PROTEIN IMPORT INTO SKELETAL MUSCLE MITOCHONDRIA: EFFECTS OF AGING AND CHRONIC CONTRACTILE ACTIVITY

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ABSTRACT

Properties of skeletal muscle, such as substrate metabolism, fatigue resistance, and maximum oxygen consumption, are intimately linked to the bioenergetics of the mitochondrial network found throughout this highly metabolic tissue. Recently, connections between sarcopenia and mitochondrial dysfunction have been made, suggesting that processes regulating mitochondrial function could be impaired due to the aging process. Mitochondrial function can be improved by increasing the content of mitochondrial proteins. The expansion of the existing network requires the expression of both nuclear and mitochondrial genomes, and nascent proteins must be correctly assembled into their appropriate compartment and complexes. Thus, to incorporate proteins synthesized in the cytosol, the mitochondrial protein import pathway exists to facilitate mitochondrial biogenesis. We hypothesize that this import pathway may be susceptible to the aging process, resulting in impaired mitochondrial turnover and leading to dysfunction of the organelle. Second to this, if mitochondrial function could be improved by chronic exercise in these aged animals, it is likely that muscle performance could also be ameliorated. Whether mitochondrial biogenesis is facilitated by enhancements in the import of mitochondrial proteins in senescent animals also remains to be determined. In addition, a paucity of studies have investigated whether the two pools of skeletal muscle mitochondria, the subsarcolemmal (SS) and intermyofibrillar (IMF) subfractions, respond similarly to age or chronic contractile activity. The following series of experiments were designed in order to test these hypotheses.

We utilized 6 and 36 month-old F344BN rats to represent the aging paradigm. Using electron microscopy we observed that mitochondrial content declined in senescent animals. Thus, we assessed the effect of physiological aging on the import of two matrix-destined mitochondrial proteins, precursor ornithine carbamoyltransferase (pOCT) and precursor malate dehydrogenase (pMDH), into freshly isolated mitochondria from skeletal muscle of young and old animals. We observed no change in the rate of pMDH import into SS and IMF mitochondria due to age. However, we noted a 16% elevation in pOCT import ($p<0.05$) in the IMF, while no change was observed in the SS mitochondria isolated from senescent animals when compared to young. Immunoblotting analyses reveal that the expression of import machinery components was not affected by age. We detected that the protein expression of a cytosolic chaperone, MSF-L, was elevated by 97% in aged skeletal muscle ($p<0.05$). In addition to these import assays, we obtained the cytosolic fraction from skeletal muscles from the two age groups in order to reconstitute, in part, the cellular environment. Despite the elevations observed in cytosolic chaperone content, import assays supplemented with the cytosolic fraction illustrated an approximate 40% attenuation of pOCT import ($p<0.05$) into IMF mitochondria, and this effect was similar between the age groups. Incubation of pOCT with the cytosolic fraction isolated from skeletal muscle revealed the presence of degradation factors which acted to reduce the amount of pOCT. The degradation of pOCT was 2.5-fold greater ($p<0.05$) when the precursor protein was incubated with cytosol isolated from senescent animals compared to that from young animals.

Our next goal was to investigate the effect of chronic stimulation on skeletal muscle of aged animals. We subjected the young and aged animals to 7 days of *in vivo* chronic contractile activity (CCA) to elicit mitochondrial biogenesis. Electron micrographs of the extensor digitorum longus muscles obtained from stimulated legs revealed an elevation in the content of SS mitochondria of both young and old animals, when compared to control. We also noted morphological changes in the IMF subfraction within stimulated muscle of young animals, while the effect of CCA was not as noticeable in the IMF subfraction of aged animals.

The import of pOCT was analyzed in SS and IMF mitochondria isolated from the control and stimulated muscles. We observed 78% and 31% increases in the import of pOCT induced by CCA in SS mitochondria isolated from young and senescent animals ($p<0.05$), respectively. Interestingly, while we detected an effect of CCA on pOCT import in IMF mitochondria from young animals (31% increase versus control; $p<0.05$) we did not observe an increase pOCT import in the IMF mitochondria from aged animals. We conducted protein expression analyses to profile any alterations in the import machinery components, as well as the content of cytosolic chaperones in response to chronic stimulation and aging. We observed an increase in the expression of import machinery components due to 7 days of CCA in young animals ($p<0.05$), although this effect was not noted in aged animals. We also did not detect an effect of CCA on the expression of cytosolic chaperones in either age group. However, we saw significant 1.5-

to 2.0-fold increases in the content of these chaperones in response to age ($p<0.05$), when compared to young animals.

These data, when considered together, suggest that the impairments observed in mitochondrial function in aged animals may not be related to alterations in the mitochondrial import pathway. However, future studies directed at the assessment of the import of mitochondrial proteins targeted to different compartments will provide more insight on this matter. The availability of precursor proteins in the cytosol may be susceptible to post-translational modifications other than protein import such as degradation by cytosolic factors.

Chronic contractile activity of skeletal muscles in aged animals resulted in elevated mitochondrial content. However, the import due to CCA was attenuated along with the expression of import machinery components in senescent animals. We noted that CCA-induced alterations in protein import seem to preferentially affect the SS, rather than the IMF mitochondrial population. Thus, in aged animals, a blunted capacity to adapt to CCA could limit the full potential of inductions in mitochondrial biogenesis.

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ABBREVIATIONS

$\Delta\psi$	mitochondrial inner membrane potential
$[Ca^{2+}]_i$	intracellular calcium
β-GPA	β-guanidinoproprionic acid
3'UTR	3' untranslated region
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AIF	apoptosis inducing factor
AMP	adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
BrdU	5-bromo-2-deoxyuridine
Ca^{2+}	calcium
CaMK	calcium/calmodulin-dependent protein kinase
CaMKII	calcium/calmodulin-dependent protein kinase II
CaMKIV	calcium/calmodulin-dependent protein kinase IV
CCA	chronic contractile activity
cDNA	circular deoxyribonucleic acid
CLFS	chronic low-frequency stimulation
CnA	calcineurin
CON	control
COX	cytochrome c oxidase
CREB	cyclic AMP responsive element binding protein
CSA	cross-sectional area
DNA	deoxyribonucleic acid
Drp1	dynamin-related protein
EDL	extensor digitorum longus
EndoG	endonuclease G
ERK2	extracellular signal-regulated kinase-2
ETC	electron transport chain
F344BN	Fischer 344 Brown Norway
FGF	fibroblast growth factor
Fig.	figure
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G_0	quiescence
G1	gap phase 1
GFP	green fluorescence protein
GIP	general import pore
HGF	hepatocyte growth factor

Hsp60	heat shock protein 60
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
IGF-1	insulin growth factor-1
IM	inner membrane
IMF	intermyofibrillar
IMP1	inner membrane protease 1
IMS	intermembrane space
JNK	c-Jun-N-terminal kinase
MAPK	mitogen-activated protein kinase
MDH	malate dehydrogenase
MHC	myosin heavy chain
MIP	mitochondrial intermediate peptidase
MISP1	mitochondrial inner membrane space protease 1
MPCs	muscle precursor cells
MPP	mitochondrial processing peptidase
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
mtHsp70	mitochondrial heat shock protein 70
mtPTP	mitochondrial permeability transition pore
MTS	mitochondrial targeting signal
MSF	mitochondrial import stimulating factor
NMJ	neuromuscular junction
NRF	nuclear respiratory factor
NUGEMPS	nuclear genes encoding mitochondrial proteins
OCT	ornithine carbamoyltransferase
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
PAM	presequence translocase-associated motor
PAX7	paired box gene 7
PI3K	phosphoinositide-3 kinase
PIM	protein import machinery
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PKC	protein kinase C
pMDH	precursor malate dehydrogenase
pOCT	precursor ornithine carbamoyltransferase
POLG	polymerase gamma
ROS	reactive oxygen species
RNC	ribosome-nascent chain complex
rRNA	ribosomal ribonucleic acid
S6K1	p70 S6 kinase-1

SAM	sorting and assembly
SDS	sodium dodecyl sulfate
SS	subsarcolemmal
sTIM	small translocases of the inner membrane
TA	tibialis anterior
Tfam	mitochondrial transcription factor A
TIM	translocases of the inner membrane
TOM	translocases of the outer membrane
tRNA	transfer ribonucleic acid
type IIa	fast-oxidative muscle fiber
type IIb	fast-glycolytic muscle fiber
VDAC	voltage dependent anion channel
VO_2max	maximum oxygen consumption

INTRODUCTION

Mitochondrial biogenesis induced by a variety of environmental stimuli is a complex process involving the transcriptional coordination between nuclear and mitochondrial genomes and the post-translational event known as mitochondrial protein import. Aging results in large-scale decrements in skeletal muscle performance that may be related to impaired mitochondrial function. The decrease in mitochondrial content and function in skeletal muscle of aged individuals could be due to, in part, alterations in the mitochondrial protein import pathway. In lieu of the large number of studies concentrating on the association between mitochondrial dysfunction and aging, the regulation of mitochondrial biogenesis during aging is not well understood in skeletal muscle. In fact, no study has investigated the effects of age on subsarcolemmal and intermyofibrillar mitochondrial protein import in skeletal muscle.

Chronic endurance activity is a well known stimulus that results in increased mitochondrial biogenesis in skeletal muscle leading to enhanced muscle performance. Changes in ATP availability, intracellular calcium and kinase activation converge on the nuclear genome, resulting in the up-regulated expression of genes coding for mitochondrial proteins. We have demonstrated that chronic muscle stimulation increases the rate of precursor protein import, the expression of protein import machinery components and the content of cytosolic chaperones in adult animals. However, no studies have investigated if these adaptations occur to the same degree in senescent animals. In addition, the effects of age and chronic contractile activity on skeletal muscle mitochondria have been investigated in whole muscle, rather than in the two populations

of skeletal muscle mitochondria. Thus, the purposes of my thesis were to determine whether:

1) alterations in the mitochondrial protein import pathway occur in response to senescence;
2) cytosolic fractions, isolated from skeletal muscle of young and old animals, influence preprotein import into SS and IMF mitochondria;
3) the plasticity of skeletal muscle in response to chronic contractile activity is intact in senescent animals and,
4) mitochondrial biogenesis induced by chronic contractile activity is due to alterations in the mitochondrial protein import pathway in senescent animals.

REVIEW OF LITERATURE

1.0 Skeletal muscle

1.1 Muscle plasticity

The biochemical and metabolic characteristics of skeletal muscle have a large potential to change due to the extremely malleable and responsive nature of this tissue to a wide range of external and internal stimuli. As a result, remodelling of skeletal muscle occurs out of the necessity to meet altered energy needs. This adaptive characteristic of skeletal muscle, shared by many other organs like cardiac muscle and the liver, is referred to as plasticity. Internal stimuli causing widespread changes to muscle include, but are not limited to, aging, endocrine hormones and hypoxia while external stimuli include physical activity, inactivity, nerve injury and altitude. However, the main focus throughout this review of literature is the effects of aging and chronic endurance activity on the plasticity of skeletal muscle.

Depending on the type of exercise, skeletal muscle adaptations occur over a period of time, starting with the acute responses of the cell (i.e. up-regulation of mRNA expression), followed by the concomitant changes in muscle protein synthesis, and manifesting as the adaptations seen at the phenotypic level (i.e. increased force production or fatigue resistance). Chronic endurance training consisting of long duration, aerobic, moderate-intensity exercise, results in increased capillary-muscle fiber ratio, VO_2max, fatigue resistance and mitochondrial enzyme activities (23; 90; 131; 221). These adaptations are beneficial to muscle in that oxygen delivery and metabolite removal is enhanced, increased oxygen consumption promotes continued provision of

ATP through oxidative phosphorylation and lactic acid production is delayed. Adaptations in skeletal muscle induced by chronic endurance exercise occur predominantly through the conversion of fast-glycolytic fibers into fast-oxidative fibers. Indeed, the greatest metabolic transformations occur in the tibialis anterior, extensor digitorum longus and mixed gastrocnemius muscles (52; 131; 154; 155).

1.2 Experimental models of exercise

Mechanisms of exercise-induced adaptations have been uncovered, although other unknown pathways likely still exist and remain to be discovered. In order to elucidate these mechanisms, strategies have focused on determining whether repeated endurance exercise elicits changes at the level of kinase activation, leading to up-regulated gene expression. Other research focuses include identifying post-transcriptional and post-translational modifications, such as decreased mRNA and protein degradation, and determining the locations of important transcription factors within the cell during resting and exercising conditions.

1.2.1 Voluntary exercise

Voluntary exercise protocols are employed to uncover the mechanisms through which chronic endurance exercise brings about changes within skeletal muscle (6; 8; 54; 130). The exercise stimulus can range from running wheels and treadmill running for animal groups. In humans, exercise protocols include treadmill running, cycle ergometry, seated rowing and hand-crank ergometry. These models of exercise offer a much more representative idea of how chronic endurance exercise affects skeletal muscle, when

compared to in vitro cell culture exercise models (45; 57; 91; 92), because the full architecture and systemic factors are present. However, inconsistencies can occur in using these models of exercise. The intensity of exercise may vary between the treatment groups and even within the group if this parameter is not rigorously controlled. It is also apparent that motivation and encouragement play a large part in volitional VO_2max determination trials when using human subjects (14). If the stimulus of exercise varies, the data collected throughout the study may result in erroneous interpretation. To circumvent these confounding factors, an exercise stimulus can be delivered in the form of chronic low-frequency electrical stimulation (CLFS) onto the nerves which innervate skeletal muscle.

1.2.2 Chronic low-frequency stimulation (CLFS)

Chronic low-frequency electrical stimulation solves the issue of whether the exercise stimulus is the cause for adaptations observed in skeletal muscle. The first reported use of CLFS in a mammalian model was by Pette et al. using long-term stimulation of fast-twitch mixed muscles of rabbits. CLFS induced changes in blood flow, capillary to muscle fiber ratio, metabolic enzymatic activities, contractile properties, sarcoplasmic reticulum proteins and resulted in the conversion to the slow-oxidative muscle phenotype (153; 155-160; 162-164). The repeated delivery of neural activity, similar to the pattern of a slow-oxidative α-motor neuron, to the muscle units of fast-twitch muscle resulted in these changes without causing any damage to skeletal muscle. From the work of this group, many other researchers employ CLFS to reproduce inductions of mitochondrial

protein content (174), enzyme activity (203), mitochondrial volume density (2; 3; 119; 120) and transcription factor activities (66) in order to conduct in-depth investigations of their underlying mechanisms.

1.3 Muscle regeneration

Prior to birth, skeletal muscle arises from the committed differentiation of muscle progenitor cells into myoblasts which in turn fuse to become myotubes, the immature form of myofibers (233). Due to the post-mitotic nature of skeletal muscle, post-natal turnover of myofibers is not preceded by clonal replication. Instead, in the event of muscle damage, regeneration and replacement of muscle fibers depends on the supply of quiescent, mononuclear muscle precursor cells (MPCs), termed satellite cells, that are found beneath the basal lamina and above the sarcolemmal membrane (132). Environmental stimuli such as resistance exercise, mechanical stress, and CLFS trigger the activation of satellite cells, that is, the entry into G1 phase of the cell cycle from the quiescent state (G_0). Moreover, numerous pathways involving the activation of various kinases transduce the mechanical signals induced by environmental stimuli. Some examples of signalling pathways implicated in satellite cell activation include c-Jun-N-terminal kinase (JNK), p38 MAPK, extracellular signal-regulated kinase-2 (ERK2), p70 S6 kinase-1 (S6K1) and phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) (227). Morphological indications of activation include increased satellite cell volume, enlargement of the nucleus, diffuse chromatin and enlargement of organelles. Presently, the identified molecular markers of activation are derived from experimental models

tracing the increased expression of genes that regulate myogenesis. These include an increase in the expression of Rad (76), Myf5, MyoD, (46; 47), c-jun and c-fos (98) after the onset of activation. When considering the nitric oxide/hepatocyte growth factor (HGF) pathway of satellite cell activation, binding of HGF to c-met receptors found on satellite cells is another marker of activation (12).

After activation, satellite cells rapidly proliferate, indicated by an increase in BrdU incorporation into replicating DNA within the nuclei (187). During this stage, approximately 6 hours after activation, the increased expression of myoD and Myf5 drive satellite cells into becoming committed myoblasts which further proliferate. Molecular markers of proliferating myoblasts include the expression of myocyte nuclear factor (63) and paired box gene 7 (Pax7) (189). Although once studied and purported to activate satellite cells, anabolic hormones and other growth factors, such as insulin growth factor-1 (IGF-1) and fibroblast growth factor (FGF), actually promote the proliferation and differentiation of satellite-cell derived myoblasts (13).

Subsequently, committed myoblasts undergo differentiation, marked by an increase in myogenin, MRF4 and p27 (227). For the purposes of muscle regeneration, differentiation of myoblasts refers to exiting the cell cycle, which allows for the fusion and donation of nuclei to existing muscle fibers. Another scenario of differentiation is the fusion of myoblasts to form *de novo* muscle fibers (75). Centrally located nuclei, the expression of embryonic myosin heavy chain (MHC) and a disintegrin and metalloprotease protein referred to as ADAM12 (62) are markers of regenerated muscle. Not all myoblasts

undergo differentiation, as a set of myoblasts serves to renew the original satellite cell population. Up-regulation of myostatin plays an important role in returning these satellite cells back into the quiescent state (133). Thus, this unique muscle cell population serves as the line of defence against muscle atrophy and muscle fiber loss during the lifetime. How satellite cells may be affected by the aging process is discussed in detail below.

1.4 Myonuclear domain: hypertrophy versus atrophy

Confocal microscopic images reveal that a single muscle fiber can contain up to hundreds of myonuclei (9). The ability of skeletal muscle to readily adapt to environmental and internal perturbations could be linked to the presence of multiple myonuclei which mediate changes in muscle phenotype such as hypertrophy or increased metabolic enzyme activities. A relationship between myonuclei and the cross-sectional area (CSA) of a muscle fiber is explained by the term myonuclear domain. Myonuclear domain is a theoretical concept, describing that a fixed amount of cytoplasm within the muscle fiber is controlled by the transcriptional action of a single myonucleus located at the periphery of the muscle cell (9; 186). Chronic activity, such as CLFS or resistance training, results in the activation of satellite cells leading to an increase in myonuclear number and expression of contractile apparatus proteins. Subsequently, alterations of the muscle fiber CSA occur to maintain myonuclear domain and results in the preservation of muscle fiber size or hypertrophy. Conversely, muscle fiber atrophy occurs as a result of chronic muscle disuse, likely due to a decrease in the expression of contractile apparatus proteins and a loss in myonuclear number. The exact mechanisms leading to myonuclear

loss remain to be uncovered however the selective loss in nuclei within the muscle fiber could be mediated by a process known as apoptosis which has been linked to muscle atrophy caused by inactivity (193) and denervation (193).

Apoptosis, which is known as programmed cell death (230), is mediated by several pathways. Mitochondria play an important role in regulating apoptosis as the release of cell death factors housed within the organelle (i.e. apoptosis inducing factor (AIF), endonuclease G (EndoG) and cytochrome c) are potent stimulators of apoptosis. The release of these apoptotic proteins occur in response to cellular stressors such as reactive oxygen species (ROS) (197), chronic elevations in intracellular Ca^{2+} concentration and gamma irradiation (149). The ultimate manifestation of apoptosis is fragmentation and degradation of nuclear DNA, and likely resulting in myofiber shrinking to maintain the myonuclear domain size. Therefore, it is important to understand whether mitochondria have an explicit role in muscle fiber CSA. This knowledge could be applied in order to treat conditions such as muscular dystrophy and aging where fiber atrophy is a hallmark characteristic of the disorder.

2.0 Mitochondria: Powerhouses of the cell

As mentioned, the muscle fiber is unique because of numerous myonuclear content, contractile apparatus and specialized sarcoplasmic reticulum network. An additional feature to the myofiber is a significant contribution of mitochondria to the overall cell volume, which is most notable in slow-oxidative muscle fibers. Studies suggest that the Rickettsiales bacterium may have existed in a symbiotic relationship within a simple

eukaryotic host, allowing for various cellular exchanges, resulting in the transfer of mitochondrial DNA to the host genome (60; 118) and leading to the evolution of mammalian mitochondria. The relationship between mammalian mitochondria and its ancestor is supported by a vast number of studies that identify bacterial, fungal and yeast homologues in mammalian mitochondria (38; 118; 152). The transfer of genetic information from the mitochondrial genome to the nuclear genome is evidenced by the fact that over 90% of the genes coding for mitochondrial proteins exist in nuclear DNA compared to the less than 10% of genes coded by mtDNA.

2.1 Subcellular populations of mitochondria

Skeletal muscle mitochondria is distributed below the surface of the sarcolemmal membrane and interspersed between the myofibrils. Because of these two cellular locations the mitochondria can be further divided into the subsarcolemmal (SS) and the intermyofibrillar (IMF) mitochondrial subfractions. In addition, these two subfractions demonstrate differential biochemical properties such that IMF mitochondria have a higher rate of oxygen consumption, ATP content, preprotein import and enzyme activity when compared to SS mitochondria (43). SS mitochondria demonstrate a greater sensitivity to perturbations like CLFS, disease states and muscle disuse. The resulting adaptations are more pronounced in this subfraction when compared to the IMF mitochondria (3; 5; 203). In addition, SS mitochondria tend to generate more ROS when compared to the IMF mitochondria (4). Mitochondria conduct vital functions in the cell, contributing to calcium homeostasis, cholesterol synthesis, heme synthesis, providing a

steady supply of ATP and regulating apoptosis. Because of these cellular responsibilities, it is vital that the IMF and SS mitochondria are correctly assembled within the muscle fiber so that their function is not impaired.

2.2 Transcriptional regulation of mitochondrial biogenesis

Mitochondrial biogenesis is the expansion of the existing mitochondrial network leading to increased mitochondrial enzymatic activities and is mediated through a variety of pathways that remain to be fully elucidated. However, it is well accepted that the activation of a host of transcription factors causing increased gene expression is a common theme to mechanisms of mitochondrial biogenesis currently understood. A well known transcription factor, referred to as the master regulator of mitochondrial biogenesis, is peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) (117; 173; 229). PGC-1α does not directly bind to the promoter regions of nuclear genes but it binds with other transcription factors to enhance their activity in regulating the expression of nuclear genes encoding mitochondrial proteins (NUGEMPS) (171). Nuclear respiratory factor 1 and 2 (NRF-1, -2) are two known binding targets of PGC-1α, which result in the increased expression of NUGEMPS (172). Mitochondrial biogenesis also requires the increased expression of mtDNA encoded proteins. PGC-1α binding with NRF-1 results in increased expression of mitochondrial transcription factor A (Tfam), and the protein is imported into the mitochondrial matrix where it can bind to mtDNA to increase transcription of genes and mtDNA copy number (80). Although mitochondria house many enzymes and complexes, one of the most representative

measures of biogenesis is cytochrome c oxidase (COX) activity (33; 57; 91; 113) due to the fact that this large multi-subunit complex of the electron transport chain requires the assembly of ten nuclear-encoded and three mitochondrial-encoded proteins (199).

2.3 Exercise-induced mitochondrial biogenesis

Regular and frequent bouts of endurance exercise result in chronic alterations in intracellular calcium concentration and the turnover of energy stores within skeletal muscle. In addition, short-term adaptations occur in response to acute exercise yet it is evident that repeated bouts of exercise and the subsequent recovery periods confer the greatest adaptations to endurance exercise (82). Mitochondrial biogenesis is induced in order to confer skeletal muscle with an enhanced ability to generate a greater supply of ATP to meet the increased demands. The induction of mitochondrial biogenesis by calcium signalling and ATP turnover pathways is discussed further below. A summary of these mechanisms is illustrated in Figure 1.

2.3.1 Calcium-mediated mitochondrial biogenesis

Although skeletal muscle contraction is mediated by the release of calcium into the cell from the sarcoplasmic reticulum, transient changes in intracellular Ca^{2+} resulting from repeated bouts of contractile activity brings about the activation of secondary messengers in the cell. These calcium-sensitive messengers, namely the calcium/calmodulin-dependent protein kinases (CaMKs), calcineurin (CnA) and protein kinase C (PKC), coordinate signalling cascades throughout the muscle fiber which induce the activation of transcription factors important to the expression of mitochondrial

proteins involved in substrate metabolism. Calcium-mediated signaling cascades have been implicated in the up-regulation of mitochondrial gene expression as they converge on NRF-1 (148), cAMP responsive element binding protein (CREB) and PCG-1α (70; 108) in the nucleus. *In vitro* studies utilizing a calcium ionophore illustrated the role of CaMKIV on the initiation of signaling cascades upstream to CREB activation which induced mitochondrial biogenesis (56; 57; 91). Another study revealed that a mitogen-activated protein kinase (MAPK), p38, was activated by CaMKII, leading to inductions in mitochondrial protein synthesis (228). However, mitochondrial biogenesis is still intact in CamKIV null animals in response to exercise (8), suggesting that other pathways are also induced during exercise that play equally important roles in mediating mitochondrial adaptations.

2.3.2 AMP-mediated mitochondrial biogenesis

ATP turnover also mediates the induction of mitochondrial biogenesis. During sustained muscle contractions, like those in prolonged endurance exercise, available ATP is consumed while the level of AMP in the cell increases. The increase in AMP results in the activation of an energy sensor known as 5' AMP-activated protein kinase (AMPK) (59) and a growing body of evidence illustrates a direct link between AMPK activation and the induction of mitochondrial biogenesis (72; 82; 180). In response to both acute and chronic exercise, activated AMPK results in the induction of PGC-1α gene transcription and protein synthesis, in addition to promoting protein-protein interactions between PGC-1α and NRF-1 (21). AMPK knockout animals do not gain enhancements in

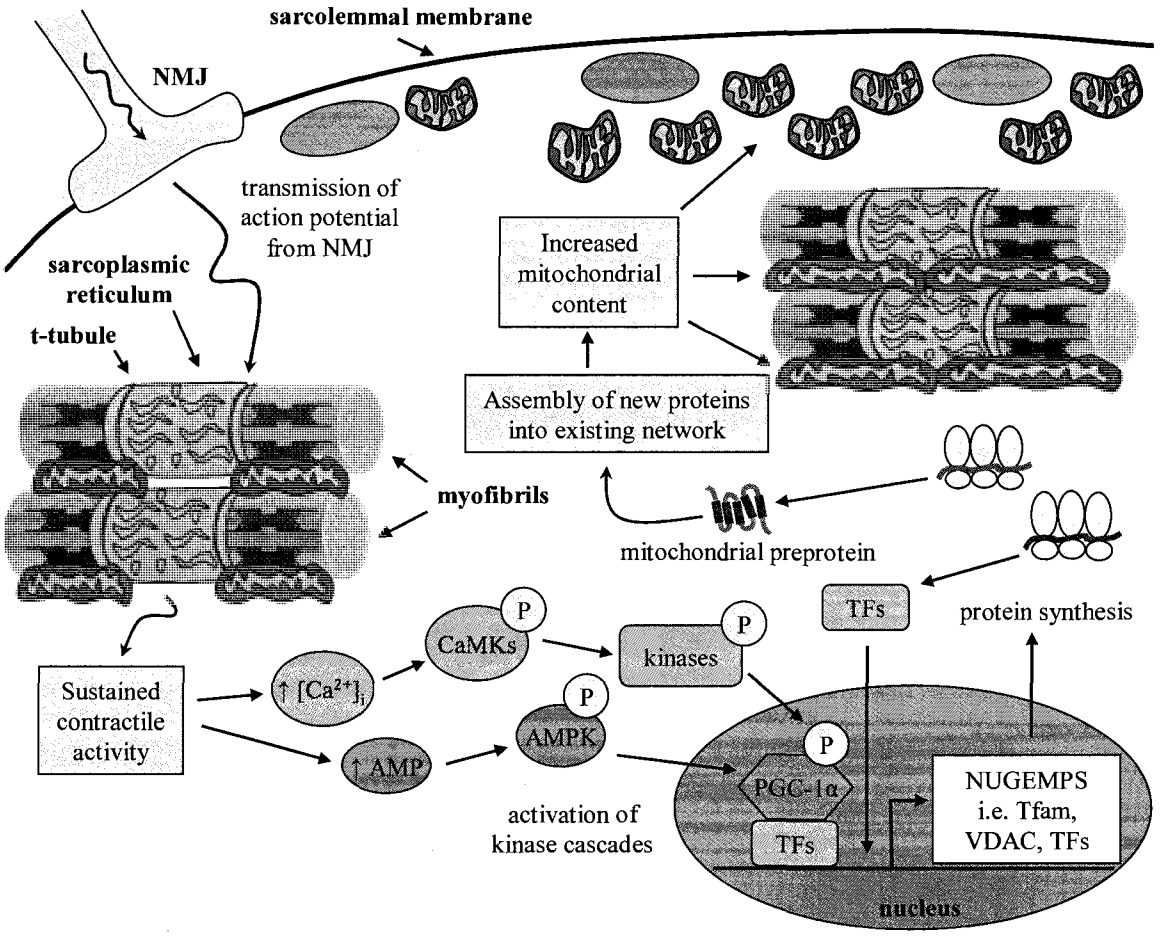

Figure 1: Mechanisms of exercise-induced mitochondrial biogenesis.

Transmission of the action potential from the neuromuscular junction (NMJ) to the t-tubule and sarcoplasmic reticulum results in an elevation in intracellular calcium concentration ([Ca2+]i) allowing for muscle contractions to occur. Sustained contractile activity results in the turnover of energy sources and an increase in AMP levels. Calcium and AMP can induce the activation of specific kinase cascades involving the CaMKs and AMPK, respectively. These kinase cascades activate downstream kinases that induce an increase in the activity of transcription factors and co-factors (i.e. PGC-1α). These factors bind to nuclear DNA to initiate the transcription of NUGEMPS and other transcription factors. The mRNA transcripts are translated and nascent transcription factors can bind to nuclear DNA to increase the expression of other mitochondrial proteins. Mitochondrial preproteins are assembled into the existing SS and IMF mitochondrial networks resulting in increased content and function. Abbreviations: AMP, adenosine monophosphate; AMPK, 5' AMP-activated protein kinase; CaMKs, calcium/calmodulin activated protein kinases; IMF, intermyofibrillar; NUGEMPS, nuclear genes encoding mitochondrial proteins; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SS, subsarcolemmal; Tfam, mitochondrial transcription factor A; TFs, transcription factors; VDAC, voltage-dependent activated channel (Adapted from Adhihetty et al., 2006).

mitochondrial biogenesis in response to conditions mimicking exercise (235) which further strengthens the role of AMPK in mediating adaptations to exercise. Electrical stimulation of muscle cells also results in AMPK activation upstream of increased PGC-1α protein expression (16). Similar effects on mitochondrial biogenesis are reproduced using pharmacological agents that induce AMPK activation (91; 147; 175; 180; 226) such as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside and β-guanidinoproprionic acid (AICAR and β-GPA, respectively) that elicit increases in the expression of Kreb's cycle enzymes (226), uncoupling protein 3 (175) and Tfam (147).

3.0 Effect of age on skeletal muscle

In the late stages of life there are changes in skeletal muscle which lead to decrements in the quality of life and independence for many of the elderly. The aging population is growing at an increased rate which has stimulated a large body of research to uncover the mechanisms that underlie the changes in muscle function with age. These changes manifest as decreases in muscle strength (1), maximal oxygen consumption (190; 209), and power (74). In addition, aging is also associated with a well established pattern of lean muscle atrophy seen over the lifetime of animals and humans that is known as sarcopenia.

3.1 Sarcopenia

The term sarcopenia has Greek derivations, meaning poverty or lack of flesh, and was described by Irwin H. Rosenberg. The most defining feature of aging skeletal muscle is an approximate 10% loss in lean muscle per decade of life that is most noticeable in the

fifth decade (184; 185). Changes in skeletal muscle CSA result in the concomitant loss in muscle strength and is most pronounced in the major muscle groups in the lower limbs containing a significant portion of type IIa and IIb fibers. In concert with the muscle atrophy there is an increase in non-muscle tissue occupancy of intramuscular fat (204) and fibrosis (30) when comparing young and old individuals (184).

The exact mechanisms leading to the loss of muscle mass and function have yet to be clearly defined although sarcopenia is hypothesized to be linked to decreased protein synthesis, anabolic hormone levels, physical activity, and to an increase in malnutrition (39), muscle denervation (193) and inflammatory cytokine levels. However, these factors may have interacting effects on sarcopenia, making the elucidation of mechanisms leading to muscle atrophy difficult to interpret. A theory gaining support suggests that sarcopenia is linked to skeletal muscle mitochondria, as they play an essential role in mediating the loss in myonuclear number via apoptosis (4; 168) and resulting in a drastic reduction in myofiber CSA (127) that may be irreversible. Thus, apoptosis of myonuclei and nuclei within other cell types in close proximity of skeletal muscle (i.e. components of the neuromuscular junction) can play an important role in remodelling aging skeletal muscle that ultimately leads to muscle fiber atrophy.

3.2 Effect of age on muscle regeneration

The loss in muscle plasticity and regenerative capacity may also be significant contributors to the decrements in muscle function as we age. For example, after periods of prolonged muscle disuse, skeletal muscle mass of aged animals is not significantly

restored to baseline values (61; 69; 234). Because of their role in muscle maintenance and regeneration it is reasonable to assume that satellite cell activation or proliferation is impaired during the aging process. Satellite cells in skeletal muscle of aged animals have a diminished capacity to proliferate in response to external stimuli (24; 61; 188). This may be due to replicative senescence (179) or increased propensity to remain in cell cycle arrest (114; 124). However, satellite cells may behave in this manner due to systemic factors within the host environment that affect the responsiveness of MPCs to hypertrophic stimuli. Heterochronic parabiosis experiments revealed that after muscle injury, regeneration of young and old skeletal muscle was restored to the same degree (44). The extent of muscle regeneration was not similar in isochronic parabiosis experiments between two aged animals. Transplantation of muscles from aged skeletal muscle into young animals also have a comparable regenerative capacity, but not vice versa, (35) indicating that systemic factors inhibit the ability of satellite cells in aged muscle to regenerate. Further research will be necessary to elucidate these systemic factors and the dependent pathways affected by age that have this profound impact on satellite cell function.

3.3 Effect of age on muscle plasticity

Declining skeletal muscle function in the elderly raises questions about whether plasticity in response to acute and chronic bouts of exercise is compromised. It is logical to hypothesize that aging skeletal muscle may fail to adapt to resistance or endurance exercise due to the decreases in protein turnover and muscle regenerative capacity.

However, when aged subjects adopted a long duration program, at an intensity of 70% VO_2max, increases in VO_2max, mtDNA content, cardiolipin and Kreb's cycle enzymes (138) were elicited. Indeed, other endurance exercise studies reveal similar adaptations in mitochondrial volume (95) and enzyme activity (41; 42) indicating that plasticity is preserved in skeletal muscle of aged individuals. Yet, the main limitation in previous studies is the lack of a comparison to the adaptive potential of young subjects. When comparing the adaptive capacity of skeletal muscle, aged skeletal muscle exhibits a slower response of adaptation to endurance activity, compared to adaptations observed in young, when the same exercise stimulus and duration are used (198). Therefore, elevations in mitochondrial enzyme activity and content do occur for aging skeletal in response to a standard exercise stimulus although not to the same degree as young.

Other studies report that aging skeletal muscle requires a longer time period to achieve the same degree of adaptations as young. In fact, studies that utilize chronic endurance activity or CLFS for periods up to 50 days show that alterations in muscle fiber transitions and mitochondrial enzyme activities between the age groups is quite similar (161; 176; 194; 195). Thus, with these data combined, it is clear that the aging population can receive the same benefits of exercise as younger counterparts so long as the exercise regime is maintained over a longer period of time to overcome an attenuated cellular response.

3.4 Mitochondria and aging

While mitochondrially-mediated apoptosis can lead to myonuclear loss, other aspects of mitochondrial function can lead to devastating effects on cellular integrity. In particular, these organelles generate almost 95% of reactive oxygen species (ROS) during cellular respiration. Thus, mitochondria are implicated in the mitochondrial theory of aging, which was first proposed by Denham Harman (73). The basis of this model purports that the generation of ROS over the lifetime induces oxidative damage of the phospholipid membranes and proteins within mitochondria. Oxidative damage has significant effects on the cellular integrity of highly metabolic, long-lived and post-mitotic tissues such as brain, heart and skeletal muscle. The effect of ROS is exacerbated by its potential to induce mutations in mtDNA, which is located in close proximity to the source of ROS generation, has no protective histones and has substantially less repair mechanisms than nuclear DNA. Thus, ROS-induced accumulations in faulty proteins, oxidized fatty acids and mutated mtDNA would result in a progressive, feed-forward, and irreversible cycle of cellular dysfunction that leads to the onset of phenotypes associated with aging.

3.4.1 The effects of mtDNA mutations and ROS on aging

Investigations into the link between aging, mtDNA mutations and ROS are guided by a few key questions. Is mitochondrial dysfunction a result of ROS generation or vice versa? Is the combined accumulation of dysfunctional mitochondria and ROS over the lifetime synergistic on the decrements in cell function causing aging? The answers

throughout the literature seem to be equivocal. ROS generation increases with age (17; 32; 34; 53; 85), is paralleled by an increase in mtDNA mutations and impaired mitochondrial function eventually leading to the downfall of skeletal muscle function (7; 11; 19; 22; 31; 36; 78; 134; 150; 196; 219; 220). Interestingly, increasing mtDNA mutations by utilizing genetically altered mice lacking DNA polymerase gamma (POLG) activity resulted in early onset of aging related phenotypes and severe deficiency in ATP synthesis but occurring in the absence of increased ROS production or DNA damage (112; 210). If deficient DNA POLG activity is a potential culprit behind aging, it would be interesting to determine if ectopically enhancing DNA POLG activity results in extended lifespan in normal animals. Importantly, these studies revealed clear indications of elevated apoptosis in the mutator mice. This study does not fully discount the contributions of ROS on aging as the artificial mutation setting did not investigate whether normal aging resulted in elevated levels of oxidized DNA POLG. In addition, ROS can cause disruptions involved in pathways responsible for mitochondrial morphology, such as the assembly of newly synthesized proteins encoded by nuclear DNA (18). Future research focusing on the link between mitochondrial morphology and ROS, in addition to their relationship with aging may be fruitful. A recent study illustrated a definite role for ROS in producing mutations and mitochondrial dysfunction (126). However, this model did not illustrate any cumulative or synergistic effects of dysfunctional mitochondria and ROS on lifespan. As a result of the abovementioned studies, there is much criticism regarding the mitochondrial theory of aging (49; 177).

However, the associations between dysfunctional mitochondria, mutations in mtDNA, apoptosis and aging remain to be strong themes in the description of mechanisms causing sarcopenia.

3.4.2 Effect of age on mitochondrial function and content

The changes in mitochondrial content and function with age have been widely documented in the literature. The role of mitochondria in promoting sarcopenia was uncovered by studies showing that muscle fibers containing dysfunctional mitochondria were atrophied compared to fibers that did not (7; 78). Histochemical analyses of skeletal muscle reveal an increase in ragged red fibers exhibiting elevated levels of succinate dehydrogenase and low content of cytochrome c oxidase (15). Functional analyses reveal decreased activity of electron transport chain (ETC) complexes, respiratory rate, ATP synthesis (126; 190) and activities of enzymes that play important roles in fatty acid and glucose metabolism (190). In addition, mitochondrial mRNA content (222) and protein synthesis (183; 190; 209) is reduced along with the decrements in mitochondrial volume and mtDNA copy number with age. However, other reports illustrate some contrasting findings in the age-related changes in mitochondrial content. Mitochondrial protein expression and mtDNA copy number increase with age (129), while other groups state modest or no change in the activities of mitochondrial enzymes found in aging skeletal muscle (123). These differential results may arise from the lack of consistency of animal age comparisons, animal strains, tissue preparation methods and representation of the data.

4.0 Degradation of existing mitochondria

Several pathways play a housekeeping role in degrading dysfunctional mitochondria, ensuring that faulty proteins do not accumulate which can lead to impaired bioenergetics of the organelle. These degradation processes include the ubiquitin-proteasome pathway, selective autophagy of mitochondria (mitophagy) and the intramitochondrial ATP-dependent proteases. Through these mechanisms, mitochondria and selected components of the organelle can be repaired or eliminated to maintain cellular function and integrity. A summary of these degradation pathways is illustrated in Figure 2.

4.1 Ubiquitin-proteasome pathway

The ubiquitin-proteasome is a well-characterized system which degrades proteins in the cell, is found throughout all tissues and has been implicated as a means of degrading unnecessary mitochondrial proteins. This degradation pathway is important during the removal of mitochondria during mammalian embryo fertilization. Mitochondria located within sperm are selectively tagged with ubiquitin protein after fertilization is complete, leading to the degradation of this organelle by the 26S proteasome (144; 201). The resulting genome of the fertilized embryo contains only maternal mitochondrial DNA. A more recent role of ubiquitin has been discovered for mitochondrial fission events as a mitochondrial E3 ligase, MARCH5, is required for dynamin related protein 1 (Drp1)-dependent mitochondrial fission (99).

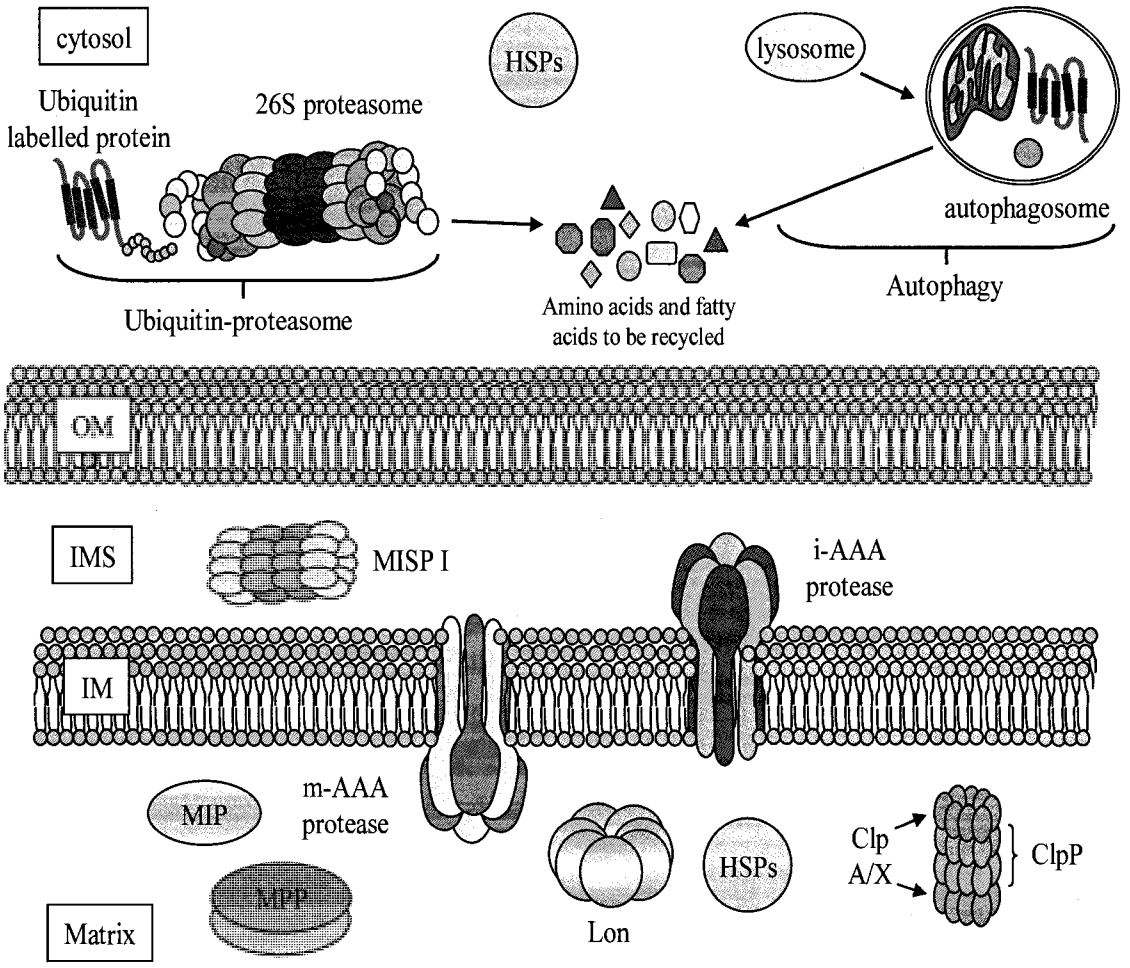

Figure 2: Pathways and proteins involved in mitochondrial turnover.

Several processes are involved in the repair or removal of damaged mitochondrial proteins. Located in the cytosol are the autophagy and ubiquitin-proteasome pathways which break down mitochondria and other proteins into basic amino acids and fatty acids while heat shock proteins (HSPs) can refold damaged proteins. Intramitochondrial proteases also process proteins that are either dysfunctional, damaged or in excess. In the intermembrane space (IMS) are the MISP1 and i-AAA proteases. In the mitochondrial matrix are the m-AAA, Lon and ClpP-A/X proteases. MIP and MPP are proteases involved in the proteolytic cleavage of imported matrix proteins. Similar to the cytosolic forms, HSPs can refold proteins within the matrix into their functional forms. Abbreviations: HSPs, heat shock proteins; IM, inner membrane; MISP1, mitochondrial intermembrane space protease 1; MIP, mitochondrial intermediate peptidase; MPP, mitochondrial processing peptidase; OM, outer membrane (Adapted from Leidhold and Voos, 2007).

Finally, it has been suggested that the proteasome may be recruited to eliminate fragmented mitochondrial proteins that cannot be incorporated back into the existing network. Inhibition of the proteasome has the potential to promote cell death, through caspase-dependent apoptosis and depolarization of the mitochondrial membrane potential (51). Thus, the ubiquitin-proteasome pathway serves as a vital function used by the cell to remove unwanted gene products to protect viability.

4.2 Mitophagy

Selective mitochondrial autophagy, or mitophagy, involves the recruitment of vesicles and lysosomes which completely surround damaged or dysfunctional organelles for their removal by hydrolytic digestion. This process most likely serves as a protective mechanism by the cell to eliminate any faulty mitochondria which, if left alone, could cause impaired substrate metabolism and oxidative damage. In addition, new research reveals that the induction of mitophagy may influence cell survival when apoptosis is impaired (40; 231). A number of contributors are important to this process including the autophagy-related gene proteins (ATG proteins), beclin-1 and LC3 (178). In the initial stages, ATG5 and ATG12 proteins mediate the formation of the isolation membrane around the targets to be degraded. Beclin-1 and LC3 are recruited in the process to further elongate and form the membrane to completely surround the internal contents. The completed double-membrane structure and its constituents are referred to as the autophagosome. Subsequently, lysosome fusion to the autophagosome occurs, allowing

for the delivery of hydrolases which degrade the internal contents and rendering amino acids and fatty acids for recycling (135).

Current research suggests that mitophagy is regulated by specific factors in the cell. The opening of the mitochondrial permeability transition pore (mtPTP) is linked to the formation of autophagosomes, followed by subsequent removal of the depolarized mitochondria during states of nutrient deprivation (182). Blocking the opening of the mtPTP using cyclosporin A causes a suppression of mitophagy (100). The exact mechanisms occurring after mtPTP opening that influence mitophagy remain unclear. Another indication that mitophagy is a selective process is the identification of a novel protein named Uth1p which is localized to the outer membrane of mitochondria undergoing mitophagy. In yeast, the deletion of the Uth1p gene results in decreased removal of mitochondria by mitophagy and decreased longevity (102; 103). These results suggest that mitophagy is not a random process and may protect cells from premature aging and mortality.

4.3 ATP-dependent proteases

Another class of cellular proteases, named the ATP-dependent proteases because of their requirement of ATP to catalyze degradation, include Lon, Clp, AAA-metalloproteases and MISP1. These proteases and their exact functions are only partially understood as the literature has focused on the bacterial orthologues, while the mammalian homologues are beginning to be uncovered.

The Lon protease shows substrate specificity for oxidatively damaged aconitase, a mitochondrial matrix enzyme (27). By recognizing minor damage to aconitase and possibly other matrix enzymes, Lon protease degrades damaged proteins. Lon has been identified in a wide variety of tissues, including liver, brain, cardiac and skeletal muscle (121; 205). This protease may be important to the maintenance of mitochondrial function as its activity enhances mitochondrial biogenesis (122) and mtDNA replication (58). In addition, down-regulation of Lon has detrimental effects on cell viability, through rampant caspase-3 activation, leading to increased cell death (28).

When products of the nuclear or mitochondrial genome are in surplus, the second class of proteases, the AAA metalloproteases, quickly process extra proteins, thereby preventing aggregation of unassembled products to maintain organelle integrity. There are two well characterised members of the AAA metalloprotease family, the m-AAA protease and i-AAA protease (115). These function on the matrix and intermembrane space sides of the inner membrane, respectively. The m-AAA protease structural arrangement consists of a hexameric complex of paraplegin and AFG3L2 proteins. These subunits may assemble into either homo-oligomeric (AFG3L2) or hetero-oligomeric complexes (AFG3L2 and paraplegin) (107). The i-AAA protease has a similar arrangement to the m-AAA protease, consisting of Yme1 proteins in a homo-hexameric complex. This complex degrades subunits of cytochrome c oxidase and also has the ability to bind to misfolded proteins to promote their insertion into the inner membrane (67).

The third member of this ATP-dependent protease family is the Clp protease complex, which is formed by two proteins serving different functions. The components of this complex are ClpP and ClpA/X, the substrate recognition and ATP-hydrolyzing units, respectively. In this arrangement, this complex is analogous to the 26S proteasome and degrades proteins within the matrix (128). Finally, the last member of the protease family is a protein named mitochondrial intermembrane space protease (MISP1). MISP1 has characteristics of degradation very similar to the 20S proteasome, and cross-reacts with an antibody for the 26S subunit. Similarities of its peptide sequence to that of the AAA-protease family, particularly the i-AAA protease, have also been identified (26).

5.0 Mitochondrial protein import

The mitochondrion is a unique organelle as it contains its own DNA (mtDNA) which encodes proteins of the ETC. The human mtDNA genome contains 16,569 kilobases, which encode 13 mitochondrial proteins, 22 tRNA and 2 rRNA genes (202). However, mtDNA encodes for approximately ten percent of the total mitochondrial proteome. As hundreds to thousands of proteins make up the mitochondrion (151; 169; 170), the nuclear genome must account for all other mitochondrial proteins. Thus, the two genomes must coordinate in response to many cellular perturbations to orchestrate the precise assembly of new mitochondrial proteins in order to meet the energy needs of the organism. The task of chaperoning, processing and assembling newly synthesized mitochondrial proteins is done by the mitochondrial protein import pathway (PIM) and is likely a key regulator of mitochondrial biogenesis. Nuclear-encoded proteins destined for

the mitochondria cannot passively incorporate into the mitochondrion and must be guided through several specialized import networks (166). This post-translational event mediates the translocation of nuclear-encoded proteins into the mitochondrion for proper processing and assembly. Proteins that are destined to make up the mitochondrion can be inserted in any one of four components of the organelle: the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM) and the matrix. A summary of the components involved in the import of proteins is depicted in Figure 3.

5.1 Mitochondrial targeting signals

Proteins synthesized in the cytosol contain targeting signals which direct the preprotein towards the mitochondrial network (118; 223). The mitochondrial targeting signal (MTS) is an important component of the import pathway and two types have been identified, each having an influence on the mode of import through the mitochondrion (215; 216). The first MTS is characterized by a positively charged, N-terminal presequence ranging from approximately 10-80 amino acids in length (165) and nascent proteins containing this MTS are often destined for the mitochondrial matrix. While the presequences do not share a specific common motif it is apparent that these MTS have the ability to form α-helices, due to the presence of hydrophobic regions located in their polypeptide sequences, resulting in an amphiphatic structure (225). This amphiphatic α-helix arrangement is exposed to the aqueous cytosol, IMS and matrix environments allowing the hydrophobic bases to be protected inside the α-helix (140). The second MTS is characterized by internal targeting sequences found along the preprotein that are

hydrophobic in nature, necessitating chaperone assistance while traveling through the aqueous environments. All OM, and many of the IM proteins, contain internal signals that are recognized by the PIM. In addition to the MTS, a crucial step must take place before the precursor proteins can enter the mitochondrion. Often, these preproteins cannot pass through the import machinery in the OM unless they are in an unfolded conformation. This is mediated by the cytosolic chaperones.

5.2 Cytosolic chaperones

For preproteins synthesized in the cytosol, an unfolding process is required to make the preprotein import-competent. Hsp70, a member of the heat shock protein family, is responsible for recognizing nascent preproteins and unraveling them so that they can be recognized by other cytosolic chaperones. These chaperones will assist in directing preproteins to the PIM located on the OM. Mitochondrial import stimulating factor (MSF) is a cytosolic chaperone that binds to preproteins with N-terminal presequences and directs the preproteins to a specific OM receptor. The ATP-powered chaperone heat shock protein 90 (HSP90) is known to associate with the hydrophobic domains of preproteins containing internal MTSs (232) and delivers them to another specific receptor on the OM (86). The literature on the interactions of these chaperones with their respective preprotein is equivocal. Some report that Hsp70 directs matrix-destined precursors to the OM translocation pore without requiring cellular energy (68; 81; 83) and that MSF requires ATP to interact with preproteins containing N-terminal presequences or internal MTSs. Conversely, Young and colleagues describe that ATP-

dependent Hsp70 is a chaperone that interacts with internal MTSs (232). Others imply that only Hsp70 and Hsp90 serve chaperone roles (146), and only a handful of investigators refer to the role of MSF in the import pathway (71; 81; 106; 139; 203). While the exact knowledge of how these cytosolic factors operates still remains to be revealed, it can be concluded that chaperones are involved in assisting many precursor proteins with their import into the mitochondrion.

5.3 Co-translational import model

In addition to the post-translational model of protein import, another model exists that describes the translocation of nuclear-encoded preproteins into the mitochondria. This co-translational import model states that proteins can associate with the PIM on the OM while still being processed by ribosomes (143; 217). A signaling sequence located within the 3' untranslated region (3' UTR) of the mRNA coding for the mitochondrial protein directs the transcript to an assortment of ribosomes juxtaposed to the OM of the mitochondria. As the mRNA transcript is being translated, the growing polypeptide chain is tightly coupled with the import machinery and directed through the OM so that the preprotein can reach other components of the PIM (25). Collectively, the ribosome assembly machinery, the nascent polypeptide and the interaction with the PIM is named the ribosome-nascent chain complex (RNC) (125). This co-translational mechanism of delivering nuclear-derived gene products mediates the relatively fast import rate of proteins with largely hydrophobic regions and the import of proteins in the absence of cytosolic chaperones.

5.4 The TOM complex

Once bound to the precursor proteins, the cytosolic chaperones bring the proteins to the translocases of the outer membrane (TOM) complex. This complex exists as a multiunit network containing several proteins embedded in the outer membrane that includes Tom20, Tom22 and Tom70 as the outer membrane receptors. Tom40, Tom5, Tom6 and Tom7 are the other components of the TOM complex that are collectively known as the general import pore (GIP), with Tom40 serving as the channel through which all preproteins travel through (20). Tom7 is necessary for the initial stages of Tom40 biogenesis (94; 141). Tom20, stabilized in the OM by Tom6, recognizes MSF and presequence preproteins while Tom70 recognizes Hsp70 and Hsp90, thus accepting the preproteins containing internal MTSs (86; 232). Both receptors transfer the preprotein to Tom22. This receptor, with the assistance from Tom5 (213), directs the preproteins to Tom40 for membrane translocation towards the IMS. At this point of the import process, four divergent pathways can be taken for protein assembly in the subcompartments of the organelle.

5.5 The SAM complex

All OM proteins are synthesized in the cytosol, contain an internal MTS, and must be imported into the mitochondrion. Some preproteins can be laterally transferred into the OM by Tom40, while others, such as the β-barrel variety (i.e. VDAC and Tom40) require the transfer to another network within the OM named the sorting and assembly machinery (SAM) complex. Discovery of the SAM complex occurred when Tom40

import studies showed that the precursor traveled into the IMS first (141). Purification of the SAM complex revealed its components Sam37, Sam35, Sam50 which all interact with the new protein for its assembly. Another component, Mdm10, associates with Tom5 during the late stages of TOM complex assembly (166; 167). Conversely, Tom7 disassembles the existing TOM complex, allowing new Tom40 proteins to be assembled (137). The SAM complex is vital for assembly of the OM proteins, illustrated by the finding that knock-down expression of Sam50, the channel forming protein of the SAM complex, resulted in a drastic decline in TOM40 assembly (110).

5.6 The intermembrane space chaperones

The small translocases of the inner membrane (sTIM) chaperone proteins Tim9, Tim10, Tim8 and Tim13 are the family of IMS chaperones which facilitate the movement of preproteins through the IMS. These sTIM proteins are synthesized as proteins containing internal sequences that target them to the IMS. Assembly of sTIM proteins involves protein refolding that is facilitated through the activity of Mia40 (37; 181) and Erv1 (208). During assembly of the precursor sTIM proteins, specific complexes are formed such that Tim9 pairs with Tim10, and Tim8 pairs with Tim13 (104). Functionally, each pair serves to shield the hydrophobic domains of preproteins during transport through IMS to the IM translocase complexes with substrate specificity (84; 213). In addition, sTIM complexes serve to chaperone the OM proteins destined for the Sam complex (224).

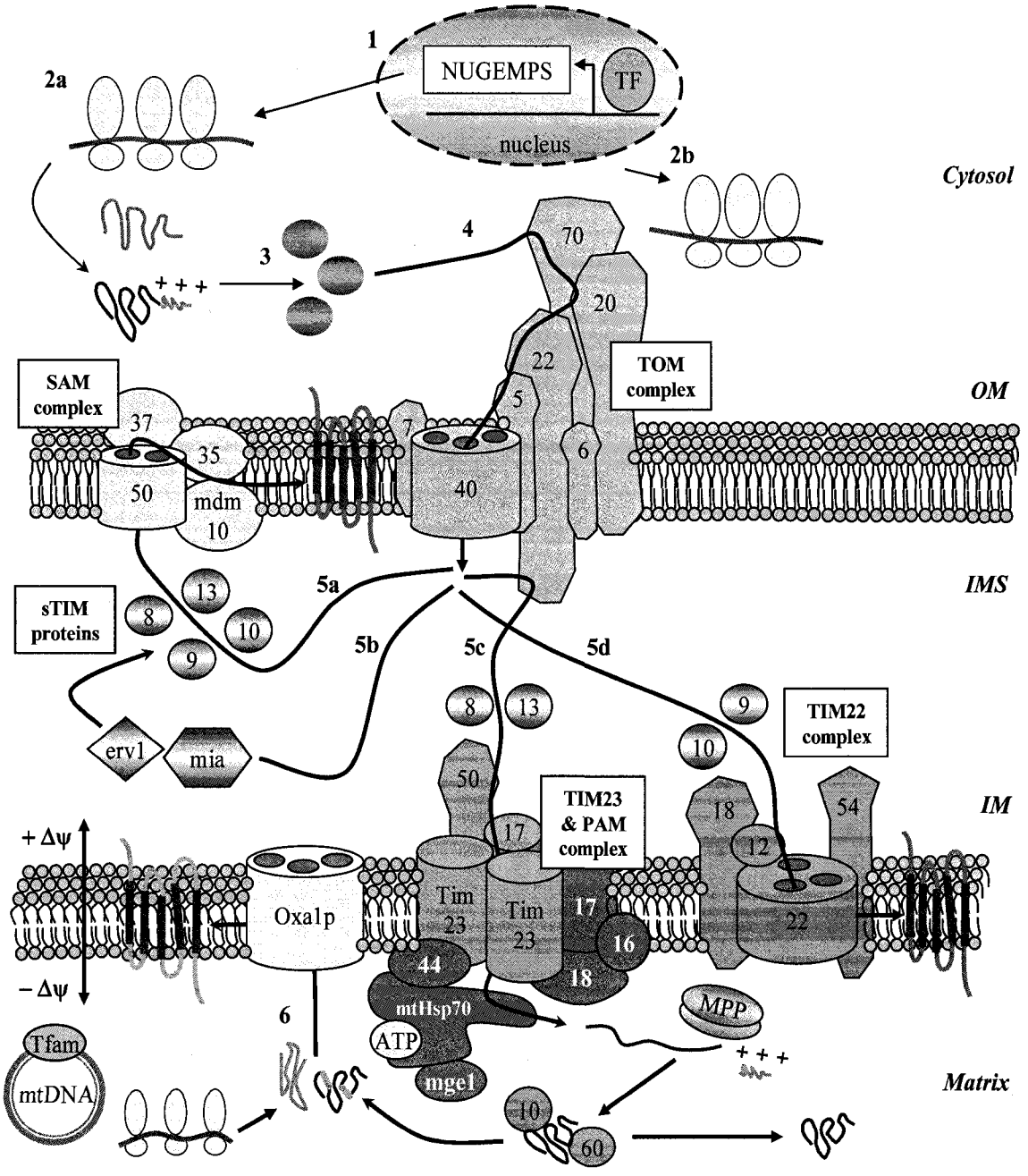

Figure 3: The mitochondrial protein import pathway.

1. Mitochondria are composed of proteins encoded by genes located in nuclear and mitochondrial DNA. *2.* After transcription, mRNA is translated on ribosomes located (a) distant to, or (b) justaxposed on the mitochondrial outer membrane. *3.* Nascent proteins bind with cytosolic chaperones for unfolding and transport to the TOM complex. *4.* Proteins are recognized by the TOM receptors and translocated into the

IMS. At this point of the import process four divergent pathways exist. *5a.* Outer membrane-destined proteins are chaperoned by sTIM proteins and carried to the SAM complex for processing and insertion. *5b.* Proteins destined for the IMS are processed by Mia and Erv1. *5c.* Matrix-destined proteins are chaperoned to the TIM 23 complex (light proteins) resulting in passage through the import channel that is driven by $\Delta\psi$. The protein is pulled into the matrix by the PAM complex (dark proteins). The leader sequence is cleaved off by MPP and then the remaining protein is refolded by heat shock proteins (10 and 60). *5d.* Proteins containing internal targeting signals are chaperoned to the TIM22 complex for lateral transfer into the IM. *6.* Proteins located in the matrix needing export into the inner membrane are assembled by Oxap1. Abbreviations: ATP, adenosine triphosphate; IM, inner membrane; IMS, intermembrane space; MPP, mitochondrial processing peptidase; mRNA, messenger ribonucleic acid; mtDNA, mitochondrial deoxyribonucleic acid; mtHsp70, mitochondrial heat shock protein 70; NUGEMPS, nuclear genes encoding mitochondrial proteins; Oxap1, oxidase assembly protein 1; OM, outer membrane; PAM, presequence-translocase associated motor; SAM, sorting and assembly machinery; sTIMS, small translocases of the inner membrane; TF, transcription factor; Tfam, mitochondrial transcription factor A; TIM, translocases of the inner membrane; TOM, translocases of the outer membrane (Adapted from van der Laan et al., 2006).

5.7 The TIM22 complex

Many of the mitochondrial proteins that contribute to the biogenesis of the IM contain internal MTSs. These preproteins are imported and assembled into the mitochondrion using the network of proteins named the Tim22 complex or carrier translocase. The transfer of these preproteins into the IM is dependent on the specific $\Delta\Psi$ across the IM (109). Precursor proteins are shuttled across the aqueous IMS by Tim9-Tim10 (116) and brought to Tim12, the component of this complex which provides a docking site for the sTIM chaperones and bound preproteins. Once the precursor protein is accepted by the Tim22 complex and if the required $\Delta\Psi$ is present, the IM protein is laterally transferred into the IM and incorporated as a functional protein. The functions of the last two components of this complex, Tim18 and Tim54, are not well understood but their structures suggest complex stability (88; 105). Additionally, a recent report demonstrated that Tim54 is essential for the assembly of the Yme1 protein of the i-AAA protease (88).

5.8 The Tim23 complex

The second translocase network in the IM is the TIM23, or presequence translocase complex, which mediates the passage of proteins containing N-terminal presequences. These MTSs are recognized by Tim50, the receptor of the complex, which then directs the protein through the translocation channel formed by Tim23 and Tim17. This translocase complex can exist in two modular forms; the $TIM23^{SORT}$ and $TIM23^{MOTOR}$ forms (214). Through the first form, preproteins are laterally transferred into the IM using $\Delta\Psi$ (142). The unique structural feature of these preproteins is a combination of the presequence MTS and internal MTS. The presequence interacts with the PAM complex and the internal MTS serves as a stop signal to arrest the protein in the Tim23 channel. Tim21 is a component of this complex that plays a predominant role during the lateral transfer and insertion of the protein, as it brings complexes along the IM in close proximity of the $TIM23^{SORT}$ complex. After lateral transfer, the preprotein is processed by the inner membrane protease 1 (IMP1) (64) ($TIM23^{SORT}$ complex is not pictured in Figure 3). Tim21 is not present in the $TIM23^{MOTOR}$ complex. Instead, this complex is tethered to the presequence translocase-associated motor (PAM) complex, and mediates the translocation of preproteins to the mitochondrial matrix. Although the Tim23 channel could provide a source for inner membrane permeability, $\Delta\Psi$ is maintained by Tim50. When preproteins are not present, the IMS domain of Tim50 interacts with the IMS domain of Tim23, thereby closing the translocation channel (136).

5.9 The PAM complex

Matrix-destined and some IM proteins must be transferred through the IM for processing before becoming functional. The translocation subunits of the $TIM23^{MOTOR}$ complex are referred as the PAM complex due to their involvement in the translocation of presequence preproteins into the matrix. Energy requirements of the $TIM23^{MOTOR}$ and Pam complexes are dependent on $\Delta\Psi$ and ATP (111). Components of the Pam complex are mtHsp70, Tim44, Pam16, Pam17, Pam18 and Mge1.

The major component responsible for translocation of preproteins into the matrix is mitochondrial heat shock protein 70 (mtHsp70), and a variety of models exist that describe the action of this protein. The Brownian ratchet model states that mtHsp70 interacts with proteins passing through the translocation channel with a tight association, preventing retro-translocation of proteins. This forces the preprotein to move forwards into the matrix due to electrophoretic forces provided by the $\Delta\Psi$ (191). The power-stroke model involves an ATP-induced allosteric change in mtHsp70 conformation, allowing it to bind and actively pull in the precursor protein through successive rounds of mtHsp70 binding (65). De Los Rios et al (50) describe another model, called entropic pulling, that combines both the Brownian and power-stroke models, stating that mtHsp70 prevents retro-translocation and pulls in the precursor protein with successive rounds of ATP hydrolysis.

The identified components of the PAM complex have a variety of interactions with the $TIM23^{MOTOR}$ complex during protein import. Tim44, an adaptor protein, brings

mtHsp70 to receive the incoming preprotein (93). Mge1 is a nucleotide exchange factor that enhances mtHsp70 activity (218). Rounds of ATP consumption by mtHsp70 and $Tim23^{MOTOR}$ complex stability is enhanced by Pam18 (213), a protein that is regulated by Pam16 (48; 55). The final key member of the Pam complex is Pam17, a protein that strongly links Pam16 and Pam18 with the $TIM23^{MOTOR}$ complex (212).

After the precursor protein is brought into the matrix by the PAM complex it can follow one of two paths. Presequence precursors become functional following their interaction with two members of ATP-independent peptidases, mitochondrial processing peptidase (MPP) and mitochondrial intermediate peptidase (MIP). These proteases recognize and cleave the presequence allowing the protein to be refolded by either Hsp60 or Hsp10 (83; 200) into its mature and active state. Cleavage of the mitochondrial targeting sequence by MPP is sufficient for most proteins, although for others, the action of MPP is followed by MIP to further modify the proteins before they are refolded (97).

5.10 The export complex

Some proteins targeted to the matrix are merely modified in the aqueous compartment before they are assembled into the IM. These IM preproteins, and proteins that are encoded by mtDNA, access a route of export in order to reach their final destination. The final complex in the IM comprises the export machinery, also referred to as the oxidase assembly (OXA) complex. Presently one component, oxidase assembly 1 protein (Oxa1p), has been identified (79) and this nuclear-encoded protein forms the export channel in the IM, mediating the insertion and lateral transfer of proteins into their

appropriate multi-subunit complex along the IM (10; 77; 89; 200). The role of this protein was identified when researchers discovered that its ubiquitous expression in the IM was essential for assembly of complexes I and IV (145).

6.0 Effect of age on mitochondrial turnover

In normal circumstances, mitochondrial turnover is a constant and purposeful process. Thus, the overall mitochondrial function and morphology depend on the balance of new protein synthesis and assembly with the degradation of damaged or improperly assembled proteins. Impairments of the ability of degradation pathways to remove whole or damaged compartments of mitochondria could lead to devastating effects on the cell. These effects likely manifest as decreased ATP synthesis, increased ROS generation, accumulated mtDNA mutations and cell death. A summary of the pathways and proteins involved in protein quality control is illustrated in Figure 4.

Interestingly, the detrimental effects of impaired protein quality control are quite similar to alterations observed in skeletal muscle of aging individuals. Mitochondrial turnover appears to be impaired with age due to deficiencies in mitochondrial protein synthesis, despite elevations in the mRNA levels. It is possible that decreased degradation of faulty proteins is a cause for age-related phenotypes in metabolically active tissues. To corroborate this hypothesis, studies have illustrated that with increasing age, Lon protease expression and activity is reduced, indicated by an accumulation of dysfunctional aconitase (27; 29). Indeed, in ATG5 null pancreatic cells, the importance of autophagy in maintaining insulin secretion and mitochondrial respiration, as well as protecting proteins

against oxidative damage by ROS, has been illustrated (211). Conversely, an imbalance of accelerated degradation of mitochondrial protein with decreased biogenesis could also serve as a regulator of mitochondrial function in aging skeletal muscle.

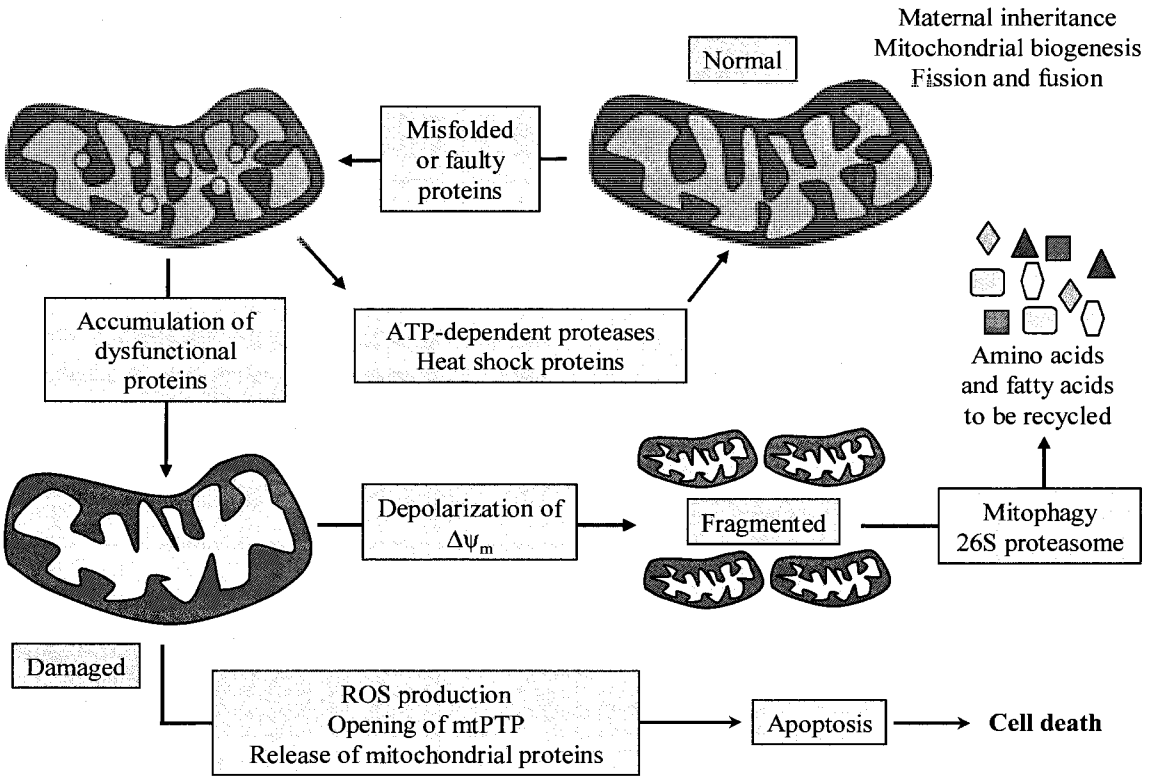

Figure 4: Protein quality control pathways involved in mitochondrial turnover.

Mitochondria are in a continuous state of remodelling due to their intimate involvement in cell homeostasis. In the event of damage, HSPs and intramitochondrial proteases (small circles within the organelle) can refold and repair faulty proteins to maintain mitochondrial function. Accumulation of damaged proteins can occur if these quality control elements fail. This may lead to the fragmentation of mitochondria that contain dysfunctional proteins from the reticular network. These mitochondria can be degraded by the autophagy or ubiquitin-proteasome pathways to obtain amino acids and fatty acids for other metabolic processes. Dysfunctional mitochondria that are resistant to degradation can generate elevated levels of ROS, inducing the opening of the mtPTP promoting the release of proteins that initiate apoptosis, which lead to cellular death. Abbreviations: $\Delta\psi$, mitochondrial inner membrane potential; HSPs, heat shock proteins; mtPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species (Adapted from Mijaljica et al, 2007).

Whether autophagy (mitophagy), the ubiquitin-proteasome pathway, and the activities of the intramitochondrial proteases are altered during aging remain to be elucidated. However, emerging research implicates a potential role for lipofuscin, a non-degradable protein, in the progressive decline in protein turnover that occurs in aging tissue. Lipofuscin localizes within vesicles throughout tissues in aged individuals, which may exhaust the availability of vesicles to form the autophagasome (87; 96; 101; 192; 206; 207).

7.0 Conclusion

The process of aging and its effects on skeletal muscle remains to be fully investigated in order to uncover the mechanisms leading to sarcopenia. The importance of mitochondria in maintaining cellular homeostasis warrants research that focuses on the age-dependent alterations in these pathways. This may provide new insight on therapeutic strategies to ameliorate skeletal muscle function and performance in the aging population.

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MANUSCRIPT

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Protein import into skeletal muscle mitochondria: effects of aging and chronic contractile activity.

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ABSTRACT

Sarcopenia is an age-related phenomenon associated with mitochondrial dysfunction, mtDNA mutations and mitochondrial-mediated apoptosis. Therapeutic strategies such as exercise could improve mitochondrial function in skeletal muscle of aging individuals. However, the processes regulating exercise-induced mitochondrial biogenesis in aged animals are not well characterized. We utilized chronic contractile activity (CCA) in aged rats to determine if changes in protein import kinetics occur in skeletal muscle, to evaluate the plasticity of subsarcolemmal and intermyofibrillar mitochondria and to identify if mitochondrial biogenesis was facilitated through changes in the rate of protein import. We also reconstituted the cellular environment to assess the influence of cytosolic chaperones on import. Mitochondrial content declined in aged, when compared to young animals. However, the import of proteins into the mitochondrial matrix was unaltered with age. We observed a 2.5-fold increase in precursor ornithine carbamoyltransferase (pOCT) degradation in cytosolic fractions obtained from senescent animals. CCA elicited 78% and 32% increases in protein import in SS and IMF mitochondria of young animals ($p<0.05$), respectively, and these adaptations were likely mediated by changes in the expression of import machinery. Adaptations in protein import and expression of machinery components were attenuated with age, as a 31% increase in import induced by CCA was evident only in SS mitochondria of aged animals. In summary, the induction of protein import in muscle due to exercise is blunted when compared to young animals, which may indicate a reduced potential for adaptation with age.

INTRODUCTION

Aging is associated with a plethora of physiological changes within skeletal muscle, such as the loss of muscle strength, mass and function (7). These alterations in skeletal muscle are cumulatively referred to as sarcopenia, involving fiber atrophy, as well as the progressive loss of fibers after the fifth decade (48; 49). It is hypothesized that some of the changes in aged skeletal muscle are due to a reduction in the number of myonuclei through apoptosis (36). An important locus for the initiation of apoptotic signaling is the mitochondrion. Within mitochondria reside proteins, which upon release from the organelle, can implement a cascade of proteolytic events that converge onto the myonuclei. A hallmark characteristic of apoptosis is the fragmentation of nuclear DNA, which compromises cell viability and ultimately leads to cell death (5). The intimate connection between mitochondrial function and the viability of skeletal muscle suggests that this organelle could play a significant role in the progression of sarcopenia. Studies reveal an inverse relationship between mitochondrial biogenesis and aging such that as an individual increases in age, mitochondrial biogenesis decreases (8; 17; 18; 34; 47; 50).

Mitochondrial biogenesis refers to the expansion of the existing organelle reticulum, leading to increases in mitochondrial volume and/or improvements in mitochondrial function within the cell (28; 30). This process requires contributions from the mitochondrial and nuclear genomes, necessitating coordinated communication so that multi-subunit protein complexes are properly assembled. Since the mitochondrial genome only codes for thirteen proteins involved with the electron transport chain, the nuclear

genome must account for the rest. Proteins encoded by nuclear DNA are synthesized in the cytosol and are subsequently imported into the mitochondrion via the protein import machinery (PIM) (54; 60; 62). Protein import requires the assistance of cytosolic chaperones, such as heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90) and mitochondrial import stimulation factor (MSF) (38; 39), which bind to and unfold the preprotein, and direct it to the translocases of outer membrane (TOM) complex of the mitochondrion. Once associated with TOM complex receptors (i.e. Tom70, Tom20 and Tom22), the preprotein can be further directed and assembled into one of the four subcompartments of the organelle.

Matrix-destined proteins contain N-terminal presequences that serve as mitochondrial targeting signals. These presequences direct the preproteins to a specialized network of translocases located in the inner membrane referred to as the Tim23 translocase complex. Preproteins are further guided into the matrix with the assistance of the presequence translocase-associated motor (PAM) complex, consisting of Pam16, Pam17, Pam18, Tim44, Mge1 and mtHsp70. The preprotein is further processed to its mature form once inside the matrix by mitochondrial processing peptidase (MPP), and refolded by the chaperonins heat shock protein 10 (Hsp10) and heat shock protein 60 (Hsp60). Considering the proportion of mitochondrial proteins that must be imported, processed and assembled into the mitochondrion by the PIM, the protein import pathway serves as a mechanism to regulate the mitochondrial content and function to match the energy needs of skeletal muscle (27). A great deal of research has been devoted to studying this

pathway in yeast and plant mitochondria, with a relatively small amount of work focused on mammalian studies (15; 16; 22; 23; 31; 32; 57; 58). Previous research from our laboratory suggests that the protein import pathway may be affected by the aging process. Craig and Hood demonstrated age-dependent increases in the rate of preprotein import into cardiac mitochondria when compared to young animals (16). In addition, the protein expression of cytosolic chaperones was elevated in aged cardiac muscle. However, whether these alterations in the import of matrix-destined proteins and the expression of machinery components also occur equally in the subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria within skeletal muscle is not yet established. Therefore the first purpose of our study was to determine whether the protein import pathway, and its associated components, was impaired in skeletal muscle of senescent animals.

It is well established that the skeletal muscle phenotype is sensitive to external perturbations such as chronic endurance activity (1) and muscle disuse conditions (51). In response to repeated bouts of endurance exercise there is an increase in the mitochondrial density and function in skeletal muscle (29), allowing for both higher oxidative capacity and fatigue resistance. The converse is true for chronic periods of muscle disuse, such that mitochondrial enzyme activities and muscle fiber cross-sectional area decrease, particularly within type II fibers (35). Previous research also suggests that muscle plasticity may be affected by the aging process. Aged animals appear to have an attenuated response to exercise, and do not adapt to the same degree as their young

counterparts (13; 47; 53). Aged muscle also exhibits characteristics of decreased muscle respiratory capacity (11), fatigue resistance, and mitochondrial enzyme activities (4; 25). As a therapeutic modality, chronic endurance exercise could attenuate these decrements in muscle function by eliciting an increase in mitochondrial biogenesis in aged animals, leading to improvements in muscle function such as a decrease in apoptotic susceptibility (2) and an increase in oxygen consumption (24). In order to investigate this within the context of aged animals, we employed an *in vivo* chronic contractile activity (CCA) protocol to provide a standard exercise stimulus known to induce mitochondrial biogenesis. The number of studies investigating the effects of CCA on aged animals is extremely small (43; 45; 52). Thus, our second objective was to determine if aged animals could adapt to a standard stimulus of CCA via changes in the mitochondrial protein import pathway, and to compare the degree of adaptation to young animals also undergoing CCA.

METHODS

Animals

Male Fischer 344xBrown Norway hybrid (F344BN) rats were studied at two different ages: 6 months of age (young) and 35-38 months of age (referred to in this study as either aged, old or senescent). These rats are ideal for the investigation of age-related decrements in muscle mass and function as they display a higher resistance to age-related pathologies, have a longer lifespan compared to other strains and most importantly, demonstrate decreases in muscle mass, average fiber cross-sectional area, and peak iosmetric tetanic tension (6). The animals were anaesthetized with a ketamine and xylazine cocktail (0.2 ml/100 g weight) and the tibialis anterior (TA) and extensor digitorum (EDL) muscles were excised from the hindlimb for mitochondrial isolation and electron microscopy, respectively. The *in vivo* chronic contractile activity model employed another group of F344BN rats studied at similar ages. Young and senescent animals were surgically prepared with an implantable stimulator within the peritoneal cavity. Wires leading from the stimulator were tunneled subcutaneously and sutured adjacent to the common peroneal nerve innervating the left TA and EDL muscles. The right hindlimb served as the non-stimulated internal control muscle for this study. The animals were allowed to recover for approximately one week prior to the onset of CCA. The CCA stimulation protocol consisted of impulses delivered at 10 Hz (duration of 0.1 msec) for 3 hours each day over 7 days. After 7 days, the animals were sacrificed and the TA and EDL of both the control and stimulated hindlimbs were harvested for

mitochondrial isolation and electron microscopy, respectively. For the import assays supplemented with the cytosolic fraction, we chose to utilize male Sprague-Dawley rats ranging from 300-350 g were utilized (2.25-2.50 months of age). Thus, for each import assay the mitochondria were obtained from the same animal in order to evaluate the effect of the cytosolic fraction on pOCT import. Tibialis anterior muscles were extracted and processed using the methods described above to obtain IMF mitochondrial subfraction.

Muscle extraction, cytosolic fractionation and mitochondrial isolation

The mitochondria corresponding to the young and aged F344BN, and young Sprague-Dawley rats were isolated with homogenization and differential centrifugation as described previously (12). Briefly, the TA muscle was cleaned of connective tissue and fat, minced briefly, weighed and then homogenized. The SS and IMF mitochondrial subfractions were obtained by differential centrifugation and each pellet was resuspended in a medium containing 100 mM KCl, 10 mM MOPS and 0.2% BSA. Freshly isolated mitochondria were used for *in vitro* protein import assays and aliquots of mitochondrial extracts were stored at -20°C for immunoblotting analyses. To obtain the cytosolic fraction, the supernate was removed after the final centrifugation used to isolate the SS mitochondria and centrifuged at 100,000 *g* for 1 h at 4°C. The supernate was concentrated in an ultrafiltration cell with a molecular weight cutoff at 10 kDa (Amicon, Beverly, MA) to a volume of <1 ml. The cytosolic fraction was stored at -20°C for import and degradation assays, and immunoblotting analyses. The protein concentration values of

isolated mitochondria and cytosolic fractions were determined with the Bradford method (64).

Electron microscopy

The stimulated and contralateral control EDL muscles were excised and cut at mid-belly to obtain 2-3 mm serial sections. Muscle samples were incubated on ice for 1 hour in 3.0% glutaraldehyde buffered with 0.1 M sodium cacodylate. Sections were then washed three times in 0.1 M sodium cacodylate buffer before being post-fixed for 1 hour in 1% osmium tetroxide in 0.1 M sodium cacodylate at room temperature. Muscle sections were then dehydrated by washes with 30%, 50%, 80% and 100% ethanol and then in ethanol-propylene oxide for 1 hour, followed by 100% propylene oxide for 1 hour. Subsequently, muscle sections were left overnight in a propylene oxide-Epon resin mixture in a glass dessicator. Groups of muscle fibers were then dissected from the sections, embedded in fresh resin and incubated at 60°C for 48 hours. Ultrathin sections (60 nm) were cut, collected on copper grids, stained with uranyl acetate and lead citrate. Electron micrographs were obtained using a Philips EM201 electron microscope.

DNA isolation and In vitro transcription

The plasmids containing the full-length cDNAs encoding precursor ornithin carbamoyltransferase (pOCT) and precursor malate dehydrogenase (pMDH) were isolated from bacteria using an alkaline lysis method. The cDNAs resulting from this preparation were linearized with Sac I (pOCT) and BamHI (pMDH) at 37°C for 2 hours. Plasmid DNA was extracted with phenol and precipitated in ethanol overnight at -80°C.

DNA, at a final concentration of 0.8 μg/μl, was transcribed with SP6 RNA polymerase, ribonucleoside triphosphate substrates and a cap analog $m^7G(5')ppp(5')G$ at 40°C for 90 min. The mRNA was extracted with phenol and precipitated in ethanol at -80°C overnight. The mRNA pellet was resuspended in sterile distilled water and adjusted to a final concentration of 2.8 μg/μl. Aliquots were stored at -20°C for use in *in vitro* import assays.

In vitro translation and protein import

The pOCT and pMDH mRNAs were translated and labeled with the use of a rabbit reticulocyte lysate system in the presence of [^{35}S]-methionine. Freshly isolated SS and IMF mitochondria and the translated radiolabeled precursor proteins were equilibrated separately at 30°C for 10 min. The translated precursor proteins were added to the mitochondrial samples and incubated at 30°C to initiate the protein import reaction. Final import reactions consist of 25 μg of mitochondria and 12 μl of the lysate containing the radiolabeled precursor proteins. Equal aliquots of the import reaction were withdrawn at 0, 5, 10 and 20 min to determine basal pOCT and pMDH import rates of the young and senescent animals. Equal aliquots of the import reaction were withdrawn at 5 and 20 min to obtain import rates in mitochondria from young, senescent, control and CCA conditions. For import reactions containing the cytosolic fraction, the translation products were preincubated with 0 (control), 7.5 or 15 μg of cytosol for 10 min at 30°C before the addition of freshly isolated IMF mitochondria to initiate the import reaction. Mitochondria were then recovered by centrifugation through a 20% sucrose cushion for

15 min at 4°C. Pellets were resuspended, lysed and then separated using either 8% (OCT) or 12% (MDH) SDS-PAGE. After electrophoresis, gels were boiled for 5 min in 5% TCA, rinsed for 30 seconds in distilled water, followed by rinsing in 10mM TRIS (5 min) and 1 M sodium salicylate (30 min). Gels were subsequently dried for ~ 1 hour at 80°C and exposed overnight to a Kodak Phosphor screen. Total intensities were quantified using the Quantity One software (Bio-Rad laboratories). The extent of import was measured as the percent of processed mature protein (mOCT and mMDH), relative to the total protein available, per minute of the import incubation.

Degradation of pOCT

Radiolabelled pOCT was incubated with cytosol isolated from young and senescent animals over 120 minutes at 30°C. The final reaction contained 2.5 µl of the lysate containing the radiolabeled precursor proteins and 150 µg of cytosolic proteins. At 0, 30 and 120 min time points, equal aliquots were withdrawn and placed on ice to establish time- and age-dependent degradation of pOCT. Samples were then prepared for SDS-PAGE and subjected to autoradiography. The Quantity One software was employed to obtain total intensities and the extent of degradation was measured by comparison to the time-matched control condition where equal volumes of vehicle were incubated with the pOCT.

Immunoblotting

Mitochondrial extracts (25-35 µg of protein) were separated by 12% SDS-PAGE and then transferred onto a nitrocellulose membrane. Membranes were blocked in 5% skim

milk for one hour and then incubated overnight at 4°C with primary antibodies directed against Tim23 (1:250), Hsp60 (1:1000), mitochondrial Hsp70 (mtHsp70; 1:1000), adenine nucleotide translocase (ANT; 1:7500), Hsp70 (1:1000), Hsp90 (1:1000), MSF-L (1:2500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000). Membranes were probed with horseradish peroxidase-conjugated rabbit, mouse or goat anti-rabbit secondary antibodies. Proteins were visualized using an enhanced chemiluminescence kit, and quantified densitometrically using SigmaGel software (Jandel Scientific, San Rafael, CA). Loading in the mitochondrial and cytosolic fractions were corrected by the total intensities of corresponding ANT and GAPDH, respectively. Antibodies were acquired from Abcam (GAPDH) and Stressgen Bioreagents (mtHsp70, Hsp60, Hsp70, Hsp90). Antibodies for ANT and MSF-L were purchased from Dr K. B. Freeman (McMaster University, Ontario) and Dr. K. Mihara (Kyushu University), respectively.

Statistics

All data are expressed as means ± SEM. GraphPad Prism 4 computer software was used to perform statistical analyses. The two-way ANOVA was used to analyze all import assays, PIM protein expression, and the effect of CCA. The unpaired Student's t-test was used for the protein expression analyses of basal cytosolic chaperones and degradation assay data.

RESULTS

Import of preproteins into SS and IMF mitochondria is not reduced with age

Autoradiograms illustrating the extent of pOCT protein import into IMF and SS mitochondria isolated from young and senescent animals are shown in Fig. 1A. Protein import into SS mitochondria was not different between age groups (Fig 1B). However, import of pOCT was 16% higher ($p<0.05$) in IMF mitochondria isolated from senescent, when compared to young animals. Similar to our previous report (58), protein import was approximately 53% and 72% greater in IMF mitochondria ($p<0.05$) than in SS mitochondria isolated from young and senescent animals, respectively. Autoradiograms illustrating the extent of pMDH protein import into IMF and SS mitochondria isolated from young and senescent animals are shown in Fig. 2A. There was no effect of age on the import of pMDH into either the SS or IMF mitochondria (Fig. 2B). The import of pMDH was 37% higher in IMF mitochondria when compared to SS mitochondria, however this effect was only seen in young animals ($p<0.05$).

Components involved in the preprotein import pathway are not affected by age

Tim23, mtHsp70 and Hsp60 are PIM components which are intimately involved in the translocation process of matrix-destined proteins. Representative western blots of these proteins are shown in Fig. 3A. Quantification of repeated experiments revealed there were no significant changes in the expression Tim23, mtHsp70 or Hsp60 in SS mitochondria in response to age (Fig. 3B) when compared to young animals. These findings were similar in the IMF mitochondria (Fig. 1. additional data; appendix B). In

addition, there was no difference in the expression levels of these proteins between SS and IMF mitochondria within both young and old animals.

Cytosolic chaperone expression is affected by age

Cytosolic chaperones facilitate the import of proteins containing presequence and internal mitochondrial targeting signals (38; 39; 60). We hypothesized that the expression of Hsp70, Hsp90 and MSF-L would be affected by age. Representative western blots of these chaperones are shown in Fig. 4A. Quantification of experiments revealed that the expression of MSF-L was 97% higher ($p<0.05$) in cytosol isolated from senescent, when compared to young animals (Fig. 4B). There were no age-dependent effects in the protein expression profiles of Hsp70 and Hsp90.

Import of pOCT is reduced in the presence of the cytosolic fraction

The elevated expression of MSF-L may be an indication that the protein import process is enhanced in aged animals. In addition, we have previously reported that factors present in the cytosolic fraction can influence the rate of protein import measured *in vitro* (15; 57). To evaluate these in the context of aging, we measured protein import in the presence and absence of cytosolic fractions isolated from muscle of young and senescent animals. Representative autoradiograms and quantification of repeated experiments are shown in Fig. 5. The addition of 7.5 μg and 15 μg of cytosolic proteins, isolated from skeletal muscle of young animals, resulted in 28% and 47% decreases, respectively, in pOCT import into IMF mitochondria when compared to control (Fig. 5B; $p<0.05$). We observed that the addition of 7.5 μg and 15 μg of cytosolic proteins, isolated from skeletal

muscle obtained from old animals, resulted in 35% and 50% decreases, respectively, in pOCT import into IMF mitochondria when compared to control (Fig. 5B; $p<0.05$). However, we did not detect a significant effect of age.

Degradation of pOCT by cytosolic proteins is dependent on age

To understand the cause of the inhibition of preprotein import by cytosolic proteins, we aimed to determine whether pOCT was sensitive to degradation factors found in the cytosol from young and senescent skeletal muscle. pOCT was incubated with cytosolic fractions for up to 120 minutes to assess the extent of degradation. Representative autoradiograms are shown in Fig. 6A. Quantification of experiments revealed a 2.5-fold greater rate of pOCT degradation in the cytosol obtained from aged animals when compared to young ($p<0.05$; Fig. 6B).

Effect of CCA on pOCT import into mitochondria is different between age groups

We then investigated the effect of CCA on protein import in both young and old animals. Autoradiograms illustrating the extent of pOCT protein import into SS and IMF mitochondria isolated from young and senescent animals are shown in Fig. 7A. CCA elicited 78% and 31% increases in protein import in SS mitochondria isolated from young and old animals, respectively ($p<0.05$; Fig. 7B). In the IMF mitochondria, import of pOCT into IMF mitochondria isolated from young animals increased by 32% ($p<0.05$; Fig. 7C) in response to CCA when compared to control. This effect of CCA was not observed in IMF mitochondria isolated from senescent animals.

Expression of PIM components is enhanced by CCA in young animals

To examine the mechanisms underlying the differential effect of CCA on protein import in young and old animals, we measured the expression of key components of the PIM. Representative western blots of Tim23 and mtHsp70 are shown in Fig. 8A. Quantification of experiments revealed that CCA induced 2.1-fold and 1.9-fold increases in the expression of Tim23 and mtHsp70, respectively, in SS mitochondria isolated from young animals (Fig. 9B; $p<0.05$). CCA also induced 52% and 73% increases in the expression of Tim23 and mtHsp70, respectively, in the IMF mitochondria isolated from young animals when compared to control (Fig. 9C; $p<0.05$). In contrast, there was no effect of CCA on Tim23 and mtHsp70 protein expression in either mitochondrial subfraction isolated from senescent animals, when compared to control.

Expression of cytosolic chaperones is not affected by CCA

To evaluate the affect of age and CCA on chaperone expression, we measured the levels of cytosolic Hsp90 and MSF-L. Representative western blots are shown in Fig. 9A. No effect of CCA on the expression of these chaperones was observed in either young or old animals. However, the expression of MSF-L was approximately 2.0-fold higher in senescent (Fig. 9B, $p<0.05$), when compared to young animals. A similar pattern was observed for Hsp90, as the level of expression was 45% greater in the cytosolic fraction of aged animals, compared to their younger counterparts (Fig. 9B, $p<0.05$).

Mitochondrial content is enhanced with CCA

We employed transmission electron microscopy to assess changes in mitochondrial content occurring in response to both age and CCA. As expected, mitochondrial content was higher in young animals (Fig. 10A) compared to aged animals (Fig. 10C). CCA induced large increases in mitochondrial content in both young and old animals (Fig. 10B and 10D, respectively). Within the IMF mitochondria of young and old animals, CCA resulted in a restructuring of the mitochondrial network, evidenced by reticular-like structures of mitochondria between the myofibrils (Fig. 10B and 10D) when compared to their respective controls.

DISCUSSION

Adverse and widespread reductions in muscle function and quality are age-related phenomena collectively known as sarcopenia. Proposed mechanisms of sarcopenia include denervation of muscle fibers via degeneration of the α-motoneuron, decreased satellite cell number, reduced regenerative capacity, decreased protein synthesis and diminished levels of circulating hormones (19; 41; 44; 55; 61). However, recent associations between elevated mitochondrial dysfunction, mitochondrial DNA (mtDNA) mutations and mitochondrial-mediated apoptosis in sarcopenic skeletal muscle fibers have lent support to an important role for mitochondria in contributing to this loss of muscle mass (3; 9; 35). Decreases in mitochondrial content and function are evident in aged skeletal muscle (4; 10; 16; 33; 37; 46; 50). This may be caused by an impairment of the mechanisms responsible for maintaining or augmenting mitochondrial content and function, otherwise known as mitochondrial biogenesis. Biogenesis of mitochondria is achieved through the increased transcription and translation of mitochondrial proteins that are encoded by both the nuclear and the mitochondrial genomes. Additionally, the incorporation of new proteins into the appropriate organelle compartment is mediated by a highly specialized series of complexes, cumulatively referred to as the mitochondrial protein import pathway (PIM) (60). Because of the importance of this pathway in mediating the translocation of approximately 1500 proteins, it plays a key role in the overall synthesis of the mitochondrial network. Impaired import of new proteins may lead to faulty multi-subunit complex assembly resulting in defective mitochondria that

are inefficient in producing ATP, have enhanced ROS production, and are more susceptible to apoptosis. Evidence suggests that these are characteristics of mitochondria from aging muscle (8-10; 14), which could contribute substantially to the sarcopenia of aging. Thus, we investigated whether skeletal muscle undergoing sarcopenia would exhibit decrements in the mitochondrial protein import pathway.

We have recently reported that aged animals exhibit declines in muscle mass and peak tetanic force, confirming the extent of sarcopenia in our senescent animals (10). In addition, cytochrome oxidase activity is reduced by 30% (10). As shown in the present study, electron microscopy verified that mitochondrial content was reduced in senescent, when compared to young animals. Despite these decrements in mitochondrial content, our data reveal that the import of new proteins into the matrix remains intact with age. Interestingly, a modest, age-dependent increase in pOCT import in IMF mitochondria was noted, an effect that has been previously reported in cardiac mitochondria (16). Within mitochondria, several factors are known to affect protein import into the organelle. These include 1) expression of the PIM, 2) the rate of ATP synthesis, and 3) the mitochondrial membrane potential ($\Delta\psi$) (57-59). Coincident with the similar rates of protein import between young and old animals, our analyses revealed that the content of selected PIM components (Tim23, mtHsp70, Hsp60) was not affected by age. Thus, the age-dependent elevation of pOCT import in IMF mitochondria is not mediated by alterations in these proteins, although the expression of other components (i.e. the Tom and PAM complexes) could be altered in response to age. In addition, the rate of ATP

synthesis, as reflected by State 3 and State 4 respiration, is greater in IMF mitochondria compared to the corresponding SS subfraction (10) which could account for the differential rates in protein import measured between these organelle subfractions. However, we have shown that respiration rates in the presence of glutamate and ADP were unaffected by age (10). Finally, the inner membrane potential ($\Delta\psi$), which serves to drive the positively charged N-terminus of the preprotein towards the negatively charged matrix environment, could influence the rate of protein import. We observed an age-associated 50% decrease of the $\Delta\psi$ in the SS mitochondria in these senescent animals, while the $\Delta\psi$ was unchanged with age in the IMF subfraction (10). Since pOCT import was not reduced in SS mitochondria from aged animals, we presume that the $\Delta\psi$ remained sufficient to support the insertion of pOCT into the matrix compartment in this subfraction.

In vivo, mitochondrial preproteins interact with cytosolic chaperones that primarily mediate the unfolding and transport of newly synthesized proteins. Thus, within the intact cell, another locus of regulation on protein import lies within the cytosol. We therefore investigated whether the content of selected cytosolic chaperones was altered during senescence. Our observations indicate that the expression of MSF-L was elevated in aged animals, suggesting that the capacity for cytosol-mediated precursor protein movement toward the organelle is augmented with age. When considered alongside the maintained rate of protein import in mitochondria from aged animals, these data indicate the possibility of a compensatory up-regulation in protein import in aged animals. However,

the final content of mitochondrial proteins within the organelle is also a function of the rate of degradation of newly synthesized cytosolic precursor proteins. When we reconstituted the cellular environment consisting of mitochondria and cytosolic fraction, we observed that there was an impairment of pOCT import in both young and old animals. This could be mediated by the degradation of mitochondrial precursor proteins in the cytosol by the ubiquitin-proteasome system (40; 56; 63). We noted that the degradation of pOCT was time-dependent, and proceeded at a greater rate in the cytosolic fraction obtained from aged animals. This could contribute to the inhibition of protein import observed in the presence of cytosol, and could attenuate the potential benefit of the higher MSF-L expression observed in senescent animals. Alternatively, the inhibitory effect of cytosol on protein import could also be due to the presence of factors which promote refolding or aggregation of precursor proteins, making them import incompetent. This possibility remains to be explored.

Sarcopenia could be reversed through treatment modalities known to improve the quality of skeletal muscle. For example, chronic endurance exercise is a potent stimulus for inducing a number of cellular alterations leading to enhanced mitochondrial content, muscle oxygen consumption, fatigue resistance and decreased apoptotic susceptibility (2; 20; 21; 29; 42). These beneficial adaptations could confer positive benefits to skeletal muscle of aged individuals. However, whether endurance exercise can enhance mitochondrial biogenesis via alterations in the import pathway in sarcopenic skeletal muscle is not currently known. To investigate this, we also evaluated the role of exercise,

in the form of chronic contractile activity (CCA) on the plasticity of skeletal muscle in aged animals. CCA, rather than locomotory exercise, was employed to ensure that both age groups experienced the same frequency, intensity and duration of exercise, independent of whole animal behavioural characteristics. We have previously shown that CCA induces appreciable increases in the rate of protein import into both SS and IMF mitochondria (22; 26; 57), along with an increase in COX activity and mitochondrial content (2).

Since we have shown that COX activity (10) and mitochondrial content (Fig. 10) are reduced in senescent animals, we wished to determine whether CCA could reverse this decline in aged animals. We observed that 7 days of CCA resulted in a pronounced increase in the SS mitochondrial content, while there was a modest increase in the IMF mitochondrial content in aged animals. In addition, CCA elicited 30% and 20% increases in COX activity of homogenates prepared from young and old stimulated EDL muscles, respectively ($p<0.05$; Fig. 2. additional data; appendix B). We next investigated whether comparable changes in protein import kinetics were responsible for these observed adaptations in mitochondrial content and function. Our data indicate a differential response to CCA between young and old animals. We observed that CCA induced greater increases in import kinetics in mitochondria from young, when compared to old animals. We also detected that CCA preferentially affected the SS, when compared to the IMF mitochondria. CCA also elicited elevations in the expression of PIM components in the SS and IMF mitochondria of young animals, while the expression of these was

unchanged in senescent animals. Thus, increased PIM component expression plays an important role in the elevated protein import kinetics induced by CCA. We also ascertained that the expression of cytosolic chaperones was not responsive to CCA. Our data suggest that CCA-induced elevations in protein import *in vivo* into the SS and IMF mitochondria may not rely on a parallel increase in chaperone content, although the activity of these chaperones could certainly be enhanced. Another possible adaptation could be related to CCA-induced reductions in the degradation of preproteins within the cytosol. Future research will be warranted to test these hypotheses.

In summary, our study illustrates that while mitochondrial biogenesis declines with senescence, the import of matrix proteins into isolated mitochondria remains unaffected with age. In a reconstituted cytosol-mitochondrial environment, protein import is also influenced by the activity of factors housed within the cytosol, causing increased susceptibility of preproteins to degradation and/or aggregation in both young and old animals. The higher degradation rate of preproteins within the cytosolic fraction of senescent animals could reduce protein import *in vivo*. In response to CCA, we demonstrate that the plasticity of mitochondria from senescent animals is reduced, indicated by an attenuation of protein import, along with a reduced induction of PIM components. This decrease with age preferentially affected IMF mitochondria. The results of our study suggest that the onset of sarcopenia does not stem from alterations in the kinetics of protein import as a property of the isolated organelle. However, further investigation is required to determine whether mitochondrial-cytosolic interactions could

attenuate protein import with senescence, possibly in a mitochondrial compartment-specific (e.g. matrix versus outer membrane) manner, leading to an overall decline in mitochondrial biogenesis with age.

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FIGURE LEGENDS

Fig. 1. Effect of age on pOCT import in SS and IMF mitochondria. (A) Representative autoradiograms from SS (top) and IMF (bottom) mitochondrial protein import assays. Upper band represents preprotein OCT (pOCT) and the lower band represents the mature form of OCT (mOCT). Lanes: Time course of pOCT import; TL, 5 μl of translated product not incubated with mitochondria. (B-C) Graphical representation of experiments conducted in SS (B) and IMF (C) skeletal muscle mitochondria isolated from young and old animals (n=7-12; * $p<0.05$ vs. SS; ¶ $p<0.05$ vs. young IMF).

Fig. 2. Effect of age on pMDH import in SS and IMF mitochondria. (A) Representative autoradiograms from SS (top) and IMF (bottom) mitochondrial protein import assays. Upper band represents preprotein MDH (pMDH) and the lower band represents the mature form of MDH (mMDH). Lanes: Time course of pOCT import; TL, 5 μl of translated product not incubated with mitochondria. (B-C) Graphical representation of experiments conducted in SS (B) and IMF (C) skeletal muscle mitochondria isolated from young and old animals (n=4; * $p<0.05$ vs. SS).

Fig. 3. Effect of age on the protein expression of mitochondrial PIM components. (A) Representative western blots of Hsp60, mtHsp70 and Tim23 content in SS mitochondria isolated from young (Y) and old (O) animals. Adenine nucleotide translocase (ANT) was used as a control for loading. (B) Graphical representation of experiments using 35 μg of mitochondrial protein per lane (n=5).

Fig. 4. Effect of age on chaperone protein expression. (A) Representative western blots of Hsp70, Hsp90 and MSF-L content in the cytosolic fractions isolated from skeletal muscle of young (Y) and old (O) animals. (B) Graphical representation of experiments using 30 μg of cytosolic protein per lane. GAPDH was used as a control for loading. (n=7-10; * $p<0.05$).

Fig. 5. Influence of the cytosolic fraction on the import of pOCT. (A) Representative autoradiogram of a 20 min protein import assay. Radiolabelled pOCT (12 μl) and IMF mitochondria (25 μg) were supplemented with the addition of 0 μg (control = CON), 7.5 μg or 15 μg of the cytosolic fraction. Cytosolic fractions were isolated from the skeletal muscle of young and old animals. (B) Graphical representation of experiments (n=6-9; * $p<0.05$ vs. CON; † $p<0.05$ vs. 7.5 μg).

Fig. 6. The effect of age on pOCT degradation. (A) Representative autoradiogram illustrating the degradation of pOCT by the addition 150 μg of the cytosolic fraction isolated from young (Y) and old (O) animals. (B) Graphical quantification of experiments represented as a percent of control (0 μg of cytosolic proteins; n=6-9; * $p<0.05$). Equation for the linear functions are: pOCT degradation by cytosolic fraction of young animals = 1.0052-0.0017*time, r=0.66; pOCT degradation by cytosolic fraction of old animals = 1.0087-0.0043*time, r=0.87.

Fig. 7. Effect of 7-day CCA on pOCT import. SS and IMF skeletal muscle mitochondria were isolated from stimulated (CCA) and control (CON) muscles obtained from young and old animals. (A) Representative autoradiograms from SS (top) and IMF (bottom)

mitochondrial protein import assays. Upper band represents preprotein OCT (pOCT) and the lower band represents the mature form of OCT (mOCT). Lanes: Time course of pOCT import; TL, 5 μl of translated product not incubated with mitochondria. (B-C) Graphical representation of experiments conducted in SS (B) and IMF (C) skeletal muscle mitochondria isolated from young and old animals (n=9-12; * $p<0.05$ vs. CON).

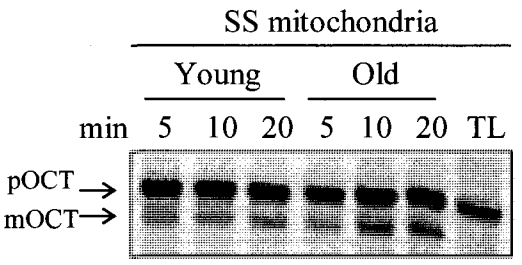
Fig. 8. Effect of 7-day CCA on the protein expression of PIM components. (A) Representative western blots of Tim23 and mtHsp70 content in SS and IMF mitochondria isolated from control (CON) and stimulated (CCA) legs of young and old animals. Adenine nucleotide translocase (ANT) was used as a control for loading. (B-C) Graphical representation of experiments using 35 μg of mitochondrial protein per lane (n=9-11; * $p<0.05$ vs. CON).

Fig. 9. Effect of 7-day CCA on cytosolic chaperone protein expression. (A) Representative western blots of MSF-L and Hsp90 content in control (CON) and stimulated (CCA) skeletal muscle cytosolic fractions obtained from young and old animals. GAPDH was used as a control for loading. (B) Graphical representation of experiments using 30 μg of cytosolic protein per lane (n=5-7; * $p<0.05$ vs. young).

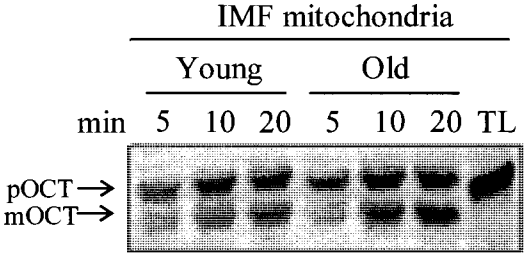
Fig. 10. Effect of age and 7-day CCA on EDL mitochondrial content. Muscles were obtained from control and chronically stimulated legs of young and old animals. SS and IMF mitochondrial populations (dark grey areas) are located below the sarcolemmal membrane (edge of muscle fiber) and between the myofibrils (below the SS mitochondria). Scale bar is located at the lower right of each picture represents 1 μm.

FIGURE 1

A


SS mitochondria
Young
Old
min 5 10 20 5 10 20 TL
pOCT
mOCT

B


IMF mitochondria
Young
Old
min 5 10 20 5 10 20 TL
pOCT
mOCT

C

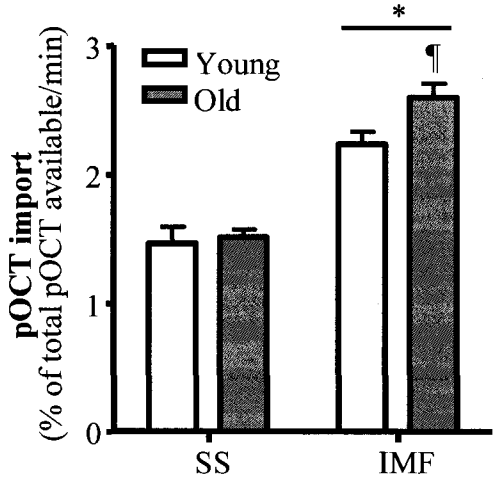

*
¶
Young
Old
pOCT import
(% of total pOCT available/min)
3
2
1
0
SS
IMF

FIGURE 2

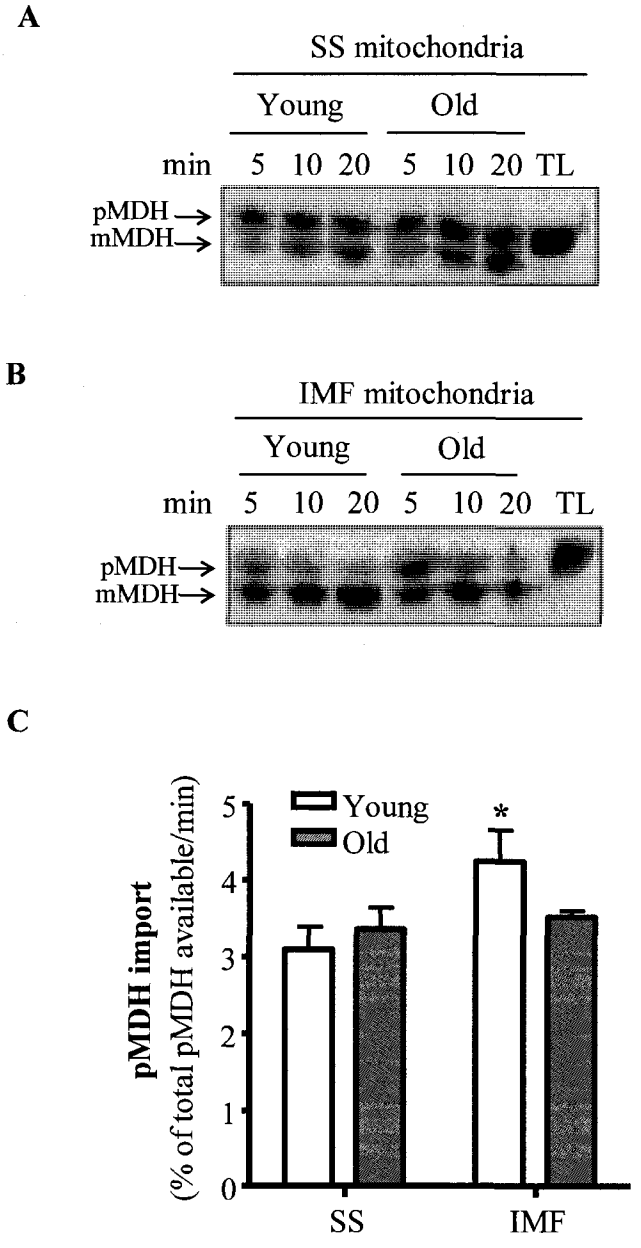
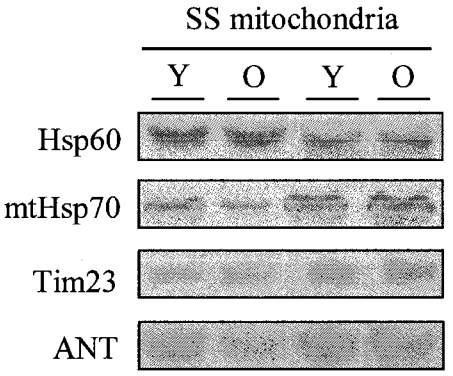

A
SS mitochondria
Young
Old
min 5 10 20 5 10 20 TL
pMDH
mMDH
B
IMF mitochondria
Young
Old
min 5 10 20 5 10 20 TL
pMDH
mMDH
C
pMDH import
(% of total pMDH available/min)
Young
Old
*
0
1
2
3
4
5
SS
IMF

FIGURE 3

A


SS mitochondria
Y
O
Y
O
Hsp60
mtHsp70
Tim23
ANT

B

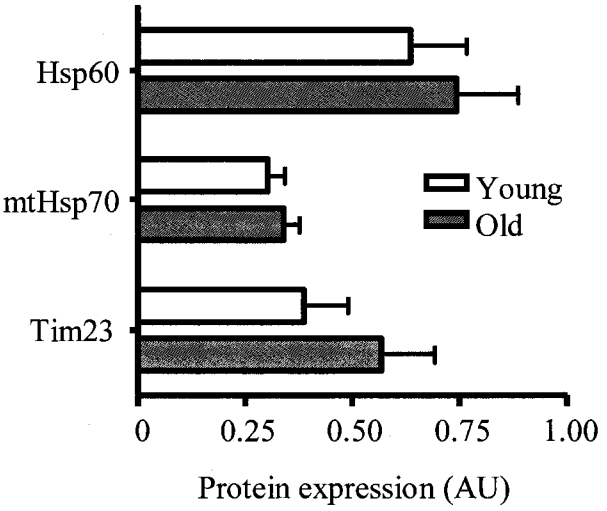
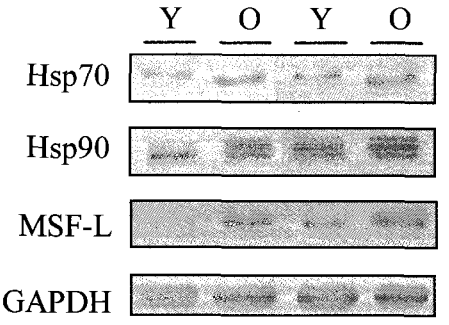

Hsp60
mtHsp70
Tim23
Young
Old
0
0.25
0.50
0.75
1.00
Protein expression (AU)

FIGURE 4

A


Y
O
Y
O
Hsp70
Hsp90
MSF-L
GAPDH

B

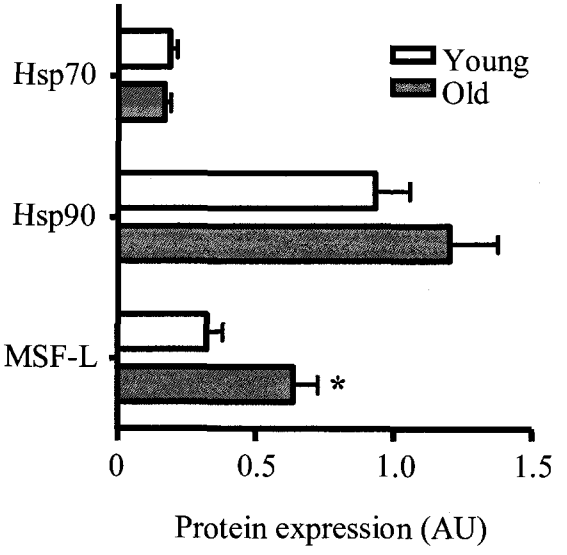
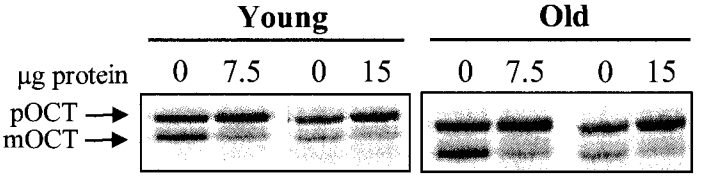

Young
Old
Hsp70
Hsp90
MSF-L
*
0
0.5
1.0
1.5
Protein expression (AU)

FIGURE 5

A


Young
Old
µg protein
0 7.5 0 15
0 7.5 0 15
pOCT
mOCT

B

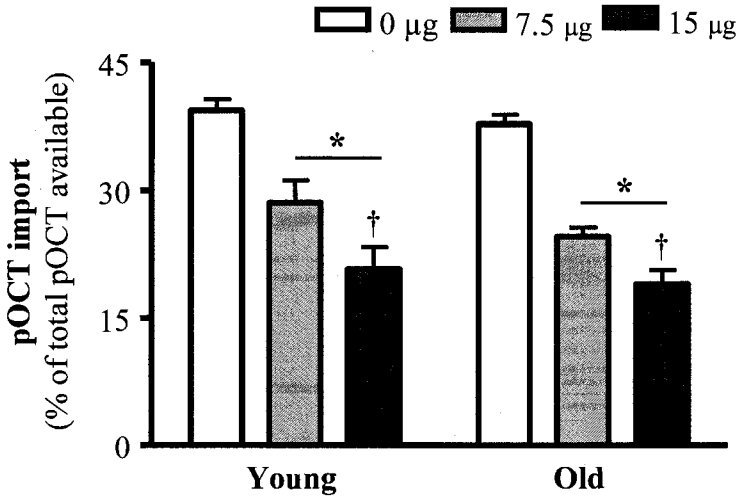

0 µg
7.5 µg
15 µg
pOCT import
(% of total pOCT available)
45
30
15
0
*
†
*
†
Young
Old

FIGURE 6

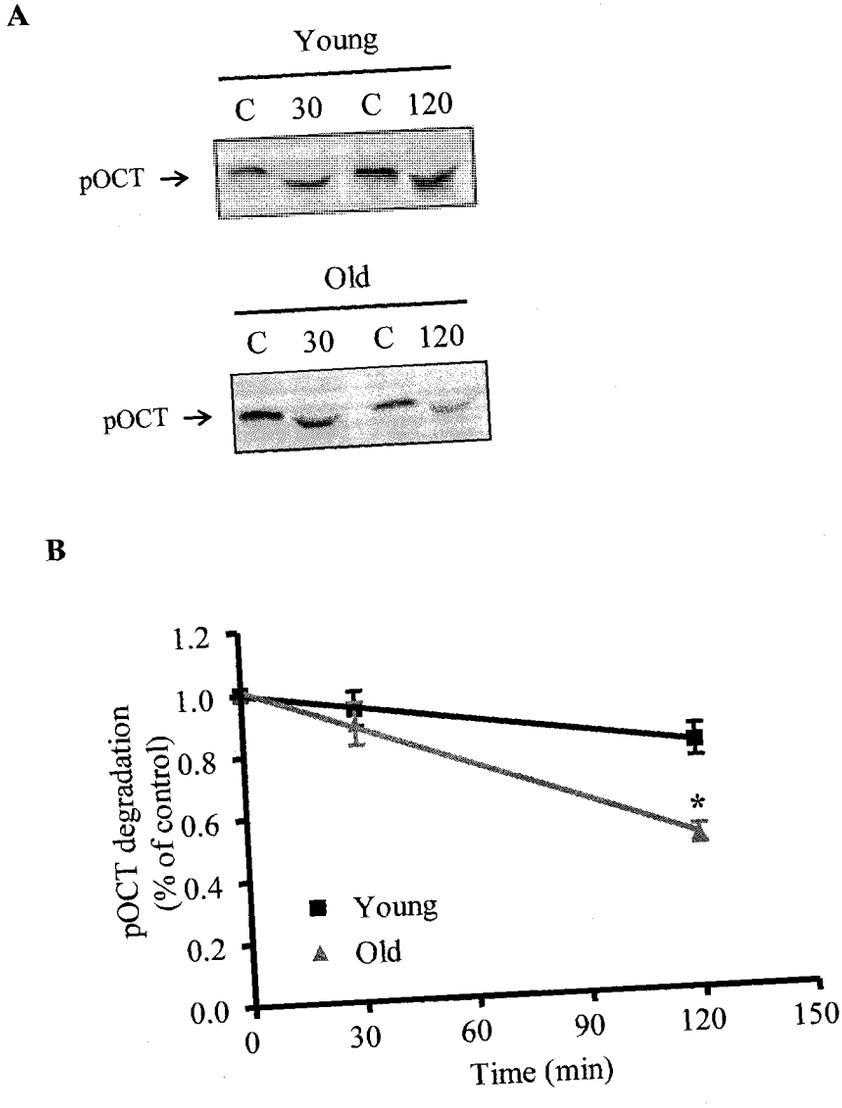

A
Young
C 30 C 120
pOCT →
Old
C 30 C 120
pOCT →
B
pOCT degradation (% of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Young
Old
*
0 30 60 90 120 150
Time (min)

FIGURE 7

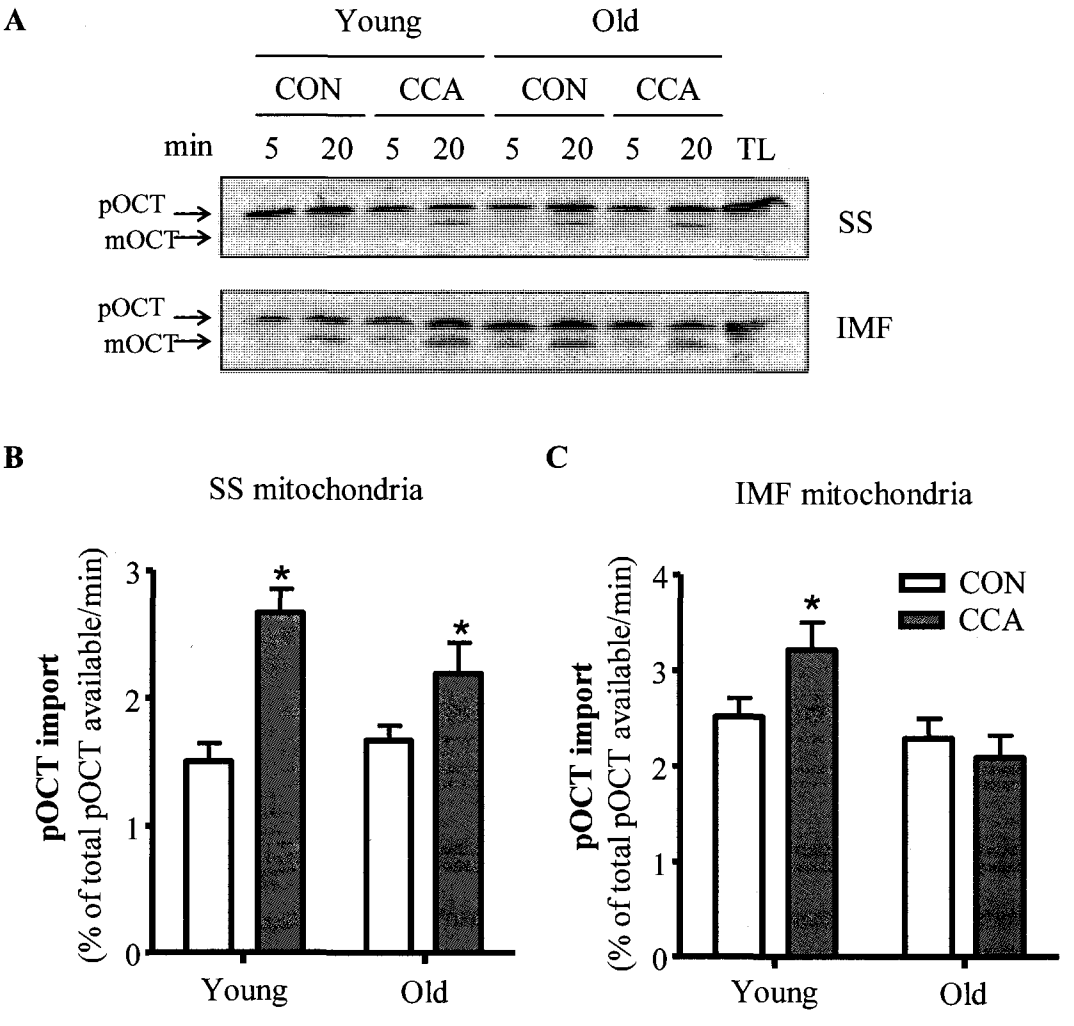

A
Young
Old
CON
CCA
CON
CCA
min
5
20
5
20
5
20
5
20
TL
pOCT
mOCT
SS
pOCT
mOCT
IMF
B
SS mitochondria
pOCT import
(% of total pOCT available/min)
3
2
1
0
*
*
Young
Old
C
IMF mitochondria
pOCT import
(% of total pOCT available/min)
4
3
2
1
0
*
CON
CCA
Young
Old

FIGURE 8

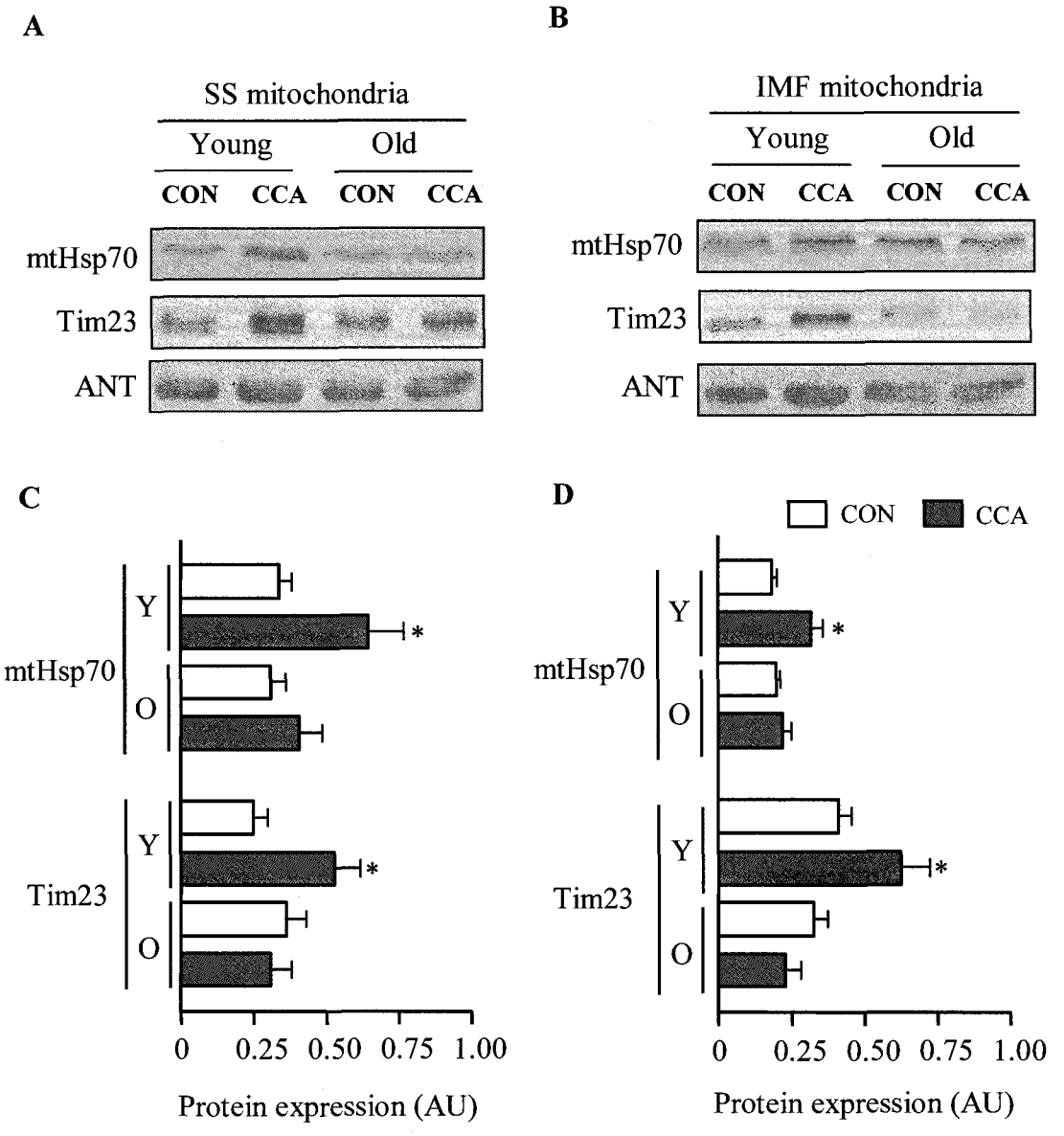
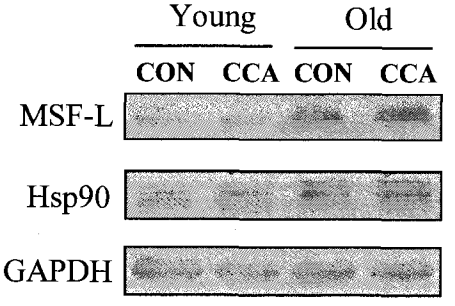

A
SS mitochondria
Young
Old
CON
CCA
CON
CCA
mtHsp70
Tim23
ANT
B
IMF mitochondria
Young
Old
CON
CCA
CON
CCA
mtHsp70
Tim23
ANT
C
mtHsp70
Y
O
Tim23
Y
O
*
*
0
0.25
0.50
0.75
1.00
Protein expression (AU)
D
CON
CCA
mtHsp70
Y
O
Tim23
Y
O
*
*
0
0.25
0.50
0.75
1.00
Protein expression (AU)

FIGURE 9

A


Young
Old
CON
CCA
CON
CCA
MSF-L
Hsp90
GAPDH

B

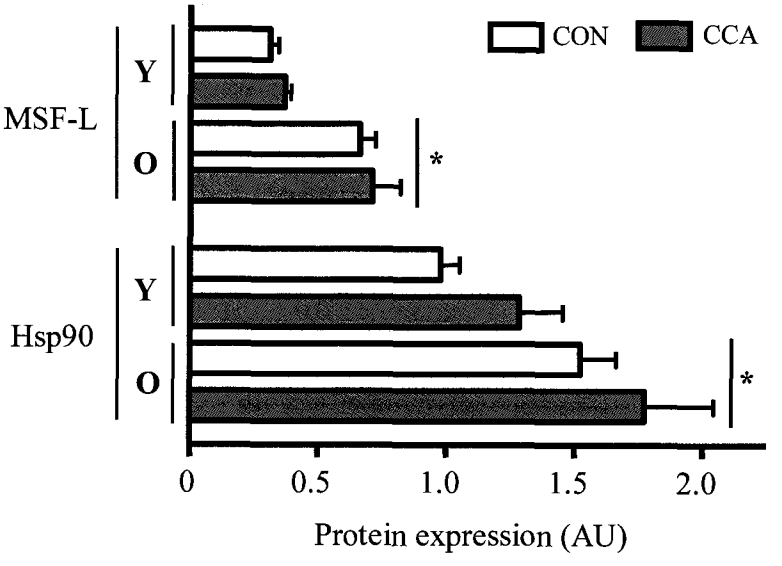
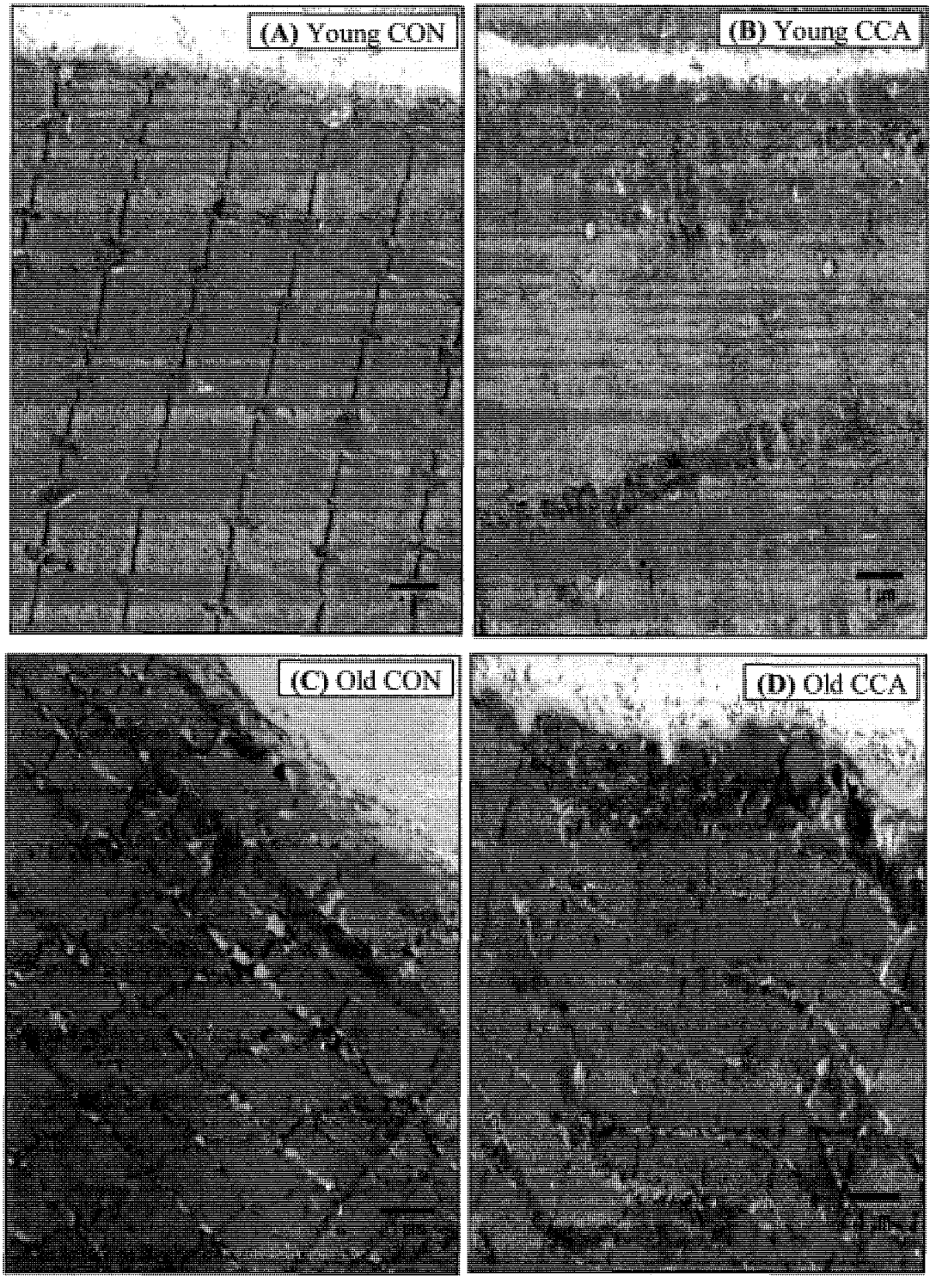

CON
CCA
MSF-L
Y
O
*
Hsp90
Y
O
*
0
0.5
1.0
1.5
2.0
Protein expression (AU)

FIGURE 10



SUMMARY

AND

FUTURE WORK

The first purpose of my thesis was to identify a potential connection between the presence of mitochondrial dysfunction and a regulatory process of mitochondrial biogenesis in skeletal muscle of senescent animals. A second purpose focused on examining the plasticity of skeletal muscle of these aged animals and to see whether alterations in the import of proteins were underlying mediators of adaptations elicited by an exercise-like stimulus.

We compared 6 and 36 month-old F344BN rats to determine adaptations, at the level of protein import, caused by senescence and chronic contractile activity. Prior to the stimulation protocol, we first assessed the effect of age on parameters of mitochondrial biogenesis and the protein import pathway. We confirmed that mitochondrial biogenesis was lower in aged animals when compared to young animals. Our analyses of the import of matrix-destined proteins suggest that the import pathway in aged animals is intact, as the rate of pOCT and pMDH import into SS and IMF mitochondria did not decline when compared to observations made in young animals. Reconstitution of the cellular environment revealed that other post-translational modifications of precursor proteins, upstream of their interaction with import machinery components, may be accelerated in aged animals. Enhanced degradation of preproteins may serve as a potential cause for declined mitochondrial content and function associated with the sarcopenia of aging.

In response to CCA, we demonstrate that while mitochondrial biogenesis is elicited, the plasticity of skeletal muscles in senescent animals is blunted in comparison to young animals. This was indicated by a lower degree of adaptation in pOCT import in the SS

mitochondria, and an absence of change in the IMF mitochondria in aged animals. There was also no increase in the protein expression of the import machinery components measured. Thus, our data suggest that the attenuated plasticity of both the SS and IMF mitochondria with age may represent a limiting factor in the possible benefits that could be attained through chronic contractile activity.

To complement the data observed in the present study, short-term goals could include:

1) elucidating the factors that mediated the CCA-induced alterations in protein import observed in SS mitochondria of aged animals. Ideally, this would include measurements of cardiolipin, the expression of other import machinery components relevant to the translocation of matrix-destined proteins, the activities of intramitochondrial proteases, such as MPP and MIP, the binding activities of cytosolic chaperones to precursor proteins, and lastly the degradation of precursor proteins within the cytosolic fraction obtained from stimulated muscles.
2) investigating whether inductions in PGC-1α, Tfam and other transcriptional regulators of mitochondrial biogenesis are elevated in response to CCA in the senescent animals. This will provide information on the transcriptional-activity of the nuclear genome within senescent animals elicited by CCA and allow for inferences to be made about the import of nuclear-encoded proteins.
3) determining if the activation of various kinases (i.e. AMPK), elicited by CCA or an acute exercise bout, is impaired in senescent animals. This allows for the understanding for attenuated mitochondrial biogenesis in these aged animals.

Recommendations for long-term goals using our current techniques for measuring mitochondrial protein import would include:

1) examining the affect of age and/or chronic contractile activity on the import of nuclear-encoded COX subunit precursor proteins into the mitochondrion. This may grant a clear indication of whether impaired import of these proteins is responsible for the observed alterations in COX activity.
2) investigating the effect of age and/or chronic contractile activity on the import of proteins into other mitochondrial compartments such as the TOM complex proteins and Bcl-2 (outer membrane), Cytochrome c (intermembrane space), and the TIM complex proteins, ATP synthase and COX complex proteins (inner membrane).
3) determining the underlying processes in the cytosolic fraction which resulted in precursor protein degradation using pan or specific inhibitors. Inhibitors that would be useful would include lactacystin or epoxomicin for the activity of the 26S proteasome, 3-methyladenine or wortmannin to inhibit autophagy and calpeptin or calpastatin-agonists to counteract degradation caused by calpains.
4) experimenting with other forms of endurance exercise (i.e. treadmill running) or subject animals to a longer period of CCA (i.e. beyond 7 days) to investigate whether these protocols elicit adaptations in mitochondrial protein import in a similar fashion to our present CCA protocol.

5) establishing a true aging effect and its influence on the plasticity of skeletal muscle in animals of gradually increasing age. Comparisons made between 6, 12, 18, 24, 30 and 36 month-old animals would provide insight on the time-dependent alterations that occur in mitochondrial biogenesis and the pathways which regulate this process.

Long-term studies investigating the involvement of the protein import pathway in mediating mitochondrial biogenesis could be further supplemented using novel techniques which measure the extent of protein import *in vivo*, rather than *in vitro*. These techniques include:

1) assessing the import of tagged precursor proteins which require adenoviral transfection into skeletal muscle. Fusion tags, such as FLAG, His, Myc and GFP, could be fused to mitochondrial proteins and cloned into muscle specific expression vectors. The resulting transfection (via intramuscular injection) of the adenoviral vector would allow for sensitive measurements of *in vivo* protein import in young and aged animals. This will require immunoprecipitation and immunoblotting of whole muscle homogenates for these fusion tags and mitochondrial proteins, respectively.
2) measuring protein import via microscopy techniques. Using a similar construction described above or by fusing the mitochondrial targeting signal of a matrix-destined protein to a reporter (i.e. GFP) and inserting the fusion protein into a vector. Transfection of skeletal muscle would result in the expression and

incorporation of the fusion protein *in vivo*. Sectioning of the whole muscle after the transfection period would allow for immunohistochemical analyses probing for the tag protein. Fluorescent probes conjugated to antibodies directed at endogenous mitochondrial proteins could indicate whether co-localization with the GFP signal occurred and indicate the efficiency of import into mitochondria.

APPENDIX A

Data tables and statistical analyses

Table 1: Effect of age on pOCT import in SS and IMF mitochondria

	pOCT import/min young SS			
	5'	10'	20'	Mean
1	1.92	0.82	0.63	1.12
2	2.92	1.10	0.79	1.60
3	2.18	1.46	0.77	1.47
4	3.02	1.70	0.73	1.82
5	2.26	1.07	0.44	1.26
6	1.42	0.87	0.64	0.98
7	1.35	1.08	0.66	1.03
8	1.22	1.26	0.55	1.01
9	2.47	1.50		1.98
10	2.54	1.35		1.94
11	0.99			0.99
12	2.34			2.34
Mean	2.05	1.22	0.65	**1.46**
SD	0.67	0.28	0.12	**0.47**
SEM	0.19	0.09	0.04	**0.14**

	pOCT import/min old SS			
	5'	10'	20'	Mean
1	2.50	1.31	0.72	1.51
2	1.88	1.45	0.77	1.37
3	2.60	1.50	0.88	1.66
4	1.86	1.53	0.89	1.43
5	2.55	1.47	0.78	1.60
6	2.69	1.25	1.03	1.66
7	3.18	1.77	0.75	1.90
8	2.23	1.59	0.84	1.55
9	1.91	1.71	1.08	1.56
10		1.14	1.05	1.09
11		1.60	0.99	1.29
12				
Mean	2.38	1.48	0.89	**1.51**
SD	0.45	0.19	0.13	**0.21**
SEM	0.15	0.06	0.04	**0.06**

	pOCT import/min young IMF			
	5'	10'	20'	Mean
1	3.72	2.57	1.51	2.60
2	2.80	2.20	1.44	2.15
3	2.52	2.02	1.29	1.94
4	4.10	1.59	1.19	2.29
5	3.11	2.08	1.10	2.09
6	2.86	2.02	1.19	2.02
7	3.69	2.41	1.16	2.42
8	3.67	2.48	1.33	2.50
9	3.53		1.73	2.63
10			1.66	1.66
11				
Mean	3.33	2.17	1.36	**2.23**
SD	0.53	0.32	0.22	**0.31**
SEM	0.18	0.11	0.07	**0.10**

	pOCT import/min old IMF			
	5'	10'	20'	Mean
1	3.48	2.36	1.56	2.47
2	4.20	2.16	2.01	2.79
3	3.78	1.90	1.58	2.42
4	2.62	2.67	1.27	2.18
5	3.07	2.72	1.58	2.46
6	2.62	2.02	2.02	2.22
7	2.69	2.33	1.71	2.24
8	3.54		1.72	2.63
9	3.94		1.59	2.77
10	3.34			3.34
11	3.03			3.03
Mean	3.30	2.31	1.67	**2.60**
SD	0.55	0.31	0.23	**0.36**
SEM	0.16	0.12	0.08	**0.11**

pOCT import: Two-way factorial ANOVA (age x mitochondrial subfraction)

Source of Variation	% of total variation	P value
Interaction	1.78	0.1500
Mito	60.97	**<0.0001**
Age	3.05	0.0614

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.2738	0.2738	2.154
Mito	1	9.402	9.402	73.96
Age	1	0.4710	0.4710	3.705
Residual	40	5.085	0.1271	

Bonferroni post hoc tests

SS versus IMF mitochondria

Age	Difference	t	P value
Young	**0.7683**	**5.033**	**P<0.001**
Old	**1.085**	**7.134**	**P<0.001**

Young versus Old

MITO	Difference	t	P value
SS	0.04924	0.3309	P > 0.05
IMF	**0.3655**	**2.346**	**P < 0.05**

Table 2: Effect of age on pMDH import in SS and IMF mitochondria

	Young - pMDH import in SS					Old - pMDH import in SS			
	5'	10'	20'	Mean		5'	10'	20'	Mean
1	5.06	3.31	1.97	3.44	1	5.11	3.27	2.12	3.50
2	4.45	3.09	2.20	3.25	2	5.63	3.81	2.18	3.87
3	5.06	3.31	1.97	3.44	3	5.11	3.27	2.12	3.50
4	2.69	2.58	1.45	2.24	4	3.86	2.40	1.29	2.52
Mean	4.31	3.07	1.90	**3.09**	Mean	4.93	3.19	1.93	**3.35**
SD	1.12	0.34	0.32	**0.58**	SD	0.75	0.58	0.43	**0.58**
SEM	0.56	0.17	0.16	**0.29**	SEM	0.38	0.29	0.21	**0.29**
	Young - pMDH import in IMF					Old - pMDH import in IMF			
	5'	10'	20'	Mean		5'	10'	20'	Mean
1	6.54	4.64	2.44	4.54	1	5.24	3.37	1.91	3.51
2	3.89	3.34	1.81	3.01	2	6.13	3.06	2.01	3.73
3	6.54	4.64	2.44	4.54	3	5.24	3.37	1.91	3.51
4	7.28	4.70	2.58	4.86	4	3.47	4.09	2.30	3.29
Mean	6.06	4.33	2.32	**4.24**	Mean	5.02	3.47	2.03	**3.51**
SD	1.49	0.66	0.35	**0.83**	SD	1.11	0.44	0.18	**0.18**
SEM	0.75	0.33	0.17	**0.42**	SEM	0.56	0.22	0.09	**0.09**

pMDH import: Two-way factorial ANOVA (age x mitochondrial subfraction)

Source of Variation	% of total variation	P value
Interaction	13.65	0.1215
MITO	24.18	**0.0466**
AGE	3.16	0.4385

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.9653	0.9653	2.777
MITO	1	1.710	1.710	4.918
AGE	1	0.2233	0.2233	0.6422
Residual	12	4.171	0.3476	

Bonferroni post hoc test

SS versus IMF mitochondria

AGE	Difference	t	P value
Young	**1.145**	**2.746**	**P < 0.05**
Old	0.1625	0.3898	P > 0.05

Table 3: Effect of age on the protein expression of mitochondrial import machinery components

mtHsp70				Tim23				Hsp60		
Young	SS	IMF		Young	SS	IMF		Young	SS	IMF
1	0.235	0.386		1	0.583	1.305		1	0.765	0.416
2	0.465	0.553		2	0.674	0.931		2	0.645	0.036
3	0.264	0.287		3	0.164	0.925		3	0.901	0.753
4	0.265	0.350		4	0.182	0.521		4	0.146	0.083
5	0.277	0.253		5	0.336	0.447		5	0.733	0.600
Mean	**0.301**	**0.366**		Mean	**0.388**	**0.826**		Mean	**0.638**	**0.378**
SD	**0.093**	**0.117**		SD	**0.232**	**0.349**		SD	**0.290**	**0.314**
SEM	**0.042**	**0.052**		SEM	**0.104**	**0.156**		SEM	**0.130**	**0.141**
mtHsp70				Tim23				Hsp60		
Old	SS	IMF		Old	SS	IMF		Old	SS	IMF
1	0.441	0.600		1	0.933	1.280		1	1.166	0.423
2	0.377	0.325		2	0.790	0.935		2	0.495	0.376
3	0.260	0.376		3	0.393	0.459		3	0.816	0.435
4	0.374	0.500		4	0.280	0.849		4	0.363	0.472
5	0.249	0.220		5	0.446	0.384		5	0.879	0.346
Mean	**0.340**	**0.404**		Mean	**0.568**	**0.781**		Mean	**0.744**	**0.410**
SD	**0.083**	**0.149**		SD	**0.279**	**0.367**		SD	**0.319**	**0.050**
SEM	**0.037**	**0.066**		SEM	**0.125**	**0.164**		SEM	**0.143**	**0.022**
* data corrected by ANT										

mtHsp70: Two-way factorial ANOVA (age x mitochondrial subfraction)

Source of Variation	% of total variation	P value
Interaction	0.00	0.9953
Age	3.21	0.4557
Mito	8.86	0.2222

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.00000045	0.00000045	0.00003511
Age	1	0.007488	0.007488	0.5843
Mito	1	0.02067	0.02067	1.613
Residual	16	0.2050	0.01282	

Tim23: Two-way factorial ANOVA (age x mitochondrial subfraction)

Source of Variation	% of total variation	P value
Interaction	2.92	0.4311
Mito	24.44	**0.0328**
Age	1.07	0.6315

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.06328	0.06328	0.6525
Mito	1	0.5298	0.5298	5.463
Age	1	0.02319	0.02319	0.2391
Residual	16	1.552	0.09698	

Bonferroni post hoc test

SS versus IMF mitochondria

Age	Difference	t	P value
Young	0.4380	2.224	P > 0.05
Old	0.2130	1.081	P > 0.05

Hsp60: Two-way factorial ANOVA (age x mitochondrial subfraction)

Source of Variation	% of total variation	P value
Interaction	0.41	0.7648
Age	1.48	0.5714
Mitochondria	27.18	**0.0248**

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.006661	0.006661	0.09265
Age	1	0.02401	0.02401	0.3340
Mitochondria	1	0.4407	0.4407	6.130
Residual	16	1.150	0.07190	

Bonferroni post hoc test

SS versus IMF mitochondria

Age	Difference	t	P value
Young	-0.2604	1.536	P > 0.05
Old	-0.3334	1.966	P > 0.05

Table 4: Effect of age on chaperone protein expression

Young	HSP70	HSP90	MSF-L		Old	HSP70	HSP90	MSF-L
1	0.22	1.18	0.49		1	0.16	1.16	0.82
2	0.20	0.73	0.40		2	0.21	0.69	0.45
3	0.11	0.78	0.36		3	0.19	1.12	1.01
4	0.23	0.68	0.18		4	0.11	1.10	0.41
5		1.29	0.11		5		1.96	0.80
6			0.39		6		2.15	0.55
7					7		0.90	0.42
8					8		0.61	
9					9		1.13	
Mean	**0.19**	**0.93**	**0.32**		Mean	**0.17**	**1.20**	**0.64**
SD	**0.05**	**0.28**	**0.15**		SD	**0.04**	**0.52**	**0.24**
SEM	**0.05**	**0.17**	**0.08**		SEM	**0.02**	**0.17**	**0.09**
* data corrected by GAPDH								

Hsp70: Unpaired t-test (two-tailed p value)

P value	0.6165
Are means different? (P < 0.05)	no
t, df	t=0.5279 df=6

Hsp90: Unpaired t-test (two-tailed p value)

P value	0.3111
Are means different? (P < 0.05)	no
t, df	t=1.058 df=12

MSF-L: Unpaired t-test (two-tailed p value)

P value	**0.0168**
Are means different? (P < 0.05)	**Yes**
t, df	**t=2.817 df=11**

Table 5: Influence of the cytosolic fraction on the import of pOCT

pOCT import with cytosolic fraction (young)					pOCT import with cytosolic fraction (old)			
	0 µg	7.5 µg	15 µg			0 µg	7.5 µg	15 µg
1	42.5	28.9	17.5		1	41.7	20.1	19.0
2	41.4	23.5	15.2		2	35.8	26.4	18.5
3	47.9	38.3	29.9		3	42.2	25.8	16.9
4	40.9	26.6	18.7		4	40.9	21.0	15.0
5	38.6	20.3	15.4		5	37.7	23.7	10.8
6	38.9	33.3	27.3		6	38.1	20.8	17.1
7	38.2				7	33.2	26.5	23.8
8	33.7				8	32.1	27.7	20.1
9	43.3				9	41.5	28.5	28.9
10	32.4				10	37.3		
11	34.4				11	43.5		
12	40.7				12	38.6		
13					13	36.5		
14					14	34.1		
15					15	32.3		
16					16	34.8		
17					17	31.5		
18					18	48.1		
Mean	**39.4**	**28.5**	**20.7**		Mean	**37.8**	**24.5**	**18.9**
SD	**4.4**	**6.6**	**6.3**		SD	**4.5**	**3.2**	**5.2**
SEM	**1.3**	**2.7**	**2.6**		SEM	**1.1**	**1.1**	**1.7**

pOCT import: Two-way factorial ANOVA (age x concentration)

Source of Variation	% of total variation	P value
Interaction	0.27	0.7388
Concentration	70.77	**<0.0001**
Age	1.48	0.0736

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	14.37	7.185	0.3045
Concentration	2	3763	1881	79.72
Age	1	78.56	78.56	3.329
Residual	54	1274	23.60	

Bonferroni post hoc tests

0 µg vs. 7.5 µg

Age	Difference	t	P value
Young	**-10.93**	**4.498**	**P<0.001**
Old	**-13.27**	**6.693**	**P<0.001**

0 µg vs. 15 µg

Age	Difference	t	P value
Young	**-18.74**	**7.716**	**P<0.001**
Old	**-18.87**	**9.516**	**P<0.001**

7.5 µg vs. 15 µg

Age	Difference	t	P value
Young	**-7.817**	**2.787**	**P < 0.05**
Old	**-5.600**	**2.445**	**P < 0.05**

Table 6: The effect of age on pOCT degradation

pOCT degradation				
Young	0'	30'	120'	slope
1	1.00	0.80	0.75	0.125
2	1.00	0.79	0.81	0.095
3	1.00	1.10	0.70	0.15
4	1.00	0.95	0.64	0.18
5	1.00	1.10	0.98	0.01
6	1.00	0.91	0.89	0.055
7				
8				
9				
Mean	1.00	0.94	0.80	**0.10**
SD	0.00	0.14	0.13	**0.06**
SEM	0.00	0.06	0.05	**0.03**

pOCT degradation				
Old	0'	30'	120'	slope
1	1.00	0.67	0.46	0.27
2	1.00	0.70	0.43	0.29
3	1.00	1.10	0.39	0.31
4	1.00	0.86	0.59	0.21
5	1.00	0.81	0.50	0.25
6	1.00	1.07	0.42	0.29
7	1.00	1.01	0.68	0.16
8	1.00	1.17	0.53	0.24
9	1.00	0.64	0.44	0.28
Mean	1.00	0.89	0.49	**0.25**
SD	0.00	0.20	0.09	**0.05**
SEM	0.00	0.07	0.03	**0.02**

pOCT degradation

Young versus Old: Unpaired student's t-test (two-tailed)

P value	**0.0001**
Are means signif. different? (P < 0.05)	**Yes**
t, df	**t=5.424 df=13**

Table 7: Effect of 7-day CCA on the import of pOCT into SS mitochondria

SS Young	CON			SS Young	CCA		
	5'	20'	average		5'	20'	average
1	1.30	0.63	0.97	1	3.95	1.53	2.74
2	2.83	1.09	1.96	2	3.46	1.32	2.39
3	2.24	0.80	1.52	3	5.89	1.96	3.93
4	1.77	0.49	1.13	4	2.93	1.08	2.00
5	1.47	0.54	1.01	5	2.81	1.49	2.15
6	2.92	1.35	2.14	6	4.41	1.85	3.13
7	3.40	1.02	2.21	7	4.92	2.07	3.50
8	2.83	0.66	1.75	8	3.28	1.37	2.32
9	2.18	0.83	1.50	9	3.73	1.84	2.78
10	2.43	0.90	1.67	10	2.83	1.20	2.02
11	2.04	1.05	1.55	11	4.12	2.10	3.11
12	0.71	0.53	0.62	12	2.54	1.44	1.99
Mean	2.18	0.83	**1.50**	Mean	3.74	1.60	**2.67**
SD	0.77	0.27	**0.49**	SD	0.99	0.35	**0.64**
SEM	0.22	0.08	**0.14**	SEM	0.29	0.10	**0.18**

SS Old	CON			SS Old	CCA		
	5'	20'	average		5'	20'	average
1	1.82	0.58	1.20	1	2.61	0.84	1.72
2	2.78	1.06	1.92	2	3.81	1.57	2.69
3	2.61	0.94	1.78	3	4.23	1.45	2.84
4	1.70	0.73	1.22	4	2.75	0.79	1.77
5	2.05	0.93	1.49	5	1.72	0.82	1.27
6	2.62	1.22	1.92	6	2.26	1.02	1.64
7	2.27	0.97	1.62	7	2.62	1.29	1.95
8	2.80	1.77	2.28	8	4.97	2.22	3.59
9	2.32	0.87	1.59	9	3.25	1.25	2.25
Mean	2.33	1.01	**1.67**	Mean	3.14	1.25	**2.19**
SD	0.41	0.34	**0.35**	SD	1.03	0.46	**0.73**
SEM	0.14	0.11	**0.12**	SEM	0.34	0.15	**0.24**

pOCT import in SS mitochondria: Mixed two-way ANOVA (age x treatment)

Factorial = Age (Young/Old)

Repeated measures = Treatment (CON/CCA)

Source of Variation	% of total variation	P value
Interaction	4.88	**0.0175**
AGE	1.15	0.4814
CCA	33.36	**P<0.0001**
Subjects (matching)	42.3925	0.0089

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	1.076	1.076	6.769
AGE	1	0.2538	0.2538	0.5158
CCA	1	7.356	7.356	46.27
Subjects (matching)	19	9.348	0.4920	3.095
Residual	19	3.021	0.1590	

Bonferroni post hoc tests

Control versus Chronic contractile activity

AGE	Difference	t	P value
Young	**1.169**	**7.182**	**P<0.001**
Old	**0.5222**	**2.778**	**P < 0.05**

Table 8: Effect of CCA on pOCT import in IMF mitochondria

IMF Young	CON			IMF Young	CCA		
	5'	20'	average		5'	20'	average
1	3.4	1.7	2.5	1	4.8	2.4	3.6
2	2.8	0.7	1.8	2	3.2	0.8	2.0
3	4.4	2.1	3.3	3	6.9	2.7	4.8
4	2.6	1.1	1.9	4	4.3	1.9	3.1
5	2.1	0.9	1.5	5	3.7	2.4	3.0
6	4.9	2.3	3.6	6	4.3	2.7	3.5
7	4.2	2.1	3.1	7	6.9	2.8	4.9
8	4.4	1.1	2.8	8	1.4	1.8	1.6
9	3.9	1.5	2.7	9	4.6	2.1	3.4
10	2.4	1.3	1.8	10	3.4	1.5	2.4
11	2.7	1.9	2.3	11	3.3	1.9	2.6
12	3.7	2.1	2.9	12	5.1	2.3	3.7
Mean	3.5	1.6	**2.5**	Mean	4.3	2.1	**3.2**
SD	0.9	0.5	**0.7**	SD	1.6	0.6	**1.0**
SEM	0.3	0.2	**0.2**	SEM	0.4	0.2	**0.3**

IMF Old	CON			IMF Old	CCA		
	5'	20'	average		5'	20'	average
1	2.5	1.1	1.8	1	3.1	1.0	2.0
2	2.6	0.8	1.7	2	2.3	0.6	1.4
3	4.6	1.9	3.3	3	4.9	1.1	3.0
4	2.4	0.6	1.5	4	2.4	0.8	1.6
5	2.8	1.4	2.1	5	3.1	0.9	2.0
6	3.7	1.5	2.6	6	2.0	0.6	1.3
7	2.7	1.2	2.0	7	3.0	0.9	2.0
8	4.3	1.7	3.0	8	5.4	1.3	3.4
9	3.6	1.6	2.6	9	3.0	1.2	2.1
Mean	3.3	1.3	**2.3**	Mean	3.2	0.9	**2.1**
SD	0.8	0.4	**0.6**	SD	1.2	0.3	**0.7**
SEM	0.3	0.1	**0.2**	SEM	0.4	0.1	**0.2**

pOCT import in IMF mitochondria: Mixed two-way ANOVA (age x treatment)

Factorial = Age (Young/Old)

Repeated measures = Treatment (CON/CCA)

Source of Variation	% of total variation	P value
Interaction	6.78	**0.0096**
AGE	15.38	**0.0382**
CCA	2.09	0.1264
Subjects (matching)	58.8976	0.0028

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	2.083	2.083	8.279
AGE	1	4.725	4.725	4.962
CCA	1	0.6429	0.6429	2.555
Subjects (matching)	19	18.09	0.9522	3.785
Residual	19	4.780	0.2516	

Bonferroni post hoc tests

Control versus Chronic contractile activity

AGE	Difference	t	P value
Young	**0.7000**	**3.419**	**P<0.01**
Old	-0.2000	0.8459	P > 0.05

Young versus Old

Treatment	Difference	t	P value
CON	-0.2278	0.5294	P > 0.05
CCA	**-1.128**	**2.621**	**P < 0.05**

Table 9: Effect of CCA on mtHsp70 expression in SS mitochondria

mtHsp70 expression in SS mitochondria						
Young	CON	CCA		Old	CON	CCA
1	0.235	1.047		1	0.441	0.710
2	0.465	0.604		2	0.377	0.558
3	0.264	0.483		3	0.260	0.366
4	0.265	0.467		4	0.374	0.580
5	0.277	0.839		5	0.249	0.245
6	0.567	0.959		6	0.390	0.169
7	0.458	0.128		7	0.328	0.215
8	0.121			8	0.689	
9	0.294			9	0.392	
10	0.471			10	0.087	
11	0.544			11	0.064	
12	0.120			12	0.061	
13				13		
14				14		
Mean	**0.340**	**0.647**		Mean	**0.309**	**0.406**
SD	**0.155**	**0.323**		SD	**0.181**	**0.210**
SEM	**0.045**	**0.122**		SEM	**0.052**	**0.080**
Data corrected by ANT						

mtHsp70 expression: Two-way mixed ANOVA (age x treatment)

Factorial = Age (Young/Old)
Repeated Measures = Treatment (CON/CCA)

Source of Variation	% of total variation	P value
Interaction	2.31	0.1406
Age	9.32	0.2071
Treatment	14.30	**0.0020**
Subjects (matching)	62.9077	0.0027

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.0448	0.0448	2.490
Age	1	0.1805	0.1805	1.779
Treatment	1	0.2768	0.2768	15.38
Subjects (matching)	12	1.218	0.1015	5.640
Residual	12	0.2159	0.01799	

Bonferroni post hoc test

Control versus Chronic contractile activity

Age	Difference	t	P value
Young	**0.2789**	**3.889**	**P<0.01**
Old	0.1189	1.658	P > 0.05

Table 10: mtHsp70 expression in IMF mitochondria

mtHsp70 expression in IMF mitochondria						
Young	CON	CCA		Old	CON	CCA
1	0.193	0.434		1	0.300	0.294
2	0.276	0.359		2	0.163	0.160
3	0.144	0.203		3	0.188	0.320
4	0.175	0.397		4	0.250	0.279
5	0.126	0.202		5	0.220	0.132
6	0.265	0.320		6	0.218	0.140
7	0.273			7	0.218	0.217
8	0.186			8	0.180	
9	0.178			9	0.209	
10	0.177			10	0.161	
11	0.120			11	0.130	
12	0.100			12	0.147	
13				13		
14				14		
Mean	**0.184**	**0.319**		Mean	**0.199**	**0.220**
SD	**0.060**	**0.098**		SD	**0.047**	**0.078**
SEM	**0.017**	**0.040**		SEM	**0.014**	**0.030**
Data corrected by ANT						

mtHsp70 expression: Two-way mixed ANOVA (age x treatment)

Factorial = Age (Young/Old)
Repeated Measures = Treatment (CON/CCA)

Source of Variation	% of total variation	P value
Interaction	1.44	0.3626
Age	2.26	0.5283
Treatment	12.65	**0.0159**
Subjects (matching)	64.3590	0.0234

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.002881	0.002881	0.8956
Age	1	0.004526	0.004526	0.4217
Treatment	1	0.02532	0.02532	7.872
Subjects (matching)	12	0.1288	0.01073	3.337
Residual	12	0.03860	0.003216	

Bonferroni post hoc test

Control versus Chronic contractile activity

Age	Difference	t	P value
Young	**0.08043**	**2.653**	**P < 0.05**
Old	0.03986	1.315	P > 0.05

Table 11: Effect of CCA on Tim23 expression in SS mitochondria

Tim23 expression in SS mitochondria						
Young	CON	CCA		Old	CON	CCA
1	0.583	0.145		1	0.933	0.170
2	0.674	0.230		2	0.790	0.119
3	0.164	0.710		3	0.393	0.481
4	0.182	0.646		4	0.280	0.133
5	0.336	0.868		5	0.446	0.306
6	0.043	0.789		6	0.081	0.078
7	0.119	0.190		7	0.095	0.309
8	0.174	0.547		8	0.246	0.380
9	0.097	0.612		9	0.207	0.777
10	0.174			10	0.246	
11	0.169			11	0.135	
12	0.226			12	0.210	
13	0.297			13	0.413	
14	0.265			14	0.582	
Mean	**0.250**	**0.526**		Mean	**0.361**	**0.306**
SD	**0.179**	**0.271**		SD	**0.256**	**0.222**
SEM	**0.048**	**0.090**		SEM	**0.068**	**0.074**
Data corrected by ANT						

Tim23 expression: Two-way mixed ANOVA (age x treatment)

Factorial = Age (Young/Old)
Repeated Measures = Treatment (CON/CCA)

Source of Variation	% of total variation	P value
Interaction	10.35	**0.0173**
Age	2.65	0.3356
Treatment	20.52	**0.0018**
Subjects (matching)	42.9875	0.1189

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.1929	0.1929	7.044
Age	1	0.04936	0.04936	0.9855
Treatment	1	0.3825	0.3825	13.97
Subjects (matching)	16	0.8014	0.05009	1.829
Residual	16	0.4381	0.02738	

Bonferroni post hoc test

Control versus Chronic contractile activity

Age	Difference	t	P value
Young	**0.3526**	**4.520**	**P<0.001**
Old	0.05978	0.7664	P > 0.05

Table 12: Tim23 expression in IMF mitochondria

Tim23 expression in IMF mitochondria						
Young	CON	CCA		Old	CON	CCA
1	0.652	0.482		1	0.640	0.289
2	0.466	1.056		2	0.467	0.185
3	0.463	0.408		3	0.230	0.210
4	0.260	1.026		4	0.425	0.155
5	0.224	0.612		5	0.384	0.066
6	0.457	0.303		6	0.327	0.062
7	0.326	0.435		7	0.147	0.195
8	0.655	0.663		8	0.391	0.311
9	0.221			9	0.125	0.590
10	0.675			10	0.104	
11	0.195			11	0.112	
12	0.309			12	0.197	
13	0.325			13	0.316	
14	0.530			14	0.678	
Mean	**0.411**	**0.623**		Mean	**0.324**	**0.229**
SD	**0.170**	**0.282**		SD	**0.187**	**0.160**
SEM	**0.045**	**0.100**		SEM	**0.050**	**0.053**
Data corrected by ANT						

Tim23 expression: Two-way mixed ANOVA (age x treatment)

Factorial = Age (Young/Old)
Repeated Measures = Treatment (CON/CCA)

Source of Variation	% of total variation	P value
Interaction	4.70	**0.0047**
Age	26.46	**0.0171**
Treatment	1.96	**0.0502**
Subjects (matching)	59.8778	P<0.0001

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.09902	0.09902	10.74
Age	1	0.5575	0.5575	7.071
Treatment	1	0.04134	0.04134	4.486
Subjects (matching)	16	1.262	0.07885	8.554
Residual	16	0.1475	0.009217	

Bonferroni post hoc test

Control versus Chronic contractile activity

Age	Difference	t	P value
Young	**0.1727**	**3.815**	**P<0.01**
Old	-0.03711	0.8200	P > 0.05

Table 13: Effect of CCA on cytosolic chaperone expression

	Hsp90					MSF-L			
	Young		Old			Young		Old	
	CON	CCA	CON	CCA		CON	CCA	CON	CCA
1	1.177	1.411	1.162	1.604	1	0.493	0.431	0.818	0.581
2	0.729	0.811	1.387	1.068	2	0.397	0.406	0.446	0.693
3	0.782	1.726	1.121	2.667	3	0.359	0.304	1.013	0.725
4	0.685	0.986	1.100	1.505	4	0.185	0.390	0.405	1.109
5	1.290	1.502	1.956	2.037	5	0.108	0.331	0.797	0.464
6	1.039		2.152		6	0.389		0.549	
7	0.648		0.904		7	0.322		0.424	
8	1.296		1.221		8	0.288		0.673	
9	0.886		1.131		9	0.219		0.925	
10	1.178		1.562		10	0.248		0.472	
11	1.073		1.007		11	0.178		0.584	
12			1.791		12	0.469		0.877	
13			2.173		13	0.404			
14			2.648		14				
Mean	**0.980**	**1.287**	**1.522**	**1.776**	Mean	**0.312**	**0.372**	**0.665**	**0.714**
SD	**0.244**	**0.378**	**0.535**	**0.606**	SD	**0.119**	**0.053**	**0.214**	**0.243**
SEM	**0.074**	**0.169**	**0.143**	**0.271**	SEM	**0.033**	**0.024**	**0.062**	**0.109**

Hsp90 expression: Two-way factorial ANOVA (age x treatment)

Source of Variation	% of total variation	P value
Interaction	0.01	0.9590
Age	38.83	**0.0031**
Treatment	3.90	0.2914

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.0007723	0.0007723	0.002717
Age	1	3.357	3.357	11.81
Treatment	1	0.3370	0.3370	1.186
Residual	17	4.833	0.2843	

Bonferroni post hoc test

Young versus Old

Treatment	Difference	t	P value
CON	**0.8143**	**2.522**	**P < 0.05**
CCA	0.7900	2.343	P > 0.05

MSF-L expression: Two-way factorial ANOVA (age x treatment)

Source of Variation	% of total variation	P value
Interaction	0.36	0.6815
Age	59.22	**P<0.0001**
Treatment	0.65	0.5812

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	1	0.004484	0.004484	0.1741
Age	1	0.7406	0.7406	28.75
Treatment	1	0.008130	0.008130	0.3156
Residual	18	0.4637	0.02576	

Bonferroni post hoc test:

Young versus Old

Treatment	Difference	t	P value
CON	**0.3997**	**4.253**	**P<0.001**
CCA	**0.3420**	**3.369**	**P<0.01**

APPENDIX B

Additional data

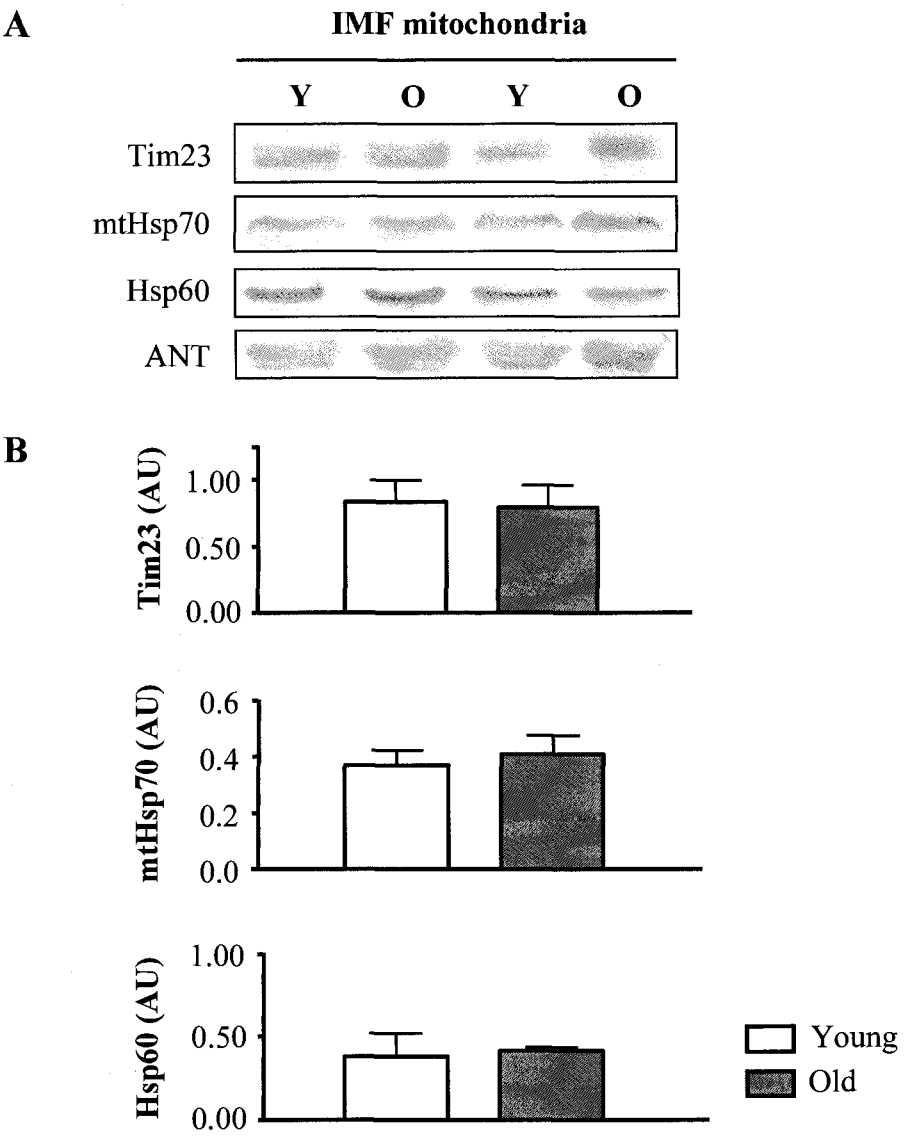


Fig. 1. Effect of age on the protein expression of mitochondrial PIM components. (A) Representative western blots of Tim23, mtHsp70 and Hsp60 content in IMF mitochondria isolated from young (Y) and old (O) animals. Adenine nucleotide translocase (ANT) was used as a control for loading. (B) Graphical representation of experiments using 35 μg of mitochondrial protein per lane (n=5).

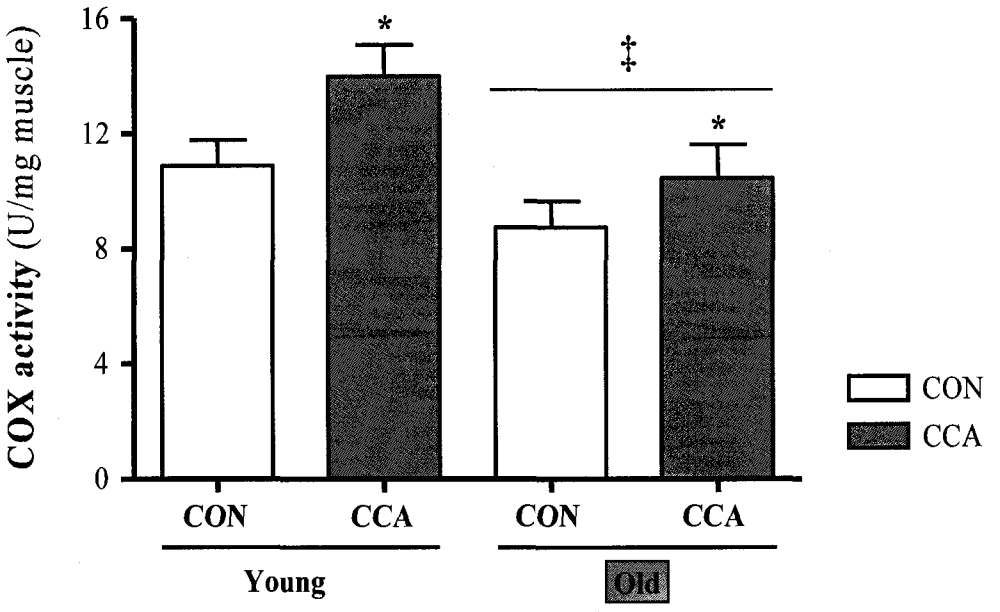


Fig. 2. Effects of age and 7-day CCA on cytochrome c oxidase activity. Whole muscle homogenates were prepared from control (CON) and stimulated (CCA) extensor digitorum longus muscles obtained from young and old animals. Values are expressed as units of COX activity per gram of tissue (n=9-16; * $p<0.05$ vs. internal control; ‡ $p<0.05$ vs. young).

APPENDIX C

Methods

ALKALINE LYSIS PLASMID DNA PREPARATION

Solutions

A) LB medium: 10 g tryptone
5 g yeast extract
10 g NaCl

Make up to 1 L with H_2O and autoclave. Store at room temperature until needed.

B) Ampicillin (AMP) stock: 50 mg/ml in ddH_2O Stored at -20 °C
Filter sterilize and aliquot into sterile eppendorfs
Use 2 µl for each ml of LB

C) LB + Agar plates: 5 g tryptone
2.5 g yeast extract
5 g NaCl
7.5 g agar
1 ml of 50 mg/ml AMP

Add ingredients and stir to dissolve in 500 ml of ddH_2O. Autoclave solution and allow to cool while stirring. Use a sterile thermometer to check temperature and add AMP when solution reaches below 50°C (final concentration of AMP is 100 mg/ml). Stir for ~1-2 min and immediately pour into sterile plates

D) M9 medium (10X stock): 30 g Na_2HPO_4
15 g KH_2PO_4
5 g NH_4Cl
2.5 g NaCl
480 ml H_2O

Combine ingredients and dissolve in 480 ml of ddH_2O. Autoclave, cool and store at 4°C.

E) Supplemented M9 medium

The following solutions combined with the M9 stock solution provides a supplemented M9 medium which is used on Day 2, step 1. Autoclave 437 ml H_2O in a 2 L Erlenmeyer (1 Erlenmeyer/plasmid), and allow to cool overnight. On day 2, add the following sterile supplements to each flask:

48 ml M9 medium (10X stock)
10 ml 20% casamino acids
2.5 ml 40 % glucose
2.5 ml 1 mg/ml thiamine
0.5 ml 1 M $MgSO_4$
1.0 ml 50 mM $CaCl_2$

Stock solutions for supplemented M9 medium

20% (w/v) casamino acids	100 g/500 ml, autoclave, store at 4°C
40% (w/v) glucose	40 g/100 ml, filter sterile, store at 4°C
1 mg/ml thiamine	filter sterile, store at 4°C in a dark bottle
1 M $MgSO_4$	12.04 g/100 ml, autoclave, store at 4°C
50 mM $CaCl_2$	277.45 mg/50 ml, autoclave, store at 4°C

F) Solution I: Stock solutions

20 ml 0.25 M EDTA	9.31 g /100 ml, pH to 8, autoclave
12.5 ml of 1M Tris-HCl	12.11 g/100 ml, pH to 8, autoclave
456 ml H_2O	

Autoclave and allow to cool. Add 11.25 ml of 40% glucose. Store at 4°C.

G) Lysozyme: make fresh the day of the experiment.

28 mg/ml (Sigma L-6876) dissolved in Solution I.

H) Solution II: Stock solutions

1.6 ml 10 M NaOH	40 g/100 ml, store at room temperature
8.0 ml 10% SDS	10 g/100 ml, store at room temperature
70 ml H_2O	

Make fresh the day of the experiment. No sterilizing.

I) Solution III: Stock solution

180 ml 5 M potassium acetate	122.7 g/250 ml
34.5 ml glacial acetic acid	
85.5 ml H_2O	

Autoclave and allow to cool. Store at 4°C.

J) Solution IV: Stock solutions

16.7 ml 3 M sodium acetate — 12.3 g/50 ml, pH 6.0
25 ml 1 M Tris-HCl — 12.11 g/100 ml, pH to 8, autoclave
458 ml H_2O

Check that final the pH = 8.0 (if around 7.9, then add 1.5 ml 1 M NaOH). Store at 4°C.

K) Lithium chloride: 84.8 g/250 ml, autoclave, store at 4°C.

L) Ribonuclease A:

10 mg/ml pancreatic RNase A (Sigma R-6513). Aliquot and store at -20°C.

M) TEN: Stock solutions

5 ml 1 M Tris-HCl — 12.11 g/100 ml, pH to 8, autoclave, store at RT
2 ml 0.25 M EDTA — 9.31 g/100 ml, pH to 8, autoclave, store at RT
1 ml 5 M NaCl — 29.22 g/100 ml, store at RT

Add to 450 ml dH_2O, pH to 7.5, and then volume up to 500 ml. Autoclave. Store at 4°C.

N) TE Buffer: Working solution

10 mM Tris base — 242.3 mg/200 ml
1 mM EDTA — 74.4 mg/200 ml

Combine powders and dissolve in 150 ml ddH_2O, pH to 8 and volume to 200 ml. Autoclave and store at room temperature.

O) Ethanol:

100% anhydrous ethyl alcohol (Commercial Alcohols Inc.). Store at -20°C.

Protocol

Day 1

1. Inoculate 10 ml of LB medium in a 125 ml Erlenmeyer flask containing the selective antibiotic. Shake overnight at 37°C.

Day 2

1. In a 2 L Erlenmeyer flask, pour the 10 ml growth culture evenly into 500 ml of the supplemented M9 medium.

2. Grow with vigorous shaking (200 rpm) at 37°C until the OD_{600} = 0.4 (2-3 hours). Spectrophometrically check the OD by removing 1 ml aliquots.

3. Add 0.5 ml of 135 mg/ml chloramphenicol (dissolved in absolute ethanol). Incubate overnight at 37°C with shaking (200 rpm). This step is done to stop chromosome replication and protein synthesis, and amplify the plasmid replication to thousands of copies/cell.

Day 3

1. Distribute each plasmid culture into 2 x 250 ml sterile centrifuge bottles.

2. Spin 5 min at 11,000 g at 4°C. Discard supernate and drain the pellets on a paper towel.

3. Resuspend the 2 pellets in a total of 6 ml of solution I (i.e. add 6 ml to one bottle, resuspend by pipetting up and down, then transfer to the second bottle and resuspend the pellet).

4. Transfer to a 50 ml sterile centrifuge tube.

5. Add 1.5 ml of lysozyme solution to each tube (1 tube/plasmid). Vortex and incubate on ice 30 min.

6. Add 18 ml of solution II to each tube. Cover with parafilm and invert the tubes gently to mix several times (3-4). Avoid excessive agitation as this will shear chromosomal DNA. Yellowish and clear layers will form. Incubate on ice 5 min.

7. Add 14 ml of solution III to each tube. Cover with parafilm and invert sharply several times (7-8). A white clot of genomic DNA will form. Incubate on ice 20 min.

8. Spin at 27,000 g (Beckman JA25.50 rotor) for 20 min at 4°C to pellet cell debris (the failure to form a compact pellet is usually a consequence of inadequate mixing of the bacterial lysate with solution III). Pour the supernate into a fresh labelled tube. Cover and invert several times. Sometimes suspended materials and precipitates form upon mixing. To remove this:

9. Spin again as above for 5-10 min and transfer to new tubes to remove suspended material (1 tube/plasmid).

10. Repeat this centrifugation step as necessary to remove all suspended material. Usually steps 8 and 9 are done. If some material remains, suck it off with a Pasteur pipette.

11. Split the supernate into two 50 ml tubes (2 tubes/plasmid). Balance and add 25 ml cold 100% ethanol to each tube. Cover with parafilm and mix well.

12. Incubate overnight at -20°C.

Day 4

1. Spin at 23,000 g for 15 min at 4°C.

2. Discard the supernate and drain the pellet on a paper towel.

3. Add 4.5 ml of solution IV to one tube. Resuspend and transfer to second tube. Resuspend by pipetting.

4. Add 3 ml 8M LiCl to each tube (1 tube/plasmid, now 4 tubes total).

5. Incubate on ice 20 min.

6. Spin at 23,000 g for 15 min at 4°C.

7. Transfer the supernates to new 50 ml tubes (1 tube/plasmid) and add 25 ml cold 100% ethanol. Cover with parafilm and mix well. Store at -20°C overnight.

Day 5

1. Spin at 23,000 g for 15 min at 4°C.

2. Discard the supernate and drain the pellet on a paper towel. Resuspend each pellet in 3 ml TEN. Transfer each to a 15 ml Corex tube (1 tube/plasmid).

3. Add 12.5 µl pancreatic RNase A per tube and incubate at 37°C for 20 min.

4. Add 1 volume (3 ml) of phenol (Sigma P4557). Vortex and spin for 5 min at 5,000 g at 4°C.

5. Transfer the upper aqueous phase to a new tube and add 1 volume of P:C:I (Sigma P3803). Vortex and spin as above.

6. Repeat step 24.

7. Transfer the upper phase to a new tube and add 1 volume of C:I (24:1 v/v; Chloroform, Caledon; Isoamyl alcohol, Sigma I9392). Vortex and spin as above.

8. Transfer the upper aqueous phase to a new tube and add 1 volume of ether (Caledon). Vortex and spin as above.

9. Discard the upper ether phase and add 1 volume of ether. Vortex and spin as above.

10. Repeat step 9.

11. Repeat step 9.

12. Discard the upper ether phase and add 150 µl 5M NaCl + 5 ml cold 100% ethanol. Cover with parafilm and mix well.

13. Incubate overnight at -20°C.

Day 6

1. Spin at 16,000 g for 15 min at 4°C.

2. Discard the supernate and vacuum dry the pellet.

3. Resuspend in 0.4-1 ml of TE Buffer. For example, use 0.2 ml for resuspension, and transfer to an eppendorf tube. Wash the tube with 0.2 ml and combine the volumes.

4. Measure the DNA concentration using the DPA assay.

5. Check the plasmid on an agarose gel.

ELECTRON MICROSCOPY

Solutions

A) 3% glutaraldehyde in 0.1 M sodium cacodylate buffer
Dilute stock glutaraldehyde in 0.1 M sodium cacodylate

B) 0.2 M sodium cacodylate (stock solution)
Make up 0.2 M stock solution in sterile water
Dilute to 0.1 M working solution with sterile ddH_2O

C) 1% Osmium tetroxide (OsO_4)
1 part 4% OsO_4
1 part sterile ddH_2O
2 parts 0.2 M sodium cacodylate

D) Graded ethanol washes
Dilute 100% ethanol (EtOH) to obtain 30%, 50% and 80% solutions

E) EtOH:Propylene oxide
Combine 100% EtOH with 100% propylene oxide (PO) in 1:1 ratio

F) Epon resin

Epon	2.88 g
DDSA	1.55 g
NMA	1.43 g
DMP-30	0.1 ml

G) PO:Epon resin
Combine PO and Epon resin in 1:1 ratio; must be made fresh

Fixation procedure

Note: All steps except muscle extraction must be conducted in the fumehood area

Day 1

1) Slice muscle into small, thin sections no more than 2mm in width. Orient strips depending on if you want cross-sectional or longitudinal EM pictures.

2) Incubate muscle sections in 1 mL 0.1 M Na cacodylate w/ 3% glutaraldehyde for 1 hour on ice.

3) Using a glass pasteur pipet, remove fixative and replace w/ 1 mL 0.1 M Na cacodylate. Let muscle sit for 15 min on ice. Remove wash buffer with glass pasteur pipet. Repeat two more times for a total of three washes.

4) Incubate for 1 hour in 1 mL 1% OsO_4 at room temperature.

* see below for further instructions if you need to break up this day into two

5) Remove OsO_4 and add 1 mL of 30% ethanol for 15 min @ RT.

6) Remove 30% EtOH and add 1 mL of 50% ethanol for 15 min @ RT.

7) Remove 50% EtOH and add 1 mL of 80% ethanol for 15 min @ RT.

8) Remove 80% EtOH and add 1 mL of 100% ethanol for 1 hour @ RT.

9) Remove muscle sections from plastic eppendorfs and place into glass vials containing 1 mL of PO:EtOH for 1 hour @ RT.

10) Remove and add 1 mL of 100% propylene oxide for 1 hour @ RT.

11) Remove and add 1 mL of PO:Epon resin for 1 hour @ RT.

12) Uncap vials and let evaporate slowly overnight in a glass dessicator @ RT.

Day 2

13) Embed tissues in fresh resin (ensure proper label is embedded in the resin)

14) Incubate at 60°C for 48 hours or over the weekend.

Day 4 (or 5)

13) Store resin blocks for further processing for EM.

* Muscle sections can be incubated overnight in 0.1 M sodium cacodylate after the 1 hour of osmium tetroxide incubation. Wash three times in 0.1 M sodium cacodylate prior to the overnight incubation (at 4°C). Continue on with the alcohol dehydration steps on the following day.

SKELETAL MUSCLE MITOCHONDRIAL ISOLATION

REFERENCES: Krieger et al., J. Appl. Physiol. 48: 23-28, 1980.
Cogswell et al. Am. J. Physiol. 264: C383-C389, 1993.

SOLUTIONS:

A) Buffer 1	100 mM KCl 5 mM $MgSO_4$ 5 mM EDTA 50 mM Tris base
B) Buffer 1 + ATP	Buffer 1 + 1 mM ATP
C) Buffer 2	100 mM KCl 5 mM $MgSO_4$ 5 mM EGTA 50 mM Tris base 1 mM ATP
D) Resuspension medium	100 mM KCl 10 mM MOPS 0.2% BSA

ISOLATION PROCEDURE:

This isolation procedure has been determined to be an optimal method for the isolation of pure and intact IMF and SS mitochondria through differential centrifugation. The cytosolic fraction can also be isolated from skeletal muscle. The entire procedure is done at 4°C (everything to be kept on ice).

1: Set out materials (put centrifuge tubes, scissors, forceps, Teflon pestle, watch glass and beakers on ice).

2: Remove the muscle sample and put it immediately in a beaker containing 10-20 ml Buffer 1 (no ATP), on ice. Repeat this for 3 subsequent rinse steps using Buffer 1.

3: Quickly blot the excess Buffer 1 from the muscle tissue on gauze and trim away fat and connective tissue. Place the muscle on the watch glass (on ice). Proceed to thoroughly mince the muscle with forceps and scissors, until the tissue is well mixed and no large pieces are remaining.

4: For each centrifuge tube, take between 1.0 g and 1.5 g of the minced tissue and record the exact weight. Place the sample in a capped 50 ml plastic centrifuge tube (on ice).
---Repeat this step for each sample---

5: Add a 10-fold dilution of Buffer 1 + ATP to each tube.
(Tissue weight * 10 - tissue weight = ml of solution to add for a 10-fold dilution).

6: Homogenize the samples using the Ultra-Turrax polytron (7 mm probe; 40% power output; 10 sec exposure). During the time the samples are being polytroned, move the plastic tube vigorously in a circular motion. Rinse the shaft with 0.5 mL of Buffer 1 + ATP. Be sure to clean the shaft (ddH_2O) before homogenizing the next sample.

7: At a centrifuge setting of 800 g, with Beckman JA25.50 rotor, spin homogenate samples for 10 min. This is the step that divides the IMF and SS mitochondrial fractions. The supernate will contain the SS mitochondria and the pellet will contain the IMF mitochondria. To differentiate between the two fractions, the SS fraction will be labelled with step numbers ending in "-SS" and the IMF fraction will be labelled with step numbers ending in "-IMF". Steps to isolate the cytosolic fraction will be labelled with step numbers ending in "-CYT".

SS mitochondrial isolation:

8-SS: Filter the supernate through a single layer of cheesecloth into a second set of 50 ml plastic centrifuge tubes. Spin tubes at 9000 g for 10 min to recover the mitochondria. The remaining pellet contains the IMF mitochondria and other cell constituents (see further isolation steps below).

9-SS: Upon completion of the spin, reserve the supernate to isolate the cytosolic fraction. Gently resuspend the pellet in 3.5 ml of Buffer 1 + ATP using a 1000 µl pipette. This is called the "wash step". Since the mitochondria are easily damaged, it is important that the resuspension of the pellet is done carefully.

10-SS: Repeat the centrifugation (9000 g for 10 min) and discard the supernate.

11-SS: Resuspend the SS mitochondrial pellet in a small volume of Resuspension Medium using a 1000 µl pipette. The volume depends on the amount of pellet isolated, however an approximate volume would be 0.4 ml. Resuspend the pellet gently to prevent damage to the mitochondria.

12-SS: Measure and record the final volume of the resuspended SS mitochondrial sample, and store in a labelled eppendorf tube. Keep the samples on ice while proceeding to isolate the IMF fraction.

IMF mitochondrial isolation:

8-IMF: Gently resuspend the pellet (from step 8) in at least a 10-fold dilution of Buffer 1 + ATP using the Teflon pestle.

9-IMF: Homogenize the resuspended pellet for 10 sec (as done previously in step 6).

10-IMF: Spin at 800 g for 10 min and discard the resulting supernate.

11-IMF: Resuspend the pellet in at least a 10-fold dilution of Buffer 2 using a Teflon pestle.

12-IMF: Add the appropriate amount of nagarse to the resuspended homogenate (this protease releases the IMF mitochondria from the myofibrils). The calculation for the appropriate volume is 0.025 ml/g of tissue. Mix the homogenate gently and let stand on ice for exactly 5 min.

13-IMF: After 5 min, dilute the nagarse by adding 20 ml of Buffer 2. Spin the diluted samples at 5000 g for 5 min.

14-IMF: Discard the resulting supernate. Gently resuspend the pellet in at least a 10-fold dilution of Buffer 2 with a Teflon pestle.

15-IMF: Spin the samples at 800 g for 10 minutes. This spin allows the IMF mitochondria to remain in the supernate.

16-IMF: Upon the completion of the spin, pour the supernate into another set of 50 ml plastic tubes (on ice). Discard the pellet. Spin the supernate at 9000 g for 10 min.

17-IMF: Discard the supernate and resuspend the pellet in 3.5 ml of Buffer 2 using the 1000 µl pipette. This is the "wash step" for the IMF mitochondria. Spin samples at 9000 g for 10 min.

18-IMF: Discard the supernate. Gently resuspend the pellet in a small volume Resuspension Medium using a 1000 µl pipette. The volume depends on the size of the resulting pellet, however an approximate volume is 0.45 ml.

19-IMF: Using a 1000 µl pipette, measure and record the final volume of the resuspended IMF sample and store in labelled eppendorf tubes.

Cytosolic fraction isolation:

1-CYT: Pour the reserved supernate from step 9 into an 8.9 ml polyallomer centrifuge tube (Beckman). Repeat for each sample. Spin tubes at 4°C, in an ultracentrifuge (Beckman 50 TI rotor) at 100 000 g for 1 hr.

2-CYT: Pour supernate into an ultrafiltration cell (4°C; Amicon) to concentrate the cytosolic fraction to a volume of < 1 ml (molecular weight cutoff: 10 kDa).

3-CYT: Transfer the concentrated cytosolic fraction to a labelled eppendorf tube. Store at -20°C until needed.

PROTEIN IMPORT INTO SKELETAL MUSCLE MITOCHONDRIA

REFERENCE: Takahashi and Hood, J. Biol. Chem. (1996) 271, 27285-27291.

The majority of nuclear-encoded mitochondrial proteins require a mechanism of import. Proteins destined for this organelle also require a presequence. This will provide a "passport" containing instructions for the final destination for that protein. Once the protein has reached its final destination, the presequence is cleaved, leaving a "mature" protein. By taking advantage of this change in protein size, we are able to establish a means by which imported proteins can be discerned. This can be accomplished by radiolabelling the precursor proteins in vitro then allowing them to be imported. The samples can be electrophoresed on a polyacrylamide gel and will appear as two bands (precursor and mature) when the gel is exposed to film.

A: DNA linearization

Note: Good quality DNA is needed for this preparation. This can be obtained using an alkaline lysis DNA isolation method.

1. Linearize DNA

Combine the following in an eppendorf:

40 µl DNA at 5 µg/µl (200 µg)
5 µl 10x Enzyme buffer
5 µl Restriction enzyme

Incubate for 1 hour at 37°C.

2. Phenol extraction/ethanol precipitation

a) Add appropriate volume of sterile dH_2O so that the final volume equals 400 µl.
b) Add 400 µl phenol (Sigma P4557). Mix by inversion and spin in microfuge for 30 sec.
c) Withdraw and transfer upper phase to a sterile eppendorf.
d) Add 400 µl P:C:I (Sigma P3803). Mix by inversion and spin in microfuge for 30 sec.
e) Withdraw and transfer upper phase to a sterile eppendorf.
f) Add 400 µl C:I (24:1, v:v). Mix by inversion and spin in microfuge for 30 sec.
g) Withdraw and transfer upper phase to a sterile eppendorf.
h) Add 40 µl 3 M sodium acetate and 1 ml of cold 100% ethanol.
i) Mix by inversion and precipitate at -80°C overnight.
j) Spin 10 min at 4°C in microfuge and discard the supernate.
k) Gently wash pellet with 400 µl 70% ethanol.

l) Spin 3 min at 4°C, discard the supernate.
m) Dessicate pellet.
n) Resuspend the pellet in 30-50 µl TE, pH 8.0.

3. Measure DNA concentration

a) Read the O.D. at A_{260} to determine the concentration of DNA.
b) Dilute the DNA to 0.8 µg/µl in sterile TE Buffer (pH 8.0).

B: In vitro transcription

1. Combine the following in the order indicated:

60.8 µl plasmid (0.8 µg/µl in TE)
8.4 µl dH_2O
5.2 µl NTP (10 mM)
10.0 µl ATP (10 mM)
11.6 µl 7-MGG (1 mM)
15.6 µl Mix 1
5.2 µl RNA guard
4.8 µl of appropriate RNA polymerase
121.6 µl total volume

Incubate for 90 min at the optimum temperature for the polymerase (i.e. 37°C for T7, 40°C for SP6). The reaction volume can be modified, provided proportions are maintained.

2. Phenol extraction/ethanol precipitation

a) Bring volume up to 400 µl with sterile dH_2O (using 280 µl)
b) Proceed exactly as described in step 3, above.
c) Resuspend the pellet in 25-40 µl sterile dH_2O

3. Measure mRNA concentration

a) Read the O.D. at A260 to determine the concentration of mRNA
b) Dilute mRNA to 2.8 µg/µl in sterile dH_2O. Store at -20 °C.

C: In vitro translation

Promega Technical Manual: "Rabbit Reticulocyte Lysate System" Part #TM232.

1a) Combine the following:

	1 Rx (µl)	% of Rx
Lysate	11.8	64.1%
AA(-met)	0.4	2.2%
st. dH_2O	3.97	21.1%
^{35}S-met	1.33	7.2%
mRNA	1.0	5.4%
TOTAL	18.5	

Notes:
i) The Promega manual suggests that lysate should be thawed slowly on ice. It also suggests that the number of freeze/thaw cycles be limited to two.
ii) The volume of mRNA can be adjusted to optimize translational efficiency by altering the dH_2O volume accordingly.

b) Incubate for 25-60 min. at 30 °C to obtain a translation produce (TP) (note: time may vary with mRNA)
c) Record ^{35}S use (15 µCi/µl).

D: Protein import
Isolated mitochondria (see protocol above) should be resuspended in a resuspension buffer (100 mM KCl, 10 mM MOPS and 0.2% BSA, pH 7.4).

a) Pre-incubate the mitochondria and TP, separately, for 10 minutes @ 30^0C, and then combine TP and mitochondria to initiate import reaction.
b) Incubate @ 30^0C for a time course (i.e. 0, 5, 10, 20 min).
c) To recover the mitochondria following import, spin the entire volume through a sucrose gradient (600 µl) for 15 min at 4^0C.
d) Remove the supernate and discard into the radioactive waste.
e) Resuspend pellets in 25 µl breaking buffer.
f) Add 25 µl 1x lysis buffer and 10 µl sample dye.
g) Denature samples for 5 min @ 95°C, then quick cool on ice. Spin down samples prior to loading in the radioactive specified microcentrifuge.
h) Apply the samples to a SDS polyacrylamide gel (range of 8-12%) and electrophorese to separate proteins. A sample pipette plan is provided below (typically 10 lanes can be run on SDS-PAGE gels):

Lane	1	2	3	4	5	6	7	8	9	10
Time (min)	RM	0	5	10	20	0	5	10	20	TL
Import Rx (µl)	0 µl	25								0 µl
TP (µl)	0									5 µl
1x LB (µl)	0 µl	25								45 µl
SD (µl)	0 µl	10								

D. Fluorography

a) Remove gel from chamber and cut out the appropriate section of the gel. Identify the orientation of the gel.
b) Boil the gel in approximately 200 ml of 5% TCA for 5 min. in metal container, over the Bunsen burner in the fume hood.
c) Using a spatula, transfer the gel to a radioactive Tupperware container and rinse the gel briefly in dH_2O (approximately 30 seconds).
d) Wash in 10 mM Tris-base for 5 min. on shaker (approximately 100 ml).
e) Wash in 1 M salicylic acid for 30 min. on shaker (approximately 100 ml).
f) Dry the gel for 1 hr. at 80°C.
g) Combine all wash solutions in the metal container and boil down the liquid. Let cool and then dispose of in ^{35}S waste.

E. Acquiring autoradiogram

a) Place dried gel into an imaging cassette. Lay the white side of the Kodak Phosphor imaging screen overtop the gel. Close the cassette and lock cover in place.
b) Image the screen overnight. Use the Pharos Fx Plus Molecular Imager (Biorad) to acquire signal for [^{35}S]-Methionine.
c) Quantify import using Quantity One software (Biorad) by dividing the signal obtained for the mature protein by the total available radiolabelled protein (i.e. the sum of the signals obtained for the precursor and mature proteins).

Solutions for Transcription and Translation

Note: Because mRNA is involved in both transcription and translation, sterile conditions should be adhered to at all times. Use sterile glassware, eppendorfs, dH_2O, etc. Autoclave or sterile filter solutions where indicated.

10 X Proteinase K buffer	For 10 ml	
500 mM NaCl	0.2922g	or 5 ml of 5 M NaCl
50 mM EDTA	0.1861g	or 0.5 ml of 0.5 M EDTA, pH 8
100 mM Tris-HCl	0.1576g	or 1 ml of 1 M Tris, pH 8
3.5 ml sterile dH_2O		
-pH solution to 8.0		

20 mM HEPES Use 0.4766g/100 ml, pH to 7.0, autoclave.

1 M HEPES Use 23.83 g/100ml, pH to 7.9, autoclave. Store at RT.

NTPs	MW(g/mol)	For 50 mM (stocks)
CTP	483.2	24.16 mg/ml
GTP	523.2	26.16 mg/ml
UTP	484.1	24.21 mg/ml
ATP	551.1	27.56 mg/ml

Note: These NTPs should be made up in 20 mM HEPES. Make up 1 ml of each as stock. Filter each sterile (using sterile acrodisk).

10 mM NTPs	For 500 µl
10 mM GTP	100 µl of 50 mM GTP stock
10 mM CTP	100 µl of 50 mM CTP stock
10 mM UTP	100 µl of 50 mM UTP stock
20 mM HEPES (pH 7.0)	200 µl of 20 mM HEPES(pH 7.0)
- store in 50 µl aliquots at -20 °C	

10 mM ATP	For 500 µl
10 mM ATP	100 µl of 50 mM ATP
20 mM HEPES (pH 7.0)	400 µl of 20 mM HEPES (pH 7.0)
- store in 50 µl aliquots at -20 °C	

7-MGG (Pharmacia 27-4635-02)
1 mM stock is made from Pharmacia pellet. 25U is ordered; added to this is 1208 µl of 20 mM HEPES (pH 7.0), yielding a 1 mM stock. Aliquot into 300 µl, store at -20 °C.

Mix 1	For 1200 µl
0.167 M HEPES (pH 7.9)	200 µl of 1 M HEPES (pH 7.9)
0.083 M $MgAc_2$	100 µl of 1 M $MgAc_2$
1.667 M Kac	500 µl of 4 M KAc
1.667 mM spermidine	100 µl of 20 mM spermidine
0.042 M DTT	100 µl of 0.5 M DTT
200 µl of dH_2O	
- store in 100 µl aliquots at -20 °C	

Phenol - Sigma P4557

Phenol:Chloroform:Isoamyl alcohol - Sigma I9392

Chloroform – Caledon 3000-1-40

Isoamyl alcohol - Sigma I9392

TE Buffer	Working solution
10 mM Tris base	242.3 mg/200 ml
1 mM EDTA	74.4 mg/200 ml

Combine powders and dissolve in 150 ml ddH_2O, pH to 8 and volume to 200 ml. Autoclave and store at room temperature.

RNA guard - Pharmacia #27-0815-01

Lysate - Fisher Scientific (Promega) PRL 4960

^{35}S-met - Amersham SJ-1515

T7 RNA Polymerase - Boehringer 881767 (20 U/ µl)
SP6 RNA Polymerase - Boehringer 1487671 (20 U/ µl)

Solutions for Import

PMSF
Prepare stock of 130mM in DMSO (i.e. 22.65 mg PMSF / ml DMSO). Store at -20 °C.

Breaking Buffer	for 100ml	
0.6 M Sorbitol	25 ml of 2.4 M Sorbitol	-store at 4 °C
20 mM HEPES	2 ml of 1 M Hepes (pH 7.4)	
72 ml dH_20		

- Store at 4°C (can leave up to 1 month)

2.4 M Sorbitol Use 43.73 g / 100 ml. Store at 4 °C.

2.5 M KCl Use 18.64 g / 100 ml. Store at 4 °C.

1 M $MgCl_2$ Use 2.033 g / 10 ml. Store at 4 °C.

1 M HEPES (pH 7.4) Use 238.3 mg/ml, pH to 7.4. Store at 4 °C.

Sucrose cushion (make fresh per experiment)	for 25 ml
0.6 M Sucrose	5 g
0.1 M KCl	1 ml of 2.5 M KCl
2 mM $MgCl_2$	50 µl of 1 M $MgCl_2$
20 mM HEPES	0.5 ml of 1 M HEPES (pH 7.4)

Solutions for Fluorography

5% TCA Use 200g / 4 L

10 mM Tris-base Use 4.846 g / 4 L

1 M Salicylic acid Use 640.4 g / 4 L

PROTEIN IMPORT SUPPLEMENTED WITH CYTOSOLIC FRACTION

Isolated mitochondria should be resuspended in a resuspension buffer (100 mM KCl, 10 mM MOPS and 0.2% BSA, pH 7.4).

The import of preproteins into mitochondria in the presence of the cytosolic fraction is similar to the protocol outlined in "PROTEIN IMPORT INTO SKELETAL MUSCLE MITOCHONDRIA" with only a few modifications as listed below.

1. Prepare the cytosolic fraction

a) Determine the protein concentration of cytosolic fractions using the Bradford method. Adjust so that final concentration is 1 µg/µl.
b) Pipette 7.5 µl and 15 µl of the cytosolic fraction (1 µg/µl) into pre-labelled eppendorfs (ensure you have labelled a sufficient number of eppendorfs for your time course).
c) Pipette 7.5 µl and 15 µl of Buffer 1 (no ATP) into pre-labelled eppendorfs (ensure you have labelled a sufficient number of eppendorfs for your time course). These will serve as the control conditions.

2. Import reaction

a) During the last stages of the mitochondrial isolation, prepare the translation product (TP) using the protocol outlined above.
b) After the translation is complete, combine the TP and cytosolic fractions together. Keep on ice until needed.
c) Determine mitochondrial protein content and adjust to a final concentration of 4 µg/µl. Pre-incubate the mitochondria and the TP-cytosolic fraction mix, separately, for 10 minutes @ 30^0C.
d) Combine the TP-cytosolic fraction mix and mitochondria to initiate import reaction.
e) Incubate @ 30^0C for 20 min (note: time of import reaction will vary).
f) To recover the mitochondria following import, spin the entire volume through a sucrose gradient (600 µl) for 15 min at 4^0C.
g) Remove the supernate and discard into the radioactive waste.
h) Resuspend pellets in 25 µl breaking buffer.
i) Add 25 µl 1x lysis buffer and 10 µl sample dye.
j) Denature samples for 5 min @ 95°C, then quick cool on ice. Spin down samples prior to loading in the radioactive specified microcentrifuge.
k) Apply the samples to a SDS polyacrylamide gel (range of 8-12%) and electrophorese to separate proteins.

A sample pipette plan is provided below (typically 10 lanes can be run on SDS-PAGE gels):

Lane	1	2	3	4	5	6	7	8	9	10
		----------YOUNG----------				----------OLD----------				
Time (min)	RM	B7.5	C7.5	B15	C15	B7.5	C7.5	B15	C15	TL
Import Rx (µl)	0 µl	25---------->								0 µl
TP (µl)	0---------->									5 µl
1x LB (µl)	0 µl	25---------->								45 µl
SD (µl)	0 µl	10---------->								

D. Fluorography

a) Remove gel from chamber and cut out the appropriate section of the gel. Identify the orientation of the gel.
b) Boil the gel in approximately 200 ml of 5% TCA for 5 min. in metal container, over the Bunsen burner in the fume hood.
c) Using a spatula, transfer the gel to a radioactive Tupperware container and rinse the gel briefly in dH_2O (approximately 30 seconds).
d) Wash in 10 mM Tris-base for 5 min. on shaker (approximately 100 ml).
e) Wash in 1 M salicylic acid for 30 min. on shaker (approximately 100 ml).
f) Dry the gel for 1 hr. at 80°C.
g) Combine all wash solutions in the metal container and boil down the liquid. Let cool and then dispose of in ^{35}S waste.

E. Acquiring autoradiogram

a) Place dried gel into an imaging cassette. Lay the white side of a Kodak Phosphor imaging screen overtop the gel. Close the cassette and lock cover in place.
b) Image the screen overnight. Use the Pharos Fx Plus Molecular Imager (Biorad) to acquire signal for [^{35}S]-Methionine.
c) Quantify import using Quantity One software (Biorad) by dividing the signal obtained for the mature protein by the total available radiolabelled protein (i.e. the sum of the signals obtained for the precursor and mature proteins).

Additional solutions (different from previous protocol)

Buffer 1
100 mM KCl
5 mM $MgSO_4$
5 mM EDTA
50 mM Tris base

CYTOSOLIC FRACTION-MEDIATED DEGRADATION OF MITOCHONDRIAL PREPROTEINS

The final reaction contains:

- 5 µl ^{35S}Met-labelled precursor protein
- 10 µl cytosolic fraction proteins (10 µg/µl, 100 µg in total)
- 15 µl Buffer 1
- 10 µl Sample Dye
- 30 µl 1x Lysis Buffer

PROCEDURE

1. Thaw products for the *in vitro* translation on ice. For each reaction, pipette into eppendorfs the following (total volume = 60 µl):

Lysate	64.1 %	38.46 µl
AA	2.20 %	1.32 µl
mRNA	5.40 %	3.24 µl
dH_20	21.1 %	12.66 µl
^{35}S-Met	7.20 %	4.32 µl

2. Incubate the translation reaction for the appropriate time needed to obtain a sufficient translation product (TP) (i.e. 30 min for pOCT).

3. During translation, adjust concentrations of cytosolic fractions to 10 µg/µl. Aliquot 10 µl per eppendorf for each condition. Aliquot 10 µl of Buffer 1 for each control condition into eppendorfs (i.e. 3 for the young cytosolic fraction, and 3 for the old).

4. Once translation is complete pre-incubate cytosolic fraction/Buffer 1 and TP, separately, at 30°C for 10 min.

5. To start degradation assay, add TP (5 µl) each eppendorf containing the cytosolic fraction/Buffer 1 (final volume is 15 µl). Stagger this process to facilitate the removal of samples from the water bath. Note: A control sample is put on ice immediately after mixing Buffer 1 and TP together (i.e. CON, 0 min). This sample is used as a reference to the quality of the translation reaction.

6. Remove the 30 min sample from the water bath after the time course is complete and put on ice immediately. Repeat for 120 min samples.

7. Add 25 µl of 1x LB w/ 5% beta-mercaptoethanol and 6.5 µl sample dye to each eppendorf. Boil at 95°C for 5 min. Spin down volume and load onto 12% SDS-PA gels.

8. Run gel until blue dye is close to the bottom of the glass plate. Do not allow sample to completely run through the gel, as this may contaminate the apparatus with radioactivity.

9. Once electrophoresis is complete, subject the gel to fluorography (see protocol "Protein import into skeletal muscle mitochondria").

10. Place dried gel into an imaging cassette. Lay the white side of a Kodak Phosphor imaging screen overtop the gel. Close the cassette and lock cover in place.

11. Image the screen overnight. Use the Pharos Fx Plus Molecular Imager (Biorad) to acquire signal intensities for [^{35}S]-Methionine. Determine percent degradation of precursor proteins:

    1 - (cytosolic fraction ÷ buffer control) = % pOCT degradation

    Example:

	Intensity	% Buffer 1	% pOCT degradation
CON (0 min)	65401	-	-
Buffer (30 min)	45577	-	-
Cytosol (30 min)	30668	67	33
Buffer (120 min)	23205	-	-
Cytosol (120 min)	10624	46	54

APPENDIX D

Contributions

Throughout the duration of my MSc degree, I contributed to the following studies:

1. Chabi B, Ljubicic V, Menzies KJ, **Huang JH**, Saleem A and Hood DA. Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. *Aging Cell* 7: 2-12, 2008.

2. **Huang JH** and Hood DA. The effects of aging and chronic contractile activity on the mitochondrial protein import pathway in skeletal muscle. (Abstract presented at the annual Ontario Exercise Physiology Conference, Barrie, Ontario, Canada, 2007).

3. Joseph AM, **Huang JH**, Adhihetty PJ, Ljubicic V and Hood DA. Mitochondrial protein import and assembly dynamics in response to chronic contractile activity in skeletal muscle of young and aged animals (Abstract submitted for the annual Experimental Biology Conference, San Diego, LA, 2008).

4. Ljubicic V, Adhihetty PJ, Joseph AM, **Huang JH**, Saleen A, Uguccioni G, Menzies KJ and Hood DA. Plasticity of aged skeletal muscle: chronic contractile activity-induced adaptations in muscle and mitochondrial function (Abstract submitted for the annual Experimental Biology Conference, San Diego, LA, 2008).

5. **Huang JH** and Hood DA. Exercise-induced adaptations in protein import kinetics are attenuated in skeletal muscle mitochondria of senescent animals. (Abstract presented at the annual Ontario Exercise Physiology Conference, Barrie, Ontario, Canada, 2008).

The following people have been instrumental to the preparation of this manuscript:

1. Vladimir Ljubicic and Peter Adhihetty were responsible for all animal preparations: electrode implantation, initiation and termination of chronic contractile activity, surveillance,

2. Anna-Maria Joseph was responsible for the isolation of the cytosolic fractions which were used in the protein import and pOCT degradation assays. In addition, she taught me the techniques required to isolate plasmid DNA and to assess the import of proteins into mitochondria using the described *in vitro* methods.

3. Giulia Uguccioni was responsible for the COX activity data shown in appendix B (Fig. 2).