selective parameters of PTP function in isolated mitochondria has allowed for a thorough characterization of PTP function, in the process of isolating organelles form their integrated cellular environment, artifact may become introduced in the measurements.

Furthermore, the results presented in this thesis were derived from work obtained by exposing isolated mitochondria to high concentrations of Ca^{2+}. *In vitro*, these conditions reveal increased susceptibility to PTP opening in mitochondria from soleus muscle fibers, however, it is likely that such conditions are not encountered by mitochondria *in vivo*. Finally, it should be considered that although similarities in physiological function between the rat and human are well established, the results obtained in the present studies may be species- and/or model-specific and therefore not entirely transferable to humans.

PTP opening *in vitro* is followed by water influx across the mitochondrial membrane caused by the high protein concentration of the mitochondrial matrix. This causes mitochondrial swelling and results in dramatic impairments in mitochondrial function including the collapse of membrane potential across the inner membrane required to drive ATP synthesis. However, it is worth mentioning that some reports suggest that swelling is not a necessary consequence of pore opening (Pfeiffer *et al.*, 1995) since swelling results from the influx of water across the inner mitochondrial membrane but only in the presence of an osmotic imbalance. Indeed, studies performed *in vitro* have

shown that mitochondrial swelling following PTP opening could be completely prevented by counterbalancing the osmotic pressure developed by the matrix proteins by adjusting the concentration of non-diffusible molecules. The authors suggest that this 'adjustment' reflects a condition that is certainly more representative of the conditions existing in living cells.

In conclusion, based on careful observation and interpretations of the data acquired from experimental investigations in the rat, we have attempted to further characterize and contribute to a better explanation of various aspects of the cellular processes operant during conditions of muscle atrophy. As is often the case, the elucidation of one question instigates the conception of numerous more. Although the past few years have seen great contributions to the identification of intracellular signaling processes implicated in muscle atrophy, there is still much to be learned about this fascinating realm of skeletal muscle physiology. The objective of ongoing studies in this area will be to further dissect the various signal transduction pathways that play a role in the atrophy process in the efforts to construct an integrated, unifying picture to better understand muscle atrophy.

Chapter 6: References

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