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A mitochondrial perspective on striated muscle physiopathology: insights from sepsis, denervation, and dystrophinopathies.

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Résumé

La mitochondrie est de plus en plus reconnue pour sa contribution à la dégénerescence musculaire. Les dysfonctions mitochondriales, en plus de causer une défaillance énergétique, contribuent à la signalisation apoptotique, stimule la production de ROS et peuvent induire une surcharge calcique. Ces caractéristiques sont tous reliées à certains types de myopathies. Cette thèse met en lumières comment certaines dysfonctions mitochondriales peuvent intervenir dans la pathogenèse de diverses myopathies. Nous démontrons que les dysfonctions mitochondriales sont impliqués dans l'atrophie dû à la perte d'innervation. Par contre, la désensabilisation de l'ouverture du pore mitochondrial de transition de perméabilité, via ablation génétique de cyclophiline-D, ne prévient ni la signalisation apoptotique mitochondrial ni l'atrophie. Nous avons aussi observé des dysfonctions mitochondriales dans le muscle atteint de dystrophie musculaire de Duchenne qui furent améliorés suite à une transfection de PGC1-α, laquelle résulta aussi en une amélioration de la pathologie. Finalement, nous démontrons que le recyclage de mitochondrie par les voies de mitophagies et de contrôles de la qualité impliquant Parkin et possiblement d'autres voies de signalisation inconnues sont cruciales au recouvrement cardiaqe lors d'un choc septique.

Mots clés: mitochondrie, ROS, mPTP, respiration, signalisation apoptotique, atrophie, muscle squelettique, dénervation, dystrophie musculaire de duchenne, PGC1- α, biogenèse, parkin, mitophagie, septicémie, métabolisme cardiaque.

Summary

Mitochondria are increasingly being recognized for their role in contributing in cellular damage. Mitochondrial dysfunctions, in addition to causing energy failure, contribute to apoptotic signaling, stimulate ROS production and calcium overload. These are all features of various types of myopathies. This thesis sheds light on how mitochondrial dyfunctions may contribute to the pathogenesis in certain myopathies that have been found to show mitochondrial abnormalities. Specifically, we found that although mitochondrial dysfunctions are involved in denervation-associated atrophy, desensitizing mitochondrial permeability transition pore opening through genetic ablation of CyclophilinD does not prevent mitochondrial apoptotic signaling nor atrophy in this model of chronic inactivity. We also observed mitochondrial dysfunctions in the Duchenne dystrophic muscle that were improved after PGC1-α transfection, which also resulted in an amelioration of the disease presentation. Finally, we found that mitochondrial recycling, led by Parkin and alternate mitophagy pathways a crucial component of cardiac recovery in sepsis.

Keywords: mitochondria, ROS, mPTP, respiration, apoptotic signaling, atrophy, skeletal muscle, denervation, duchenne muscular dystrophy, PGC1-α, biogenesis, Parkin, mitophagy, sepsis, cardiac metabolism.

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List of abbreviation

Ca^{2+}: Calcium
ROS: Reactive Oxygen Species
PTP: Permeability Transition Pore
CypD: Cyclophilin D
WT:Wild-type
DMD: Duchenne muscular Dystrophy
PGC1-α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
QC: Quality Control
LPS: Lipopolysaccharide
mtDNA: mitochondrial Deoxyribonucleic Acid
OMM: Outer mitochondrial Membrane
TOM/TIM: Translocase Outer/Inner Membrane
VDAC: Voltage-Dependant Anion Channel
ETC: Electron Transport Chain
ATP/ADP: Adenosine Tri/Diphoshate
NRF: Nuclear Respiratory Factor
TFAM: Mitochondrial Transcription Factor A
AMPK: Adenosine Monophosphate activated Kinase
SIRT1: NAD-dependent deacetylase silent mating type information regulation 2 homolog
NADH: Nicotinamide Adenine DInucleotide
FADH: Flavin Adenine Dinucleotide
SOD: Superoxide Dismutase
ANT: Adenine Nucleotide Translocase
CYPD: Cyclophilin D
BH: Bax Oligomer
HSP: Heat Shock Protein
ER: Endoplasmic Reticulum
LC3: Light-Chain
ATG: Autophagy-related Genes
mTOR: mammalian Target Of Rapamycin
Drp: Dynamin-Related Protein
Mfn: Mitofusin
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
OXPHOS: Oxidative Phosphorylation
TLR: Toll-Like Receptor

List of tables and figures:

1 Foreword

This thesis was completed under the supervision of Yan Burelle whose research program focuses on the role of mitochondrial (dys)functions in normal physiology and their implication in the pathogenesis of various diseases in striated muscle. Over the years, his laboratory has developed a unique expertise in the assessment of multiple facets of mitochondrial function (respiration, ROS dynamics, Ca^{2+} regulation, cell death signalling, biogenesis and mitophagy) using various approaches *in vivo*, *in situ* and *in vitro*. These approaches have been used not only in basic studies using animal models (Picard, 2008, Am J Physiol), but also in tissues from patients in the context of translational research (Picard, 2008, Am J Respir Crit Care Med). The research program in the laboratory is articulated around two main axes. The first research axis is focused on mitochondrial functional abnormalities associated with genetic and acquired cardiomyopathies, on their role in disease progression, and on testing novel therapeutic strategies targeted to these organelles. The second research axis is focused on the implication of mitochondria in various pathologies affecting skeletal muscle including disuse and muscular dystrophies. This broad scope of interest is well reflected in the present thesis, which includes three articles exploring diverse but inter-related questions on the role of mitochondrial dysfunction in cardiac and skeletal muscle pathogenesis.

1.1 Introduction to the articles:

1.1.1 Study 1:

The first article, which was published in *The Journal of Physiology* in 2011, focused on the implication of mitochondrial dysfunction in muscle atrophy and degeneration following loss of innervation. Prior to this work, several reports (Rasola, 2007, Apoptosis / Halestrap, 2005, Nature), including ours (Csukly, 2006, J. Physiol 2006), provided evidence that mitochondria contributed to the activation of muscle proteolysis and apoptosis through the permeabilization of their membranes and the subsequent release of pro-apoptotic factors. However, the mechanisms

involved in mitochondrial membrane permeabilization, in particular the role of the permeability transition pore (PTP), was not clearly delineated. In this first study we therefore used transgenic mice harboring a ubiquitous knockout of the PTP regulating protein cyclophilin-D (CypD) to determine whether CypD-dependent opening of the PTP played a role in muscle apoptotic signaling and atrophy. We observed that denervation in wild type (WT) mice induced muscle atrophy, a strong increase in the propensity of mitochondria to opening of the PTP *in vitro*, and activation of mitochondria-dependent apoptotic proteolytic signaling. Moreover, although lack of CypD decreased the overall propensity to opening of the PTP of the normal muscle in vitro, it did not prevent denervation-induced sensitization to permeability transition, muscle atrophy or the activation of apoptotic signaling. Our study therefore provided direct evidence that CypD, and by extension opening of the PTP, was dispensable for atrophy and activation of apoptotic proteolytic signalling induced by denervation

1.1.2 Study 2:

After completion of this first study, we turned our attention to Duchenne Muscular Dystrophy (DMD), the most frequent form of dystrophy in humans. Although DMD is primarily caused by mutation of the cytoskeletal protein dystrophin, it is suggested by our laboratory (Ascah, 2011, Am J Physiol Heart Circ Physiol / Burelle, 2010, J Mol Cell Cardiol) and others (Reutenauer, 2008, Br J Pharmacol), that mitochondrial dysfunction, a presumed secondary consequence of dystrophin deficiency, could play a role in the pathogenesis of myocyte necrosis. However, the extent to which the multiple facets of mitochondrial function are altered in DMD remained uncertain due to the lack of detailed assessment. Importantly, at the time this study was initiated, the group of Bruce Spielgman had shown that crossing of *mdx* mice (the murine model of DMD) with mice overexpressing PGC1α, a master regulator of mitochondrial biogenesis, improved skeletal muscle pathology (Handschin, 2007, Genes Dev). However, the underlying mechanisms were not explored in details. In the second study presented in this

thesis, which was published in the *Journal of Physiology* in 2012 we therefore performed a detailed assessment of mitochondrial function in skeletal muscle from young *mdx* mice. Short-term overexpression of the transcriptional co-activator PGC1α, achieved by *in vivo* plasmid transfection, was then performed to determine whether this intervention could prevent mitochondrial impairment and mitigate associated biochemical abnormalities after the onset of necrosis. Our results showed the presence of multiple mitochondrial abnormalities in dystrophin-deficient muscle including a lower mitochondrial biomass and oxidative capacity, a greater ROS buffering capabilities, and an increased vulnerability to Ca^{2+}-induced opening of the mitochondrial permeability transition pore complex (PTP). Importantly, we showed that PGC1α gene transfer restored mitochondrial biomass, normalized the susceptibility to PTP opening, increased the capacity of mitochondria to buffer Ca^{2+} and reduced the activity levels of the Ca^{2+}-dependent protease calpain as well as caspases 3 and 9. Our results thus provided novel information on the nature of mitochondrial dysfunction in DMD as well as new mechanistic information regarding the beneficial effects of PGC1 α overexpression upon dystrophic muscles.

1.1.3 Study 3:

Over the course of this Ph.D. thesis, several studies, mostly in the field of cell physiology have contributed to advance our knowledge on mitochondrial quality control (QC) mechanisms, which, together with mitochondrial biogenesis, is responsible for the maintenance of optimal function of the mitochondrial pool within cells. Among the mitochondrial QC mechanisms, mitophagy, a process whereby defective mitochondria are selectively enclosed in autophagosomes for subsequent degradation into lysosomes, has attracted major interest. This stemmed in part from the discovery of Richard Youle and others that the E3-ligase Parkin played an important role in identifying defective mitochondria for autophagic degradation (Narendra, 2009, Autophagy).

The molecular mechanisms regulating mitophagy as well as its role in muscle physiopathology currently remains undefined. However, considering the centrality of this process in the life cycle of mitochondria, we reasoned that mitophagy may be important for the maintenance of optimal mitochondrial and muscle function. In the third study presented in this thesis, which is under review in the journal *Autophagy*, we tested the hypothesis that Parkin is important for the maintenance of normal mitochondrial function in the heart and adequate response to mitochondrial injury under stress conditions. To this end, the cardiac and mitochondrial phenotype of wild type mice and mice harbouring a deletion of exon 3 of the Parkin gene was assessed under normal conditions, and in response to sepsis induced by *E. Coli* lipopolysaccharide (LPS). This clinically relevant stress model was used because it has previously been associated with mitochondrial dysfunctions (Supinsky, 2009, Am J Physiol Regul Integr Comp), compensatory mitochondrial biogenesis (Suliman, 2004, Cardiovasc Res) and activation of autophagy (Hickson-Bick, 2006, Shock), three features suggesting an important implication of mitochondrial QC in the cardiac response to sepsis. Our results showed a lack of cardiac pathology in young Parkin-deficient mice under normal baseline conditions, despite the presence of multiple but mild mitochondrial abnormalities. However, when challenged with LPS, Parkin-deficient mice displayed impaired recovery of mitochondrial function and hemodynamics compared to WT mice. Importantly, we observed that LPS induced the recruitment of the autophagy machinery to mitochondria even in the absence of Parkin, likely through compensatory mechanisms involving Bnip3 and Nix, two BH3-only protein of the Bcl-2 family with a dual role in apoptosis and mitophagy. We also found that non-selective macroautophagy was enhanced in parkin-deficient compared to controls both at baseline and in response to LPS presumably to compensate for a mitophagy deficit. Overall, this study provided several novel information in this emerging field of research namely that i) mitophagy is strongly induced as part of the normal cardiac response to sepsis, ii) Parkin is important for the maintenance of normal mitochondrial functions in the heart and iii) in absence of Parkin, the alternate mechanisms to recruit the autophagy machinery to mitochondria are present but are likely less optimal as they associate with impaired

mitochondrial and cardiac recovery from sepsis. Of note, we have obtained convincing experimental evidence suggesting similarities between the heart and skeletal muscle with respect to the role of Parkin. However, the results were judged too preliminary to be included in the body of this thesis.

1.2 Introduction to the literature review:

Considering that the three studies included in this thesis are revolving around the role of mitochondrial dysfunctions but are not focused on a single type of muscle (heart *vs* skeletal muscle) or physio-pathological condition (denervation, DMD, sepsis), we have opted for an introduction divided in two sections. In the first section we provide an overview of key mitochondrial properties and functions that are central to myocyte homeostasis and for understanding of the work presented in this thesis. This includes a general description of oxidative phosphorylation, generation and buffering of reactive oxygen species, and regulation of cell death through mitochondrial membrane permeabilization, which have been investigated in all of our three studies. We also describe the process of mitochondrial biogenesis on which we have focused more particularly in our study investigating the effect of PGC1α in the *mdx* mouse muscle. Finally, we provide an introduction to the mechanisms of mitochondrial quality control (QC), which are emerging as important in the normal turnover of mitochondria and maintenance of optimal mitochondrial function in cells. We specifically focus on autophagy and mitophagy, two processes that we have investigated in the third study on sepsis-induced mitochondrial and cardiac dysfunction as well as in other collaborative studies performed during the course of this Ph.D. program but that were not included here (Pauly, 2012, Am J Pathol / Mofarrahi, 2012, Plos one)

In the second part of the introduction, we provide an analysis of how alterations of these various mitochondrial functions may contribute to pathogenesis of the three pathological conditions investigated by this thesis namely, denervation

disorders, muscular dystrophies and sepsis-induced dysfunction. In doing so we also highlight the rationale underlying our work.

2 Review of Literature

2.1 Mitochondrial properties and functions and their implication in striated muscle pathology

Mitochondria sit at the intersection of metabolism and cellular signaling for it is the primary site of energy production in the cell and for it emits signals that may alter the metabolism, modify gene expression, regulate the activity of different proteases or even trigger cellular death (Di Lisa, 2009, Pharmacol Rep). Since these processes are intimately linked to skeletal muscle (Dirks, 2006, Ageing Res Rev) and myocardial remodeling (Gustafsson, 2008, Cardiovasc Rev) in both health and disease, elucidating how mitochondria may be integrated in various pathophysiological models has, and continues to be an important research focus. In this section of the thesis, we present key mitochondrial properties and functions that have been classically investigated in heart and skeletal muscle in this context. These include: 1) tissue oxidative capacity and the process of oxidative phosphorylation, 2) the generation of reactive oxygen species (ROS) by the respiratory chain, 3) mitochondrial permeability transition and induction of various forms of cell death including necrosis, apoptosis, and autophagy, and, 4) regulation of cellular calcium homeostasis. We next provide an overview of how alterations of these mitochondrial properties/functions are considered to contribute to select skeletal and cardiac muscle pathologies, emphasizing mainly on those that were studied in the present thesis (*i.e.* denervation, DMD and sepsis).

2.1.1 The mitochondrial structure

Mitochondria are doubled-membrane organelle believed to have evolved from the endosymbiotic inclusion of bacteria specialized in energy production into a proto-eukaryotic cell 1.2 billion years ago (Sagan, 1993, J NIH Res). There are different levels of evidence supporting this notion. Firstly, the double membrane of the mitochondria speaks to this possible evolution from bacteria that would have been phagocytized. Secondly, the circular mitochondrial DNA with motifs reminiscent of those observed in certain bacteria also support this hypothesis on the origin of mammalian cells. The outer membrane (OMM) plays an important part

in controlling mitochondrial permeability. It contains numerous transport proteins (TOM, VDAC, CPT, …) that allow a coordinated transport of cytosolic constituents into the mitochondria that are necessary to maintain proper mitochondrial functions. The OMM also consitutes a site of physical interaction with other cellular organelles through protein-protein interactions including the cytoskeleton, the endoplasmic/sarcoplasmic reticulum and adjacent mitochondria.

In the intermembrane space, energy transfer players, such as creatine kinase, or important signaling molecules can be found. The two membranes are sometimes linked directly with the formation of complex polypeptide structures that span both layers and optimize the coordination of bilayer transport by linking outer and inner translocating proteins (TOMs and TIMs) as well as the lipid transporters carnitine palmitoyl transferase (CPT-I and CPT-II).

The inner membrane appears to be more impermeable than the outer membrane, this facilitates the formation of an electrochemical gradient and maintenance of the integrity of the matrix where the Krebs cycle and β-oxidation take place and where the fragile mtDNA also lays. The inner membrane is characterized by the presence of cristae, which increases total surface area and maximizes the density of the electron transport chain enchased in the membrane. These cristae are invaginated and give rise to internal compartments that may be built around ATP synthase, giving rise to focal proton gradients and a more efficient energy production system (Mannella, 2006, Biochim Biophys Acta). Cristae junctions are channels that allow exchanges between these isolated compartments and may impact mitochondrial functions. Precisely, they may facilitate ADP diffusion when its absence becomes a limiting factor to respiration, or may even facilitate cytochrome c mobilization and efflux during apoptosis signaling (Mannella, 2013, J Mol Cell Cardiol). Hackenbrock and colleagues observed two main IMM conformations, condensed or orthodox, and may easily go through interconversion in response to different stimuli, with the help of fusion-fission machinery (Hackenbrock, 1966, J Cell Biol / Frezza, 2006, Cell). It is now well accepted that

changes in IMM topology as well as overall mitochondrial morphology also impact other mitochondrial functions such as ROS production and calcium handling (Picard, 2013, Am J Physiol).

2.1.2 Tissue oxidative capacity and the process of oxidative phosphorylation

2.1.2.1 The electron transport chain

Within each mitochondrion, the energy currency of the cell, ATP, is generated through oxidative phosphorylation, a process whereby redox energy contained within nutrients is extracted through a series of oxido-reduction reactions and subsequently used to drive the synthesis of ATP. Thanks to the pioneering work of Hans Krebs in the 1930s, we now know that the carbon-based substrates, lipids and carbohydrates, are sent to the mitochondria for oxidation. There, the dehydrogenase enzymes of the Krebs cycle and β-oxidation will oxidize these substrates into reducing equivalents (NADH and $FADH_2$) that will be used to feed electrons into the electron transport chain (ETC) (Krebs, 1937, Biochem J). The electrons provided by NADH will enter the ETC at complex I (NADH dehydrogenase) (See Figure 1). Within complex I, the flavin mononucleotide is the first recipient of the electron, which is then transferred onto ubiquinone (Q), via Fe-S clusters, to form the reduced ubiquinol (QH_2). The free energy released by the passing of the electron to a lower energy level is used in moving protons against their natural gradient from the mitochondrial matrix towards the intermembrane space. The Q-cycle loop is closed when ubiquinol (QH_2) is recycled back into the oxidized ubiquinone (Q) by giving off its electron to complex III. Inside the complex, also known as cytochrome b-c1 reductase, another Fe-S cluster awaits the arrival of the electron so that it can be passed onto cytochrome c. Once again, the free energy released from electron transfers in the complex is harnessed to pump protons into the intermembrane space. Cytochrome c, much like ubiquinone does between complex I and III, serves as an electron shuttle between complex III and IV. When cytochrome c reaches complex IV (cytochrome c oxidase), it is oxidized by a copper-containing

subcomplex. Next, the electron travels through different hemoproteins until it reaches the ultimate electron acceptor, oxygen. This measurable consumption of oxygen is termed mitochondrial respiration (Chance, 1972, FEBS Lett). Accordingly, complex IV is the third and final site where protons are pumped into the intermembrane space. The electrochemical gradient resulting from the accumulation of H^+ in the intermembrane space creates a membrane potential ($\Delta\psi$) across the inner mitochondrial membrane. This energy is used to produce a motive force within complex V (ATP synthase) to rephosphorylate ADP into ATP, creating a situation where the higher membrane potential, the higher the phosphorylation capacity. Peter Mitchell first described this phenomenon in his chemosmotic theory, work for which he received the Nobel Prize in the late 1970s (Mitchell, 1961, Nature). Electrons snatched from $FADH_2$, a substrate heavily produced by the lipid oxidizing β-oxidation, follow a nearly identical path to NADH in the ETC. One discerning aspect of $FADH_2$ however, which can also be seen in Figure 1 is its entrance via complex II (succinate dehydrogenase). The bypass of complex I renders $FADH_2$ a less efficient substrate, as it cannot contribute to complex I proton pumping although complex III and IV pumping remains intact. Therefore, it can be estimated that for any given level of O_2 reduction, $FADH_2$ can only contribute about two-thirds of the normal proton pumping capacity of NADH. Therefore, it is said that the P/O ratio, where the Phosphorylating capacity (P), or the number of ATP molecules that can be formed for each molecule of oxygen that is consumed (O) of NADH and $FADH_2$ is 3 and 2 respectively. The well-respected Peter Hinkle suggests however slight deviations from these generally acceped values (Hinkle, 2005, BBA bioenergetics).

Figure 1: The Electron Transport Chain

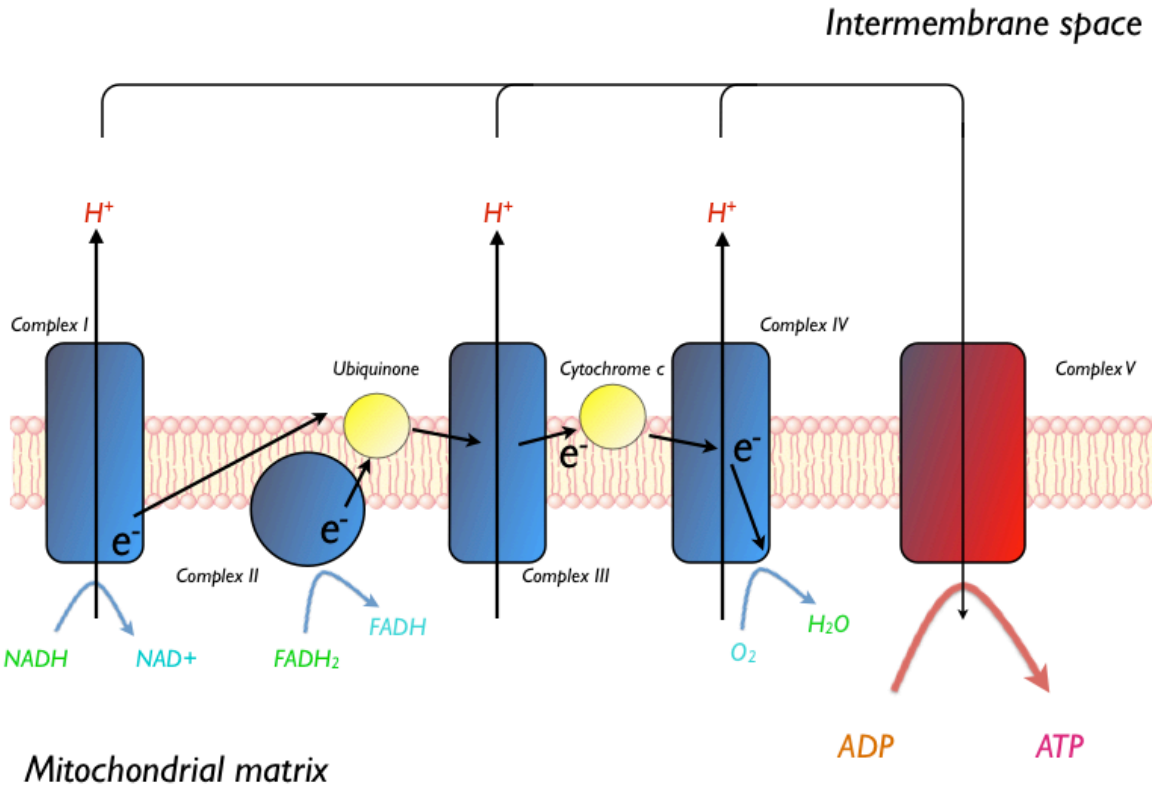


Figure 1: The Electron Transport Chain
The chain of oxido-reduction exchanges taking place in the electron transport chain allows the creation of an electrochemical gradient across the inner mitochondrial membrane, which is then used to create a motive force at ComplexV, linking ATP production and mitochondrial respiration.

2.1.2.2 Mitochondrial volume

Mitochondria are the most important contributor to aerobic energy production. Accordingly, tissue oxidative capacity is largely determined by mitochondrial volume density (Larsen, 2012, J Physiol). With the inner membrane relatively saturated with ETC, the most effective way to increase oxidative capacity is by increasing mitochondrial volume via biogenesis. The gold standard to assess mitochondrial volume density is measurement of the mitochondrial fractional area using electron microscopy images. Nowadays, 3D reconstruction of the mitochondrial network using fluorescent dyes also offers an interesting perspective to appreciate the cellular distribution of the organelle (Picard, 2013, J Appl Physiol). Popular indirect indexes of mitochondrial density that have shown a good correlation to mitochondrial fractional area, include tissue content of the inner membrane phospholipid, cardiolipin (Larsen, 2012, J Physiol), the enzymatic activity of mitochondrial enzymes involved in the krebs cycle (i.e. citrate synthase), or in the electron transport chain (i.e. cytochrome oxidase). These indexes consistently indicate that mitochondrial volume has an important degree of plasticity. In the face of heightened contractile activity, muscle mitochondrial volume increases (Tonkonogi, 2002, Exerc Sport Sci Rev); whereas chronic inactivity causes a loss in the organellar biomass (Adhihetty, 2007, J Appl physiol). Indeed, in the human vastus lateralis, it is not unusual to observe a 50% increase in mitochondrial density after a few weeks of exercise training (Hoppeler, Pflugers Arch, 1973). Conversely, Adhihetty and colleagues witnessed a 50% decrease in mitochondrial density after 6 weeks of denervation in rat skeletal muscle (Adhihetty, 2007, J Appl Physiol). We observed an even more abrupt reduction in the diaphragm, where a decrease in mitochondrial volume of similar magnitude occurred in a matter of hours after human patients were put on mechanical ventilation (Picard, 2011, Am J Respir Crit Care Med). Although the scale of these changes in response to varying metabolic demands may lead one to believe that environmental factors are the main determinant to mitochondrial volume in muscles, the reality is that mitochondrial tissue content is mostly range-bound by pre-determined genetic and epigenetic

factors. Indeed, it appears that early during development, muscles become differentiated according to their metabolic profile and this largely determines mitochondrial content (Tiivel, 2000, Mol Cell Biochem). Correspondingly, as seen in Figure 2, in highly oxidative tissues, such as the heart, where oxidative energy demand is great, mitochondria may represent up to 35% of total cell volume (Laguens, J Cell Biol, 1971). On the other hand, in highly glycolytic tissues, such as the gastrocnemius, where aerobic energy production is nearly dispensable, the total volume mitochondria occupy in the cell may be as low as 1%. The range of mitochondrial content in muscles of varying metabolic profile is outlined in Figure 2.

Table 1: Mitochondrial fractional density in striated muscle

Muscle	Range of mitochondrial fractional density (%)	Reference
Gastrocnemius	1-3	Macdougall, 1979, Med Sci Sports
Vastus Lateralis	2-8	Hoppeler, 1973, Pflugers Arch
Soleus	8-12	Hoppeler, 1987, J Physiol
Diaphragm	18	Weibel, 1984, Harv Uni Press
Heart	30-38	Laguens, 1971, J Cell Biol

Table 1: Mitochondrial fractional density in striated muscle
Range of mitochondrial fractional density, determined by electron microscopy across different muscles reported in various studies.

2.1.2.3 Mitochondrial biogenesis & PGC-1 α

Peroxisome proliferator activated receptor gamma coactivator-1-alpha (PGC-1α), long considered the master regulator of mitochondrial biogenesis, is overexpressed in periods of mitochondrial volume expansion but also decreases in periods of attrition (Adhihetty, 2007, J Appl Physiol), confirming its role in dictating oxidative capacity. PGC-1α directly coactivates a variety of transcription factors including PPARs, thyroid hormone receptors, glucocorticoid receptors, estrogen and estrogen related receptors (ERRs), myocyte enhancing factor-2 (MEF-2) as well as the forkhead O-box (FOXO) (Canto, 2009, Curr Opin Lipidol). Thus, the activation of PGC-1α results into a transcriptional program that characterizes the entire metabolic profile of the cell. To this effect, ectopic overexpression of PGC-1α in the muscle of mice has been shown to effectively transform fast twitch glycolytic muscles into slow twitch oxidative muscles (Lin, 2002, Nature); making PGC-1α the coordinator of metabolic plasticity in muscle (Hood, 2006, J Exp Biol).

PGC-1α coordinates mitochondrial biogenesis by acting as a coactivator in the transcription of nuclear-encoded proteins that will be incorporated into the mitochondria. The vast majority of these gene products are under the transcriptional control of nuclear respiratory factors (NRFs) and represent the bulk of the proteins necessary to synthesize new mitochondria, but other important transcription factors regulated by PGC-1α, such as YY1, are also involved (Scarpulla, 2012, BBA). Before mitochondria can become fully functional entities, these proteins need to be imported through multisubunit membrane complexes and assembled into complex polypeptides with the help of various molecular chaperones (Scarpulla, 2011, Biochim Biophys Acta). However, out of the 37 proteins encoded in the mtDNA, 13 are integral parts of the ETC complexes and are absolutely required for normal biochemical functions of the ETC (Wallace, 2005, Ann Rev Genet). Four of the five ETC complexes require bi-genomic expression, leaving succinate dehydrogenase as the sole complex that is completely derived from nuclear DNA. Additionally, PGC-1α also serves to increase mitochondrial protein synthesis.

Indeed, amongst the nuclear proteins under the transcriptional control of PGC-1α, are factors such as TFAM, TFBs as well as an RNA polymerase, all used in the transcription of the mitochondrial genome (Scarpulla, 2012, Trends endocrin metab). Consequently, it is thought that the transcription of these two distinct compartments is coordinated by PGC-1α. Surprisingly, Leick and colleagues revealed that PGC-1α was dispensable to mitochondrial biogenesis although its absence somewhat hinders the process (Leick, 2008, Am J Physiol Endocrinol Metab). One potential explanation is that PGC-1β, which has been shown to share a high degree of overlap in transcriptional targets with PGC-1α, may alleviate these blockades in the absence of PGC-1α (Lin, 2002, J Biol Chem). Although they are both necessary to attain normal physiological function (Lai, 2008, Genes Dev), single knockout of either activators will only cause mild metabolic disorders in baseline animals but the deficits become aberrant in the face of mounting stress. Meanwhile a complete deficit of both PGC-1α/β is lethal The maintenance of this redundance through evolution may be be explained by PGC-1β having upstream regulators distinct from PGC-1α. Nonetheless, this may be confounding in the interpretation of the true individual functions of both coactivators (Scarpulla, 2012, Trends endocrin metab). As indicated by the differences in mitochondrial volume density in different muscles, the need for aerobically derived ATP varies a great deal across tissues. Interestingly, PGC-1α and β content seems to vary across different muscles in a pattern similar to distributions of mitochondrial content, confirming their role in maintaining higher levels of oxidative capacity (Lin, 2002, Nature).

The transcriptional activity of PGC-1α is intensively regulated by post-transcriptional modifications, including phosphorylation, methylation and acetylation (Rodgers, 2008, FEBS lett). Given, the role of PGC-1α in determining cellular oxidative functions, it is not surprising to note that energy sensors such as AMP-activated protein kinase (AMPK) and Sirtuins play an important role in this mode of regulation. AMPK and Sirtuins are respectively activated by high AMP/ATP and NAD/NADH ratios. These energy and stress sensors are important contributors to homeostasis and are known to intervene in a plethora of signaling pathways with

impacts on physiological processes as varied as ROS production, cell proliferation, ATP production, autophagy and cell death (Canto, 2011 Physiol Bethesda). It has also been shown that AMPK and Sirtuins play a key role in the improved mitochondrial metabolism observed in models of caloric restriction (Figure 3) (Chen, 2008, Genes Dev) and in response to aerobic training (Baar, 2002, FASEB). Concordingly, the repression of these pathways in models of substrate oversupply has also been linked to poor health (Banks, 2008, Cell Metab). The link between energy supply and cellular health in addition to the varied functions of Sirtuins and AMPK have made them potential therapeutic targets in conditions as varied as diabetes, aging, cancer, and neuromuscular disorders (Canto, 2011 Physiol Bethesda). As shown in figure 3, AMPK activation results in higher PGC-1α activity via phosphorylation whereas SIRT1 will do so via deacetylation (Rodgers, 2005, Nature / Jager, 2007, Proc Natl Acad Sci).

Figure 2: Metabolic demands regulate mitochondrial volume

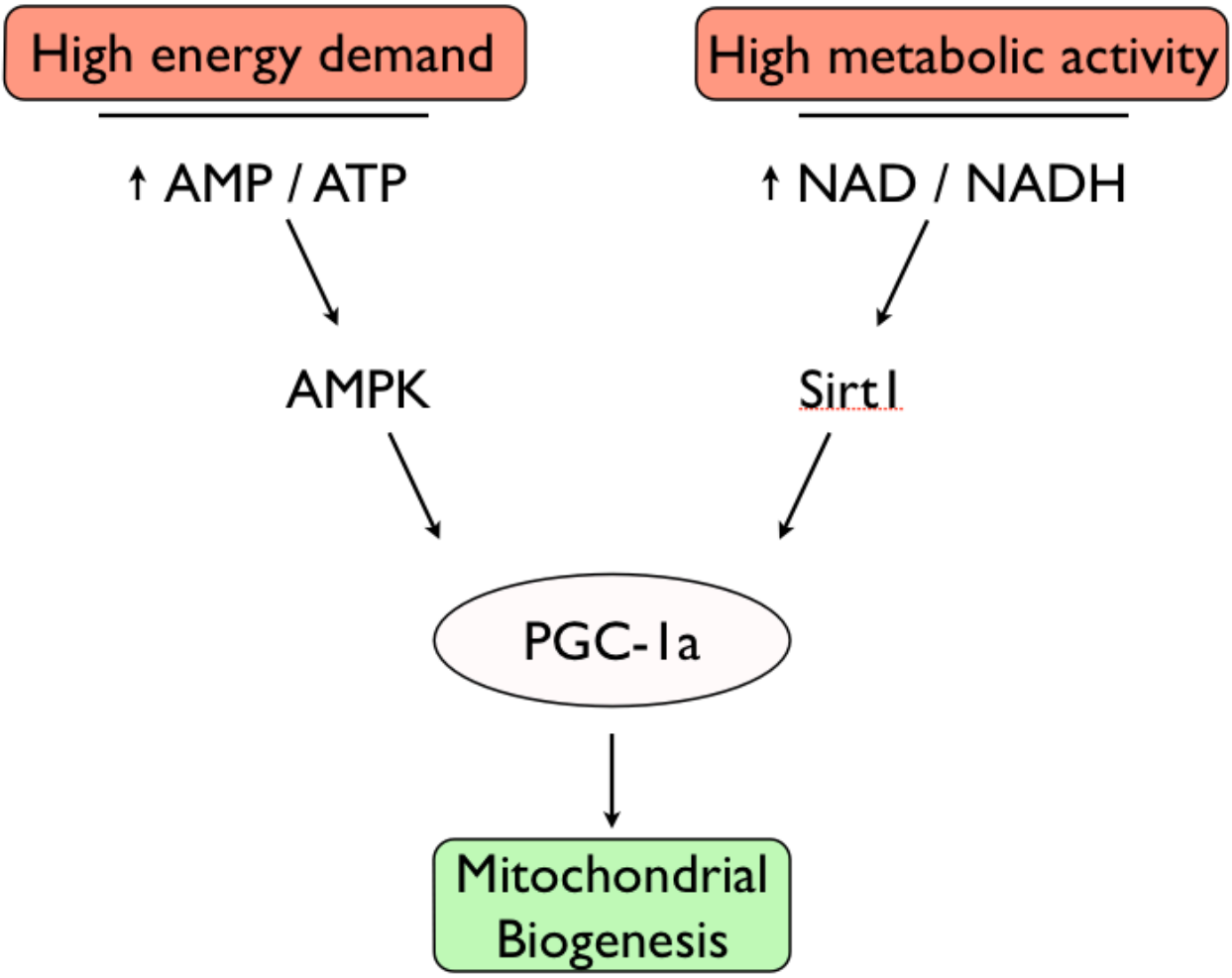


Figure 2: Metabolic demands regulate mitochondrial volume
Periods of high AMP/ATP ratios (fasting or high demand) activate AMPK. In periods of high energy demands (exercise), the increased NAD/NADH ratio leads to Sirt1 activation. Both AMPK and Sirt1 increase the transcriptional activity of PGC1, which is tied to mitochondrial biogenesis.

2.1.2.4 Mitochondrial respiratory controls

The energy and redox status do not only play a role in determining the long-term oxidative capacity of the tissues by modulating PGC-1α activity, it is also used in determining the acute rate of ATP synthesis within the mitochondria. Specifically, the redox potential (NADH/NAD) as well as the phosphate potential (ATP/ ADP • P_i) are the strongest determinant of mitochondrial respiration. A high NADH/NAD ratio indicates a high availability of substrates and pushes mitochondrial respiration higher by forcing electrons into the ETC. On the other hand, a low ATP/ADP • P_i ratio indicates elevated energy demand, which pulls mitochondrial respiration higher by providing adenylates for phosphorylation. Based on these principles, Chance and Williams began measuring oxygen consumption in isolated mitochondria using a titration protocol that permits assessment of different parameters of oxidative phosphorylation functions. The different respiratory states based on these principles were first defined by Chance and Williams in 1955 in their landmark studies and have been through a number of improvements through the years. The method was further improved with the introduction of permeabilization protocols, which allow proceeding with polarographic assessment of oxygen reduction without disrupting the cellular architecture as much as mitochondrial isolation does (Veksler, 1987, Biochim Biophys Acta).

The four main respiratory states characterized in the classic titration protocol are as follows: a) State 1: no substrates or adenylates supplemented with inorganic phosphate, b) State 2: supplemented with ADP but no substrates, c) State 3: addition of a substrate, d) State 4: ADP depleted. Whereas state 2 and 4 yield states of leak, state 3 is often considered maximal respiration and offers insight into oxidative phosphorylation capacity, mitochondrial density or gross impairments. Different substrates in combination to inhibitors can be used in state 3 to differentiate between the maximal capacities of complex I or complex II (NADH vs $FADH_2$) driven respiration. However, such measures of complex functionality rely on normal electron flow through the entire ETC, leaving direct measurements of enzymatic

activity a more reliable indicator of a specific complex dysfunction. This might be of interest when suspecting mtDNA damage as it would favorably affect complex I and IV, while complex II, which is completely derived from nDNA gene products, should be relatively unscathed.

The degree of uncoupling is a key component of mitochondrial respiratory control and may provide important insights into certain pathologies. Indeed, the mitochondrial inner membrane normally leaks a small portion of the H^+ that are pumped into the intermembrane space. Although a certain degree of proton leak may be desirable, as we will see in section 2.2, there comes a point where excessive uncoupling is symptomatic of pathological or toxological oxidative phosphorylation impediments (Gnaiger, 1998, Biochim Biophys Acta). However, the best indicator of functional coupling between oxidation and phosphorylation remains the respiratory control ratio (RCR), which consists of state 3, divided by state 4 respiration (Gnaiger, 2009, Int J Biochem Cell Biol). Also, it is now common to completely uncouple oxidative phosphorylation with protonophores such as CCCP to evaluate the maximal capacity of the electron transfer system or to evaluate whether the phosphorylating process hinders maximal respiration (Steinlechner-Maran, 1996, Am J Physiol).

2.1.3 Generation of reactive oxygen species (ROS) by the respiratory chain

2.1.3.1 Reactive Oxygen Species

Free radicals are species containing one or more unpaired electrons (Halliwell, Am J Med, 1991), conferring them an important degree of reactivity (Valko, 2007, Int J Bioch Cell Biol). Due to the electronic configuration of oxygen, which is itself a radical with 2 unpaired electrons on its outermost π orbital, O_2 is a favoured recipient of unpaired electrons. Thus, the superoxide anion ($O_2^{-}\bullet$) is almost always the first step in the formation of free radicals and is accordingly referred to as the primary reactive oxygen species (ROS). $O_2^{-}\bullet$ either interacts with nitric oxide

based molecules to form reactive nitrogen species (RNS), such as peroxynitrite ($ONOO^{-}$•), or, is dismutated into the more stable non-radical ROS, hydrogen peroxide (H_2O_2), by superoxide dismutases (SOD). Hydrogen peroxide can lead to the formation of potentially damaging secondary ROS, through enzyme- or metal-catalyzed reactions (Valko, 2007, Int J Bioch Cell Biol). For example, the extremely damaging Hydroxyl radicals OH• are synthesized when H_2O_2 and O_2^{-}• come together through the iron-based Fenton and Haber-Weiss reactions (Bhatthacharjee, 2012, J Botany). Other ROS radicals derived from superoxide anions frequently observed include the perhydroxy radical (HO_2•), alkoxy radical (RO•), peroxy radical (ROO•) or hydroperoxides (ROOH) (Miller, 1990, Free Radic Biol Med).

Intracellular O_2^{-}• production originates from various sites including xanthine oxidase, NADPH oxidase, lipoxygenase, cyclooxygenase, cytochrome P-450 oxygenase, phospholipase, but most importantly from the mitochondrial electron transport chain (Bashan, 2009, Physiol Rev / Zinkevich, 2011, Am J Physiol). The ETC harbors the bulk of the machinery involved in the oxido-reduction cascade that leads to ATP production and respiration. The electron exchange sites, which are necessary to release the free energy to pump protons, are particularly prone to leak electrons onto oxygen, which we established is a competitive oxidant. Complex I and III, are especially susceptible to the untimely exchange of electrons that results into ROS formation (Brand, 2000, Exp Gerontol). While complex III was originally believed to have the highest potential for ROS output, these measurements were performed with the use of non-physiological inhibitors (Mclennan, 2000, J Bioenerg Biomemb). There is now a growing consensus that its contribution is not as important as previously thought (Hansford, 1997, J Bioenerg Biomembr / Mclennan, 2000, J Bioenerg Biomemb). It is rather complex I that is emerging as the more predominant source of mitochondrial ROS in normal physiological settings (Turrens, 1980, Biochem J / Boveris, 1976, Biochem J/ Kang, 2012, Biochem Res Int) and also seems to be the most sensitive to redox conditions (Mclennan, 2000, J Bioenerg Biomembr). It is well appreciated that complex I may leak electrons

coming directly from NADH but it appears that $FADH_2$ can also contribute to electron leakage at complex I via a thermodynamically unfavourable reverse electron flow; however, the complex II itself, long dismissed as a significant contributor to mitochondrial ROS output, may be directly involved in ROS production in such scheme (Mclennan, 2000, J Bioenerg Biomemb). There is conflicting data on where precisely the leaks occur but the literature currently indicates that the sites of exchange between ubiquinone and the complexes (I and III) to be the most likely candidates (Cadenas, 1977, Arch Biochem Biophys / Hinkle, 1967, J Biol Chem/ Andreyev, Biochem (mosc), 2005 / Lenaz, IUBMB Life, 2001). The side of the inner mitochondrial membrane where ROS are released is also a matter of debate. Nonetheless, mitochondria may emit ROS inwardly into the matrix, which could provide an explanation on how damage might be self-inflicted; but mitochondrial ROS may also be released in the intermembrane space towards the cytosol, which could contribute to cell wide oxidative damage (Mailloux, Free radic biol med, 2011 / St-pierre, J Biol Chem, 2002 / Turrens, Biosci Rep, 1997 / Iwata, science, 1998 / Han, 2001, Biochem J / de Vries, 1986, Bioenerg biomemb). However, it must be considered that most of these radicals are unable to diffuse across the mitochondrial membrane and sometimes have half-lives that are measured in microseconds.

The main difficulty in measuring mitochondrial ROS production is the presence of several enzymatic and non-enzymatic anti-oxidants in the mitochondria. Thus, any ROS output from the mitochondria results from an imbalance between production and scavenging. In fact, differences in scavenging capacity alone are sufficient to determine the muscle oxidative profile independently of ROS production (Assem, 1997, Am J Pathol). The main endogenous antioxidants are depicted in figure 4. In terms of enzymatic antioxidants, manganese superoxide dismutase in the matrix (MnSOD - SOD2) is in charge of dismutating superoxides in the mitochondria, while copper zinc SOD (CuZn-SOD1) does so in the cytosol. Other enzymatically regulated scavenging of ROS include catalase as well as different reductases, peroxidases and thioredoxins which require NAD(P)H equivalents to

regenerate reducing coenzymes (Chaudiere, 1999, Food Chem Toxicol). Among them, the highly abundant peroxiredoxins are estimated to scavenge more than 90% of mitochondrial H_2O_2 (Cox, 2009, Biochem J). Non-enzymatic antioxidants also play an important role in scavenging ROS. Unlike their enzymatic counterpart, their scavenging capacity is not adaptable and cannot be upregulated by the cell in the face of mounting ROS production as they are derived mainly from dietary sources. Nonetheless, tocopherol (Vitamin E), retinoids (Vitamin A) or ascorbates (Vitamin C) effectively offer protection against undiscriminate damage from most ROS but can't deal with superoxide nor hydroperoxides which are dealt with only by catalase and glutathione peroxidase (Powers, 1999, Clin Sports Med).

Figure 3: Mitochondrial scavenging of ROS

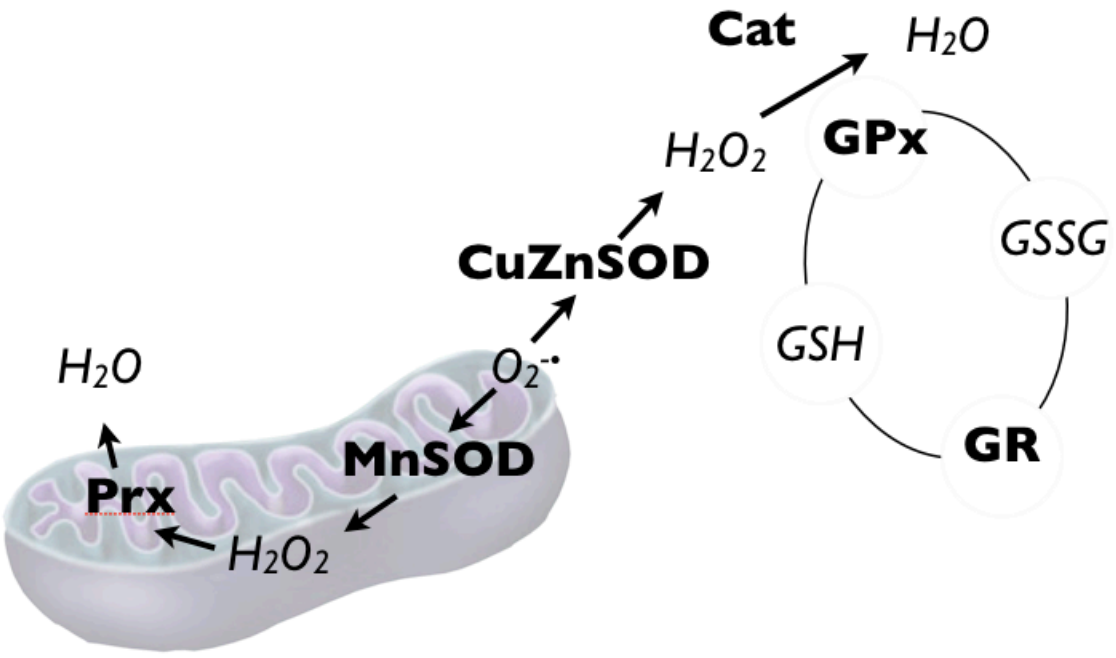


Figure 3: Mitochondrial scavenging of ROS

Oxygen anions produced in the mitochondria are detoxified into hydrogen peroxides by Mn-SOD in the mitochondria and by CuZn-SOD in the cytosol. In the mitochondria Peroxiredoxin further detoxifies peroxide into water; whereas this is done by a combination of catalase and glutathione peroxidase in the cytosol. EXPAND. Prx: Peroxiredoxin. Cat: Catalase. SOD: Superoxide dismutase. GPx: glutathione peroxidase. GR: Glutathione Reductase. GSH/GSSG reduced/oxidized glutathione.

2.1.3.2 Regulation of ROS production

In 1956, Chance was already suggesting that at least 1% of the oxygen consumed by mitochondria is the result of superoxide production. While current estimates still stand in this range (0.2% – 2.0%) (Mailloux, 2011, Free Radic biol med), observations that ROS production is not linearly related to the rate of electron flow within the ETC has re-directed research efforts towards the mechanisms regulating production. The metabolic state clearly has the most influence on acute changes in ROS production. When the redox state is high, meaning that ATP and electron-giving substrates are abundant, the demand for energy production is low but the pressure to flow electrons through the ETC is high (Skulachev, 1996, Q Rev Biophys). The resulting high membrane potential then forces ROS production unless the pressure is relieved by an alternative mechanism, in fact a strong positive correlation between membrane potential and ROS production has been observed (Korshunov, 1997, FEBS let). Exercise and the associated increased demand for ATP may relieve membrane potential by providing ADP to be phosphorylated at complex V (Boveris, 1973, Biochem J). Although this reduces the percentage of leaked electrons, exercise nonetheless results in acute oxidative stress probably due to high levels of electron flow through the ETC, which may reach 100 times those seen at baseline. During prolonged exercise, antioxidant reserves may eventually become depleted, thus exacerbating the oxidative stress accrual (McArdle, 2005, Free Rad Biol Med). Safety valves of a high membrane potential, in addition to ADP, include uncoupling proteins, which, by allowing protons back in the matrix, effectively decouple the ATP production from the oxido-reduction cascade (Brand, 2000, Exp Gerontol). It is estimated that as much as 35-50% of mitochondrial respiration in perfused rat muscle is dissipated as heat, due to proton leaking back in the matrix (Rolfe, 1996, Am J Physiol). Accordingly, a mild depolarization is thought to be a desirable phenomenon to prevent excessive ROS formation, at the expense of a slight inefficiency in ATP production.

Mitochondrial ROS production is also determined by the presence of ROS from other origins. The phenomenon termed ROS-induced ROS release (RIRR) refers to this ability of ROS signals to be amplified and may have important signalling implications (Zinkevich, 2011, Am J Physiol). Hawkins and colleagues have described the ability of extracellular NAPDH oxidase-derived ROS to induce mitochondrial ROS production (Hawkins, 2007, Mol Biol Cell), possibly to transduce an extracellular signal into a physiological response. Cross-talk between mitochondria and NADPH oxidase is not an isolated case of signal transduction as mitochondria are also believed to transduce ROS signals originating from inflammatory cells (Dikalov, 2011, Free Rad Biol Med).

2.1.3.3 ROS signaling

It is now clear that ROS intervenes in a number of signal transduction pathways as well as in gene expression. Transient elevation or low concentrations of ROS have been shown to promote survival and stimulate cell proliferation, whereas persistently high concentrations are known to cause growth arrest or even cell death (Valko, 2006, Chem Biol Interact). ROS are also known to determine protein phosphorylation by inactivation of phosphoprotein phosphatases. Thus, high ROS production leads to upregulation of signaling cascades involving MAPK- and PI3K and other kinase dependent pathways, ultimately leading to the activation of transcription factors such as AP-1, NF-κB, p53, HIF-1 and NFAT (Valko, 2006, Chem Biol Interact). Although this appears as a straightforward transcriptional program induced by ROS, there is a fine-tune control exerted by not only the concentration of ROS but also the origins and the cellular context in which they are produced. Important differences exist in the activation of the various serine/threonine kinases of the MAPK family. For example, the activation of ERK is mostly associated with mitochondrial ROS production whereas p38 activation appears more sensitive to exogenous sources (Iles, 2002, Immunol Res / Torres, 1999, Arch Biochem Biophys). For each of the aforementioned kinase as well as the transcription factors, there

exists contradicting data showing their activation as either harmful or beneficial. These discrepancies seem to lie in the nature of the exposure to oxidative stress. When the exposure is transient, there is an antioxidant, pro-survival response that leads to adaptation that will better prepare the cell to face future stress. Tapia has shown that a sublethal mitochondrial stress through moderate ROS exposure leads to improved mitochondrial health (Tapia, 2006, Med Hypoth) possibly by optimizing quality control systems. On the other hand, chronic exposure leads to depletion of the buffering capacity and, in what appears as an attempt to prevent the spread of this oxidative stress to neighboring cells, will lead the cell to apoptosis, also kown as cell suicide. This notion of adaptive stress response is the building block of the hormesis school of thought (Ristow, 2010, Exp Gerontol).

2.1.3.4 Oxidative stress

Excessive ROS exposure will cause oxidative damage to lipids, proteins and nucleic acids (Miller, 1990, Free Radic Biol Med). Polyunsaturated fatty acids (PUFA), notably present in the plasma membrane are prone to oxidation. Oxidatively modified lipids, after a series of reactions give rise to lipid peroxides such as malondialdehyde, 4-hydroxynonenal, myeloperoxidase, thiobarbituric acids (TBARs), isoprostane or lipofuscin. Their presense can alter the integrity, fluidity, permeability and the functional properties of membranes (Greenberg, 2008, J biol chem). Intracellular lipid peroxides are also known to be cytotoxic, mutagenic and to disrupt normal cellular signalling (Valko, 2006, Chem Biol Int]. In mitochondria, oxidation of cardiolipin is known to decrease the activity of respiratory complexes (Paradies, 2000, FEBS Lett / Paradies, 2002, Gene) and respiration (Wiswedel, 2010, Free Radic Res). For a detailed review of lipid peroxidation see (Niki, 2009, Free Rad Biol Med).

Although amino acids such as methionine and cysteine are most sensible to modification by ROS, all amino acids are potential targets for oxidation (Stadtman,

2006, Free Rad Res). Oxidative damage to proteins leads to the appearance of carbonylated and nitrosylated proteins, commonly used indexes of oxidative stress. Although this oxidative damage is non-lethal and reversible to some extent, certain mitochondrial proteins such as aconitase and adenine nucleotide translocase are susceptible targets of long-term accumulation of oxidative stress (Stadtman, 2004, Curr Med Chem). Again the damage done to the protein will affect both structure and functionality. Consequently, the accretion of oxidized proteins has been associated with numerous diseases (Stadtman, 2001, Ann N Y acad sci).

Both the deoxyribose backbone and the purine/pyrimidine bases of DNA can be modified by ROS, which, is an important step in mutagenesis that can underlie numerous pathologies (Dizdaroglu, 2002, Free Rad Biol Med). The level of 8-hydroxyguanine (8-OH-G) is often used as an index of the oxidative damage to the DNA. The mitochondrial DNA is even more susceptible to this kind of damage as shown by the oxidative lesions that appear to be several fold higher in the mtDNA than in nDNA (Barja, 2000, FASEB). There are numerous reasons for this 1) mtDNA has a spatial proximity to an important site of ROS production, the ETC. 2) mtDNA repair capacity is limited (Giulivi, 1995, Arch Biochem Biophys) with the nucleotide excision repair mechanism completely absent (Gredilla, 2010, Exp Gerontol) 3) mtDNA does not benefit from the histone protection that nuclear DNA enjoy (Suter, 1999, Biochem).

Overall, the accumulation of oxidative stress can be due to 1) excessive long-term ROS production 2) failure of antioxidant systems to cope with ROS production 3) a decreased ability to repair or degrade oxidized macromolecules. This may lead to disruption of important macromolecules and probably contribute to pathogenesis in various (cardio)myopathies.

2.1.4 Mitochondrial permeation, induction of cell death and calcium homeostasis

It is now well accepted that mitochondria can trigger cellular death by means other than failure of energy production or excessive ROS production. Indeed, it appears that various mode of mitochondrial permeabilization can initiate cell death signaling and result in apoptosis or necrosis. Importantly, these permeabilization processes are also used by mitochondria to help maintain cellular calcium homeostasis and appear sensitive to other mitochondrial dysfunction byproducts such as oxidative stress and ATP production failure.

Emerging evidence indicates that there is an important distinction to be made between outer and inner membrane permeabilization, with each having different modes of regulation and different physiological implications (Kroemer, 2007, Phys Rev). Although the molecular underpinnings differentiating the two remain unclear, the consensus so far establishes that outer membrane permeabilization can be performed by the insertion of Bcl-2 family BH-3 oligomers into the outer membrane while inner membrane permeabilization is achieved through opening of the mitochondrial permeability transition pore (mPTP). In some instances, the two modes of permeabilization can occur simultaneously to create a direct permeable pathway that crosses the double membrane system.

2.1.4.1 Inner mitochondrial membrane permeabilization

The mPTP, is a high conductance non-selective channel which allows equilibration of mitochondrial content across the inner membrane. Due to the large conductance of the complex, its opening results in the equilibration of ionic charges and small solutes of less than 1.5 kDa across the inner mitochondrial membrane, effectively abolishing any electrochemical gradient. Although its transient opening may be part of a normal cellular regulatory process, known as pore flickering, which may alleviate high membrane potential, different phenomena attributable to the irreversible opening of the mPTP are detrimental to cell function. These include

mitochondrial matrix swelling, oxidative phosphorylation uncoupling, increased ROS production, loss of respiratory control, induction of ATPase, triggering of cellular death and, the loss of calcium from the mitochondrial matrix (Brenner, 2012, Circ Res). Notwithstanding of these possible differences, PTP opening has an important role in physiological and pathological remodeling.

Over the timespan covered by this thesis, different theories have come and gone on the molecular identity of the mPTP. Although Ca^{2+} remain the only component known to be necessary in permeability transition (PT), numerous factors have been shown to modulate mPTP sensitivity to Ca^{2+}, triggering speculation on the potential proteins that form the mPTP. For example, Cyclosporin A can hinder PT through CyclophilinD (CypD) binding, adenine nucleotide translocase (ANT) inhibitors (atractylate and bongkrekate) also interfere with normal mPTP sensitivity which led to conclusions that they are integral part of the mPTP. However, these have all been shown to be dispensable to PT (Bernardi, 2006, FEBS J/ Beutner, 1996, Febs Lett). A long standing hypothesis led by the team of Halestrap and colleagues postulates that CypD, ANT and the voltage dependant anion channel (VDAC) are the primary elements of the mPTP (Halestrap, 2009, J Mol Cell Cardiol). Much like ANT and CypD, VDAC has also been shown to be dispensable to PT although it may modulate sensitivity (Krauskopf, 2006, Biochim Biophys Acta). However, VDAC (Sileykite, 2011, J Biol Chem), as well as other outer membrane proteins (i.e. BH3 oligomers) (Forte, 2006, Cell Death Differ), may play a particularly important role in inner membrane-outer membrane protein interactions that can modulate inner membrane permeability and also favor bilayer permeability synchronization (Bernardi, 2013, Front Physiol). Other disproven theories on the molecular identity of the mPTP include 1) an inorganic phosphate (P_i) carrier (Leung, 2008, Biochim Biophys Acta) and 2) clusters of misfolded proteins which could be modulated by chaperone proteins such as CypD (He, 2002, Febs Lett). The group of Bernardi has recently provided direct evidence that the mPTP was in fact F_0F_1 ATP synthase dimers (Giorgio, 2013, Proc Natl Acad Sci). Although there has not yet been a consensus on this new hypothesis, and that certain questions regarding the

rotenone and quinone sensitivity remain to be addressed, the first lines of evidence are rather compelling (Bernardi, 2013, Front Physiol). Indeed, purified ATP synthase dimers are susceptible to forming channels in the presence of Ca^{2+} that have a conductance similar to the one previously attributed to PTP. Additionally, these purified ATP synthase dimers form pores that can be modulated by CypD, which is suggested to do so by binding to the lateral stalk of the ATP synthase complex (Giorgio, 2009, J Biol Chem). Other characteristics of F_0F_1 ATP synthase dimers that closely resemble those of the mPTP are, among others, sensitivity to P_i, Mg^{2+}, adenine nucleotides as well as thiol oxidants.

2.1.4.2 Outer mitochondrial membrane permeabilization

There are different types of proteins with domains of BCL-2 homology (BH), those containing three BH domains (BH-3) are renown for their ability to prompt outer membrane permeabilization and apoptosis whereas those containing four BH domains are seen as anti-apoptotic. The best characterized BH-3 protein, Bax, is a cytosolic protein which is thought to transclocate into the outer mitochondrial membrane, as an oligomeric Bax containing pore in conjunction with other BH-3 proteins (t-Bid/Bak) or as an homo-oligomer upon apoptotic signals to mediate cytochrome c release (Kuwama, 2002, Cell). Although, there is conclusive evidence on the existence of such pore, the role of the PTP in this form of permeabilization remains obscure with some suggesting that the PTP can sometimes closely cooperate to this process (Green, 2004, Science) while others suggest it is dispensable. However, there are antiapoptotic BH proteins that counteract the pro-apoptotic effect of the BH-3, including Bcl-2 and Bcl-X_L, which do so by interfering with the oligomerization (Degterev, 2001, J Cell Biol). Therefore, the Bax/Bcl-2 ratio is now commonly used as an index of outer membrane permeation susceptibility to detect the apoptotic intentions of the cell.

2.1.4.3 Downstream effects of mitochondrial membrane permeabilization

Mitochondria play an important role in regulating the spatio-temporal distribution of calcium. Numerous physiological processes from enzymatic activity, to transcriptional activity are regulated by calcium signals. By either emitting or taking up calcium from these signaling cascades, mitochondria can determine the downstream outcome of a signaling wave. Short bursts of calcium release, also called sparks, have been detected during pore flickering and appear to constitute a demonstration of this process (Berridge, 2000, Nat Rev Mol Cell Biol). Conversely, the calcium uptake may itself impact mitochondrial metabolism. Numerous Krebs cycle and ETC enzymes have indeed a Ca^{2+}-dependent regulation component. Additionally, problems may arise when mitochondria are faced with excessive Ca^{2+}. Because mitochondria have a limited ability to take up the ion, there is a tipping point beyond which excess calcium triggers irreversible PTP opening. This uncontrolled release of calcium can cause the induction of a large calcium transient that can seriously damage the cell. Firstly because calpain, a calcium activated-protease, is potentially damaging to myofibers (Murphy, 2006, J Physiol). Secondly, calcium overload may disrupt excitation-contraction coupling hence hindering force generation (Verburg, 2005, J Physiol). Finally, calcium is probably the most well-described trigger of mPTP opening and can prompt a calcium-induced calcium-release. A process by which a local surge in calcium could potentially generate an amplifying calcium wave that can snowball into a cell-wide calcium release by mitochondria and cause cellular death (Ichas, 1997, Cell). The difference between the transient opening resulting in calcium sparks and the irreversible emptying of mitochondrial calcium reserves remain, however, obscure.

As seen in figure 5, mPTP opening also allows the release of several pro-apoptotic factors (Cytochrome c, AIF, EndonucleaseG, Smac/Diablo, OmiHtrA2) that are normally sequestered in the mitochondria. Cytochrome c, upon its release comes together with the cytosolic factor Apoptotic peptidase activating factor 1 (Apaf-1) to form the apoptosome and cleave procaspase 9 into caspase 9. The ensuing

activation of cysteine aspartate specific proteases (caspases) is a critical step in atrophic disorders since they are necessary for cleaving myofibrillar proteins before they can be digested by the proteasome (Du, 2004, J Clin Invest). In numerous diseases, proteasome activity has been shown to be elevated early during disease progression while signs of impaired force production or diminished oxidative capacity are not even yet apparent; meanwhile signs of mitochondrial permeabilization precede atrophy (Dupont-Versteegden, 2006, Am J Physiol Regul Integr Comp). Possibly, it may be because this proteolytic degradation is dependent on the yield fragments provided from caspase pre-digestion of myofibrillar proteins. Du and colleagues even suggested that the dependence of myofibrillar breakdown on caspase activation indicate that the permeation of the mitochondrial membrane is a key rate-limiting factor for the breakdown of the contractile apparatus (Du, 2004, J Clin Invest). As seen in figure 5, the cytosolic anti-apoptotic protein XIAP normally prevents the formation of the apoptosome, possibly in case cytochrome c inadvertently escapes the mitochondria from its normal intermembrane space localization. Thus, it may be said that the release of the second mitochondria-derived activator of caspase (Smac), a protein part of the direct IAP-binding protein with low pl (Diablo) family of proteins, that occurs during mPTP opening prevents this inhibition in a way that confirms that the leak of cytochrome c was not accidental.

The loss of cytochrome c from the intermembrane space, in addition to triggering proteolysis, can cause a surge in ROS production (Cai, 1998, J Biol Chem). As demonstrated in section 2.1.2, cytochrome c normally serves as an electron shuttle between complex III and IV. The loss of cytochrome c that occurs with membrane permeabilization decreases electron transfer to complex IV therefore increasing the reduced state of complex III and thus electron leaks. Elevated ROS production alone has been shown to be a potent inductor of apoptosis (Gardner, 1997, Free Rad Biol Med) but in the case of mitochondrial permeation mediated apoptosis, this surge in ROS production can be seen as the *coup de grace* that ensures cellular death.

Other pro-apoptotic proteins are also released upon mitochondrial permeation. Among them, AIF and EndoG, which are both re-directed to the nucleus where they cause DNA fragmentation and apoptosis (Marzetti, 2010, Biochim Biophys Acta).

In addition to the activation of proteolytic pathways and DNA fragmentation, the prolonged opening of the mPTP triggers a collapse in the mitochondrial membrane potential that effectively eliminates energy production. Experiments using potentiometric dyes have shown that even transient pore opening results in a decrease in mitochondrial membrane potential. This physiological process may be especially important when redox potential is high and that excessive ROS production threatens cell viability (De Giorgi, 2000, Cell Calcium). Again, it is currently difficult to differentiate between healthy and deadly regulation of PTP opening; however, it appears that the concerted permeabilization of both the outer and inner membrane inevitably leads to the propagation of a cell death signal (Halestrap, 2005, Nature).

Figure 4: Mitochondrial permeabilization and apoptosis

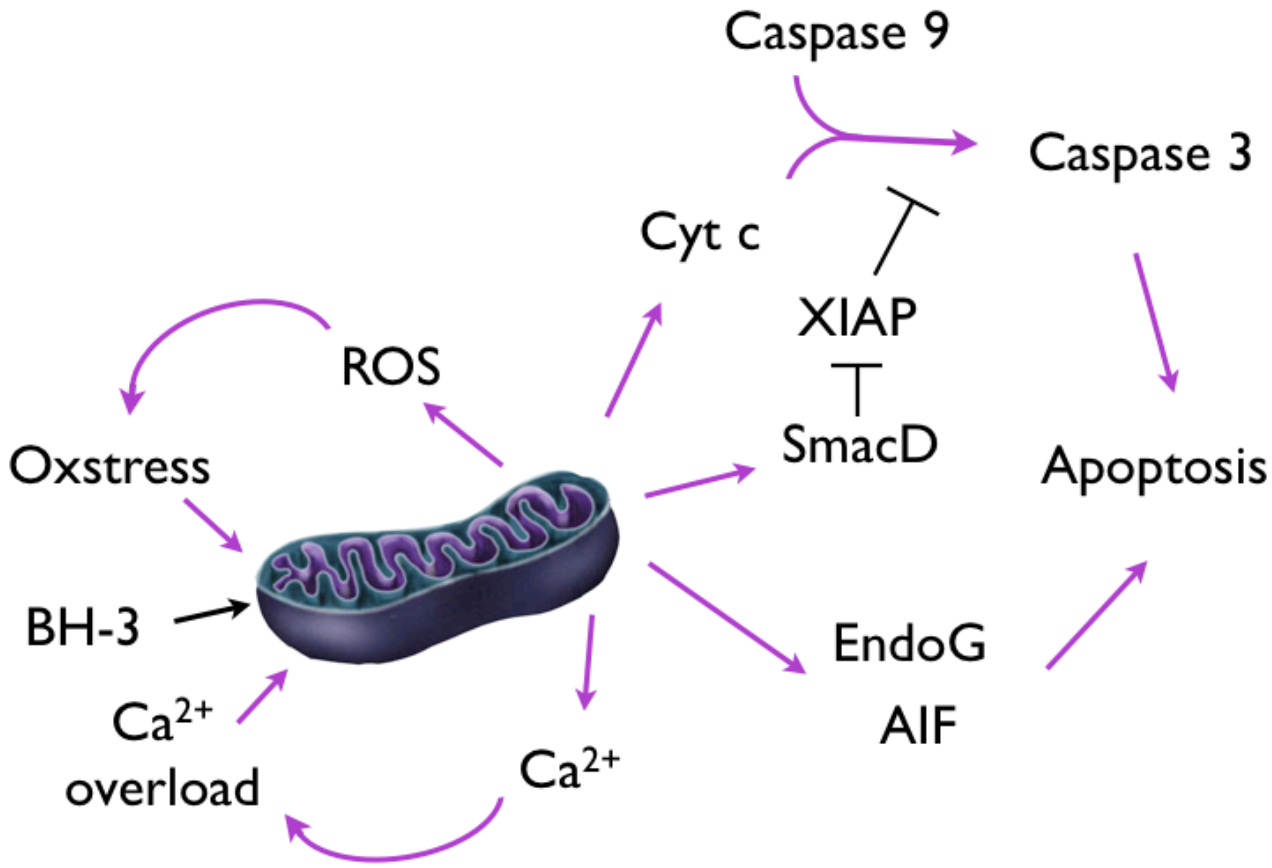


Figure 4: Mitochondrial permeabilization and apoptosis
In response to oxidative stress, calcium overload or of BH3 oligomers, mitochondrial permeabilization will cause a surge in ROS and calcium emission in addition to releasing proapoptotic factors normally sequestered in the mitochondrial lumen. Cytochrome c, with the help of SmacD will activate caspases through apoptosome formation. AIF and EndonucleaseG will both translocate to DNA and cause fragmentation.

2.1.4.4 Regulating PTP opening

There are several well-described inductors of PTP opening. We have already argued that calcium does so, in a positive feedback, amplifying wave pattern. Similarly, ROS and PTP opening tend to act synergistically in promoting cell death by mutually inducing each other. It is thought that exposure to ROS can particularly damage the putative PTP components (such as ANT), which could directly potentiate pore opening (Queiroga, 2010, J Biol Chem).

There is evidence that mitochondrial permeabilization can be controlled at different points. VDAC for example, can be inhibited by direct interaction with hexokinase, thus preventing mitochondrial permeabilization (Halestrap, 2005, Nature). Conversely, it has been postulated that some forms of VDAC modifications, including VDAC phosphorylation by various kinases can facilitate PTP opening (Kroemer, 2007, Physiol Rev). Similarly, both ANT and CypD have also been found to be potential targets for modulating sensitivity to permeabilization. However, their induction seems to lead to formation of the complex spanning the entire intermembrane space and allowing for direct exchange between the cytosol and the mitochondrial matrix. ANT for example can be induced by palmitate (Cesura, 2003, J Biol Chem) and inhibited by adenylates or bongkrekic acid (Beutner, 1998, Biochim Biophys Acta); whereas CypD is inducible by calcium or hydrogen peroxide (Baines, 2005, Nature) and inhibited by cyclosporine A (Basso, 2005, J Biol Chem).

Overall, mitochondrial membrane permeabilization is an important checkpoint in cellular degradation. However, much like ROS, mitochondrial permeabilization is increasingly suggested to be involved in mitochondrial quality control mechanisms that are intended to improve cellular health (Gottlieb, 2009, Basic Res Cardiol). Specifically, as seen in the following section, permeabilization could be a trigger signal for recruitment of autophagic machinery.

2.2 Mitophagy: An Important Mechanism of Organellar Quality Control

The progression from protein disruption to organellar stress to cellular death is normally encountered by different levels of quality control mechanisms. In the mitochondria, there are numerous chaperones, heat shock proteins and proteases that serve to maintain the structural and functional integrity of proteins. There is considerable evidence suggesting that when these mechanisms fail to cope with mounting amounts of stressed proteins; the entire organelle is discarded, most often by way of mitophagy (Gottlieb, 2009, Basic Res Cardiol). The team of Roberta Gottlieb and colleagues published a series of article in which they defend the thesis that a higher mitochondrial turnover improves the oxidative functions and the resistance of the organelle to stress (Gottlieb, 2010, Am J Physiol Cell Physiol). The model proposes that by constantly getting rid of older, potentially disruptive mitochondria through mitophagy and providing the raw materials to build brand new ones by recycling the digested organelle, mitochondrial function can be improved. Dysfunctional mitochondria are indeed re-known to produce higher amounts of ROS, are susceptible to induce apoptosis and their poor respiratory capacity put them at risk of energy catastrophe. Thus, failing of housekeeping systems can lead to accumulation of damaged mitochondria that could put the life of the cell at risk. This is somewhat in line with the vastly popular theory of mitochondrial aging that leads into a vicious cycle of accumulating dysfunctional organelles (Harman, 1972, J Am Geriatr Soc). However, in comparison to the original model of Harman, which mostly blames oxidative stress, the current model proposes that poor quality control and low turnover would cause the accumulation of deteriorating mitochondria (Gottlieb, 2011, Biochim Biophys Acta). Mitochondrial defects unequivocally present themselves during the progression of the myopathies presented in this thesis. This may pertain to failing mitochondrial quality control systems and mitophagy more specifically. Therefore we will now review the mechanics before presenting the evidence for this model being applicable in the myopathies of interest.

2.2.1 Molecular quality control

Chaperone proteins, including certain heat shock proteins (HSPs) can serve a chaperoning role in the folding of proteins from their native state into functional mature proteins. (Peisker, 2010, Biochim Biophys Acta). This aspect of their work appears to be facilitated by interactions with mitochondrial import machineries (Rassow, 1994, J Cell Biol). The main actors of this process are HSP60, also known as chaperonin, as well as HSP70 (Mortalin) and HSP10 although these proteins remain poorly understood (Voos, 2013, Biochim Biophys Acta). These HSPs and other chaperone proteins intervene to maintain protein homeostasis in already mature proteins by, for example, restoring the functional configuration of misfolded proteins or by resolubilizing aggregated proteins. Among them, the Clp proteins, including HSP78, part of the AAA+ ATP-dependent proteases family, which have drawn a lot of scrutiny for their role in resolubilization of protein aggregates (Von Janowsky, 2006, J Mol Biol / De Los Rios, 2006, PNAS / Ben-Zvi, 2001, J struct biol). Other proteases of the AAA+ family, including Pim1/LON protease, are better known for their ability to degrade dysfunctional proteins in mitochondria (Voos, 2009, Res Microbiol) In fact, the absence of Pim1/LON leads to poor mitochondrial respiration. Pim1/LON serves an even more vital role in maintaining mitochondrial homeostasis in the face of stresses that are generally recognized as pathogenic (Bender, 2011, Mol Cel Biol). Although these results have been observed in yeasts (Suzuki, 1994, Science), they speak to the importance of mitochondrial quality control in the maintenance of mitochondrial health and disease prevention. Beyond protein quality control, some of the Clp proteins have been suggested to also play a role in the maintenance of mtDNA integrity (Liu, 2004, J biol chem). When the amount of disrupted proteins starts exceeding these first line molecular controls in mitochondria, it induces an unfolded protein response (UPR), which, unlike the first line quality controls, alters the transcriptome (Aldridge, 2007, Plos One). This response is analogous to what is seen during ER stress, a somewhat better understood phenomenon than mtUPR (Hetz, 2012, Nat Rev Mol Cell Biol)

2.2.2 Aggregation

Different environmental stresses can lead to protein disruption and misfolding. Unfolded proteins can call onto molecular chaperones to attenuate the impact of such stresses on the cell or can simply be degraded via the proteasome. For a review of quality control mechanism at the molecular level see (Baker, 2011, Trends Biochem Sci). However when these cellular protection systems fail to cope with mounting amounts of unfolded proteins, the exposure of their hydrophobic sites will cause them to aggregate (Tyedmers, 2010, Nat Rev Mol Cell Biol). This can be caused by environmental stresses such as heat or excessive exposure to ROS or other aberrant post-transcriptional modifications, as well as mutations. Small protein aggregates will first be tagged with polyubiquitin chains with the intervention of ubiquitin E3 ligases. However, it appears ubiquitination is not an obligatory element in the aggresome formation (Kopito, 2000, Trend Cell Biol).

As depicted in figure 6, histone deacetylase 6 (HDAC6) is thought to serve as the hook between the polyubiquinated chains attached onto the small protein aggregates (microaggresomes) and the motor protein dynein (Kawaguchi, 2003, Cell), which is responsible for shipping the proteins to the larger aggresome-forming site along the microtubule system. Large protein aggregates (macroaggresomes) are stored in different subcellular compartments depending on their nature. For example, they can be sent to the juxtanuclear quality control compartment, where ubiquitinated proteins will be degraded by the proteasome. However, proteasomal degradation is not optimal for large protein aggregates due to its thin barrel-like structure (Snyder, 2003, J Biol Chem); thus, macroaggresomes may alternatively be sent to the insoluble protein deposits for longer-term storage and large-scale clearance via autophagy (see figure 6). The formation of aggresomes prevents widespread intracellular toxicity derived from a disparate accumulation of microaggresome that interefere with normal cell function. The concentrating of aggregates also allows for more efficient degradation by facilitating more targeted autophagy (Yao, 2010, Genes Cancer).

Figure 5: outlining the aggresome formation process

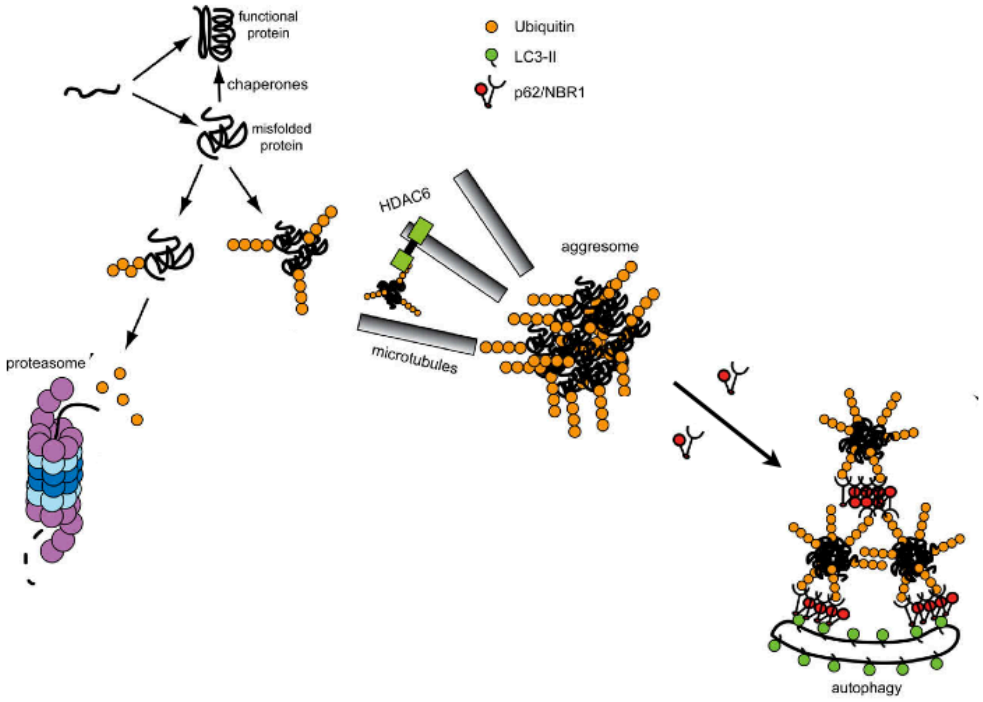


Figure 5: outlining the aggresome formation process

Misfolded proteins are ubiquitinated and are either sent for immediate degradation by the proteasome are sent for aggregation with the help HDAC6 and p62 before being degraded by macroautophagy.

*Adapted from Knaevelsrud, 2010, Febs Letters. Reproduced with permission ©

2.2.3 Autophagy

Autophagy is the process by which portions of cytoplasm, organelles, misfolded proteins or aggresomes are included in double-membraned vesicles for recycling. This process is particularly important to discard dysfunctional proteins and organelles, purging pathogens and, providing crucial nutriments during starvartion. Autophagy can be classified into selective and non-selective autophagy. Non-selective autophagy (macroautophagy) involves the sequestration of random bulk cytoplasmic components. This catabolic event probably serves in controlling cytoplasmic volume, to provide nutrients for the cell, or damaged parts of the cell. Selective autophagy on the other hand can be broken down further according to the specificity of the target cargo which may include: ribosomes (ribophagy), ER (reticulophagy), protein aggregates (aggrephagy), mitochondria (mitophagy) or even foreign material such as bacteria and viruses (xenophagy) (Liang, 2010, Curr Opin Cell Biol). In the case of selective autophagy, the cargo to be discarded is often identified by an adaptor protein added onto the ubiquitin chain attached directly on the target bulk aggregate before an autophagosome matures around it. Once the autophagosome is formed, additional material can be delivered to the autophagosome. For example, in chaperone-mediated autophagy, a misfolded cytoplasmic protein with an exposed KFERQ motif will be recognized by heat shock cognate 70 (HSC70) and targeted to lysosomal membrane protein (LAMP) 2A for inclusion into the autophagolysosome (Wang, 2013, Circ Res). The autophagosome can also engulf small specific cytoplasmic constituents in a manner that is evocative of phagocytosis (microautophagy).

2.2.3.1 Autophagosome formation

Autophagy can be induced by inactivation or dephosphorylaytion of Akt (Romanello, 2010, EMBO J), which then relieves the repressing effects of the mammalian target of rapamycin (mTOR) on autophagy. mTOR is an important metabolic sensor, in nutrient-fed states, it inactivates the expression of autophagy

related genes (Atgs) and promotes protein synthesis. Starvation can stop the repressive effect of mTOR on some of the Atgs, via dephosphorylation of Akt. Some of the important Atgs include ULK, whose activation is necessary during the initial phase of autophagosome formation, known as the phagophore nucleation (Longatti, 2010, Cell Res). Both metabolic sensors and cell cycle pathways converge toward the Akt crossroad, implying a probable role for IGF, AMPK and p53 in the initiation of autophagy (Feng, 2007, Cancer Res). The repression of Akt also causes FoxO dephosphorylation, which promotes transcriptional activity of the latter. FoxO regulates expression of both autophagic components Bnip3 and LC-3 (Menzies, 2012, mitochondrion) but also that of atrogenes including atrogin-1 and MuRF, which have been mostly related to proteasomal degradation (Lecker. 2004, FASEB J). However, It is not surprising to see crosstalk between the two prominent protein degradation systems of the cell, the latter being determined in large part by different proteins of the Bcl-2 family. The antiapoptotic B-cell lymphoma (BCL)-2 normally prevents the induction of autophagy by interacting with Beclin1, an important factor in phagophore nucleation. As discussed, earlier, various BH3 proteins can also interact with BCL-2 and are thus able to inhibit the repressive effect of BCL-2 on Beclin1 (Kubli, 2012, Circ Res). This makes BCL2/adenovirus E1B 19-kDa protein interacting protein 3 (Bnip3), Bnip3-like protein X (Nix/Bnip3L), the p53 upregulated modulator of apoptosis (PUMA) important modulators of not only Ca^{2+} homeostasis, mitochondrial function and apoptosis, but also autophagy. This suggests a tight integration of autophagy with these vital cellular functions.

The elongation process of the phagophore into a mature autophagosome will require the formation of PIP3 via a specific subclass of PI3K, named Vps34. This is followed by a signaling cascade involving the recruitment of different Atgs to the phagophore assembly site for a series of protein conjugation reactions, most notably, the lipidation of microtubule-associated protein 1 light chain 3 (LC3), the mammalian homolg of ATG8 in yeast, into LC3-II (Klionsky, 2000, Science). For extensively detailed reviews deciphering the cascade of Atgs, leading to the formation of the autophagosome, see: (Longatti, 2009, Cell Death Differ / He, 2009,

Ann Rev Genet / Klionsky, 2000, Science). LC3 is necessary for the maturation of the autophagosome, as it is proposed to bind the p62/NBR1 component, attached at the end of the polyubiquitin chain cellular that identifies the cargo to be discarded, to the phagophore. In fact, the formation of LC3 puncta, or dense accumulation of LC3 in focal areas is often used as a marker of autophagy in cellular models (Iwai-Kanai, 2008, Autophagy). The maturing phagophore has a cup shaped membrane and its elongation consists of enwrapping the target cargo until the double-membrane vesicle closes around it. The subject of autophagosome closure is actually the latest topic of interest in autophagy research; current results point to LC3, Gabarap and Atg-16L/GATE as key players in this process (Noda, 2009, Cell Death Differ). The source of the membranes that are recruited to the phagophore assembly site during growth also remain obscure. Nonetheless, some point to the endoplasmic reticulum or the mitochondria as the source of the membranes that will eventually form the autophagosome, but this remains to be determined (Juhasz, 2006, Plos Biol / Yla-Antilla, 2009, Autophagy).

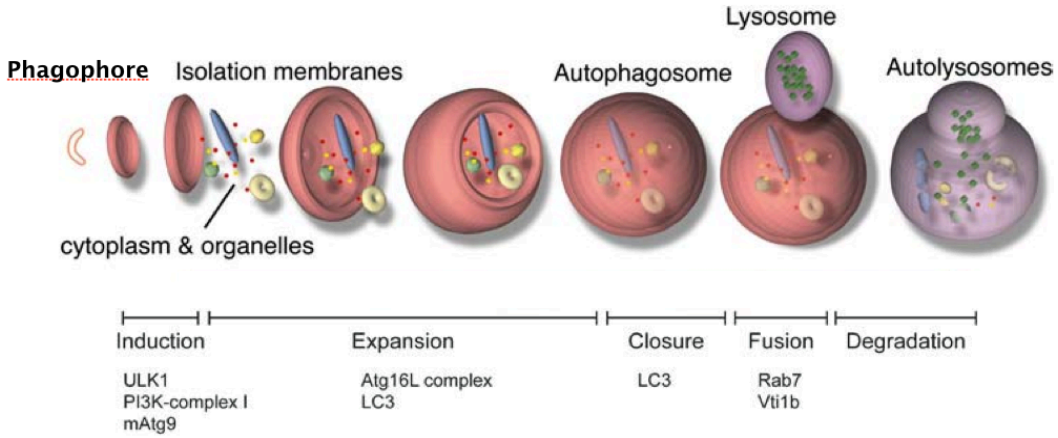
The closed autophagosome is shipped to the perinuclear region in a dynein/microtubule dependent manner. It has been proposed that the release of ATG16L leaves LC3 unbound, which allows for association with dynein (Noda, 2009, Cell Death Differ). This hypothesis makes sense as LC3 was fist identified as a microtubule associated light chain (Kuznetsov, 1987, FEBS Lett). Once delivered, the autophagosome can fuse with endosomes or lysosomes to complete its maturation into an autolysosome. This fusion process is dependent on proper vesicle tethering using common endocytic and vesicle trafficking machinery such as the GTPase containing Rab-SNARE complex and a Beclin-1 interacting protein, named UVRAG (Liang, 2008, Nat Cell Biol).

The lysosome is often seen as the intracellular digestion system, providing amino acids, lipids, and carbohydrates for the cell by degrading macromolecules. The lysosome provides a highly acidic environment that favors protein degradation. The digestion process is executed by the more than 50 digestive acid hydrolase that

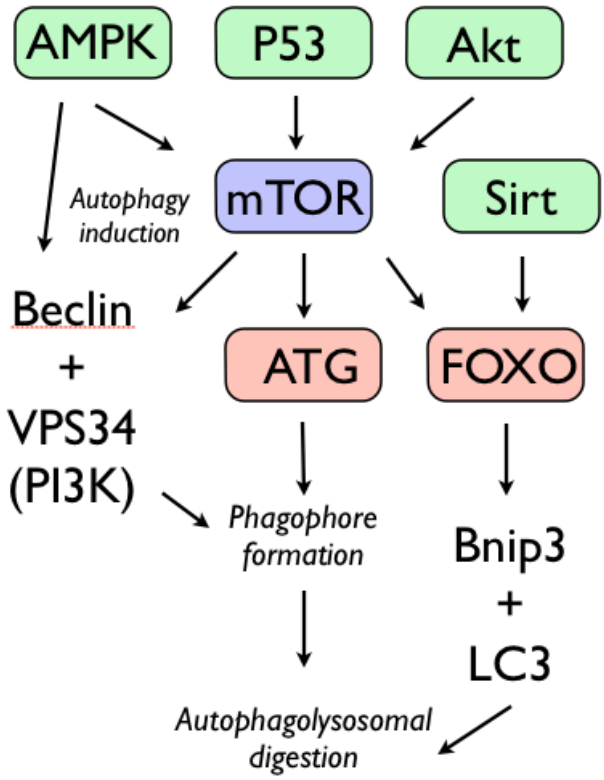
are found in the lumen of lysosomes and that are characterized by a Mannitol-6-phosphate residue (Sandri, 2011, Curr Opin Clin Nutr Metab Care). Cathepsin, one of the lysosome resident protease, is probably the most renown for it can be used as a marker of lysosomal activity but also because it can induce apoptosis if leaked from the lysosome (Johansson, 2010, Apoptosis). Following digestion, the recycling of residues is carried out by membrane bound permeases (Rajawat, 2009, Ageing Res Rev).

Figure 6: autophagy from induction to digestion

A)



B)



C)

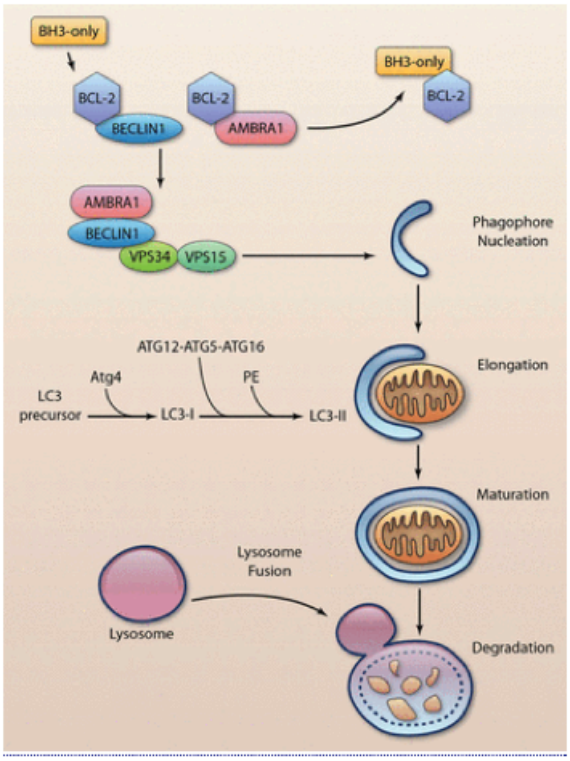


Figure 6: autophagy from induction to digestion

A) From phagophore to autophagolysosome. Adapted from Yoshimori, 2004, biochem biophys res comm. Reproduced with permission © B) Some of the important signaling pathways behing autophagy induction. C) Integrated model of signaling and expansion/maturation of the autophagosome. Adapted from Kubli, 2012, Circ Res. Reproduced with permission ©

2.2.4 Mitophagy

Mitophagy is a preferred way to selectively clear dysfunctional and potentially disruptive mitochondria. Dysfunctional organelles are often recognized as such by their inability to generate a membrane potential. Depolarized mitochondria are quickly removed from the mitochondrial network. Numerous studies have now shown that treatment of mitochondria with uncoupling ionophore CCCP will lead to fragmentation of the mitochondrial network (Figure 8B) (Duvezin-Caubet, 2006, J Biol Chem / Legros, 2002, Mol Biol Cell). This however, appears dependent on proper functioning of the fission machinery which main players include Dynamin-related protein-1 (DRP-1) and Fis-1 (Twig, 2008, EMBO J). The stabilization of Pten-induced kinase 1 (PINK1) and the ensuing recruitment of parkin to the mitochondria, is also observed after CCCP treatment. Indeed, Parkin, an E3 ubiquitin ligase, normally located in the cytosol has been shown to translocate to depolarized mitochondria using a PINK-1 dependent recruitment (Ziviani, 2010, PNAS/ Narendra, 2008, J Cell Biol). However, the relationship between the PINK1-induced translocation of Parkin and the loss of membrane potential remain unclear. Bnip3, a BH3 protein of the bcl-2 family is an interesting link as it is able to induce mitochondrial depolarization independently of the permeability transition pore. Bnip3 is also known to interact with DRP-1 (Kubli, 2007, Biochem J), and its inhibition greatly reduces mitochondrial fragmentation and mitophagy (Mammucari, 2007, Cell Metab). In physiological settings, newly fragmented mitochondria are given a chance to prove they can generate a membrane potential on their own and if they can do so, they will be re-integrated into the mitochondrial network; if not they will remain segregated and mitophagy will move forward.

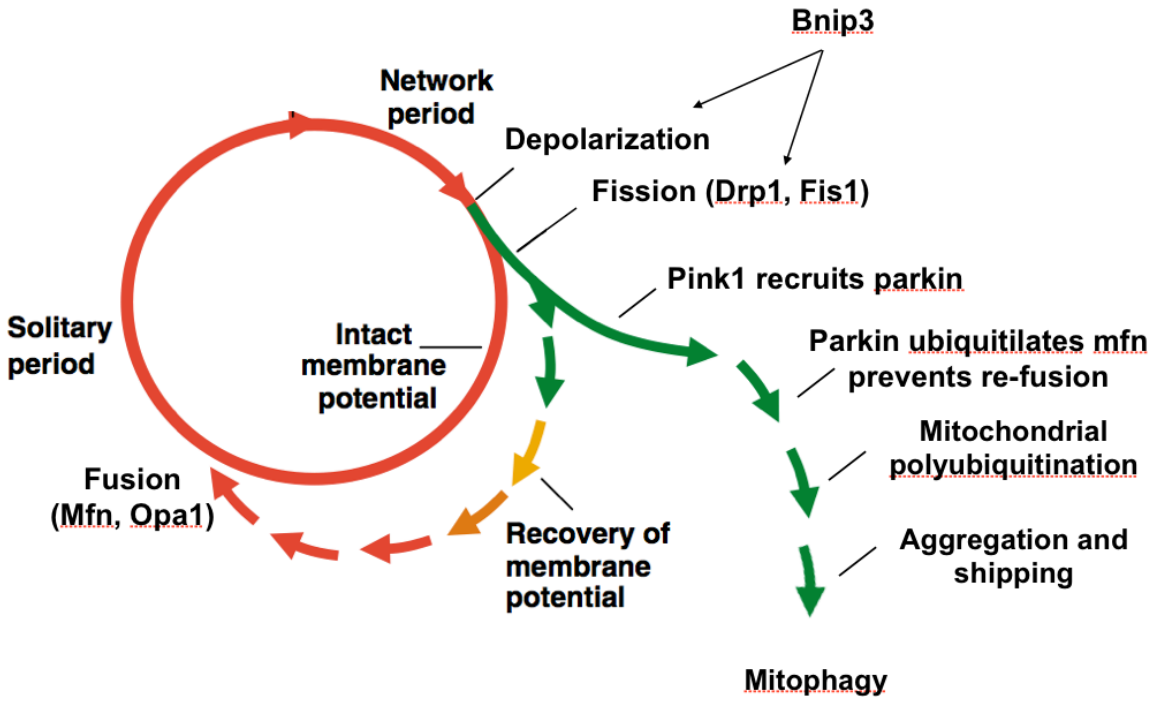
Interestingly, Parkin ubiquitinates mitofusin, possibly preventing the fissioned mitochondria to re-integrate the mitochondrial network (Gegg, 2010, Hum Mol Genet). In support of this idea, overexpression of fusion-associated proteins Mitofusin or OPA-1 prevents parkin-mediated autophagy (Ziviani, 2010, Autophagy). This idea is also supported by the finding that the atypical Rho GTPase

MIRO, which plays an important role of linking mitochondria to microtubule has been found to be a substrate of PINK-1. Once phosphorylated by PINK-1, MIRO would be targeted by Parkin for proteasomal degradation, thus disrupting the potential re-integration of dysfunctional mitochondria in the network (Wang, 2011, Cell).

Following the successful sequestration of dysfunctional mitochondria, Parkin mediates the formation lysine 63 (K63)-linked polyubiquitin chains onto the mitochondria (Olzmann, 2008, Autophagy). Parkin is also necessary in the microtubule dependent (Vives-Bauza, 2010, Proc Natl Acad Sci) translocation of dysfunctional mitochondria to the perinuclear region where they aggregate (Okatsu, 2010, Genes Cells). Following aggregation, p62 will co-localize with mitochondria to initiate autophagy by linking with LC3 (Geisler, 2010, Nat Cell Biol). Thereafter, autophagosome formation will continue as previously described with no regards on the specificity of the content. However, it was found that the absence of P62 has little effect on mitophagy (Narendra, 2010, Autophagy), raising the possibility that mitochondria can be cleared through alternate pathways. Recently, NIX/BNIP3L and BNIP3 have been found to be potential autophagy receptors for selective clearance of mitochondria and can bind directly with LC3 (Hanna, 2012, J Biol Chem / Novak, 2010, EMBO rep). However, one important discerning aspect between the PINK/Parkin and the BNIP3/NIX pathways is that the former requires the loss of membrane potential, which relies mostly on Bax/Bak whereas BNIP3 and NIX can initiate mitophagy on mitochondria retaining their membrane potential (Rikka, 2011, Cell death differ). Additionally, it has been noted that other E3 ligases may be able to prompt the ubiquitination of OMM proteins necessary to conduct mitophagy. In particular, the glycoprotein 78 (gp78), RNF185 and MARCH5 may all overlap with Parkin in regards to its role of ubiquitinating mitochondria targeted for clearance (Fu, 2013, Mol Cell Biol / Tang, 2011, Plos one).

Figure 7: Fragmentation of mitochondria for mitophagy

A)



B)

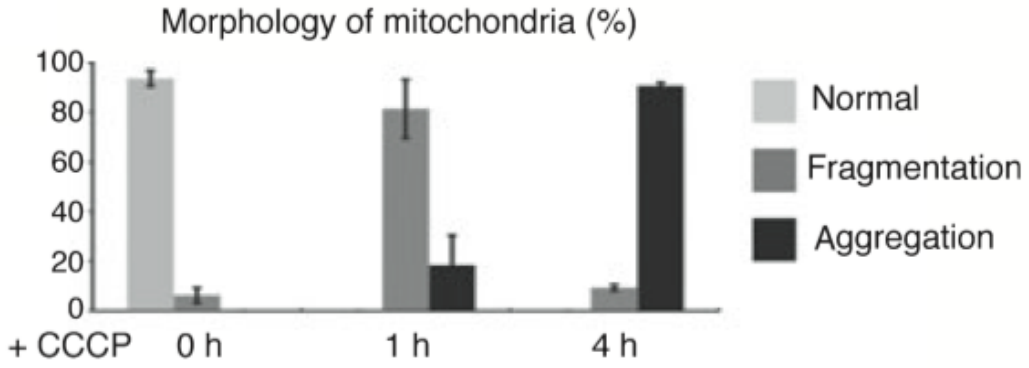


Figure 7: Fragmentation of mitochondria for mitophagy

A) Characterizing mitochondrial life cycle and steps to segregate dysfunctional mitochondria for mitophagy Adapted from Twig, 2008, Embo. Reproduced with permission © B) depicting the flow of mitochondria from normal to fragmented to aggregated in response to a depolarizing CCCP treatment. From Okatsu, 2010, genes cells. Reproduced with permission ©

2.2.5 Physiological and pathophysiological autophagy/mitophagy

Skeletal muscles are the largest available pools of amino acids. It is not a coincidence that starvation strongly induces the formation of autolysosome in muscle tissues. The product of degradation secures enough substrate for energy production and biosynthetic work. The regulating action of mTOR seems to be mostly dependent on metabolic signals and appears to be a useful tool in the maintenance of serum glucose homeostasis (Ezaki, 2011, Autophagy). In times of nutrient abundance, insulin signaling will trigger protein synthesis through mTOR phosphorylation, whereas its repression by AMPK in times of substrate deprivation, leads to autophagic digestion (Park, 2013, Autophagy). Ogata and colleagues observed a 50-fold increase in LC3 and a 6-fold increase in p62 after 48 hours of starvation in the plantaris muscle of rats. This was combined with a significant 50% decrease in phosphorylated mTOR (Ogata, 2010, Biochem Biophys Res Comm).

Accumulation of protein aggresomes is cytotoxic and is actually a hallmark of numerous neurodegenerative diseases. For example the accumulation of Lewy bodies and other inclusions are found in the neurons of Parkinson, Alzheimer's and other prominent neurodegenerative diseases (Ferrer, 2005, Brain Pathol). A simple RNA interference of the aggregation-prone proteins (Harper, 2005, Proc Natl Acad Sci) or boosting the clearance of protein aggregates have been shown to rescue the phenotype in some of the diseases (Pandey, 2007, Nature) that have been associated with the accumulation of protein aggregates (Shacka, 2008, Front Biosci).

Given the proteolytic nature of autophagy, it has been proposed that it may contribute to atrophy in various myopathic models (Schiaffino, 1972, J Ultrastruct Res). However, there is strong evidence that failing autolysosomal digestion, rather than hyperactivation, triggers the development of these diseases. For example 1) accumulation of p62 positive protein aggregates in patients suffering from sporadic inclusion body myositis (IBM) point to ineffective autophagy in this type of muscle

disease (Nogalska, 2009, Acta Neuropathol). 2) In Danon disease, a severe myopathy, the absence of the lysosomal enzyme LAMP-2 results in an inability to properly fuse the autophagosome and the lysosome in the perinuclear area, causing accumulation of autophagosomes (Saftig, 2008, Autophagy). 3) Any loss of function in the digestive enzymes of the lysosomes will most likely lead to serious lysosomal storage diseases (LSD) the most notable of them, Pompe disease (Eskelinen, 2009, Biochim Biophys Acta), 4) In Paget disease, failure of the proteasomal pathway of degradation overwhelm the autophagosomal pathways of degradation, leading to myopathy (Kimonis, 2008, Biochim Biophys Acta), 5) Accumulation of dysfunctional mitochondria and protein aggregates in the senescent muscle are also indicative of inappropriate autophagic digestion

Additionally, the group of Sandri and colleagues has reported in Atg7 $^{-/-}$, autophagy null muscle, the presence of oxidative stress and abnormal mitochondria. They have also found inclusion vacuoles such as those found in IBM, increased levels of polyubiquitinated proteins, an induction of the unfolded protein response in addition to weakness and atrophy (Masiero, 2009, Cell Metab). Overall, autophagy inhibition resulted in a myopathic phenotype, which underlines the potential role of inadequate quality control in the pathogenesis of various myopathies (Sandri, 2010, Am J Physiol Cell Physiol), including those pertinent to this thesis.

2.3 Mitochondrial dysfunction and their overall contribution to selected myopathies/cardiopathies

2.3.1 Denervation and other models of muscle disuse

Prolonged periods of muscle inactivity due to bed rest, limb immobilization or mechanical ventilation unequivocally lead to rapid muscle atrophy and concomitant changes in mitochondrial shape, number and function (Singh, 2011, Am J Physiol Cell physiol / Leivo, 1998, APMIS). Surgical denervation and nerve silencing have also been used to mimic muscle disuse in experimental settings (Siu, 2005, J Physiol). The consensus of such studies is that muscle mass of locomotor muscles is estimated to diminish by 30-40% seven days after immobilization whereas this level of atrophy in the diaphragm is achieved after only two days of mechanical ventilation (Fitts, 2001, J Exp Biol / Picard, 2012, Am J Respir Crit Care Med). It is obvious that imbalances between protein synthesis and protein breakdown underlie myofiber atrophy during disuse (Thomason, 1989, Am J Physiol Regul Integr Comp Physiol). Myofibrillar proteins appear predominantly targeted during disuse-induced atrophy translating in the loss of contractile capacity (Munoz, 1993, Metabolism). However, there are reasons to believe that these changes are derived from altered mitochondrial metabolism. It has been known since the 1960s that modifications in mitochondrial morphology that present themselves in the form of swollen and misshapen mitochondria, coincide with atrophy during denervation (Carafoli, 1964, Exp Mol Pathol). More recently, it became evident that fragmentation of the mitochondrial network through fission events plays an important part in disuse-induced mitochondrial dysfunction. In fact, mitofusin-2 loss-of-function alone induces severe mitochondrial and muscular dysfunctions (Zorzano, 2009, Appl Physiol Nutr Metab). Additionally, stimulation of fission via transfection-induced overexpression of Drp-1, an important fission inducer, resulted in spontaneous atrophy (Romanello, 2010, EMBO); thus linking fission and atrophy. At the fundamental level, fissioned mitochondria, such as seen during chronic muscle disuse, are recognized for their poor respiratory and calcium

handling functions, their higher emission of pro-apoptotic factors and ROS, as well as, facilitating their elimination by mitophagy (Powers, 2012, Am J Physiol Endo Metab).

2.3.1.1 Oxidative capacity in muscle disuse

A markedly lower mitochondrial volume underpins the lower oxidative capacity of muscle undergoing disuse atrophy (Adhihetty, 2007, J Appl Physiol). However, it remains unclear whether the loss of mitochondrial volume simply parallels the rapid loss of muscle mass or whether the appearance of mitochondrial defects exacerbate the decrease in oxidative capacity. At first glance, the 40% decrease of different mitochondrial proteins after 10 days of denervation appears to mirror (Brault, 2010, J Biol Chem) the decrease in maximal respiration (O'Leary, 2008, J Appl Physiol) and the decrease in muscle mass (Adhihetty, 2007, J Appl Physiol). However, the 30% lower maximal respiration per milligram of mitochondria isolated from denervated muscle compared to healthy muscle suggests that specific mitochondrial defects aggravate the loss of oxidative capacity normally due to the loss mitochondrial volume (O'Leary, 2012, Am J Physiol Cell Physiol). On the other hand, important research groups argue that mitochondrial oxidative impairments in denervation are experimental artifacts (Picard, Aging Cell, 2010). However, the lower enzymatic activity of mtDNA encoded complex I and IV reported in the muscle of surgically denervated animals (Ghiasi, 2012, Neurol Res) tend to support the idea of specific mitochondrial damage in addition to an overall decline in mitochondrial biomass. There is considerable evidence showing that some of these defects stem from disturbed mitochondrial turnover. Indeed, the impoverishment of the denervated muscle mitochondrial fraction in import machinery proteins (i.e. Tim23 and Tom20) as well as some key quality control protein chaperones (i.e. mtHSP70) would favor a lower protein turnover and the accumulation of protein defects (Singh, 2011, Am J Physiol). Additionally, the genetic overexpression of PGC-1a, known to promote mitochondrial turnover, and which is normally downregulated after denervation (Wagatsuma, 2011, J Physiol Biochem),

restores most of the mitochondrial defects and protects against denervation atrophy (Sandri, 2006, Proc Natl Acad Sci / Brault, 2010, J Biol Chem); demonstrating the central role of mitochondria in promoting the loss of muscle mass during inactivity.

As mitochondrial volume decreases and damage increases, the reduction in energy production capacity results in low cellular ATP levels, which may alter important signaling pathways (Max, 1972, Biochem Biophys Res). Specifically, the activation of AMPK leads to activation of FoxO, which regulates the expression of important atrogenes responsible for driving ubiquitin proteasome as well as autophagolysomal digestion (Romanello, 2010, EMBO J), thus linking mitochondrial damage to cell wide proteolysis and atrophy.

2.3.1.2 ROS production in muscle disuse

Mitochondria-derived ROS have been implicated in tissue degeneration for their role in stimulating 1) Lysosomal degradation 2) Ubiquitin Proteasome 3) Calpain 4) Caspase (Bonetto, 2009, Free Rad Biol Med). ROS are thus a key trigger in the parallel activation of these different proteolytic pathways during immobilization-induced atrophy. In addition to the activation of proteolysis, oxidative stress was also shown to accelerate muscle degeneration by attenuating protein synthesis (Patel, 2002, Eur J Biochem); heavily tilting the protein balance in favour of degradation. During inactivity, the level of substrate oxidation is greatly reduced resulting in an excess of energetic substrates combined with high levels of ATP due to failing demand. This condition, termed metabolic overload, impedes on electron flow in the ETC, forcing the formation of ROS. The accumulating lipids also facilitate their interaction with ROS and their oxidation into dangerous hydroperoxides. Such hydroperoxides have been shown to directly lead to mitochondrial dysfunction (Bhattacharya, 2009, J Biol Chem), further fueling ROS production. In fact, we have observed co-localization of mtDNA mutations with accumulating lipids in inactive muscle (Picard, 2012, Am J Respir Crit Care Med). For

all of these reasons, the recent observations by Singh and colleagues of a five fold increase in net H_2O_2 output from mitochondria isolated after 14 days of denervation compared to baseline was largely expected (Singh, 2011, Am J Physiol Cell Physiol), and is in line with the numerous evidence of oxidative stress in disused muscle (Abruzzo, 2010, Free Rad Res). In addition to providing the groundwork necessary to move forward with atrophy in models of chronic unloading by activating the aforementioned proteolytic pathways via elevated ROS production, the metabolic overload may also contribute to impaired mitochondrial turnover by downregulating the activity of important energy sensors in the AMPK/ Sirt/ PGC1-α axis (Canto, 2009, Nature).

The role of oxidative stress in mediating atrophy in models of prolonged muscle inactivity has been known for a while. Earlier studies have shown that the supplementation of Vitamin E during limb immobilization prevented about half of the normal atrophy (Appell, 1997, Int J Sports Med). However, the early studies did not make any discrepancy for the origins of these ROS. More recently, it became evident that an increase in mitochondrial ROS production is responsible for the oxidative stress observed in the inactive muscle and has a causative role in the concomitant atrophy (Min, 2011, J Appl Physiol). Min and colleagues reported that the administration of the mitochondria-specific antioxidant, SS-31, concurrently with limb immobilization largely prevented muscle atrophy, giving support to prior indications that the oxidative stress long known to exist in this model originates from mitochondrial sources. Similarly, our group has unveiled severe muscle dysfunction in mechanically-ventilated diaphragms (Picard, Am J Respir Crit Care Med, 2012), a rapidly progressing model of muscle inactivity (Powers, Crit Care Med, 2009), which were prevented by targeting specifically mitochondrial ROS production. Again, in this model we observed a rapid decrease in mitochondrial volume that was accompanied by oxidative stress and a hindering of force generation. We found various evidence that mitochondrial dysfunctions contribute to ventilation-induced diaphragm dysfunction (VIDD). First, the presence of mtDNA mutations proved that the mitochondria were abnormal beyond their expected

volume reduction. Additionally, the overexpression of the mitochondrial antioxidant peroxiredoxin3 (Prx3), largely prevented VIDD in animals mechanically ventilated for 6 hours (Picard, Am J Respir Crit Care Med, 2012). These results were corroborated by other studies in which the mitochondrial antioxidant SS-31 (Powers, Crit Care Med, 2011) also prevented the undesirable effects of mechanical ventilation on diaphragmatic functions and all point to an important involvement of mitochondrial dysfunctions in denervation-induced atrophy.

2.3.1.3 Mitochondrial permeabilization in muscle disuse

The indication that mitochondrial permeabilization and the ensuing apoptosis contribute to cellular degeneration in muscle disuse has now been convincingly demonstrated using a surgical denervation model. Bax expression and content has been shown to significantly increase during denervation (Adhihetty, 2007, J Appl Physiol / Siu, 2005 J Physiol). Even the expression of Bcl-2, known for its ability to neutralize Bax oligomerization, has been shown to be reduced in this model, probably further facilitating mitochondrial permeabilization. Previous studies by our laboratory also observed an increased susceptibility to PTP opening in this model (Csukly, 2006, J Physiol). However, its contribution to atrophy was not directly investiguated, hence justifying the first study presented in this thesis. Overall, it is not surprising that Siu and Alway, consistently found that mitochondrial pro-apoptotic factors were abundant in the cytosol of rat hindlimb muscles 14 days after surgical transection of the sciatic nerve. Among them, an astonishing 1200% increase in the cytosolic content of AIF and an increase of 400% in the ratio of cytosolic Smac-DIABLO to XIAP (Siu, 2005, J Physiol). Other studies have confirmed the presence of mitochondrial pro-apoptotic factors in the cytosol of denervated animals albeit at a much lesser extent (Csukly, 2006, J Physiol). With the role of apoptosis in contributing to denervation-induced atrophy constantly being questioned, Siu and Alway also took the time to clearly demonstrate the importance of the process in denervation atrophy by showing a robust increase in the content of

the cleaved isoform of the downstream apoptosis effectors, caspase 3 and 9, and an increase in cleaved Poly ADP-ribose polymerase (PARP) content nearing 4 times the baseline content (Siu, 2005, J Physiol / Siu, 2006, apoptosis). In a subsequent study, Siu and Alway showed that Bax knockout animals were somewhat protected from denervation-induced atrophy. The absence of Bax nearly abolished the normal rise in DNA fragmentation associated with an important decrease in the cytosolic presence of cytochrome c, suggesting that most of the apoptosis in response to this model of muscle inactivity is derived from Bax-induced mitochondrial signaling (Siu, 2006, Apoptosis). In the same study, the cytosolic levels of other mitochondrial pro-apoptotic factors normally sequestered in the mitochondria, such as Smac/DIABLO, were similar between the two genotypes raising the possibility that different factors escape the mitochondria according to different modes of membrane permeabiization. Despite the robust rise of apoptosis in normal denervation-associated atrophy, Bax deficiency and what appears to be an absence of apoptosis, does not prevent muscle atrophy. This puts in perspective the relative importance of apoptosis, which is probably responsible for a fraction of atrophy during prolonged periods of muscle inactivity; although Plant and colleagues had already revealed that the absence of caspase prevents a certain degree of atrophy in a similar model (Plant, 2009, J Appl Physiol). Apoptosis appears to work synergistically with other proteolytic pathways that can probably compensate for the lack of apoptosis. For example, O'Leary and colleagues observe increased autophagy during denervation and a further compensative increase in Bax deficient animals (O'leary, 2012, Am J Physiol) that however did not reach statistical significance in the referred study. The authors interpreted the results as a testimony of a near irrelevance of apoptosis in denervation-induced atrophy. The reality is perhaps not as clear-cut with autophagy and apoptosis probably sharing a certain level of redundancy in assuring appropriate atrophy during disuse.

There is evidence of higher intracellular calcium concentration in the denervated muscle. Although there is strong evidence that this calcium overload originate from oxidative damage to ryanodine receptors as well as SR and

sarcolemmal ionic channels/pumps, mitochondria appear to also play a role in calcium homeostasis dysregulation (Abruzzo, 2010, Free Rad Med). Indeed mitochondrial calcium content has been shown to increase four fold in the hindlimb muscles of surgically denervated rats, which prompted a decreased in mitochondrial calcium retention capacity of a similar magnitude (Csukly, 2006, J Physiol), probably exacerbating calcium overload.

2.3.1.4 Autophagy/mitophagy in muscle disuse

Autophagy is probably responsible for an important degree of atrophy during muscle inactivity. After 7 days of denervation, the protein expression of autophagy inducing factors ULK1, Beclin-1, and ATG7 were shown to average a threefold increase whereas LC3 total content rallied for an impressive 50-fold surge in otherwise healthy animals (O'leary, 2012, Am J Physiol). The contribution of autophagy in denervation-induced atrophy was later confirmed by Ju and colleagues who have shown an important increase in autophagic flux in similar experimental conditions (Ju, 2010, Autophagy). O'Leary et al also observed an important upregulation in the mRNA expression of several genes related to autophagy regulation. Importantly though, the lower protein synthesis environment that prevails during denervation, necessitates a careful examination of mRNA data. Thus, an analysis deeper than the one performed by the authors, reveals that not only is the mRNA of several autophagy related genes elevated in comparison to baseline results, but the mRNA expression of all the autophagy genes studied were higher than the expression of GAPDH, the housekeeping gene used in this study (O'Leary, 2012, Am J Physiol Cell Physiol). The upregulation of autophagy-related genes has also been corroborated both at the mRNA and protein levels in other models including hindlimb unloading in rats (Andrianjafiniony, 2010, Am J Physiol Cell Physiol/ O'Leary, 2008, J Appl Physiol). Interestingly, O'Leary and colleagues also revealed the translocation of LC-3 to mitochondria upon denervation. This step is thought to be important to initiate mitochondria-specific autophagy (mitophagy).

Thus, such observation suggests that the autophagolysosomal digestion probably plays an important part in mitochondrial dismantlement during the initial rapid phase of mitochondrial volume reduction that takes place during chronic muscle disuse (Wicks, 1991, Am J Physiol). Surprisingly however, lysosomal inhibitors did not alleviate denervation-induced atrophy, nor did deletion of autophagy-related genes; in fact, it accelerates it (Masiero, 2009, Cell Metab).

Recently, Quy et al. completely discarded autophagy as a contributor to atrophy in denervation, arguing it is actually repressed by mTOR, which also acts to accelerate proteasomal degradation (Quy, 2012, J Biol Chem). These results directly contradict the currently available data and the established dogma of autophagic participation in denervation-induced atrophy. The authors of this latest controversial study tend to shift the focus towards proteasomal degradation (Quy, 2013, J Biol Chem). These results are even more intriguing considering the degree of overlap the activation of these two pathways share, with both being highly sensitive to FOXO and TRAF6, which are both strongly upregulated during denervation; underscoring the degree of redundancy in proteolytic pathways (Zhao, 2008, Autophagy / Paul, 2011, Autophagy).

Although, mitophagy appears upregulated during denervation and is accompanied by a reduction in mitochondrial volume, it was not sufficient to prevent the accumulation of mitochondrial dysfunction. Mitophagy appears to play a protective role in denervated skeletal muscles, by clearing potentially disruptive mitochondria. Indeed, Masiero and colleagues noted that muscle atrophy during denervation nearly doubled in atg7-null animals when compared to wild-type (Masiero, 2009, Cell Metab). The authors also report that ultrastructural abnormalities and apoptosis were severely exaggerated in autophagy-deficient animals after denervation. This indicates that autophagy is able to contain an important part of muscle dysfunction and is necessary in coordinating an orderly atrophy in times of chronic inactivity.

Some of the beneficial effects of anti-caspase on denervated muscles may be due to crosstalk between the caspase-signaling pathway with autophagy. Indeed, it appears caspase, when activated, cleaves beclin-1, thus inhibiting the survival-promoting effects of autophagy (Luo, 2010, Cell Death Differ). This can be prevented by the anti-apoptotic factors member of the bcl-2 family, such as BCL-XL (Kang, 2011, Cell Death Differ / Cho, 2009, Cancer Lett).

Overall, the literature shows numerous mitochondrial dysfunctions that accumulate in the denervated muscle that is associated with cell wide damage. Mitochondrial interventions have shown a mitigated success in preserving mitochondrial functions and have at the same time alleviated cellular damage, suggesting that mitochondria may be at the center of denervation-induced atrophy.

2.3.2 Duchenne muscular dystrophy

Patients suffering from Duchenne muscular dystrophy (DMD) display muscle atrophy and dysfunction that has been mechanistically linked to excessive oxidative stress and concomitant mitochondrial dysfunctions (Grosso, 2008, Brain Dev / Tidball, 2007, J Appl Physiol). Evidently, making a case for the vicious cycle of mitochondrial dysfunction playing a role in the pathogenesis of DMD is challenging because the main pathogenic element is a nuclear mutation in the X-linked dystrophin gene. The disease results in a severe progressive myopathy and is lethal. The absence of the subsarcolemmal dystrophin protein leads to membrane instability, contractile dysfunctions and cellular death (Zhu, 2002, FASEB). Nonetheless, the oxidative deficiencies, the augmented ROS production and the Ca^{2+} imbalances known to characterize the disease also make it difficult to ignore mitochondria as potential contributors to pathogenesis.

2.3.2.1 Oxidative capacity in DMD

There are also metabolic indices that lead us to believe that mitochondrial dysfunction contribute to muscle degeneration in DMD muscle. Mitochondrial respiration has been shown to be two fold lower in human dystrophic muscle (Sperl, 1997, Mol Cell Biol) and was also seen to be significantly lower with a more appropriate sample size in the mdx mice, the murine model of DMD, albeit to a lesser extent (Kuznetsov, 1998, Mol Cell Biol). Percival and colleagues found subsarcolemmal mitochondrial content to be 40% lower in the tibialis anterior muscle of dystrophin-deficient mice (Percival, 2013, Hum Mol Genet). This is also in line with the lower content of certain TCA cycle enzymes and lower enzymatic activities of complexes I and IV (Jongpiputvanich, 2005, J Clin Neurosci/ Ohtaki, 1990, Brain Dev). However, in dystrophin deficient melanocytes, treatments with the ATP synthase inhibitor, oligomycin, resulted in depolarization whereas this treatment causes hyperpolarization in wild-type cells (Pellegrini, 2012, J Cell Physiol). Such findings suggest mitochondrial abnormalities deeper-rooted than a simple reduction in mitochondrial volume. Indeed, at a functional level, it was also recently revealed that mitochondria from dystrophin-deficient mice were uncoupled as indicated by P/O ratios that were 30% lower and also exhibited a lower maximal rate of ATP production (Percival, 2013, Hum Mol Genet). Khairallah and colleagues observed a shift of substrate utilization in the working heart from long chain fatty acids towards carbohydrates (Khairallah, 2007, J Mol Cell Cardiol). Altogether, this indicates important modifications in mitochondrial energy metabolism.

2.3.2.2 Mitochondrial Biogenesis in DMD

A lower drive for mitochondrial biogenesis and mitochondrial protein synthesis could explain the lower oxidative capacity in DMD muscle. A down regulation of both mitochondrial mRNAs and nuclear-encoded mitochondrial genes has indeed been previously observed (Gannoun-Zaki, 1995, FEBS Lett). The overexpression of mitochondrial biogenesis inducer PGC-1α re-established a normal metabolic profile

and attenuated cell death and necrotic lesions in the mdx mouse muscle (Handschin, 2007, Genes Dev), indicating that the mitochondrial abnormalities observed over the course of this disease could be derived from a lower drive for mitochondrial biogenesis. However, it remains unclear if these changes also related to improvement in mitochondrial functions, which is the question investigated in the second study of this thesis. Similarly, resveratrol, a compound believed to stimulate PGC-1α expression, when administered to mdx mice, improved the muscle pathology (Hori, 2011, J Pharmacol Exp Ther) In fact, the lower mitochondrial biogenesis may translate into poor quality control execution. Indeed, for dysfunctional mitochondria to be discarded and replaced with freshly synthesized mitochondria, proper biogenesis signals need to be in place. Although this idea of slow turnover leading to accumulation of poorly performing mitochondria in DMD remains unscathed, our group has found early indications that it may in fact be a worthy hypothesis. Specifically, we observed that AICAR treatments, renowned for their ability to induce PGC-1α expression and thus mitochondrial turnover, lead to an improvement in mitochondrial function that was also associated with improved muscle health in mdx mice (Pauly, 2012, Am J Pathol).

2.3.2.3 Oxidative stress and DMD

Oxidative stress is a hallmark feature of DMD skeletal muscle. Haycock and colleagues have found protein carbonyls to be 211% higher in the quadriceps of humans afflicted with the disease when compared with age and sex matched controls (Haycock, 1996, Neuroreport). Similarly, elevated levels of lipid peroxides are often reported (Mechler, 1984, J Neurol Sci / Chahbouni, 2010, J Pin Res). It is becoming increasingly evident that the accumulation of oxidative stress is associated with acceleration in disease progression (Lawler, 2011, J Physiol) and has been shown to parallel an important decline in mitochondrial function (Blake, 2002, Physiol Rev). The presence of mitochondrial oxidative stress more specifically, evidenced by the decrease in aconitase enzymatic activity and the lower pools of oxidized glutathione in dystrophic mitochondria (Dudley, 2006, Am J

Physiol / Khairallah, 2007, J Mol Cell Cardiol) may accelerate the myocellular degeneration derived from the absence of dystrophin. Thus, a variety of antioxidant treatments have effectively lowered oxidative stress and also led to improved muscle health in muscular dystrophy. There are reports of 1) attenutated muscle necrosis and central nucleation, 2) lower muscle damage assessed by serum levels of creatine kinase, 3) improved sarcolemmal membrane permeability and force production as well as, 4) an improvement in the histologic appearance of the dystrophic muscle, in response to different antioxidant treatments in DMD (Buetler, 2002, Am J Clin Nutr/ Messina, 2006, Am J Pathol/ Dorchies, 2006, Am J Physiol/ Whitehead, 2008, J Physiol). Although the origin of these ROS is not necessarily mitochondrial, the uptick in production appears to stem mostly from the reciprocal amplification relation that ROS and Ca^{2+} entertain, and which is believed to be mediated by mitochondria (Jung, 2008, Cardiovasc Res / Shkryl, 2009, Pflugers Arch). Furthermore, Buyse and colleagues reported that the antioxidant idebenone, a coenzymeQ10 analog, which abound in mitochondria, was effective at delaying cardiac dysfunctions in dystrophic animals. Namely, it attenuated cardiac fibrosis, inflammation and myocardial degeneration, improved filling and contractile cardiac functions as well as exercise capacity (Buyse, 2009, Eur Heart J). Idebenone treatments also resulted in improved mitochondrial respiratory chain functions, highlighting the potential role of the organelle as an important source of ROS in dystrophic muscle and a key element in determining disease progression.

2.3.2.4 Mitochondrial permeabilization in DMD

The accumulation of excess intracellular calcium may also contribute to cellular degeneration in DMD for reasons previously argued. How this extracellular calcium penetrates the cell remains nebulous. The exaggerated membrane fragility due to dystrophin deficiency is however thought to facilitate the formation of microruptures in the sarcolemma leading to abnormal Ca^{2+} influx upon contractile activity (Wang, 2005, Nat Cell Biol). The relationship between Ca^{2+} entry and mechanical stress may also suggest the implication of stretch-activated channels in

the disturbance of normal ion permeability although other calcium-permeable channels such as the transient receptor potential channel (TRPC), L-type Ca^{2+} channels and other mechanosensitive Ca^{2+} channels are also being considered (Yeung, 2005, J Physiol / Alderton, 2000, Trends Cardiovasc Med / Guharay, 1984, J Physiol / Tutdibi, 1999, J Physiol / Franco-Obregon, 2002, J Physiol). How the calcium overload leads to oxidative stress, cell death and muscle dysfunction in not completely elucidated but Jung and colleagues have made a strong case for the role of mitochondria to be a key mediator by creating an exaggerated amplification of normal calcium waves (Jung, 2008, Cardiovasc Res). Additionally, the calcium overload probably contributes to the activation of calcium sensitive proteases in DMD muscle. In addition to inducing proteolysis of the contractile apparatus (Hopf, 2007, Subcell Biochem), calpains may also prompt a deterioration in calcium homeostasis by interfering with calcium leak channels and possibly other membrane proteins (Childers, 2011, Front Pharmacol). However, Selsby and colleagues found that the calpain inhibitor (C101) failed to prevent the progression of muscular dystrophy in mice (Selsby, 2010, Am J Physiol) and similar results were also found in dystrophic canine muscle (Childers, 2011, Front Pharmacol)

Dystrophic myocyte mitochondria, constantly working against the leaky membrane to maintain cytosolic calcium homeostasis, may develop highly sensitive calcium uptake mechanism which may translate into an enhanced ability to store calcium in comparison to their wild-type counterparts (Shkryl, 2009, Pflugers Arch). However, their high calcium content comes at the price of a heightened susceptibility to 1) bidirectional amplification of ROS and Ca2+ waves 2) dissipation of the membrane potential 3) induction of cellular death signalling pathways. This higher calcium buffering capacity may lead us to believe that mitochondria evolve more resistant to calcium overload in DMD striated muscle. However, animal studies have clearly shown an increased susceptibility to PTP opening in DMD muscle in vitro (Burelle, 2010 J Mol Cell Cardiol).

Muscle degeneration in DMD is characterized by high levels of cellular death. Even though necrosis is responsible for a large portion of the cellular death, it appears that apoptotis still makes a significant contribution to cellular death. Using TUNEL detection and DNA ladder analysis, it has been reported that apoptotic nuclei account for 0.5 to 2% of total nuclei in the mdx mice muscle (Sandri, 1999, Int J Bioc Cell Biol / Friedrich, 2004, J Physiol); ten times the amount normally found in healthy muscle. Interestingly, it was found that Bax expression increased concurrently with caspase 3 in mdx skeletal muscle during waves of necrosis and that Bcl-2 overexpression has conferred a certain level of protection against apoptosis in similar dystrophinopathies (Vachon, 1997, J Clin Invest). Although caspase 12, an indicator of reticular-stress associated apoptosis, is also higher in the mdx muscle, the larger scale of the overexpression in caspase 9, in comparison to caspase 12 overexpression, in addition to the surge in caspase 9 precedes that of caspase 3 suggest that the internal mitochondrial pathway of apoptosis probably the predominant driver of the caspase 3 activation (Honda, 2007, J Muscle Res Cell Mol).

Accordingly, PTP inhibition, whether it is in the form of genetic ablation or pharmacological inhibition of cyclophilinD (Debio 025 and Cyclosporin A), much like antioxidants, greatly improved the health of the muscle in muscular dystrophies (Reutenauer, 2008, Br J pharmacol / Wissing, 2010, Neurmuscul Disord / Millay, 2008, Nat Med).

2.3.2.5 Autophagy/mitophagy in DMD

Grumati and colleagues were the first to investigate the role of defective mitophagy in models of muscular dystrophy (Grumati, 2010, Nature Med). They tested the hypothesis that the accumulation of functionally and structurally damaged mitochondria and apoptosis in collagen VI muscular dystrophy ($Col6a^{-/-}$) is due to defective autophagic clearance. At baseline, the lower levels of of LC3-II and higher accumulation of p62 are suggestive of lower autophagic flux, which was

confirmed using chloroquine-based experiments. By inhibiting lysosomal digestion in both control and Col6a$^{-/-}$ mice, they found the dystrophic muscle to accumulate much less LC3-II, which leads to believe that autophagy induction is impaired in this model. This was confirmed using a short-term fasting model in which the authors unveiled important deficiencies in the activation of autophagy. Namely, they found P62 to rapidly decrease upon fasting in wild-type mice but this decrease was much delayed in the Col6a$^{-/-}$ mice. They also found that fasting resulted in mRNA increases in Bnip3 and Beclin1 mRNA, and to cause de-phosphorylation of Akt and 4e-BP1 as well as LC3 lipidation in healthy muscle. However, this response, associated with autophagy induction in the face of short term fasting, was greatly impaired in Col6a$^{-/-}$ mice. Nonetheless, a longer-term starvation model consisting of four weeks of a low protein diet effectively induced the normal autophagic response in Col6a$^{-/-}$ mice as indicated by the traditional markers: the de-phosphorylation of Akt, the increase in beclin and Bnip mRNA, the disappearance of P62 and the lipidation of LC3. Parallel to the proper autophagy induction, the authors also observed an improvement of the dystrophic phenotype and in force production as well as an important attenuation of the mitochondrial defects that were present in baseline dystrophic muscle in response to four weeks of a low protein diet (Grumati, 2010, Nature Med). This strongly shows the role of defective autophagy in establishing the dystrophic phenotype of the two main dystrophies characterized by collagen 6 deficiencies, namely Bethlem myopathy and Ulrich congenital muscular dystrophy.

Similar results were found in DMD muscle shortly thereafter (De Palma, 2012, Cell death dis). The basal phenotype with higher levels of P62, lower levels of LC3-II, higher levels of phosphorylation in the Akt, mTOR, 4e-BP1, S6 axis is indicative of impaired autophagy. Incidentally, lower mRNA levels of downstream target genes such as ATG12, Gabarap and Bnip were also found. Similarly to Col6a$^{-/-}$ mice, DMD mice also displayed a more arduous activation of autophagy in response to short term starvation. Furthermore, the longer-term low protein diet also resulted in an improvement of the dystrophic phenotype. Specifically, they found the treatment to improve the histological appearance of the muscle by: 1) homogenizing myofiber

cross-sectionnal area which otherwise have a bi-modal distribution representative of the coexistence of atrophied and hypertrophied fibers, 2) reducing central nucleation and, 3) reducing fibrosis and collagen accumulation. De Palma et al. also report improved force production and improved membrane permeability as evidenced by the lower uptake of evans blue dye and lower apoptosis levels as indicated by TUNEL staining (De Palma, 2012, Cell death dis).

In addition to reports of impaired autophagic clearance in other forms of dystrophy (Choi, 2012, Autophagy), our group has also found evidence that increasing autophagy levels in DMD can improve the dystrophic phenotype. Indeed, we found that treatment of mdx mice for four weeks with the AMPK agonist, AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside), strongly triggered autophagy in the mdx diaphragm. The induction of AMPK, resulted in an improvement of 1) mitochondrial function, 2) force production and 3) histopathology (Pauly, 2012, Am J Pathol).

Overall, these studies show that the forced activation of autophagy yields a beneficial therapeutic response in muscular dystrophies and support the importance of a balanced autophagic flux in maintaining healthy muscle function.

2.3.3 Sepsis

Sepsis is a systemic inflammatory response to infection leading to multiple organ disorder syndrome (MODS), which affects various tissues, including the liver, kidneys, as well as skeletal and, cardiac muscles. It is the leading cause of death in the ICU where it is reported the mortality rate reaches 40-60% of affected patients (Galley, 2011, Brit J Anasth). The acute myocardial depression in response to endotoxemia is characterized by severe contractile dysfunction and cardiomyocyte injury as evidenced by the depressed cardiac output and the elevated serum levels of cardiac troponin (Balija, 2011, Curr Opin Infect Dis). Septic cardiomyopathy has been convincingly linked to profound metabolic disturbances and mitochondrial

abnormalities. In fact, the severity and outcome of the septic shock are strongly determined by the changes in both mitochondrial and cardiac functions (Brealey, Lancet, 2002/ Carré, 2010, Am J Respi Crit Care Med)

2.3.3.1 Myocardial depression

A defining feature of cardiac abnormalities during septicemia, unlike other cardiomyopathies, is the U-shaped hemodynamic response marked, as illustrated in figure 9, by two distinct phases 1) a rapid contractile inhibition combined with mitochondrial dysfunction peaking between 18-36 hours, followed by 2) a swift recovery of cardiac functions suggested to be underlined by induction of mitochondrial quality control mechanisms, including mitochondrial biogenesis. Deviation from this normal biphasic response has been associated with poor survival (Carré, 2010, Am J Respi Crit Care Med / Parker, 1984, Ann Intern Med). The temporary cardiac depression surprisingly appears to be just as important as the recovery in surviving septicemia. Parker et al. have shown that human septic shock survivors show a depressed ejection fraction marked by a dilated left ventricle in the first hours of shock whereas those who maintain normal left ventricular function tend to show poorer survival (Parker, 1984, Ann Intern Med). This was later confirmed by similar results observed in a murine model of sepsis induced by cecal ligation and puncture (CLP) (Zanotti, Chest, 2010). Some argue that the rapid decreases in mitochondrial respiratory functions and ventricular contractility may be an evolutionarily preserved adaptive response to face extreme environmental stresses. The down regulation of metabolic activity would act to preserve energy stores, which would in turn promote survival (Levy, 2005, Crit Care Med). This also impacts the cardiac metabolism, which, simultaneously to mitochondrial shutdown switches from oxidative towards more glycolytic substrates; a condition highlighted by hyperlactatemia, a decrease in coronary fatty acid oxidation and a net coronary lactate extraction (Cunnion, 1986, Circulation / Schilling, 2011, Circ Heart Fail). Although this eventually results in lower oxygen

consumption and lower ATP turnover it is somewhat paradoxical to also find even larger declines in oxygen utilization efficiency (work/ VO_2) (Khadour, 2002, Am J Physiol/ Watts, 2004, J Mol Cell Cardiol). Nonetheless, it can be suggested that myocardiocellular hibernation may be a normal response to mitochondrial OXPHOS dysfunction and the resulting cytopathic hypoxia.

Figure 8: Cardiac metabolism during sepsis

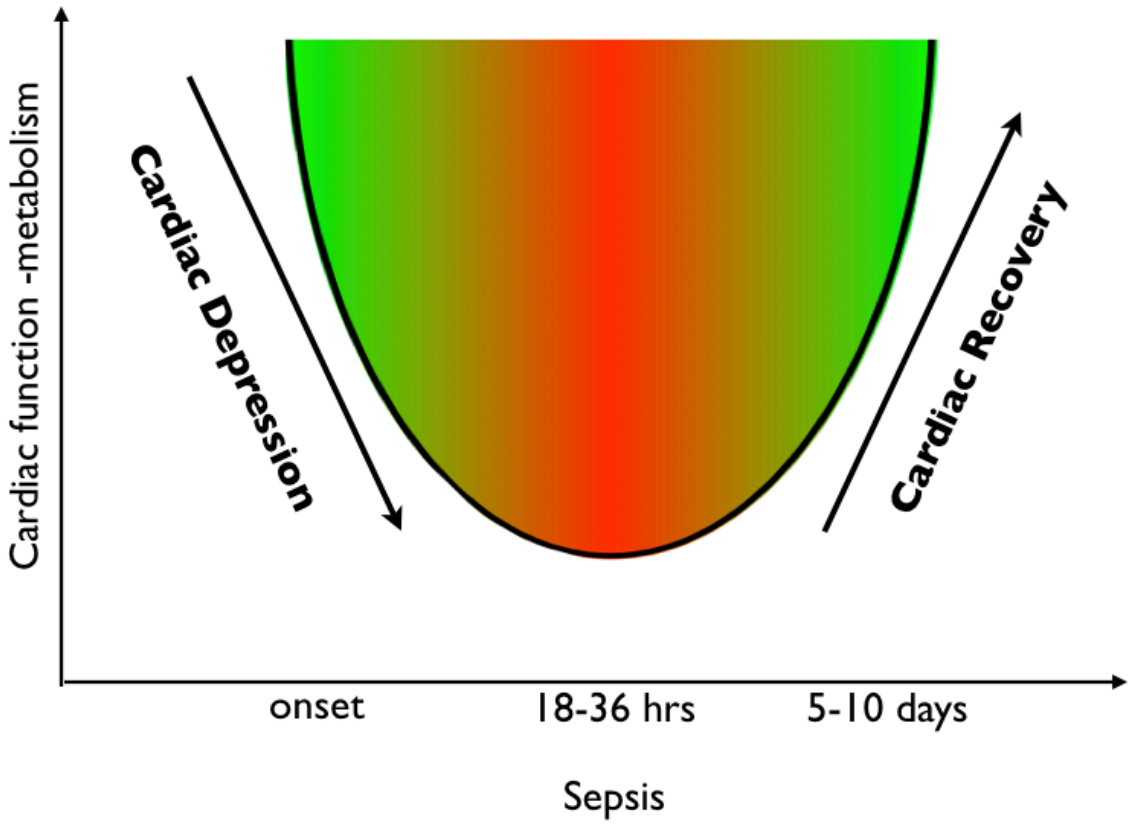


Figure 8: Cardiac metabolism during sepsis
Cardiac function declines rapidly in response to endotoxemia. The critical low point beyond which survival and non-survival are often discriminated is reached within 48 hours followed by a slow recovery which may take several days.

The strong immune response to sepsis probably underlies a large portion of the mitochondrial oxphos dysfunctions and the resulting cytopathic hypoxia. Although, inflammation does result in endothelial and circulatory dysfunction characterized by microembolisms, inflammatory cell infiltration and edema which may ultimately disrupt tissue perfusion and oxygen delivery, it appears that myocardial blood flow and cellular oxygenation remain adequate during myocardial depression (Hotchkiss, 1991, Am J Physiol). Nonetheless, it is the cellular response to increasing levels of circulating cytokines that probably mediate most of these changes resulting in hypometabolic activity. The activation of toll-like receptors (TLR), more particularly TLR-4 profoundly disturbs metabolism and is probably the mediator of both cellular damage and lower contractility in the heart in response to endotoxemia (Tavener, 2004, Circ Res / Fallach, 2010, J Mol Cell Cardiol). In particular it leads to nuclear translocation of NF-kB, which can stimulate ROS production, in addition to repressing PGC1-α (Schilling, 2011, Circ Heart Fail). TLR-4 can also induce transcription of cytokines such as TNF and IL-6 as well as increasing iNOS transcritption (Suliman, 2005, FASEB). The key role for TLR-4 was put in evidence in various studies where hindered TLR4 signaling resulted in an important attenuation of the septic phenotype (Mullarkey, 2003, J Pharmacol Exp Ther). Recently, a pharmacological intervention with the TLR-4 antagonist, eritoran tetrasodium, preserved myocardial function in mice exposed to low doses of LPS (Ehrentraut, 2011, shock).

2.2.3.2 Oxidative phosphorylation dysfunction in septic cardiomyopathy

Evidence for mitochondrial oxidative phosphorylation dysfunctions in the septic heart are overwhelming. The ~20% reduction (Watts, J Mol Cell Cardiol, 2004) in mitochondrial volume explains a portion of the diminished OXPHOS capacity but certainly cannot account for the 30-40% reduction in state 3 respiration (Supinsky, 2006, Free rad biol med / Trumbeckaite, 2001, Eur J Biochem) nor the near 50%

reduction in mechanical efficiency reported during sepsis in the myocardium (Khadour, 2002, Am J physiol heart circ). There is however a severe inhibition in enzymatic activity of ETC complexes that is paralleled by depletion of various ETC proteins. Callahan et al. observed a 50 and 67% reduction in the combined activity of complexes I and III in the myocardium of septic rabbits and baboons respectively (Callahan, 2005, Am j respir crit care med) and an approximately 40% decrease in COX activity 48h post CLP in mice (Piel, 2008, shock). This was also corroborated by findings in other muscle tissues where a heavy reduction in enzymatic activity is also regularly observed (Peruchi, 2011, J Surg Res/ Fredriksson, 2007, Crit Care Med). Although others have found more modest reductions in the enzymatic activity of these complexes (Trumbeckaite, 2001, Eur J Biochem), these findings concord with those of the team of Piandatosi who observed a substantial reduction in numerous mitochondrial ETC proteins in septic hearts in one study (Suliman, 2004, Circ Res) and an equally important depletion of mtDNA in another (Reynolds, 2009, Free Rad Biol Med / Bartz, 2011, Am J Respir Crit Care Med). This resulted into reduced ETC function and provides a mechanistic explanation for the 50% decline in the ATP generating capacity reported by Supinsky et al. (Supinsky, 2006, Free Rad Biol Med). The repeatedly observed decrease of almost 50% in the RCR ratio (Gellerich, 2002, Biosci Rep / Supinsky, 2009, Am J Physiol Regul Integr Comp) further demonstrates the existence of mitochondria OXPHOS defects and is also in line with the previously mentioned decrease in oxygen utilization efficiency in the myocardium (Watts, 2004, J Mol Cell Cardiol). Although the models, the species, the timing, the dosage and the methods of investigation vary a great deal, oxidative deficits appear to be systematic during sepsis. Although certain researchers have used an unchanged level of intracellular ATP to argue in favor of the absence of mitochondrial defects in the face of sepsis, it has to be considered that cardiac contractile and metabolic activity are heavily reduced during the hibernation that marks endotoxemia. Thus, unchanged ATP levels are not a valid measure of mitochondrial function in sepsis.

2.2.3.3 NOS hyper reactivity

One interesting hypothesis on the origin of mitochondrial deficit would be the increased activity of nitric oxide synthase (NOS), which can prompt the formation of the oxidatively damaging peroxynitrite in addition to reducing oxygen bioavailability. The inducible form of NOS (i-NOS or NOS2), is especially relevant in sepsis for it is normally associated with elevated inflammatory activity. NOS2 gene expression was shown to increase more than 5-fold in murine cardiac tissue 24 hour after a sub-lethal bacterial challenge (Reynolds, 2009, Free Rad Biol Med). This is in agreement with the well- accepted increased production of nitrite/nitrate during sepsis (Supinsky, 2006, Free Rad Biol Med). Interestingly, Vanasco and colleagues also found mitochondrial NOS (mtNOS) biochemical activity to rise nearly 40% in the heart of rats 24-48h after a 10mg/kg i.p. e.coli LPS injection whereas Xu et al. found mtNOS optical density on immunoblots of isolated mitochondria to double in response to CLP (Vanasco, 2012, Bioenerg Biomemb / Xu, 2012, Shock). As additional evidence of NOS hyperactivity underlying mitochondrial dysfunctions, Brealey and colleagues found a strong inverse relationship between tissue nitrite/nitrate and complex I activity (r= -0.75) in the skeletal muscle of septic patients in the ICU (Brealey, 2002, Lancet). Interestingly, NO is also known to be a strong cardiac depressant and reinforce the idea that NOS activity to be the missing link may between myocardial depression and the mitochondrial shutdown during the hibernation response (Stein, 1996, J Mol Cell Cardiol).

However, others are pointing to factors outside the mitochondria as the cornerstone of the metabolic depression. The lower activity of creatine kinase (CK), more precisely in the mitochondria may contribute to lower ATP generation as it would trap ATP inside the mitochondria resulting in a production inhibition. As evidence Callahan et al. found mitochondrial respiration when stimulated with ATP+Creatine in septic rat hearts to be barely 25% that of the control meanwhile mtCK activity plunged by nearly 85% in septic cardiac mitochondria isolates (Callahan, 2006, J Appl Physiol).

It is also believed that post-transcriptional modification to ETC proteins in combination with low mitochondrial turnover could contribute to mitochondrial OXPHOS dysfunction. Indeed, the elevated levels of oxidized/nytrosilated proteins in septic mitochondria could very well impair oxidative phosphorylation. The slower turnover of proteins in septic myocardium as indicated by lower mRNA turnover as well as the low levels of PGC1-α would prevent replacement of these disrupted proteins and lead to their accumulation rapidly throwing the cell into a vicious cycle of deteriorating mitochondrial function. In light of this, it is not surprising to find that PGC1-α overexpression attenuates myocardial depression (Schilling, 2011, Circ Heart Fail). This indicates that leading the lower mitochondrial biogenesis and impaired quality control mechanisms could exacerbate the mitochondrial dysfunction in sepsis by allowing the disrupted proteins to accumulate. This idea, however, remains largely unexplored.

2.2.3.4 Oxidative stress in septic cardiomyopathy

Oxidative stress is rampant in myocardial tissue during endotoxemia. Oxyblots revealed a fourfold increase in oxidized proteins barely 24 hours post streptococcus pneumonia intratracheal injection (Zang, 2012, Am J Physiol) in rodents. Celes et al. revealed an approximately tenfold increase in 4-HNE protein adducts in the hearts of animals subjected to CLP and an equally severe increase in nitrotyrosine immunoreactivity on histological specimens (Celes, 2010, Shock). Not surprisingly, the oxidative stress is also observable on mitochondria. The team of Dr Piantadosi at Duke University, a leading group on the topic of septic cardiomyopathy, observed a sixfold increase in mitochondrial MDA and a twofold increase in mitochondria protein carbonyls accompanied by a 50% depletion of mitochondrial glutathione 24 hours after e. coli LPS intraperitoneal injection (Suliman, 2004, Cardiovasc Res), a finding consistently replicated (Brealey, 2002, Lancet / Zang, 2012, Am J Physiol). This is in agreement with the results of Supinsky

and colleagues who reported protein carbonyls in mitochondria to nearly triple and lipid peroxides to more than double in septic cardiac mitochondria (Supinsky, 2006, Free Rad Biol Med) and who later replicated similar findings (Supinsky, 2009, Am J Physiol Reg Integr Comp).

Mitochondrial ROS production has been shown to be robustly elevated in the liver of septic rats (Taylor, 1995, Arch Biochem Biophys). In the heart, elevated mitochondrial ROS production has also been observed albeit a more modest yet, significant increase was reported (Zang, Am J Physiol, 2012). The multifactorial ROS production taking place in the cytosol appears to be a more important contributor to oxidative stress but probably compounds with mitochondrial overproduction to explain the markedly lower overall antioxidant capacity (Zang, Am J Physiol, 2012) and the abundant oxidative stress (Sebai, 2011, Drug Chem Toxicol). An important part of this rise in cytosolic ROS production is derived from the inflammatory response to sepsis. Thus, excessive mitochondrial and overall ROS production probably contribute to tissue injury in the septic myocardium.

Mitochondrial DNA is particularly susceptible to stress during endotoxemia, more so than nuclear DNA (Suliman, 2004, Cardiovasc Res). Sepsis has been shown to cause a rapid depletion of mitochondrial DNA in the heart and other organs characterized by important levels of mutations (Lancel, 2009, J Pharmacol Exp Ther / Bartz, 2011, Am J Respir Crit Care Med/ Suliman, 2003, Am J Respir Crit Care Med /Suliman, 2004, Cardiovasc Res/ Choumar, 2011, Antioxid Redox Signal). An increased 8-oxoguanine DNA glycosylase (OGG1) expression, an important base excision repair mechanism to remove the oxidatively damaged DNA (8-OHdG) has been observed in the liver of animals exposed to a microbial infection (Bartz, 2011, Am J Respir Crit Care Med). We could easily speculate that it is also the case in the heart because the factors responsible for driving mitochondrial biogenesis and these repair mechanism (NRF & Co) have also been found to be elevated in the septic heart (Bartz, 2011, Am J Respir Crit Care Med). The accumulation of OGG1 in

mitochondria simultaneously to mtDNA depletion indicates important oxidative damage and could also explain the rapid deterioration in oxidative phosphorylation.

Accordingly, several antioxidant therapies have shown to moderately improve sepsis survival (Berger, 2007, Crit Care Med/ Ritter, 2004, Crit Care Med/ Supinsky, 2006, Free Rad Biol Med/ Zapelini, 2008, Mitochondrion). However, mitochondria-targeted antioxidants have proved to be more effective in preventing oxidative stress, deterioration of mitochondrial and myocardial function and, fatality (Supinsky, 2009, Am J Physiol Regul Integr Comp).

2.2.3.5 Mitochondrial permeabilization in septic cardiomyopathy

Larche and colleagues convincingly demonstrated that increased susceptibility to permeability transition also contributes to the mitochondrial dysfunctions that underlie myocardial depression during shock. They show that CLP decreases resistance to a calcium challenge by more than 67% in mitochondria isolated from mice heart 18-24hours after the onset of endotoxemia. This sensitivity to mitochondrial permeabilization was heavily attenuated when interventions preventing PTP opening were implemented. Low doses of cyclosporine A or NIM811, as well as genetic overexpression of anti-apoptotic Bcl-2 appear particularly effective in preventing, mitochondrial permeabilization. These interventions also attenuated caspase 9 and caspase 3 to a lesser extent (Watts, 2004, J Mol Cell Cardiol). This is in agreement with the concept that caspase 3 can be activated via mitochondrial permeabilization through caspase 9 (internal pathway) but can also be activated through external signalling cytokines and FAS ligand, both known to be elevated during septicemia (Papathanassoglou, 2000, Shock). In agreement with this Lancel and colleagues found caspase 8, thought to be representative of this external pathway, to be elevated in the myocardium of septic animals (Lancel, 2005, Circ). Nonetheless, mitochondrial permeabilization probably contributes to caspase 3 activation because Lancel et al. also found higher levels of

cytochrome c in the cytosolic compartments in addition to an elevated Bax/Bcl-2 ratio in the myocardium of septic rodents. Caspase directly contribute to myocardial dysfunction during endotoxemia as their blockade with caspase inhibitors prevented a lot of the contractile dysfunction normally observable (Fauvel, 2001, Am J Physiol / Neviere, 2001, Am J Respir Crit Care Med). However, the role of apoptosis in mediating myocardial dysfunction is mitigated at best (Ruetten, 2001, J Am Coll Cardiol / Neviere, 2001, Am J Respir Crit Care Med). The role of caspase in eliciting impaired contractility during endotoxemia would actually pertain to its proteolytic properties by cleaving important contractile proteins such as troponin T, rather than its pro-apoptotic abilities (Supinksy, 2006, J Appl Physiol / Lancel, 2005, Circ). Nonetheless, in addition to preventing a large portion of the myocardial depression through caspase inactivation, PTP inhibitors provided an unparalleled protection against fatality, improving survival from about 25% in control CLP mice 96 hours after surgery up to 100% in most cases (CsA: 100%, NIM811: 100%, Bcl-2 overexpress: 80%) (Larche, 2006, J Am Coll Cardiol).

An intriguing report from Piel et al. showed that cytochrome c restores cox activity in the heart of septic animals (Piel, 2008, Shock). However, instead of seeing a potential curative effect here, it would be tempting to speculate that this effect is rather a representation of mitochondrial membrane damage. Normally cytochrome oxidase is sequestered away in the inner mitochondrial membrane. The ability of cytochrome c to reach COX could thus be indicative of membrane damage. The findings of Zang and colleagues showing mitochondrial membrane damage in the heart of septic rats (Zang, 2012, Am J Physiol,) corroborate this hypothetical interpretation of the results by Piel. This idea would also be in agreement with the damage observed on mitochondria by electron microscopy following endotoxemia.

Seeing how most of these mitochondrial abnormalities may be downstream of an extreme inflammatory response it is reasonable to question whether mitochondrial defects are involved in tissue injury or whether it is simply an epiphenomenon. However, mitochondria may very well exacerbate the

inflammatory response responsible for their own damage. Indeed, a time-course study indicated that mitochondrial damage may precede the inflammatory response during a bacterial challenge and may be a modulator of inflammation (Zang, 2007, Surg Infect). If mitochondrial DNA damage propagates and permeability transition calcium waves and ROS amplification cannot be contained by the quality control mechanisms, the cell, in an attempt to protect viability, could purge the mitochondrial content into the bloodstream, possibly through mitochondrial-derived vesicular carriers (MDVs) (Soubannier, 2012, Plos one). However, the presence of mtDNA in the bloodstream could also be simply derived from cell lysis. The presence of DNA and mtDNA in the bloodstream of critically ill has been known for some time (Swarup, 2007, Febs Lett). However, it is only recently that researchers have begun to appreciate the potential inflammatory implications. Once released into the plasma, these strands of mitochondrial DNA, can induce the release of proinflammatory cytokines and immunomodulators from immune cells as Damage Associated Molecular Patterns (DAMPs) (Mathew, 2012, J Alz Dis). Possibly, it is the unmethylated CpG motifs reminiscent of their bacterial ancestry they bear that invoke a strong immune response leading the activation of the inflammasome and TLR signaling (Zhang, 2010, Nature). This process has been linked to cardiac failure (Oka, 2012, Nature). Potentially, the recovery of mitochondrial function and ultimately survival are dependent on a proper containment of mitochondrial damage by proper quality control mechanisms or otherwise the exacerbation of inflammatory activity caused by the release of mtDNA will aggravate the septic cardiomyopathy. This idea would also be in line with the findings of Brealey and colleagues who observed a correlation between deteriorating mitochondrial function and fatality (Brealey, 2002, Lancet) but remains relatively unexplored.

2.2.3.6 Mitophagy in sepsis-induced cardiac dysfunction

Mitophagy appears to also play a protective role in the heart in the face of various stresses. It is well established for example that ischemia-reperfusion is a

potent trigger of mitophagy (Kubli, 2012, Circ Res). Mitochondria-containing vacuoles have been formally identified in the reperfused heart by different research groups (Hamacher-Brady, 2006, J Biol Chem / Decker, 1980, Am J. Pathol). It has been shown that successive bouts of I-R leads to improved resistance to such events. This physiological response, also known as ischemic preconditioning, is dependent on an amelioration of mitochondrial resistance to stress. This has been suggested to happen as a result of a targeted sequestration and elimination of the pools of dysfunctional organelle via mitophagy (Huang, 2011, Plos one). This adaptive response is completely abrogated in autophagy-deficient animal muscle, thus reinforcing this hypothesis (Huang, 2011, Plos One). Hsieh and colleagues actually postulate that the prevention of activation of internal apoptotic pathways is the central component of the beneficial effects of mitophagy on cell viability (Hsieh, 2009, Trends Mol Med).

During sepsis, mitophagy also appears upregulated in an attempt to promote cell survival. This is corroborated by the presence of vacuolized mitochondria, the accumulation of LC3-II and the formation of LC3 punctae in the left ventricle of mice who underwent CLP (Hsieh, 2011, Ann Surgery). However, the upregulation of mitophagy seen during sepsis is not sufficient to prevent mitochondrial and cardiac dysfunction in wild-type animals. Indeed, there are indications of different bottleneck points that are susceptible to impede with mitophagy during sepsis. In one of the rare studies of autophagy in the septic heart, the team of Hsieh noted that rapamycin relieved this proteolytic pathway of its bottlenecks, which restored mitochondrial and left ventricular contractile performance assessed by echocardiography. The same study shows that the fusion of autophagosome to lysosome may be one of those points particularly prone to saturation, although other stages of autophagy such as the phosphorylating cascades may contribute to limiting the rates of mitophagy. Precisely Hsieh et al. observed that the colocalization ratio of LC3 with LAMP1, an important lysosomal marker, was much lower in CLP mice than in sham-operated mice. Recent unpublished data from our laboratory actually point to a critical role of certain cytoskeletal proteins that could

serve as rails for delivery of the mitophagosome cargo to the lysosome for digestion. This may underlie the impaired autophagolysosome formation rate during sepsis but remains to be determined. Nonetheless, it is not yet clear how rapamycin restores LC3/LAMP1 ratio as part of its therapeutic effects in septic cardimoyopathy.

It is not unreasonable to speculate that the appearance of apoptosis in such cardiomyopathies is a spillover effect due to the overwhelming of autophagic systems. The accumulation of autophagosome is sometimes linked to apoptosis activation, which is known to be a key pathogenic trigger in various forms of cardiomyopathies (Terman, 2005, Cardiovasc Res / Martinet, 2007, Trends mol Med). In the septic heart, caspase inhibitors have resulted in significant attenuation of apoptotic activation and an improvement of the cardiomyopathy presumably by abrogating the undesirable effects of autophagic saturation. However, it is not impossible that boosting mitophagic clearance would have a similar, if not better, therapeutic effects. The positive correlation between apoptosis and poor outcome during septic cardiomyopathies lead us to believe that the more autophagic systems are overwhelmed, the more mitochondrial and cardiac functions deteriorate.

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3 Experimental Studies

3.1 Study No 1: Cyclophilin-D is dispensable for atrophy and mitochondrial apoptotic signaling in denervated muscle.

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Running head Denervation in cyclophilin-D deficient mice

Keywords: mitochondria, permeability transition pore, skeletal muscle, denervation, cyclophilin-D

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3.1.1 Non-technical summary

Activation of apoptotic cell death signalling by mitochondria has been involved in mediating skeletal muscle atrophy following denervation. However, the underlying molecular mechanisms, in particular the implication of the permeability transition pore (PTP) remains unclear. Using *Ppif*$^{-/-}$ mice, which are devoid of cyclophilin-D (CypD-KO) and thus more resistant to PTP opening, we directly tested the hypothesis that this pore is involved in atrophy and activation of apoptotic proteolytic signalling following denervation. Our results demonstrate that CypD-dependent opening of the PTP is dispensable for atrophy and activation of apoptotic proteolytic signalling induced by denervation.

3.1.2 ABSTRACT

In the present study, we specifically determined whether the regulating protein cyclophilin-D (CypD), and by extension opening of the permeability transition pore (PTP), is involved in the activation of mitochondria-derived apoptotic signaling previously described in skeletal muscle following loss of innervation. For this purpose, CypD-defficient mice (CypD-KO) and their littermate controls were submitted to unilateral sciatic nerve transection. Mitochondrial resistance to Ca^{2+}-induced opening of the PTP, and muscle apoptotic signaling were investigated 14 days post-surgery. Denervation caused atrophy, facilitated Ca^{2+}-induced opening of the PTP *in vitro* in permeabilized muscle fibres, and activation of apoptotic proteolytic cascade in the whole muscle of both mouse strains. In CypD-KO mice, mitochondrial resistance to Ca^{2+}-induced PTP opening was greater than in WT mice, both in the normal and denervated state, indicating that lack of CypD desensitized PTP opening. However, denervation in CypD-KO mice still resulted in a facilitation of PTP opening compared to normally innervated contralateral muscle, indicating that *in vitro,* additional factors could poise mitochondria from denervated muscle toward PTP opening. At the whole muscle level, lack of CypD, despite conferring greater resistance to PTP opening, did not protect against atrophy, nor

against the release of mitochondrial pro-apoptotic factors and the ensuing activation of caspases induced by denervation. All together, these results provide direct evidence that Cyp-D-dependent PTP opening is dispensable for atrophy and apoptotic signalling in skeletal muscle following denervation.

3.1.3 INTRODUCTION

Activation of apoptotic signalling by mitochondria is believed to be involved in muscle remodeling in several progressive neuromuscular diseases that are associated with atrophy, including denervation disorders, muscular dystrophy, mitochondrial myopathies, inactivity, and ageing-induced sarcopenia (Marzetti *et al.*; Siu & Alway, 2005; Aure *et al.*, 2006; Angelin *et al.*, 2007). A key event in this process is the induction of permeation pathways across the inner and/or outer mitochondrial membranes (IMM and OMM), which can occur independently or in a coordinated fashion. Several studies performed in denervation models have led to the suggestion that permeabilization of the OMM by pro-apoptotic members of the Bcl-2 protein family plays a role in this process by allowing the release of pro-apoptotic factors and subsequent activation of caspases 9 and 3 (Siu & Alway, 2005, 2006; Adhihetty *et al.*, 2007) Direct support for this hypothesis was in fact provided by experiments, which showed that genetic ablation of the pro-apoptotic Bcl-2 protein Bax attenuated denervation-induced apoptosis and atrophy (Siu & Alway, 2006).

In addition to increased susceptibility to opening of the IMM permeability transition pore (PTP), another important event in the mitochondrial cell death cascade, increased intracellular Ca^{2+} as well as mitochondrial oxidative stress, which are both the primary factors promoting PTP opening, were reported in muscle following denervation (Csukly *et al.*, 2006; Adhihetty *et al.*, 2007). We have also shown (Csukly *et al.*, 2006) that in isolated mitochondria from denervated muscle, the expression of cyclophilin-D (CypD), a mitochondrial chaperone-like protein acting as an important endogenous sensitizer of the PTP under conditions of Ca^{2+} overload and oxidative stress (Basso *et al.*, 2005), is increased. In this regard,

several studies have shown that inhibition of CypD provides efficient protection against cell death in several cell types (Crompton, 1999; Baines *et al.*, 2005). Moreover, inhibition of CypD was recently shown to ameliorate muscle pathology in several muscular dystrophies associated with Ca^{2+} dysregulation and oxidative stress, including Duchenne muscular dystrophy (Millay *et al.*, 2008), Bethlem muscular dystrophy and Ulrich muscular dystrophy (Angelin *et al.*, 2007; Palma *et al.*, 2009). However, the role of CypD, and more generally of the PTP, in denervation-induced muscle remodeling has not been established.

In the present study, *Ppif*$^{-/-}$ mice, which are devoid of CypD, were therefore used to clarify the contribution of this protein, and by extension PTP opening, to atrophy and apoptotic signaling in denervated muscle. More specifically, we hypothesized that ablation of CypD, by increasing resistance to PTP opening, would attenuate denervation-induced atrophy and activation of apoptotic signaling events located immediately downstream of mitochondrial membrane permeation.

3.1.4 METHODS

3.1.4.1 Ethics approval and animal care:

All procedures were approved by the animal ethics committee of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care. The authors have also complied with the policies and regulations outlined by *The Journal of Physiology* (REF Drummond 2009). *Ppif*$^{+/-}$ mice (provided by Dr. M Forte, University of Oregon) were bred to generate *Ppif*$^{-/-}$ (CypD-KO) and *Ppif*$^{+/+}$ (WT) mice. At 8 weeks old, animals were submitted to unilateral transection of the sciatic nerve as per (Csukly *et al.*, 2006), while sham surgery was performed on the contralateral leg. Fourteen days after the surgery, *gastrocnemius* muscles were extracted, weighed, and used for the assays described below.

3.1.4.2 Mitochondrial functional assays in permeabilized muscle fibres:

Dissection and permeabilization of fiber bundles with saponin were performed using the white part of gastrocnemius as described previously (Picard *et al.*, 2008b). Ghost fibres were prepared as per (Picard *et al.*, 2008b) by incubating saponin permeabilized bundles in a high KCl medium, which allows to extract myosin. Permeabilized myofibres and ghost fiber bundles were then kept on ice until use. All mitochondrial parameters described below were determined at least in duplicate.

3.1.4.3 Calcium retention capacity:

Ghost fibres were incubated at 23°C in q quartz microcuvette with continuous stirring in 600 µL of CRC buffer (in mM: 250 sucrose, 10 MOPS, 0.005 EGTA, 10 P_i-TRIS, pH 7.3) supplemented with glutamate-malate (5:2.5 mM) and 0.5 nM oligomycin. After addition of fibre bundles and respiratory substrates, mitochondria were exposed to a single pulse of 20 nM Ca^{2+}. Changes in extra-mitochondrial calcium concentration were monitored fluorimetrically using Calcium-green 5N.

PTP susceptibility was assessed by measuring time required for PTP opening and calcium retention capacity (CRC). CRC was calculated as the change in extramitochondrial Ca^{2+} between the pulse and PTP opening, which was identified as the reversal of the mitochondrial Ca^{2+} influx. CRC values were expressed in nmoles of Ca^{2+} per milligram of dry weight. A standard curve relating $[Ca^{2+}]$ to the fluorescence of Ca-green was performed to calculate the $[Ca^{2+}]$ in the microcuvette.

3.1.4.4 Caspase activity assay:

Caspase activity in whole muscle homogenates was measured fluorimetrically on a plate reader using specific substrates for caspase 3 (Ac-DEVD-AFC) and caspase 9 (Ac-LEHD-AFC). Activity was expressed as Dfluorescence/sec/mg protein and normalized as percentage of WT control.

3.1.4.5 Immunoblotting :

Proteins in whole lysates and cytosolic fractions were resolved on 12.5% polyacrylamide mini-gels, and transferred onto PVDF membranes as per (Siu & Alway, 2005). Membranes were then exposed to the following anti-bodies: Bax (Santa Cruz sc-526), Bcl-2 (Santa Cruz sc-492), Smac/Diablo (Abcam ab-9709), cytochrome c (BD Biosciences 556433), and LDH (Santa Cruz sc-33781), which was used as a loading control.

3.1.4.6 Statistical analyses:

Results are expressed as means ± SEM. Statistical differences were analyzed using two-way ANOVA (Statistica package). Tukey post hoc tests were used to identify the location of significant differences when the ANOVA yielded a significant F-ratio. P values inferior to 0.05 were considered statistically significant.

3.1.5 RESULTS

3.1.5.1 Morphometric data:

In WT mice, 14 days of denervation caused an atrophy of the *gastrocnemius* muscle as evidenced by a 54 % reduction in absolute muscle mass (112.6±2.7 *vs.* 61.1±1.3 mg, control *vs.* denervated respectively, $p<0.01$). However, loss of muscle mass was of similar magnitude in CypD-KO mice (53 %: 110.7±3.6 *vs.* 58.6±2.1 mg, control *vs.* denervated respectively, $p<0.01$).

3.1.5.2 Mitochondrial sensitivity to PTP opening in permeabilized muscle fibres:

In WT mice, denervation led to an expected reduction of time to PTP opening and CRC in response to the *in vitro* Ca^{2+} challenge (Figure 10), indicating an increased sensitivity to permeability transition compared to mitochondria from normally innervated muscle fibres. In mitochondria from CypD-KO mice, time to PTP opening and CRC and were 60 to 95 % greater than in mitochondria from WT mice, both in the normal and denervated state, indicating that lack of CypD generally desensitized the mitochondria to PTP opening. However, despite this relative desensitization, denervation in CypD-KO mice still resulted in a reduction of time to PTP opening (-33% in CypD-KO *vs.* -31% in control) and CRC (-55% in CypD-KO *vs.* -56% in control) compared to the normally innervated contralateral muscle fibres.

3.1.5.3 Cell death signaling in whole muscle:

At the level of the whole muscle, the activities of caspase 9 and caspase 3, two downstream consequences of mitochondrial apoptotic signaling, were consistently increased following denervation in WT mice (Figure 11.A). This was associated with an increased abundance in the cytosolic fractions of some (Smac/Diablo), but not all (cytochrome c) mitochondrial pro-apoptotic factors involved in the activation of caspases (figure 11.B), which is consistent with previously published data (Siu & Alway, 2005, 2006; Adhihetty *et al.*, 2007). However, the magnitude of changes in caspase activities, and abundance of mitochondrial pro-apoptotic factors observed

following denervation was similar in WT and CypD-KO mice, despite the fact that sensitivity to PTP opening was lower in the latter. Denervation also induced a significant increase in Bax content and a downregulation of Bcl-2 expression, which was unaffected by lack of CypD (Figure 11C).

Figure 9: Effect of denervation in Ca^{2+}-induced PTP opening *in vitro*:

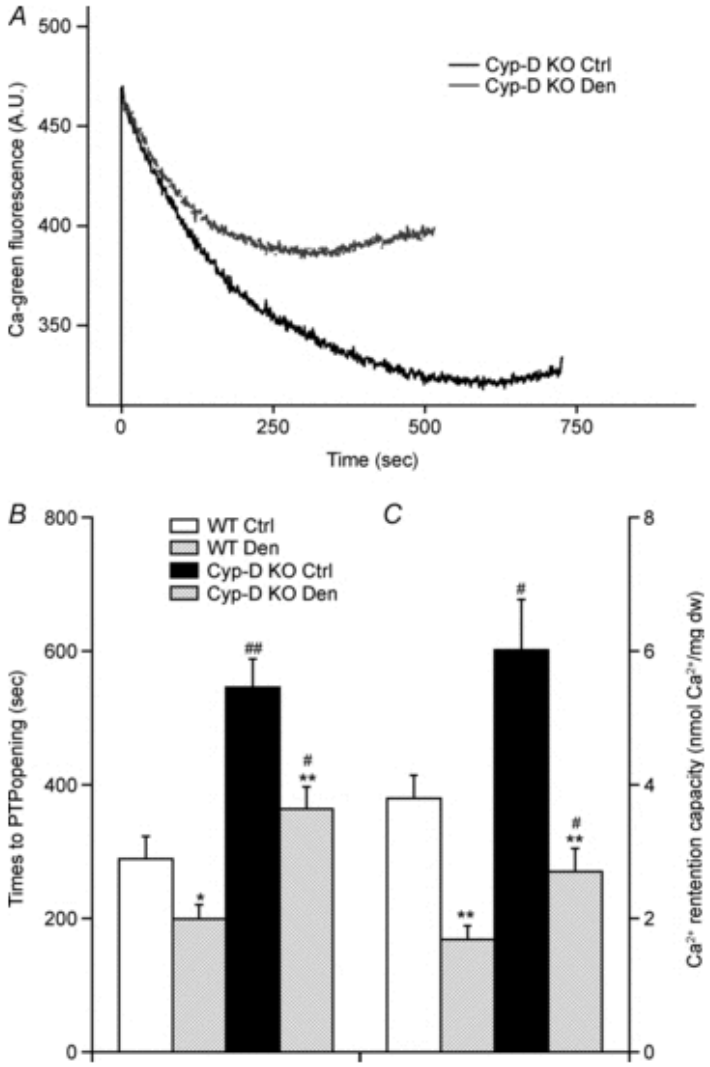

Figure 9: Effect of denervation in Ca^{2+}-induced PTP opening *in vitro*: Panel A: Typical mitochondrial Ca^{2+} uptake and release kinetics following a single pulse of 20 nmol Ca^{2+} in ghost fibres. For sake of clarity only the tracings from CypD-KO mice are shown. Panel B: Time required to trigger PTP opening following addition of a single pulse of exogenous 20 nmole Ca^{2+}. Panel C: Mitochondrial Ca^{2+} retention capacity expressed in nmol/mg dry weight. Values are means ± SEM for n= 6-9 in each group * different from control ($p<0.05$), ** different from control within the same mouse strain ($p<0.01$), # different from WT ($p<0.05$), ## different from WT within the same treatment group ($p<0.01$).

Figure 10: Effect of denervation on mitochondrial apoptotic signaling

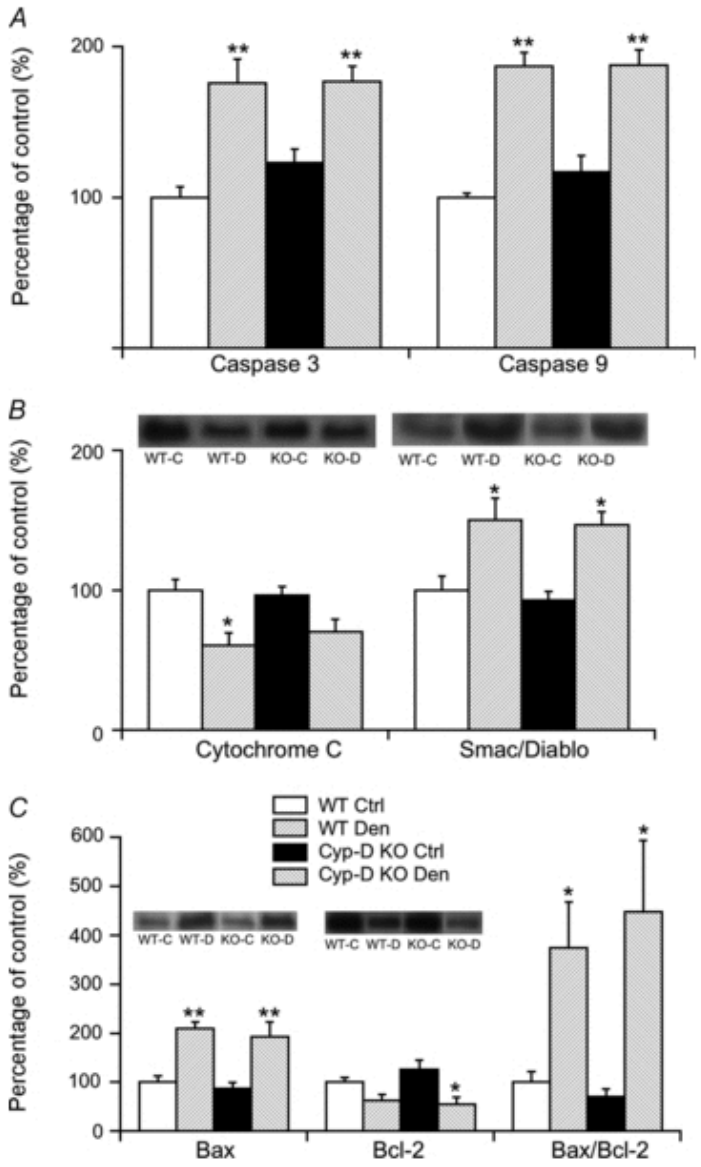

Figure 10: Effect of denervation on mitochondrial apoptotic signaling: Panel A and B respectively show the activity of caspase 3 and caspase 9 measured in the cytosolic fraction, and the amount of cytochrome c and Smac/Diablo in the cytosolic fraction from normal and denervated muscle in WT and Cyp-D deficient mice. Panel C shows the expression of Bax and Bcl-2 in whole muscle homogenate. Expression of proteins is normalized against that of LDH, which was used as loading control. Caspase activities are expressed in fluorescence units/min/mg protein. All values represent means ± SEM and are expressed as percentage of WT control values, * different from control within the same mouse strain ($p<0.05$), ** different from control within the same mouse strain ($p<0.01$).

3.1.6 DISCUSSION

Previous studies have led to the suggestion that CypD, and by extension opening of the PTP (Csukly *et al.*, 2006; Adhihetty *et al.*, 2007), could be involved in the activation of mitochondria-derived apoptotic signaling previously described in skeletal muscle following loss of innervation (Tews, 2002; Siu & Alway, 2005, 2006; Adhihetty *et al.*, 2007). However this hypothesis was never tested directly. In the present study we provide novel evidence that genetic ablation of CypD, despite enhancing resistance to PTP, confers no protection against denervation-induced muscle atrophy, and activation of apoptotic proteolytic cascades. We also show that although CypD deficiency increases the Ca^{2+} threshold for PTP opening in permeabilized fibres, sensitization still occurs in response to denervation, suggesting that in absence of CypD, additional factors poise mitochondria toward PTP opening *in vitro*.

3.1.6.1 Atrophy and mitochondrial apoptotic signalling in denervated muscle:

Membrane permeabilization, and subsequent activation of the mitochondrial apoptotic pathway, is a complex mechanism involving distinct permeation events in the OMM (mediated by Bcl-2 protein family) and IMM (mediated by PTP opening) (Crow *et al.*, 2004; Tomasello *et al.*, 2009). Although both events can occur independently, experimental evidence indicate that depending of the cellular insult, they can also occur in a coordinated fashion, with PTP opening sometime acting upstream, and sometime downstream of Bax recruitment (Tomasello *et al.*, 2009), thus making it difficult to determine the respective contribution of OMM and IMM permeabilization in a situation such as denervation, in which numerous factors promoting apoptotic signaling are present.

Studies in humans and animals models, including the present study (Figure 2.B), have reported that Bax expression is strongly upregulated in denervated muscle, while Bcl-2 expression is reduced (Tews, 2002; Siu & Alway, 2005, 2006; Adhihetty *et al.*, 2007). Furthermore, Siu et al. (Siu & Alway, 2006) showed that genetic

inactivation of Bax attenuated muscle atrophy, mitochondrial release of pro-apoptotic factors, and activation of caspase 3 and 9 measured 14 days after denervation. These previous observations contrast with our current results, which indicate that ablation of CypD, despite causing an upward shift (by 80-95%) in the threshold for PTP opening, does not have these beneficial effects on mitochondrial apoptotic signaling and muscle atrophy. Taken together these results would thus suggest that permeabilization of the OMM by Bax is the main factor triggering mitochondrial apoptotic signaling in denervated muscle, and that opening of the PTP does not play a major role, despite the existence in denervated muscle fibres of the two primary conditions known to directly promote PTP opening, namely increased intracellular [Ca^{2+}] (Joffe *et al.*, 1981; Csukly *et al.*, 2006) and oxidative stress (Abruzzo *et al.*; Adhihetty *et al.*, 2007; Bhattacharya *et al.*, 2009). Although we cannot completely rule out the possibility that the absence of CypD was insufficient to prevent PTP opening following denervation due to overwhelming conditions within muscle *in vivo*, we consider this unlikely considering that genetic ablation of CypD was previously shown to attenuate muscle pathology in other disease models associated with severe Ca^{2+} overload and oxidative stress including ischemia-reperfusion (Baines *et al.*, 2005; Nakagawa *et al.*, 2005), various muscular dystrophies (Angelin *et al.*, 2007; Millay *et al.*, 2008; Palma *et al.*, 2009), and overexpression of sarcolemmal L-type Ca^{2+} channels (Nakayama *et al.*, 2007).

In the present experiments, we observed a robust and systematic increase in caspase 9 and 3 in denervated muscle, which are hallmark of mitochondrial apoptotic signaling. However, changes in the cytosolic content of mitochondrial pro-apoptotic factors involved in the activation of these caspases were less consistent, as Smac/Diablo content was increased by denervation, while cytochrome c was paradoxically reduced. This latter phenomenon appears to be due to the fact that denervation causes a reduction of whole muscle cytochrome c content, which may limit the amount available for release in the cytosol. Indeed, in additional experiments we observed a reduction in whole muscle cytochrome c content following denervation in the two mouse strains (-40 ± 3 % compared to normally

innervated muscle, pooled data from WT and CypD-KO mice, P = 0.01, n= 7 in each group), but no change in Smac/Diablo content. In a previous study, a similar reduction in muscle cytochrome c content was reported following denervation, along with an increase in AIF content (Adhihetty *et al.*, 2007). In addition, the rise in cytosolic cytochrome c content observed following denervation in other studies (Siu & Alway, 2005) was modest and substantially lower than for Smac/Diablo and AIF. Overall, these data indicate that the measure of cytosolic cytochrome c content yields variable results in denervated muscle and may not be the best indicator of mitochondrial membrane permeabilization in this condition.

3.1.6.2 Susceptibility to PTP opening in vitro:

Previous studies conducted in CypD-KO mice have shown that CypD, acts as an endogenous "sensitizer" to Ca^{2+}-induced PTP opening (Baines *et al.*, 2005; Basso *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Our data showing that time to PTP opening and CRC were systematically higher in muscle mitochondria from CypD-KO *vs.* control mice both in the normal and denervated state are in line with these previous reports. However, despite this upward shift in resistance to PTP opening in CypD-KO mice, denervation was still able to reduce time to PTP opening as well as CRC, this indicates that additional factors can poise mitochondria toward PTP opening. However, this relative sensitization to PTP opening was observed *in vitro* under conditions in which mitochondria are exposed to Ca^{2+} overload that are well in excess of those normally observed *in vivo*.

Although the objective of this study was not to investigate the factors underlying denervation-induced sensitization in details, previously published data from our laboratory and others have shown that basal Ca^{2+} levels are higher in isolated mitochondria from denervated muscle compared to levels observed in mitochondria from normally innervated muscle (Joffe *et al.*, 1981; Csukly *et al.*, 206) bringing them closer to the Ca^{2+} threshold for PTP opening. Furthermore, a recent study (Bhattacharya *et al.*, 2009) reported that mitochondria isolated from denervated

muscle display enhanced activity of calcium-dependent cytosolic phospholipase A2 (cPLA2), which was previously shown to promote Ca^{2+}-induced PTP opening through the release of fatty acids (Broekemeier & Pfeiffer, 1995; Zoratti & Szabo, 1995). These factors could contribute to explain why in CypD-defficient mice, denervation still leads to a relative reduction in the amount of Ca^{2+} required to trigger PTP opening in mitochondria *in vitro*.

3.1.7 3.1.7 Conclusion:

Prevention of PTP opening through inhibition of CypD was recently shown to reduce cell death signaling and muscle damage in several disease models associated with Ca^{2+} dysregulation and oxidative stress (Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Angelin *et al.*, 2007; Nakayama *et al.*, 2007; Millay *et al.*, 2008; Palma *et al.*, 2009). However, results from the present study indicate that genetic ablation of CypD and the ensuing increase in resistance to PTP opening does not beneficially affect muscle atrophy, and activation of mitochondrial apoptotic signaling following the loss of muscle innervation. These novel data therefore provide direct evidence that Cyp-D-dependent PTP opening is dispensable for apoptotic signaling in skeletal muscle following denervation.

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3.2 Study No 2: PGC1α gene transfer during muscle regeneration restores mitochondrial biomass and improves mitochondrial calcium handling in post-necrotic mdx skeletal muscle.

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Running head: PGC1α and mitochondrial dysfunction in dystrophic muscle

Keywords: Duchenne muscular dystrophy, mitochondrial dysfunction, pgc-1, skeletal muscle

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3.2.1 ABSTRACT

Alterations of mitochondrial function have been implicated in the pathogenesis of Duchenne muscular dystrophy. In the present study, mitochondrial respiratory function, reactive oxygen species (ROS) dynamics and susceptibility to Ca^{2+}-induced permeability transition pore (PTP) opening were investigated in permeabilized skeletal muscle fibres of 6-week-old *mdx* mice, in order to characterize the magnitude and nature of mitochondrial dysfunction at an early post-necrotic stage of the disease. Short-term overexpression of the transcriptional co-activator PGC1α, achieved by *in vivo* plasmid transfection, was then performed to determine whether this intervention could prevent mitochondrial impairment and mitigate associated biochemical abnormalities. Compared with normal mice, *mdx* mice exhibited a lower mitochondrial biomass and oxidative capacity, greater ROS buffering capabilities, and an increased vulnerability to Ca^{2+}-induced opening of the mitochondrial permeability transition pore complex. PGC1α gene transfer restored mitochondrial biomass, normalized the susceptibility to PTP opening and increased the capacity of mitochondria to buffer Ca^{2+}. This was associated with reductions in the activity levels of the Ca^{2+}-dependent protease calpain as well as caspases 3 and 9. Overall, these results suggest that overexpression of PGC1α in dystrophin-deficient muscles, after the onset of necrosis, has direct beneficial effects upon multiple aspects of mitochondrial function, which may in turn mitigate the activation of proteolytic and apoptotic signalling pathways associated with disease progression.

3.2.2 INTRODUCTION

Duchenne muscular dystrophy (DMD) is an inherited disease resulting from mutations in the gene located at chromosome Xp21 encoding dystrophin, which leads to absence of this subsarcolemmal cytoskeletal protein from striated muscle cells (Koenig *et al.* 1987). The disease is characterized by repetitive cycles of necrosis and regeneration, which inexorably progress to overt muscle fibre loss and fibrosis as muscle repair capacities become overwhelmed (Petrof, 2002). Although

much progress has been made in our understanding of DMD (Koenig *et al.* 1987), the precise mechanisms leading to disease progression still remain unclear. The instability of the sarcolemma resulting from the lack of dystrophin is clearly a major contributor to pathogenesis (Petrof *et al.* 1993*a*); however, other factors such as increased extracellular Ca^{2+} entry leading to excessive cytosolic levels, elevated oxidative stress, and exaggerated activation of proteolytic and apoptotic signalling pathways, have also been implicated (Petrof, 2002; Ruegg *et al.* 2002; Tidball & Wehling-Henricks, 2007).

Studies in DMD patients and *mdx* mice (the most frequently employed animal model of the disease) have also shown that skeletal muscles lacking dystrophin exhibit impaired oxidative phosphorylation and reduced expression levels of numerous mitochondrial genes, leading to the suggestion that energetic deficiency is involved in disease pathogenesis (Dunn *et al.* 1993; Even *et al.* 1994; Gannoun-Zaki *et al.* 1995; Kuznetsov *et al.* 1998; Chen *et al.* 2000). Perhaps more importantly, mitochondrial dysfunction can also play a direct role in many of the above-mentioned factors suggested to cause repeated muscle injury and subsequent disease progression in DMD (Burelle *et al.* 2010). In this regard, mitochondria are known to be critically involved in the spatio-temporal regulation of intracellular Ca^{2+} (Ichas *et al.* 1997; Giacomello *et al.* 2007), the regulation of reactive oxygen species (ROS) production and scavenging (Turrens, 2003), and the triggering of cell death signalling through permeabilization of their membranes (Green & Kroemer, 2004; Zamzami *et al.* 2005; Burelle *et al.* 2010). However, the extent to which these mitochondrial functions are altered in dystrophin-deficient muscle currently remains uncertain, due in part to a lack of integrative assessments of the mitochondrial phenotype. Nevertheless, mitochondria are increasingly viewed as important contributors to muscle injury in DMD, and as potential targets for novel therapeutic interventions (Burelle *et al.* 2010). In support of this notion, recent work has shown that genetic and pharmacological inhibition of a mitochondria-dependent cell death pathway, mediated through opening of the permeability transition pore complex (PTP), significantly attenuates muscle fibre necrosis in mice

lacking dystrophin (Millay *et al.* 2008; Reutenauer *et al.* 2008), δ-sarcoglycan (Millay *et al.* 2008) and collagen VI (Irwin *et al.* 2003).

PGC1α is recognized as a dominant regulator of oxidative metabolism in many tissues, where it acts as a transcriptional co-activator of nuclear receptors and other transcription factors regulating adaptive mitochondrial responses as well as other biological programs linked to the oxidative state of cells (Kelly & Scarpulla, 2004). Recently, genetic crossing of *mdx* mice with transgenic animals overexpressing PGC1α in a skeletal muscle-specific fashion, as well as long-term overexpression of PGC1α in *mdx* mice using adenoassociated virus-mediated gene transfer, were both shown to improve multiple indices of dystrophic muscle pathology (Handschin *et al.* 2007; Selsby *et al.* 2012). However, the specific impact of PGC1α overexpression upon mitochondrial function *per se* has not been investigated in dystrophin-deficient muscle. Furthermore, in previous investigations PGC1α was overexpressed at the germline level or prior to the initial onset of myofibre damage (Handschin *et al.* 2007; Selsby *et al.* 2012). Therefore, these previous studies did not allow for testing of whether this approach has beneficial effects when instituted as a potential therapy after cycles of necrosis and regeneration have already begun, which is the more clinically relevant scenario.

Accordingly, in the present study we sought to determine the impact of PGC1α overexpression upon mitochondrial functional abnormalities and their downstream consequences in *mdx* muscles during the early regenerative phase, which follows the first wave of myonecrotic lesions. To this end, we first performed a comprehensive assessment of the mitochondrial functional phenotype prevailing at this time. We then proceeded to determine whether short-term overexpression of PGC1α, using plasmid-mediated gene transfer, was sufficient to rescue mitochondrial functional abnormalities. In particular, we tested the hypothesis that PGC1α could increase mitochondrial biomass and improve the ability of mitochondria to withstand and manage excessive Ca^{2+} loads, which are a central pathological hallmark of skeletal muscle fibres lacking dystrophin.

3.2.3 METHODS

3.2.3.1 Animal care

The 6 weeks old male *mdx* and C57BL/10ScSn mice originated from a colony maintained at the McGill University Health Centre (Montreal, Canada). All procedures were approved by the animal ethics committees of McGill University and the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care.

3.2.3.2 Mitochondrial functional assays in permeabilized muscle fibres

Dissection and permeabilization of fiber bundles with saponin were performed using tibialis anterior (TA) as described previously (Picard *et al.*, 2008b). Ghost fibres were prepared (Picard *et al.*, 2008b) by incubating saponin permeabilized bundles in a high KCl medium, which allows extraction of myosin. Permeabilized myofibers and ghost fiber bundles were then kept on ice until use. All mitochondrial parameters described below were determined at least in duplicate.

3.2.3.3 Mitochondrial respiration

Mitochondrial respiratory function was determined in an oxymeter equipped with with a Clark type electrode (Oxygraph, Hansatech Instruments) as per Picard et al. (Picard *et al.*, 2008a). The chamber was filled with 1 mL of solution B (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA (100 nM free Ca^{2+}), 6.56 $MgCl_2$ (1mM free Mg^{2+}), 20 taurine, 0.5 DTT, 50 K-methane sulfonate (160 mM ionic strength), 20 imidazole (pH 7.1), and after recording baseline oxygen content in the chamber, one bundle of 1-2mg dry weight of permeabilized myofibers was placed into the chamber, which was then sealed shut. Baseline readings were taken in presence of the complex I substrates glutamate-malate (10:5 mM, V_{GM}). The following additions where then sequentially made: the complex I blocker amytal (2 mM), ADP (2 mM, V_{ADP}), the complex II substrate succinate (25 mM, V_{succ}), the uncoupler CCCP (1 µM, V_{CCCP}), the complex III blocker antimycin-A (8 µM), the complex IV substrates N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD)-Ascorbate (0.9:9 mM, V_{TMPD}). Respiration rates were measured at 23°C under continuous stirring. At the

end of each test, fibers were carefully removed from the oxygraphic cell, blotted and dried for determination of fiber weight. Rates of O_2 consumption (JO_2) were expressed in nmoles of O_2 per minute per mg dry weight.

3.2.3.4 Enzyme activities

Activities of complex I (CI, NADH-CoQ reductase), complex II (CII, succinate dehydrogenase), complex IV (CIV, cytochrome oxidase complex), and citrate synthase (CS) were measured spectrophotometrically in a plate reader using standard coupled enzyme assays as per (Marcil *et al.*, 2006). Activities were expressed in mU/min/mg of wet muscle weight. Activities of calpain, caspase 3 and caspase 9 were determined fluorimetrically on a plate reader (excitation-emission: 400-505 nm) by measuring the cleavage of Ac-LLY-AFC (Biovision), Ac-DEVD-AFC and Ac-LEHD-AFC respectively (Enzo Life Sciences) respectively. Activities were expressed in $D_{fluorescence}$/min/ug protein.

3.2.3.5 Mitochondrial H_2O_2 release

Net H_2O_2 release by respiring mitochondria was measured in permeabilized fiber bundles with the fluorescent probe Amplex red (20 µM: excitation-emission: 563-587 nm) as described previously (Ascah *et al.*, 2011). Following preparation of permeabilized fibers, samples destined for H_2O_2 measurements were rinsed 3 times in buffer Z (in mM: 110 K-MES, 35 KCl, 1 EGTA, 5 K_2HPO4, 3 $MgCl_26H_2O$, and 0.5mg/ml BSA, pH 7.3 at 4°C). Fiber bundles (0.3-1.0 mg dry weight) were incubated at 37° C in a quartz micro-cuvette with continuous magnetic stirring in 600 µL of buffer Z (pH 7.3 at 37°C) supplemented with 1.2 U/mL horseradish peroxidase. Baseline fluorescence readings were taken in the absence of any exogenous respiratory substrates. The following additions were then made sequentially: glutamate (5 mM), succinate (5 mM), ADP (10 mM) and antimycin-A (8 µM). Rates of H_2O_2 production were calculated from a standard curve established under the same experimental conditions. All H_2O_2 measurements were performed at least in duplicate.

3.2.3.6 Mitochondrial H_2O_2 scavenging

To compare the mitochondrial H_2O_2 scavenging capacity, permeabilized fiber bundles were placed in 500 µL of buffer Z containing 50 µM pyruvate and 20 µM malate in a thermally controlled chamber set at 37°C with continuous stirring as previously described (Ascah *et al.*, 2011). An aliquot of the buffer was removed immediately after adding 40 µM of H_2O_2 and subsequently at 20, 40 and 60 secs. H_2O_2 content in aliquots was determined immediately on a fluorescence plate reader in a buffer containing 10 µM of Amplex Red and 0.5 U/mL HRP. The rate of H_2O_2 scavenging by mitochondria was determined as the difference between fluorescence levels measured at t=0 and t=60 sec. H_2O_2 levels were calculated from a standard curve performed under identical conditions. At the end of each test, fibers were carefully removed from the micro cuvette, blotted and dried for determination of fiber weight. All measurements were performed at least in triplicate and results were expressed in nmol H_2O_2 scavenged/min/mg dry weight.

3.2.3.7 Calcium retention capacity

Ghost fibers (0.3 – 1.0 mg dry fiber weight) were incubated at 23 ^{o}C in a quartz micro-cuvette with continuous magnetic steering in 600 µL of CRC buffer (in mM: 250 sucrose, 10 MOPS, 0.005 EGTA, 10 Pi-Tris, pH 7.3) supplemented with glutamate-malate (5:2.5 mM) and 0.5 nM oligomycin. Following the addition of fibers and respiratory substrates, a single pulse of 20 nmoles of Ca^{2+} was added. Time for opening of the PTP was taken as the time lapse between the addition of the Ca^{2+} pulse and the time at which Ca^{2+} release was first noted. Calcium retention capacity (CRC) was defined as the total amount of Ca^{2+} accumulated by mitochondria prior to Ca^{2+} release caused by PTP opening. CRC values were expressed per mg of dry fiber weight. Ca^{2+} concentration in the cuvette was calculated from a standard curve relating [Ca^{2+}] to the fluorescence of Ca-Green 5N (excitation-emmision: 505-535 nm) (Picard *et al.*, 2008a; Ascah *et al.*, 2011).

3.2.3.8 PGC1α gene transfer in vivo

The murine PGC1a cDNA was cloned into the mammalian expression vector pBudCE4.1 (Invitrogen), under the control of the human cytomegalovirus promoter; the human elongation factor 1a subunit promoter was used to separately drive constitutive expression of a tdTomato red fluorescent protein (Clontech) within the same vector (designated pBud-PGC1a-Tomato). The same plasmid, but in which the PGC1a cDNA was omitted (designated pBud-Tomato), was used as a control. Mice were lightly anesthetized and one TA muscle was injected with a volume of 50 µL of the pBud-PGC1a-Tomato plasmid (at a DNA concentration of 1.5 µg per µL) at three equidistant sites along the muscle length. An equal volume and concentration of the control pBud-Tomato plasmid was injected into the contralateral TA in the same fashion. The injected muscles were then exposed transcutaneously with 7 mm paddle circular electrodes to electrical stimulation (175 V/cm, 8 pulses of 20 ms at 1s intervals) using a commercial electroporator system (ECM 830; Genetronix, Inc., from BTX), as described previously (Molnar *et al.*, 2004). All animals were used for experiments 7 days after transfection. In some experiments, the whole TA muscle was homogenized for the assessment of enzymatic activities or immunoblotting as described above. For other experiments requiring the use of permeabilized fibers, transfected myofibers from both pBud-PGC1a-Tomato and control pBud-Tomato muscle groups were identified by their bright red fluorescence signal under a fluorescence microscope. Muscle regions enriched with transfected fibers were then carefully dissected (Figure 16) and permeabilized as described above.

3.2.3.9 Immunoblotting

Proteins were extracted from muscle, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride mem- branes. Protein modification induced by the lipid peroxidation by-product 4-HNE, an unsaturated aldehyde that can bind to lysine, cysteine, and histidine residues of proteins was evaluated by reacting the membranes with an antibody directed against 4-hydroxy-2-nonenal protein adducts (4-HNE, 1:1000 dilution Alpha Diagnostic Intl. Inc., San-Antonio, TX, USA). The level of cleaved casase-3 was

measured by probing the membranes with an anti-cleaved caspase 3 antibody (1:1000 #9664S Cell Signaling). Mitochondrial density was assessed by probing the membranes with an antibody directed against voltage-dependent anion channels (VDAC, 1:2000 dilution; Alexis Biochemicals, San Diego, CA, USA). The expression of lactate dehydrogenase was quantified using an anti-lactate dehydrogenase (LDH, 1:1000 dilution, Santa Cruz, Santa Cruz, CA, USA) antibody. LDH was used as a loading control because the expression of this enzyme is very high in skeletal muscle and is not affected by the disease. Equal loading of samples was confirmed by Ponceau or Coomassie Blue staining. Bands were visualized by enhanced chemiluminescence (Amersham) with film exposure times ranging from 1 to 45 min. Films were scanned and bands quantified by GelEval or FluorChem 8000 softwares.

3.2.3.10 Histology and Immunostaining

Transverse cryosections stained by haematoxylin and eosin were used for determining the percentage of centrally nucleated fibres (Briguet *et al.* 2004), with a minimum of five cross-sections per muscle each containing an average of 100 fibres. To quantify transfection efficiency, 7 µm cryosections from muscles transfected with pBud-PGC1α-Tomato and pBud-Tomato were incubated with an antibody directed against PGC1α (1:50; no. AB3242, Millipore) and a FITC-conjugated secondary antibody (1:1000; no. AF488, AlexaFluor). The slides were examined under fluorescence microscopy using fluorescein isothiocyanate filter settings, and the images were captured to computer with a cooled-CCD camera, using identical exposure parameters for all experimental conditions. Images taken at low magnification (4× Plan fluor objective) were used to quantify transfection efficiency, which was defined as the percentage of muscle area displaying increased PGC1α immunoreactivity. Quantification of PGC1α expression in transfected *vs.* untransfected fibres was quantified at higher magnification (10× Plan fluor objective)

3.2.3.11 Statistical analyses

Results are expressed as means ± SEM. Statistical differences were analyzed using one-way ANOVA (Statistica package). Tukey post hoc tests were used to identify the location of significant differences ($P < 0.05$).

3.2.4 RESULTS

3.2.4.1 Muscle histology in normal and mdx mice

In *mdx* mice, a massive wave of necrotic degeneration typically occurs between 3-4 weeks of age and is rapidly followed by a regenerative response, which results in the replacement of a large portion of damaged fibers between 5-6 weeks of age (Gillis et al. 1999). Consistent with this, TA muscle cross sections from 6 weeks old *mdx* mice presented some myofiber damage but only in discrete foci. Moreover, central nucleation, a hallmark of regeneration, was evident in numerous fibers throughout the muscle (Figure 12A-C). However, there were no major signs of muscle fiber atrophy at this age, as reported previously (Mouisel *et al.*, 2010; Kornegay *et al.*, 2012).

Figure 11: Muscle histology, mitochondrial density and respiratory function in normal and dystrophin-deficient muscle

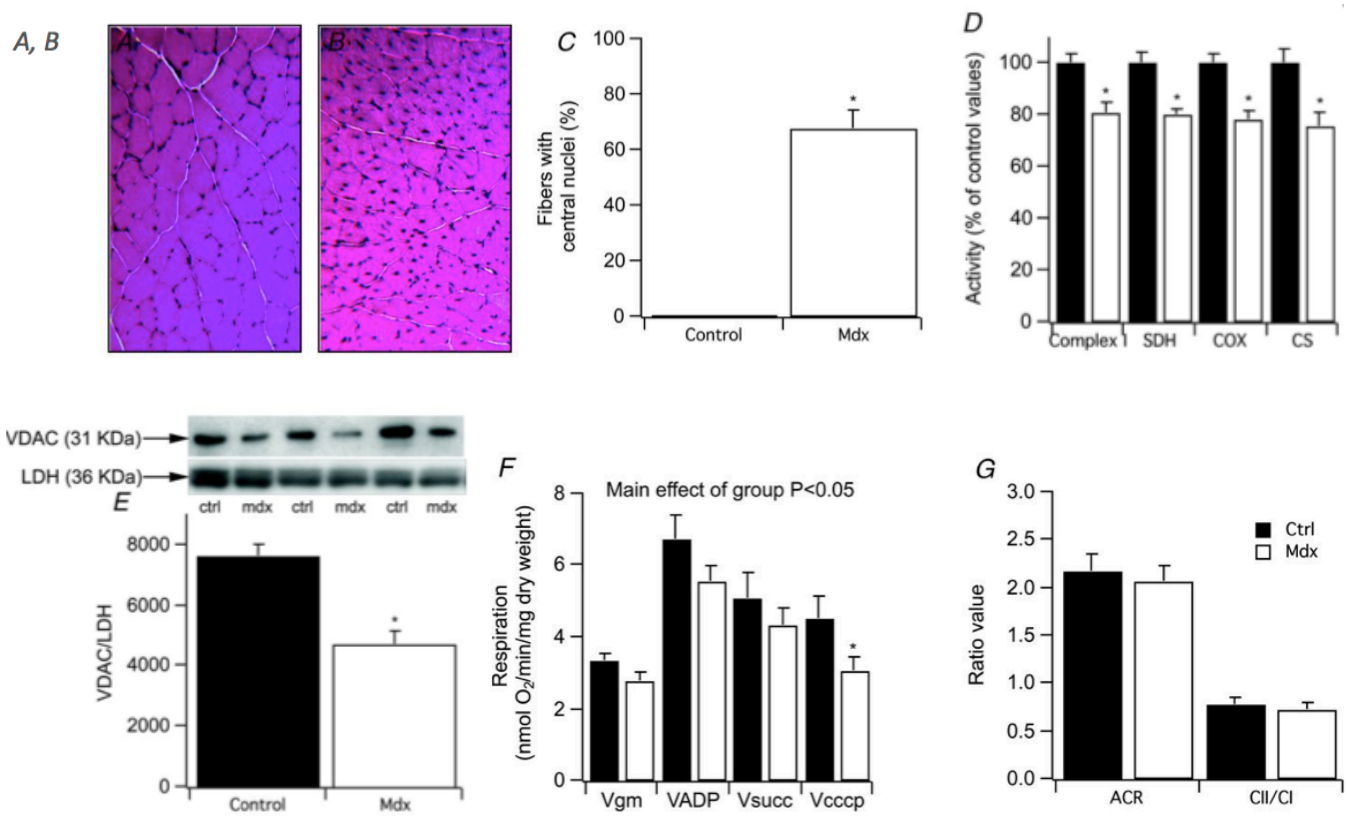

Figure 11: Muscle histology, mitochondrial density and respiratory function in normal and dystrophin-deficient muscle. Panel A and B: Representative cross-sections of TA muscle in 6 weeks-old control and *mdx* mice respectively. Panel C: Proportion of muscle fibers displaying central nucleation, a hallmark of muscle regeneration. The analysis was performed on a minimum of 5 cross-sections per muscle each containing an average of 100 fibers. Panel D: Activity of complex I, complex II (SDH), complex IV (COX), and citrate synthase (CS) in homogenates from TA muscles. Activities were measured in mU/min/mg wet muscle weight, and expressed as a percentage of values observed in the TA of normal mice. Panel E: Expression of the Voltage Dependent Anion Channel (VDAC) in whole muscle lysate normalized against the loading control LDH. Panel F: Respiration rates measured in permeabilized TA fibers. Following addition of fibers, the following addition sequence was performed: glutamate-malate (V_{gm}; 5: 2.5 mM), ADP (V_{ADP}: 1 mM), rotenone (1 μM; not shown), succinate (V_{succ}: 5 mM) and carbonyl cyanide m-chlorophenylhydrazone (V_{CCCP}; 1 M). Panel G: Acceptor control (ACR: V_{ADP}/V_{GM}) and CII/CI (V_{succ}/V_{ADP}) ratios, reflecting the coupling of oxidation to phsophorylation and the relative flux capacity between complexes I and II respectively. Black bars represent control values and open bars the *mdx* values (n = 9 in each experimental group). [a]: $P < 0.05$, significantly different from control.

3.2.4.2 Mitochondrial content and respiratory function in normal and mdx mice

To characterize the mitochondrial phenotype, enzymatic activity measurements and respirometry analyses were performed in whole muscle and permeabilized fibers. The activities of citrate synthase, and several ETC complexes (I, II, and IV) as well as the expression of the outer membrane protein VDAC were all 20-35 % lower in TA muscle from *mdx* mice as compared to non-dystrophic controls (Figure 12 D-E) which is indicative of a lower mitochondrial biomass. Respiration rates per unit of muscle weight were slightly but overall significantly lower in permeabilized TA fibers from *mdx* mice (main group effect $P<0.05$) (Figure 12F). However, this difference was absent when respiration was expressed per unit of mitochondrial marker enzyme to take into account the lower mitochondrial biomass (data not shown). As for the acceptor control ratio, and the complex II/ complex I ratio, which respectively reflect the intrinsic coupling efficiency between oxidation and phosphorylation, and the relative flux capacity between complexes I and II, no differences were observed between the two mouse strains (Figure 12G). Overall, these results indicate that mitochondrial biomass is reduced in young *mdx* muscle, but that there are no intrinsic respiratory defects detectable in mitochondria.

3.2.4.3 Mitochondrial ROS metabolism and susceptibility to PTP opening

Because substantial alterations in the integrity of the ETC complexes are required to alter respiration, overt respiratory defects often manifest at advanced disease stages (Burelle *et al.*, 2010). For this reason, we next focussed on mitochondrial ROS metabolism and susceptibility to opening of the PTP, which are more rapidly altered in response to pathological conditions in muscle (Csukly *et al.*, 2006; Marcil *et al.*, 2006; Burelle *et al.*, 2010). Figure 13A presents results from experiments in which mitochondrial H_2O_2 release was measured in permeabilized fibers under standardized respiratory states that allow identifying potential mechanisms of altered ROS release by the ETC. Mitochondrial H_2O_2 release was very low in presence of the complex I substrate glutamate, which is consistent with previous reports (Anderson & Neufer, 2006; Picard *et al.*, 2008a; Ascah *et al.*, 2011),

and no significant difference was observed between the two mouse strains. However, under conditions promoting high rates of superoxide production by the respiratory chain (*i.e.* after adding the complex II substrate succinate, or after blocking complex III with antimycin-A) as well as during active phosphorylation following addition of ADP, net H_2O_2 release was significantly lower in fibers from *mdx* mice compared to their normal counterparts. Importantly, these differences were observed even after normalizing for differences in mitochondrial biomass (Figure 13B) pointing to adaptive changes in mitochondria to limit oxidative stress.

To further evaluate this possibility the rate of scavenging of an exogenous H_2O_2 load by respiring mitochondria was measured in permeabilized fibers. As shown in Figure 13C H_2O_2 scavenging fluxes were significantly higher in permeabilized fibers from *mdx* mice as compared to those of control mice, indicating a significant upregulation in the maximal capacity of the mitochondrial anti-oxidant defences systems in *mdx* mice. Interestingly, as reported previously by our group (Dudley *et al.*, 2006a; Dudley *et al.*, 2006b), the amounts of oxidized proteins and 4-hydroxynonenal adducts were similar in the TA muscle across the two mice strain, suggesting that oxidative stress, which is known to occur in dystrophin-defficient muscle (Tidball & Wehling-Henricks, 2007), is relatively well contained at this age likely because of an adaptive anti-oxidant response.

Figure 12: Mitochondrial reactive oxygen species dynamics and oxidative damage markers in normal and dystrophin-defficient muscle.

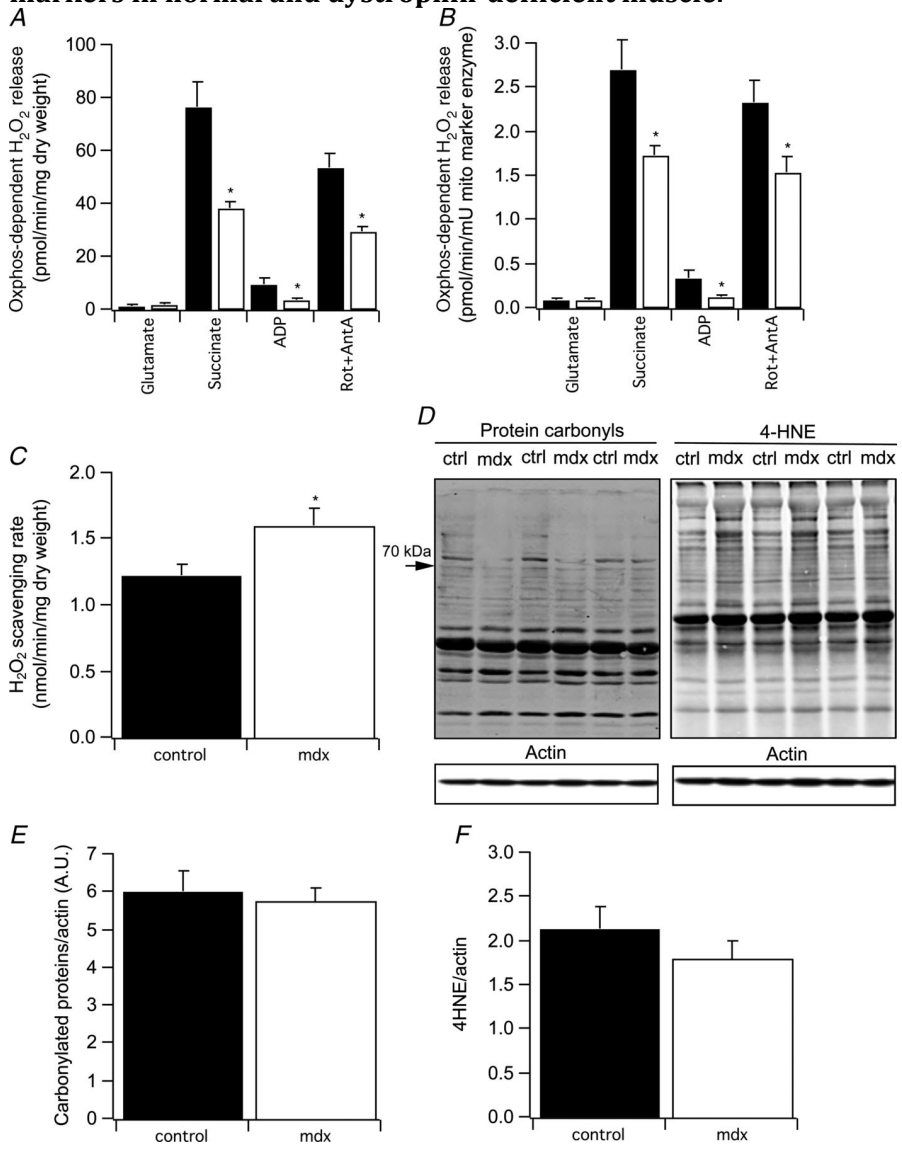

Figure 12: Mitochondrial reactive oxygen species dynamics and oxidative damage markers in normal and dystrophin-defficient muscle. Panel A-B: Net rate of H_2O_2 release by mitochondrial oxidative phosphorylation measured in permeabilized TA fibers. Rates of H_2O_2 release are expressed per mg of dry fiber weight (A) or per unit of mitochondrial marker enzyme (B) to take into account differences in mitochondrial biomass between groups. The protocol was as follows: after addition of the fibers, the following injection sequence was performed: glutamate (5 mM), succinate (5 mM), ADP (1 mM), rotenone+ antimycin A (Rot+Ant-A: 1: 8 µM). All rates presented are corrected for baseline oxidation of the Amplex red probe by fibers which was measured immediately prior these additions. Panel C: Rate of H_2O_2 scavenging by succinate-energized mitochondria following addition of 20 nmol H_2O_2. All measurements were performed in paralell in 1 control and 1 *mdx* muscle per experiment (n = 9 in each experimental group). Panel D: Representative blots showing the levels of oxidized proteins and 4-HNE adducts in whole TA muscle lysates from control and *mdx* mice at 6 weeks of age. Panel E: Densitometric analysis of oxidized protein levels normalized against the loading control actin (n=6 animals per group). Panel F: Densitometric analysis of the 70 kDa band on the 4-HNE blot normalized against the loading control actin (n=6 per group). [a]: $P < 0.05$, significantly different from control.

Considering the importance of Ca^{2+} dysregulation in the pathogenesis of muscle injury in DMD (Petrof, 2002; Ruegg *et al.*, 2002), the susceptibility of mitochondria to opening of the PTP and their ability to take up Ca^{2+} was determined in permeabilized fibers using a standardized Ca^{2+} challenge. This type of assay is known to correlate well with the propensity of mitochondria to undergo permeability transition *in vivo* when challenged by stress (Javadov *et al.*, 2003; Marcil *et al.*, 2006). As shown in Figure 14A, opening of the PTP occurred prematurely in TA fibers from *mdx* mice in comparison to fibers from control mice, indicating that mitochondria are predisposed to Ca^{2+}-induced permeability transition. The total amount of Ca^{2+} taken up by mitochondria prior to PTP opening also tended ($P = 0.07$) to be lower, mainly as a result of a lower mitochondrial density (Figure 14B). At the level of whole muscle, these observations were associated with increased activities of calpain, and caspase 3 (Figure 14D), consistent with the activation of Ca^{2+} and mitochondrial-dependent proteolytic signalling in dystrophin-defficient muscle.

Figure 13: Susceptibility to Ca^{2+}-induced PTP opening and protease activities in dystrophin-defficient muscle.

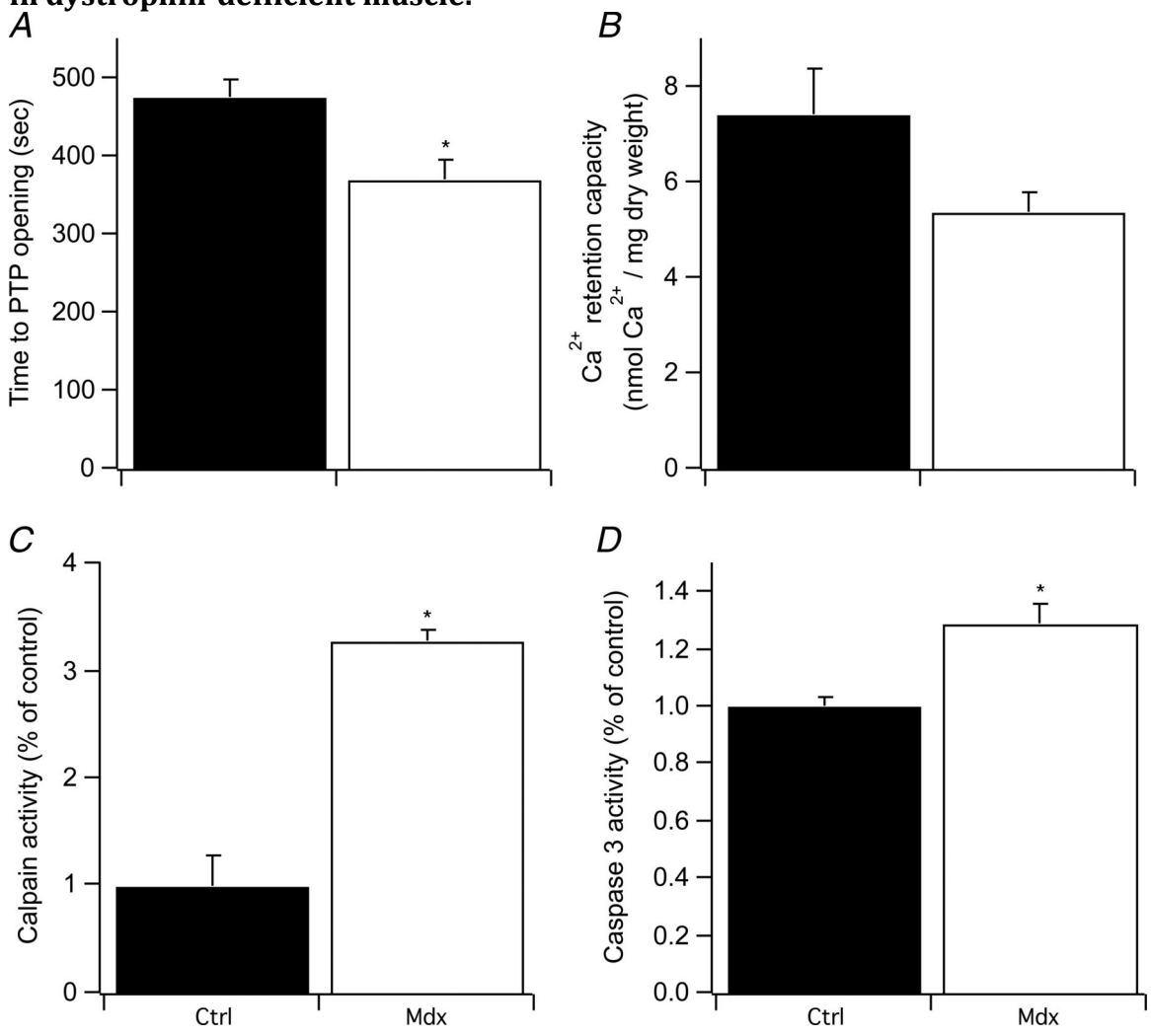

Figure 13: Susceptibility to Ca^{2+}-induced PTP opening and protease activities in dystrophin-defficient muscle. Panel A: Time required for PTP opening after the addition of a single pulse of 20 nmoles Ca^{2+} to permeabilized fibers *in vitro*. Panel B: Total amount of Ca^{2+} accumulated by mitochondria prior to opening of the PTP (n = 9 per group). Panel C and D: Calpain and caspase 3 activities in whole muscle lysates (n = 6 per group) reported relative to values obtained in control muscles. [a]: $P < 0.05$, significantly different from control.

3.2.4.4 Effect of forced expression of PGC1α on mitochondrial properties and muscle biochemistry

Based on this basic characterization of mitochondrial functions, we next determined whether short-term (i.e. 7 days) overexpression of PGC1a during the muscle regeneration phase could correct the main mitochondrial abnormalities observed, namely mitochondrial biomass, PTP sensitivity and Ca^{2+} handling capabilities. The effect of PGC1a transfection on mitochondrial anti-oxidant defences was not characterized since in TA from *mdx* mice anti-oxidant defences were already upregulated and oxidative damage was not apparent.

An expression plasmid (pBud-PGC1a-Tomato) encoding the murine PGC1a gene together with a separately transcribed red fluorescence Tomato marker gene was therefore electroporated into one TA muscle, whereas the contralateral TA was transfected in the same manner with a control plasmid (pBud-Tomato) containing the marker gene alone. This electroporation procedure resulted in a 3 fold increase in PGC1a immuno-reactivity in ~ 30 % of muscles fibers 7 days after electroporation (Figure 15). As shown in Figure 16, VDAC expression and the activities of all mitochondrial enzymes measured were increased by 20-35% in whole extracts from PGC1a-transfected muscles compared to those transfected with the control plasmid. (Figure 16A-B). Therefore, short-term overexpression of PGC1a was able to restore mitochondrial biomass to the level observed in normal TA muscles of non-dystrophic mice. In addition, dissection of transfected fibers (identified by Tomato marker gene expression in both control plasmid and PGC1a gene transfected muscles (Figure 16C)) allowed assessment of mitochondrial susceptibility to PTP opening. Compared to the values observed in *mdx* TA fibers transfected with control plasmid, fibers transfected with the PGC1a gene exhibited a greater resistance to PTP opening when faced with an exogenous Ca^{2+} challenge. Hence the time required to trigger PTP opening in response to Ca^{2+} increased (by 21 %) (Figure 16D-E), which is comparable to the level observed in muscle fibers from non-dystrophic control mice. Furthermore, PGC1a gene transfer significantly increased (by 62 %) the amount of Ca^{2+} taken up by mitochondria prior to PTP opening (Figure 16F). At

the level of the whole muscle PGC1a gene transfer also reduced the activity of calpain, caspase 3 and caspase 9 (Figure 16G). Moreover, measurements made specifically in the regions transfected with PGC1a showed that the levels of cleaved caspase 3 were reduced 5 fold compared to muscles transfected with the control plasmid (Figure 16H). Overall, these data point to an improvement in downstream cellular events linked to Ca^{2+} overload and mitochondrial permeability transition following PGC1a transfection.

Figure 14: PGC1-α expression levels in TA muscle from dystrophin-defficient muscle transfected with PGC1α and control plasmids.

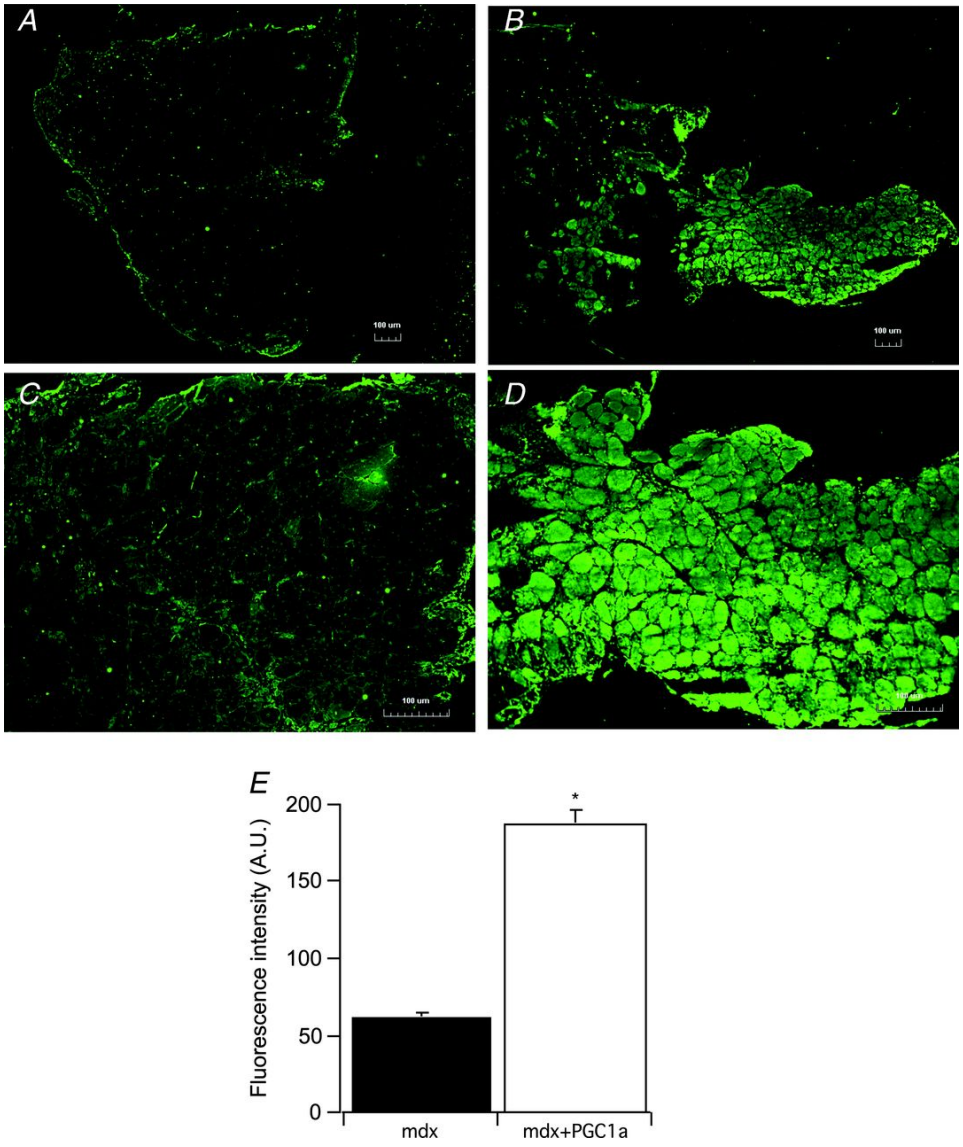

Figure 14: PGC1-α expression levels in TA muscle from dystrophin-defficient muscle transfected with PGC1α and control plasmids. Panel A-D: PGC1-α immunoreactivity in cross-sections from *mdx* mice electroporated with the control plasmid pBud-Tomato (Panel A,C) and the PGC1α - containing pBud-PGC1α-Tomato plasmid (Panel B,D). Images were taken using a 4X (Panel A-B) and a 10X (Panel C-D) objective. Panel E: PGC1-α immunoreactivity level in single fibers from transfected and untransfected muscle. Data represent means ± sem of at least 50 individual fibers in six different muscles injected with control or PGC1-α plasmids. Panel F: PGC1α content in muscles injected with control or PGC1-α plasmids.

Figure 15: Effect of PGC1α gene transfer on mitochondrial oxidative capacity, Ca^{2+} handling, and protease activities in dystrophin-deficient muscle.

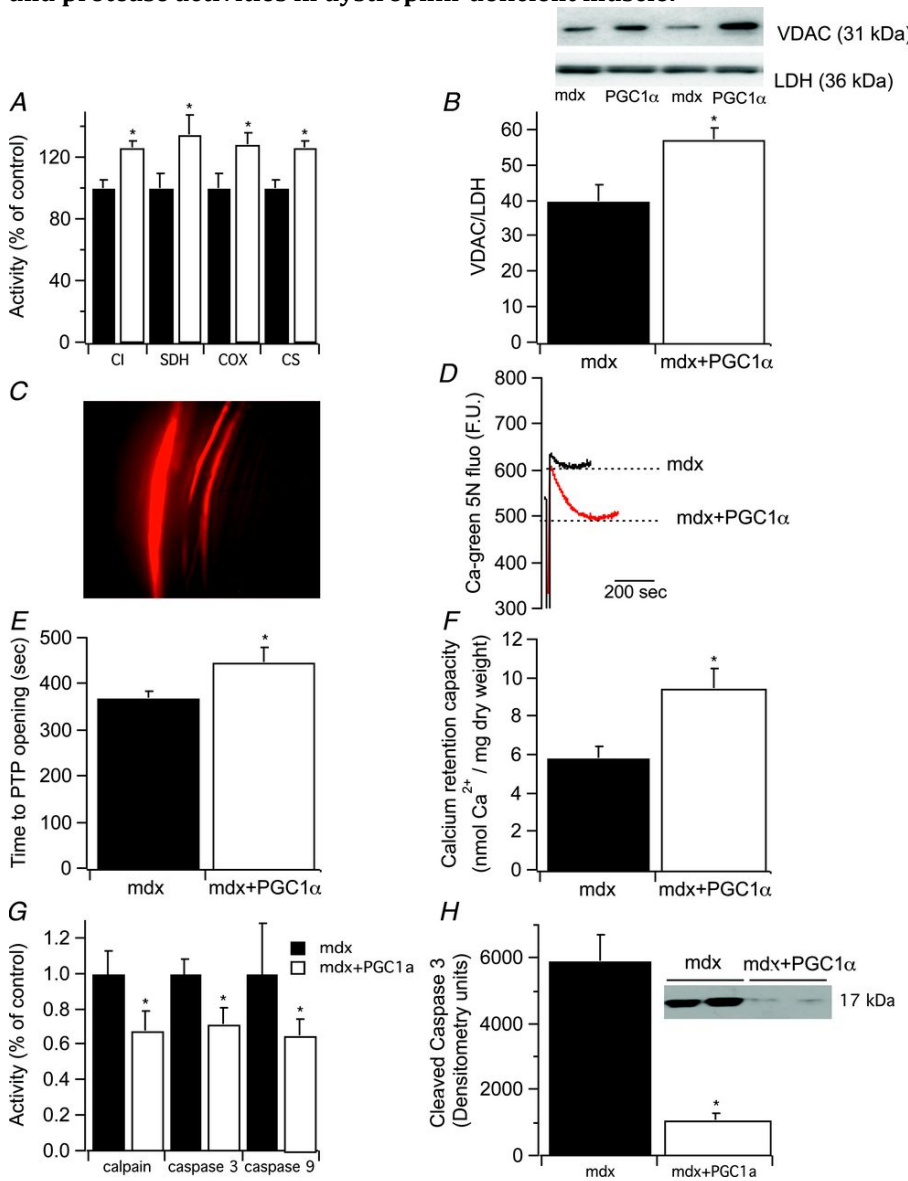

Figure 15: Effect of PGC1α gene transfer on mitochondrial oxidative capacity, Ca^{2+} handling, and protease activities in dystrophin-deficient muscle. Panel A and B: Activities of mitochondrial marker enzymes and expression of VDAC in whole lysate from TA muscle transfected with the control plasmid pBud-Tomato (*mdx:* black bars) versus the PGC1α - containing pBud-PGC1α-Tomato plasmid (*mdx*+PGC1α: open bars) (*n* = 6 per group). Panel C: Representative fluorescence image at 4X magnification of a portion of the TA muscle transfected with pBud-PGC1α-Tomato. Groups of muscle fibers transfected with the plasmid are labeled in red. The muscle border is visible on the left. Panel D: Representative traces of Ca^{2+} uptake and release measured in ghost fibers from control and PGC1α-transfected muscle. Panel E: Time required for PTP opening after the addition of a single pulse of 20 nmoles Ca^{2+} to permeabilized fibers *in vitro*. Panel F: Total amount of Ca^{2+} accumulated by mitochondria prior to opening of the PTP (*n* = 6 per group). Panel G and H: Activities of calpain, caspase 3 and caspase 9 and levels of cleaved caspase-3 in whole lysates from TA muscle transfected with the control plasmid pBud-Tomato versus the PGC1α - containing pBud-PGC1α-Tomato plasmid (*n* = 6 per group). For each animal, the activities measured in the PGC1-α gene transfected TA are expressed relative to the activities measured in the contralateral TA of the same animals transfected with the control pBud-Tomato plasmid. [a]: $P < 0.05$, significantly different from control.

3.2.5 DISCUSSION

Mitochondria are increasingly suggested to play a role in skeletal muscle pathology in DMD, and to constitute a promising target for therapy (Burelle *et al.*, 2010), yet comprehensive assessments of the mitochondrial functional phenotype at defined periods of this progressively degenerating disease are lacking. Furthermore, although overexpression of PGC1α was recently shown to ameliorate muscle pathology in *mdx* mice (Handschin *et al.*, 2007)(Selsby et al. 2012), the impact of PGC1α treatment on the mitochondrial phenotype was not addressed. In addition, PGC1α was overexpressed at the germline or immediately after birth, which did not allow testing whether this approach has beneficial effects when initiated after the onset of muscle pathology. In the present study, *mdx* mice were studied during the well-defined regenerative phase that follows the initial myonecrotic wave, which allowed to define early alterations in the mitochondrial phenotype, and to test whether short-term PGC1α can be beneficial. Our results show muscle fibers from *mdx* mice displayed multiple mitochondrial abnormalities, including a reduced mitochondrial biomass, an adaptive enhancement of H_2O_2 handling capabilities, and an increased susceptibility to opening of the PTP in response to Ca^{2+} loading *in vitro*. However, intrinsic respiratory properties were preserved at this stage of disease. We also provide novel evidence that overexpression of PGC1α for 7 days restores mitochondrial biomass to the level observed in healthy muscle, normalizes the susceptibility to PTP opening, enhances the mitochondrial capacity to buffer Ca^{2+}, and improves downstream Ca^{2+}-related abnormalities of excessive activation of proteolysis and apoptotic signalling pathways.

3.2.5.1 Alteration of the mitochondrial functional phenotype in dystrophin-deficient muscle

Previous studies have reported alterations in muscle energy metabolism in *mdx* mice, particularly with respect to oxidative capacity (Gannoun-Zaki *et al.*, 1995; Kuznetsov *et al.*, 1998; Chen *et al.*, 2000). Various mechanisms have been proposed to be responsible, but there is no definite consensus. In the present study, we

observed a loss of mitochondrial biomass in muscle from *mdx* mice, as indicated by the reduction in the activity/expression of several mitochondrial marker enzymes, and a mild depression of respiration rates per unit of muscle mass. However, there were no apparent signs of specific respiratory impairments, as judged by the absence of difference in respiration rates per unit of mitochondrial marker enzyme, acceptor control (V_{ADP}/V_0) ratio, and complex II/complex I ratio. These results thus suggest that early in the disease course, a loss of mitochondrial density appears to be the main factor contributing to impaired oxidative capacity in dystrophin-deficient muscle. Presumably, at least part of this reduction of mitochondrial content at 6 weeks old is attributable to Ca^{2+} overload and the attendant opening of the PTP. This is suggested to occur in some mitochondria (Millay *et al.*, 2008; Fraysse *et al.*, 2010), particularly during the preceding necrotic period (*i.e.* between 3-4 weeks-old), resulting in their physical disruption and rapid elimination through autophagy and the ubiquitin proteasome system (Gottlieb & Carreira, 2010; Gottlieb & Mentzer, 2010). Interestingly, several studies in fact indicate that opening of the PTP in individual organelles and the resulting loss of membrane potential constitutes one of the triggers of mitophagy (Elmore *et al.*, 2001; Carreira *et al.*, 2010). More detailed studies will be necessary to confirm this hypothesis.

Other studies performed on older animals (*i.e.* 12-24 weeks), during the more degenerative and fibrotic period of the disease, have however reported multiple signs of specific respiratory abnormalities including loss of respiratory chain complexes without accompanying changes in the activity of CS (Kuznetsov *et al.*, 1998), a lower ACR ratio (Glesby *et al.*, 1988) and reduced mRNA levels for respiratory chain complex subunits without altered mitochondrial DNA content (Gannoun-Zaki *et al.*, 1995). It is therefore possible that in addition to the loss of mitochondrial biomass associated with myopathic lesions early in the disease, alterations in respiratory chain complex assembly or activity appear as the disease progresses, possibly as a result of repetitive oxidative stress (Dudley *et al.*, 2006a).

Signs that the muscle redox status is perturbed, including an increased GSSG/GSH ratio, have been reported in 4-8 weeks old *mdx* muscle (Dudley *et al.*, 2006a; Dudley *et al.*, 2006b; Tidball & Wehling-Henricks, 2007), suggesting the presence of pro-oxidant conditions during the early disease stages. However, in accordance with previously published results (Dudley *et al.*, 2006a), we found that the levels of protein carbonyls and 4-HNE adducts were similar in muscle of control and *mdx* mice, indicating that if increased ROS production occurs, it is relatively well-contained and does not yet result in gross oxidative damage. In this regard, our results showing that mitochondria within *mdx* fibers released less H_2O_2 under standard respiratory states, and displayed an improved capacity to eliminate H_2O_2, clearly suggest that an adaptive increase in mitochondrial ROS handling capabilities contributes to limit oxidative damage in young *mdx* muscle. However, as the disease progresses, this mechanism likely become overwhelmed, thus favouring oxidative damage in older dystrophic muscles. The factors responsible for this adaptive increase in mitochondrial ROS handling capacities are currently unclear. However, Ca^{2+} is known to promote mitochondrial ROS production by increasing the reduction state of the electron transport chain (Brookes *et al.*, 2004), and by triggering transient opening of the PTP (Wang *et al.*, 2008). Therefore, dysregulation of Ca^{2+}, which is a hallmark of DMD, may induce short-term localized bursts of superoxide that are insufficient to cause oxidative damage initially, but are important enough to trigger an adaptive anti-oxidant response. This hypothesis remains to be investigated specifically.

Beside their role in energy production and redox balance, mitochondria act as important spatio-temporal buffers for cytosolic Ca^{2+} due to their high membrane potential and close proximity to Ca^{2+} channels in the sarcoplasmic reticulum and sarcolemma (Ichas *et al.*, 1997; Giacomello *et al.*, 2007; Rizzuto *et al.*, 2009). This property is relevant in the context of DMD as it may allow to limit the pathological increases in cellular Ca^{2+}. However, the extent to which mitochondria can successfully buffer cellular Ca^{2+} is ultimately set by their threshold to opening of the PTP. In the present study, we report that despite being normal with respect to basal

respiratory properties, mitochondria within permeabilized *mdx* fibers are more prone to opening of the PTP when exposed to Ca^{2+}-overload. It should however be noted that a previous study performed in 5-6 week-old *mdx* mice reported no change in the Ca^{2+} threshold for PTP opening between control and *mdx* muscle (Reutenauer *et al.*, 2008). Although the reason for this discrepancy remains unclear, one possibility is that in this study mitochondria were isolated using tissue homogenization and differential centrifugation, which tends to retain healthy mitochondria and to eliminate more fragile organelles (Picard *et al.*, 2011). This is particularly relevant for the study of the PTP, as it is well known that the sensitivity to permeability transition is heterogeneous across the population of mitochondria within a cell, and that shifts in the proportion of sensitive *vs* resistant mitochondria will directly influence the average PTP sensitivity of the whole population of mitochondria (Bernardi, Physiol review 1999). Here, the use of a less disruptive approach to study mitochondrial function in permeabilized fibers likely allowed to preserve mitochondria that were prone to Ca^{2+}-induced PTP opening. Taken together our results thus suggest that sensitization to permeability transition occurs early in the course of the disease, which may reduce the ability of mitochondria to buffer cellular Ca^{2+}, and predispose to activation of proteolytic processes and mitochondria-mediated cell death (Bernardi, 1999; Burelle *et al.*, 2010), two phenomenon that were observed in the present study (Figure 14C-D).

3.2.5.2 Effects of PGC1α gene transfer on mitochondrial function in dystrophin-deficient muscle

Our results demonstrate that plasmid-mediated PGC1α gene transfer after the first wave of myonecrosis has subsided is able to restore the loss of mitochondrial biomass normally observed in the TA muscle of *mdx* mice. Importantly, we also provide novel evidence indicating that PGC1α overexpression restores the resistance of mitochondria to Ca^{2+}-induced PTP opening, and thereby substantially improves the amount of Ca^{2+} that can be accumulated within mitochondria without triggering mitochondrial membrane permeabilization. These effects are consistent with the well-documented role of PGC1α as a master regulator of mitochondrial

biogenesis (Kelly & Scarpulla, 2004), and with results from recent cell culture models showing the ability of PGC1α to increase the distribution volume of Ca^{2+} in mitochondria, and protect against Ca^{2+}-induced cell death (Bianchi *et al.*, 2006). At the level of individual dystrophic muscle fibers, these improvements may limit injury by contributing to prevent the energy deficiency previously reported in dystrophin-deficient muscle (Dunn *et al.*, 1993; Even *et al.*, 1994; Chen *et al.*, 2000). Furthermore, PGC1α could *1)* help prevent the activation of mitochondria-mediated cell death signalling, and opening of the PTP, which are known to occur in several models of muscular dystrophy (Irwin *et al.*, 2003; Millay *et al.*, 2008; Reutenauer *et al.*, 2008), and *2)* provide muscle fibers with a mitochondrial "sink" to temporarily buffer excessive intracellular Ca^{2+} levels typically associated with DMD. This would be consistent with our observation that PGC1α gene transfer reduced the activity/levels of calpain and caspase 3 and caspase 9, which suggest an amelioration of Ca^{2+}- and mitochondria-dependent proteolytic signalling in dystrophin-deficient muscle. However, although PGC1α is a key modulator of mitochondrial functions, we cannot exclude the possibility that some of the benefits that we have observed are partly attributable to other effects of PGC1α, such as better preservation of sarcolemmal and neuromuscular junction (NMJ) integrity through the upregulation of utrophin and NMJ-related genes (Handschin *et al.*, 2007), and amelioration of calcium/clamodulin signalling (Chakkalakal *et al.*, 2006).

3.2.6 Conclusion

In summary, our results demonstrate that skeletal muscles from dystrophin-deficient animals exhibit several mitochondrial functional abnormalities early in the disease, including a lower mitochondrial biomass, a compensatory anti-oxidant response, and an increased vulnerability to mitochondrial permeabilization via PTP opening when faced with a Ca^{2+} challenge. Importantly, short-term treatment with PGC1α gene transfer during the regenerative phase following a myonecrotic wave, was able to restore mitochondrial biomass to the level observed in normal muscle, normalize the resistance to PTP opening, and increase the capacity of mitochondria

to buffer Ca^{2+}. This was further associated to a mitigation of Ca^{2+}-dependent proteolytic (calpain) and apoptotic (caspase-3/9) signalling in dystrophic muscles. Taken together, these data suggest that treatments aimed to increase PGC1α signalling may protect dystrophin-deficient muscles by stimulating mitochondrial biogenesis, improving cellular energetics, providing a Ca^{2+} sink to limit Ca^{2+}-related cellular abnormalities, and preventing the activation of mitochondria-dependent cell death pathways associated with mitochondrial permeabilization.

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3.3 Study No 3: Protective role of Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction

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3.3.1 ABSTRACT:

Mitophagy plays a vital role in the maintenance of optimal mitochondrial function. However, its roles and regulation remain ill defined in cardiac pathophysiology. Here, we tested the hypothesis that Parkin, an E3-ligase recently described as being involved in the regulation of cardiac mitophagy, is important for 1) the maintenance of normal cardiac mitochondrial function; and 2) adequate recovery from sepsis, a condition known to induce reversible mitochondrial injury through poorly understood mechanisms. Investigations of mitochondrial and cardiac function were thus performed in wild type and Parkin-deficient mice at baseline and at two different times following administration of a sub-lethal dose of *E. coli* lipopolysaccharide (LPS). LPS injection induced cardiac and mitochondrial dysfunctions that were followed by complete recovery in wild type mice. Recovery was associated with morphological and biochemical evidence of mitophagy, suggesting that this process is implicated in cardiac recovery from sepsis. Under baseline conditions, multiple cardiac mitochondrial dysfunctions were observed in Parkin-deficient mice. These mild dysfunctions did not result in a visibly distinct cardiac phenotype. Importantly, Parkin-deficient mice exhibited impaired recovery of cardiac contractility and constant degradation of mitochondrial metabolic functions. Interestingly, autophagic clearance of damaged mitochondria was still possible in the absence of Parkin through Parkin-independent mitophagy and upregulation of macro-autophagy, suggesting that redundant mechanisms partially compensate for its absence. Together, these results provide evidence that, *in vivo,* cardiac mitophagy is important to the normal maintenance of mitochondrial function, to the recovery of cardiac function following sepsis, and that redundancy exists in the regulation of this process.

3.3.2 INTRODUCTION

Mitochondrial quality control (QC) is increasingly being recognized as essential to the maintenance of normal cellular function (1,2). Mitochondrial QC is ensured, in part, by the combined action of the ubiquitin-proteasome system (UPS) (3) as well as several mitochondrial chaperones and proteases that collectively regulate normal protein folding/assembly and mitochondrial DNA (mtDNA) integrity (2). In addition to this control at the molecular level, whole organelles are constantly being recycled through the concerted action of mitochondrial biogenesis and autophagy, a process whereby damaged cellular constituents, ranging from proteins to whole organelles, are sequestered in double membrane autophagosomes and delivered to lysosomes for degradation[1]. Although initially believed to be a non-selective process, it is now well-appreciated that autophagic degradation of mitochondria (i.e. mitophagy) is, for the most part, a specifically regulated process (4).

Despite the fact that the molecular events involved in mitophagy remain poorly understood, recent studies in cell models have implicated the E3-ligase Parkin as an important regulatory factor [4]. According to the prevailing model, Parkin is recruited to depolarized mitochondria where it catalyzes ubiquitination of outer membrane proteins (5,6). This process, which requires the mitochondrial serine-threonine kinase Pink1 (7), in turn promotes the recruitment of the LC3-binding protein P62/SQSTM1and subsequent formation of the autophagosome. While this mechanism has drawn considerable attention, some reports suggest that Parkin may play additional roles in mitochondrial QC by regulating the expression of PGC1α, a master regulator of biogenesis (8), and by supporting mitochondrial genome integrity through direct physical interactions with mtDNA and/or TFAM (9). Furthermore, a recent study has provided evidence that Parkin can prevent cell death under moderate stress conditions associated with minor mitochondrial defects by activating the NF-kB pro-survival pathway through linear ubiquitination of NF-kB essential modulator (NEMO), resulting in increased OPA1 expression and maintenance of mitochondrial integrity (10).

In the heart, activation of autophagy has been reported in response to various pathological conditions in which mitochondrial dysfunction develops, including ischemia-reperfusion (11, 12) and sepsis (13, 14). However, mechanisms of mitochondrial QC and the potential role of Parkin in this process remain poorly understood. In the present study, we tested the hypothesis that Parkin is important to the maintenance of normal mitochondrial function in the heart and is required to mount adequate responses to mitochondrial injury under stress conditions. To this end, cardiac and mitochondrial phenotypes of wild type mice and mice harbouring a deletion of exon 3 of the Parkin gene were assessed under baseline conditions and in response to sepsis induced by *E. coli* lipopolysaccharide (LPS). LPS administration is a clinically relevant stress model and was used because it has previously been associated with mitochondrial dysfunction (15-17), compensatory mitochondrial biogenesis (16-18), and activation of autophagy (13, 14), three features that suggest important implications for the regulation of mitochondrial QC.

3.3.3 RESULTS

3.3.3.1 Absence of a distinct cardiac phenotype despite mild mitochondrial dysfunction in Parkin$^{-/-}$ mice:

At 12-16 weeks of age, Parkin$^{-/-}$ mice did not display a distinct cardiac phenotype. Resting heart rate, stroke volume, ejection fraction and cardiac output were similar in WT and Parkin$^{-/-}$ mouse hearts (Table 2). There was no apparent hypertrophy, as judged by absolute heart weight and heart-to-body weight ratio (Table 2). Cardiomyocyte histology also appeared mostly normal on H&E-stained ventricular cross-sections and electron micrographs (Figure 24, 25). Analyses of permeabilized cardiac fibers, however, revealed the presence of mitochondrial functional abnormalities. More specifically, respiration rates under state 2 (with complex I substrates) and state 3 conditions (with complex I or complex II substrates) were lower in fibers from Parkin$^{-/-}$ hearts compared to WT hearts (Figure 18). Net H_2O_2 release was greater in mitochondria from Parkin$^{-/-}$ hearts

under conditions promoting high rates of superoxide generation by the electron transport chain (ETC), i.e., under non-phosphorylating conditions in the presence of glutamate and succinate (Figure 18). Moreover, mitochondrial calcium retention capacity (CRC) was reduced in Parkin$^{-/-}$ hearts compared to WT hearts, indicating a greater propensity for opening of the PTP (Figure 16). Of note, tissue activity of the citric acid cycle enzyme CS and of complex I, II and IV of the ETC were similar in WT and Parkin$^{-/-}$ hearts (Figure 16). In addition, mitochondrial morphology was not noticeably different with respect to general ultrastructure, area of an individual mitochondrion, and total mitochondrial surface area (Figure 17, 25).

Table 2: Morphometric and LV-function parameters in WT and Parkin$^{-/-}$ mice

	WT (n=6)	Parkin$^{-/-}$ (n=6)
Body weight (g)	28 ± 1	26 ± 1
Heart weight (mg)	176 ± 9	173 ± 5
Heart/body weight (mg/g)	6.4 ± 0.3	6.5 ± 0.2
LV function		
SP (mm Hg)	73 ± 9	61 ± 4
DP (mm Hg)	5.8 ± 0.7	1.1 ± 1.7
dp/dt_{max} (mm Hg.sec^{-1})	4136 ± 282	4580 ± 494
dp/dt_{min} (mm Hg.sec^{-1})	-3882 ± 558	-3903 ± 391
SV (µL)	12.6 ± 0.8	12.9 ± 0.6
Qc (µL/min)	4651 ± 485	5023 ± 272
Ef (%)	59 ± 3	64 ± 6

SP: systolic pressure; DP: diastolic pressure; dp/dt_{max}: maximal rate of pressure development; dp/dt_{min}: maximal rate of relaxation; SV: stroke volume; Qc: cardiac output; EF: ejection fraction.

Figure 16: Baseline mitochondrial function in permeabilized endocardial fibers from WT and Parkin$^{-/-}$ mice

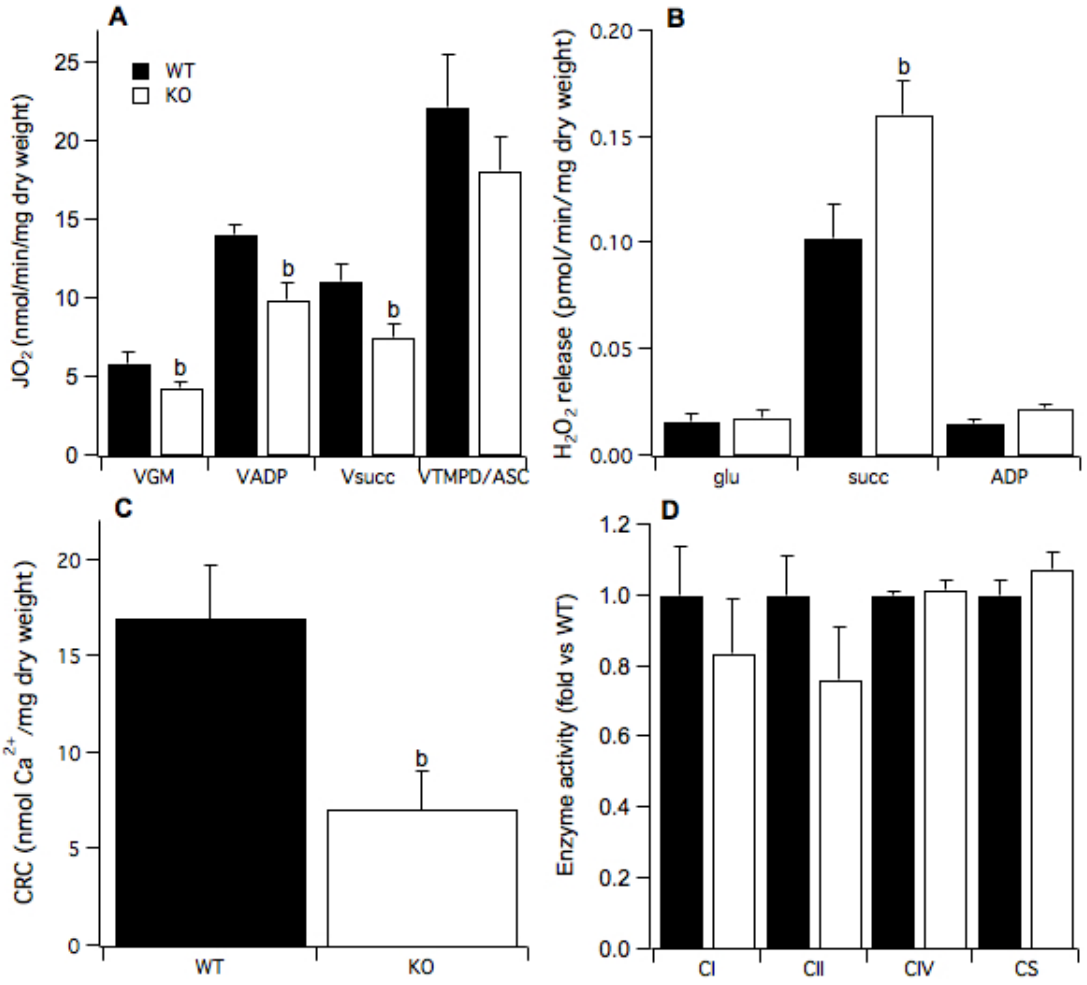

Figure 16: Baseline mitochondrial function in permeabilized endocardial fibers from WT and Parkin$^{-/-}$ mice. Panel A: Respiratory rates measured following sequential additions of glutamate-malate (V_{gm}:5:2.5 mM), ADP (V_{ADP}:2 mM), amytal (not shown: 2mM), succinate (V_{succ}: 5 mM), antimycin A (not shown:8 µM) and TMPD-ascorbate (0.9:9.0 mM). Panel B: Net rate of H_2O_2 release by the mitochondrial electron transport chain measured following sequential addition of glutamate (5 mM), succinate (5 mM) and ADP (1 mM). Panel C: Total amount of Ca^{2+} accumulated by mitochondria prior to PTP opening, during Ca^{2+} challenge experiments. Panel D: Activity levels of complex I, complex II, complex IV and citrate synthase (CS) in cardiac homogenates. All mitochondrial functions expressed per mg of dry fiber weight. Enzyme activities measured in mU/min/mg protein, expressed as a percentage of values observed in WT mouse hearts. All experiments performed in parallel in one WT and one Parkin$^{-/-}$ heart per day (*n*= 6-8hearts per experimental group). b: significantly different from WT within same experimental conditions ($P<0.05$).

Figure 17: Baseline mitochondrial morphology of hearts from WT and Parkin$^{-/-}$ mice

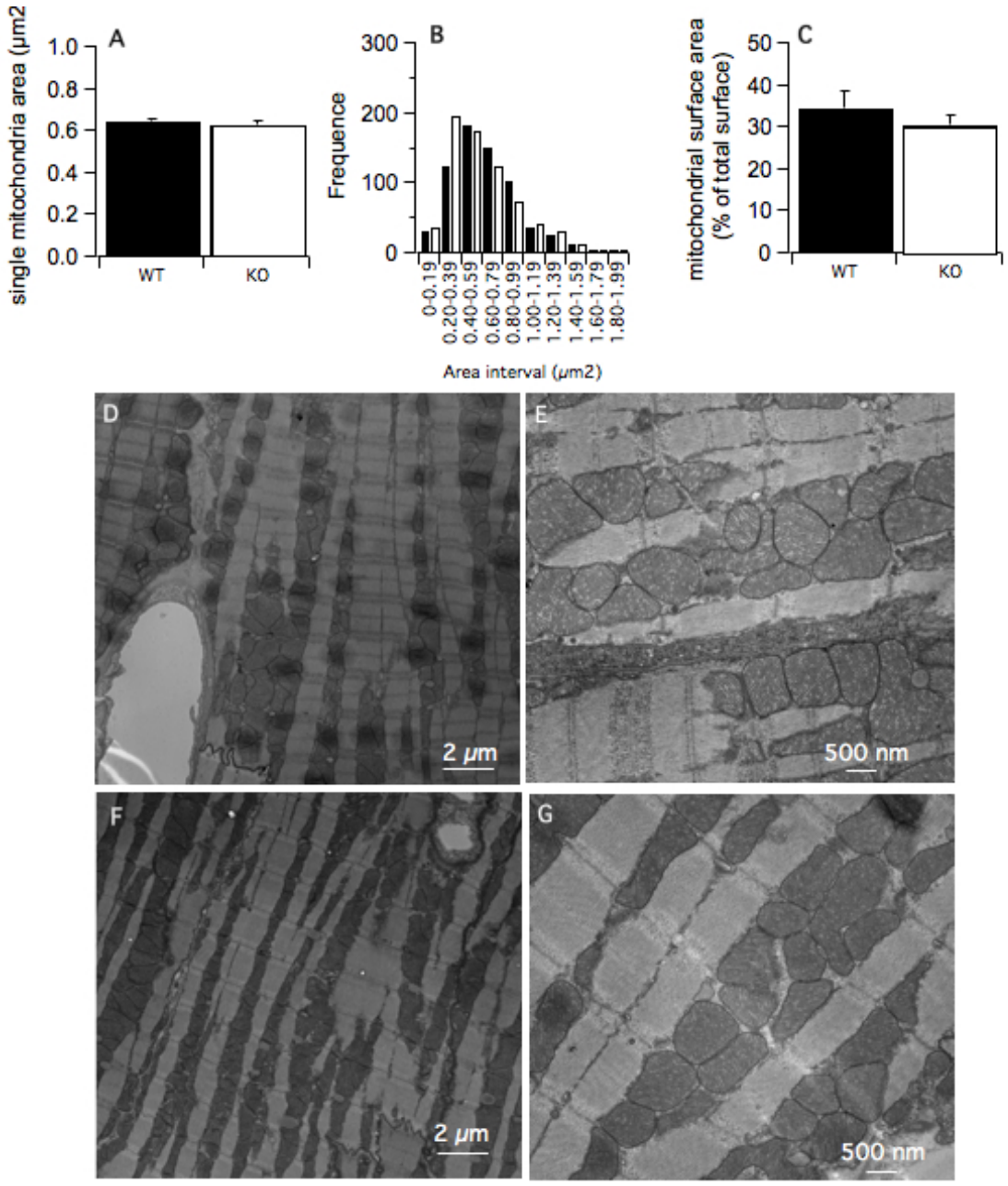

Figure 17: Baseline mitochondrial morphology of hearts from WT and Parkin$^{-/-}$ mice. Mean area (Panel A)and area distribution frequence (Panel B) of individual mitochondria in WT and Parkin$^{-/-}$ hearts. Five adjacent fields per myocyte from five different hearts per experimental group at 4600X. All mitochondria within each field included, for a total of 685 and 721 individual organelles fromWT and Parkin$^{-/-}$ hearts, respectively. Panel C: Percentage of cell surface occupied by mitochondria. Values obtained by expressing total surface area of mitochondria by total cell surface in each of above-mentioned fields. Panels D-G: Representative longitudinal sections of WT (D-E) and Parkin$^{-/-}$(F-G) hearts at 2700Xand 6500X.

3.3.3.2 Parkin is involved in recovery of cardiac and mitochondrial function following sepsis:

Given that there was no distinct cardiac phenotype at baseline, we sought to determine whether Parkin deficiency would promote cardiac and mitochondrial dysfunction in response to LPS-induced sepsis. An intraventricular Millar microcatheter was used to assess LV-performance 12 and 48 hrs after injecting WT and Parkin$^{-/-}$ mice with 5 mg/kg LPS. As shown in Figure 20, cardiac output and stroke volume were decreased by 50-60% in both mouse lines at 12 hrs. In WT mice, this was followed by complete recovery after 48 hrs. In Parkin$^{-/-}$ mice, however, cardiac output and stroke volume failed to recover. Transcript levels of brain natriuretic peptide *Nppb* (BNP) were upregulated in response to LPS, but this was more pronounced in Parkin$^{-/-}$ than in WT hearts, while the expression of atrial natriuretic peptide *Nppa* (ANP), another stress-responsive gene, was significantly greater in Parkin$^{-/-}$ hearts at baseline (Figure 18). Mortality within the 48h time period of the study was not significantly different between WT and Parkin$^{-/-}$ mice. However, no survival studies were performed beyond this time.

We then investigated whether Parkin deficiency would exacerbate LPS-induced mitochondrial dysfunction. As shown in Figure 21, the effect of LPS on the activity of mitochondrial enzymes differed markedly between WT and Parkin$^{-/-}$ hearts. In WT hearts, the activity of Complex I was transiently reduced 12 hrs after LPS injection and recovered to baseline level 48 hrs later. A similar trend was observed for the activity of Complex II, although differences did not reach statistical significance. In contrast to these observations, in Parkin$^{-/-}$ hearts, the activities of Complex I and Complex II decreased constantly over time, reaching ~50% of residual activity 48 hrs post-injection. Importantly, this differential pattern of change across mouse strains was also observed at the level of mitochondrial respiratory function (Figure 21). In WT hearts, oxidative phosphorylation capacity measured in the presence of substrates feeding Complex I, Complex II and Complex IV were all reduced 12 hrs after LPS injection, compared to baseline, but recovered completely after 48 hrs. In contrast, in Parkin$^{-/-}$ hearts, oxidative phosphorylation

capacity measured in the presence of substrates feeding Complex I and Complex IV degraded constantly over time, while Complex II-driven respiration was unaffected.

In addition to causing respiratory impairments, LPS administration also enhanced mitochondrial H_2O_2 release from muscle fibers of WT hearts, both 12 and 48 hrs after LPS injection (Figure 21). This is consistent with the suggested pro-oxidant effect of LPS-induced sepsis in mitochondria (18-20). However, no differences in mitochondrial H_2O_2 release were observed between LPS-treated WT and Parkin$^{-/-}$ mice. In WT hearts, LPS caused a transient reduction in mitochondrial CRC at 12 hrs, indicative of enhanced susceptibility to PTP opening. This was followed by recovery of CRC to baseline values at 48 hrs. However, in Parkin$^{-/-}$ hearts, CRC remained consistently low and close to baseline values following LPS treatment (Figure 21). Overall, these data suggest that Parkin is important for full recovery of cardiac and mitochondrial metabolic functions in response to sub-lethal sepsis.

Figure 18: Baseline cardiac function of hearts from WT and Parkin$^{-/-}$ mice and in response to LPS.

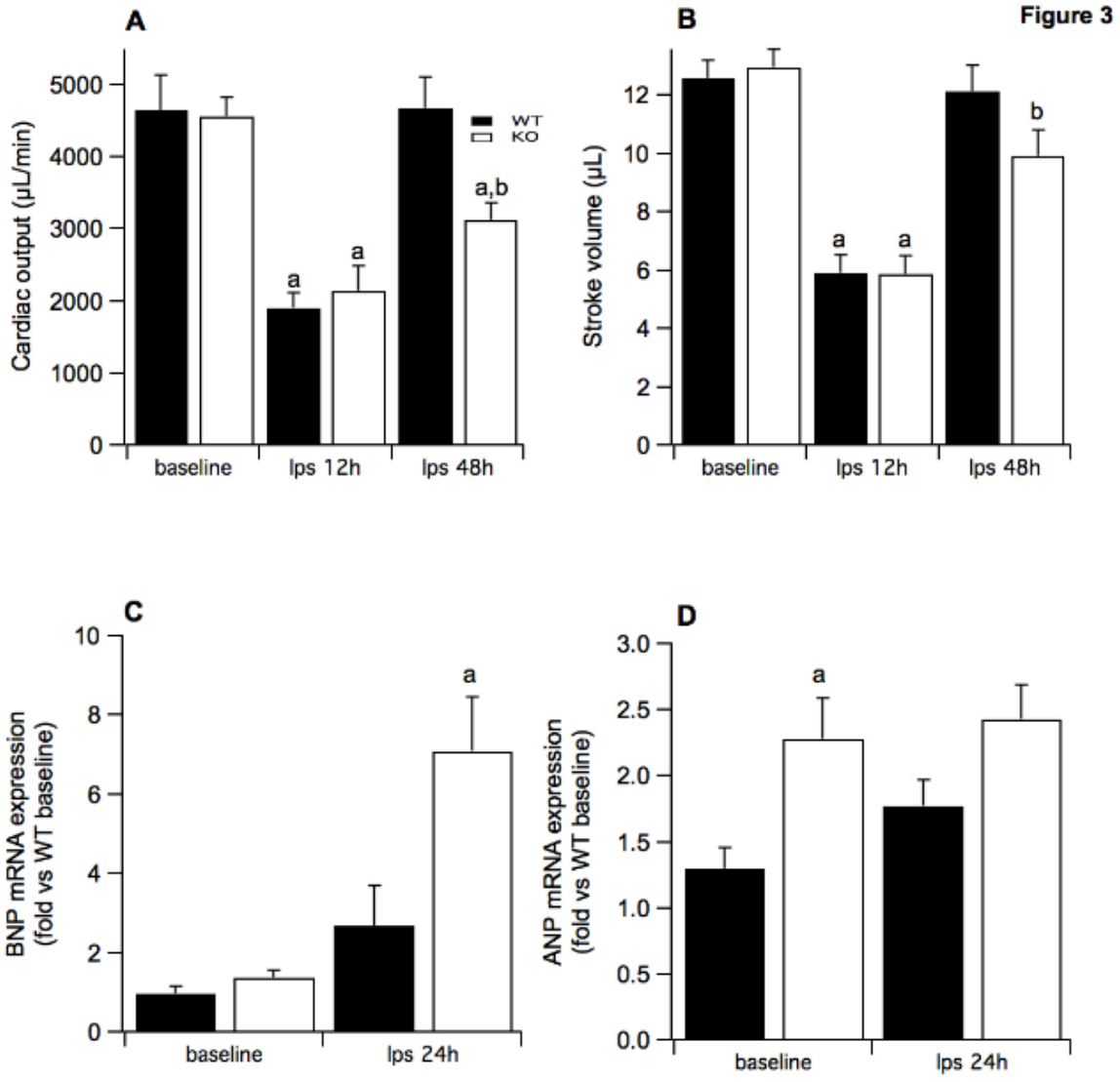

Figure 18: Baseline cardiac function of hearts from WT and Parkin$^{-/-}$ mice and in response to LPS. . Cardiac output (Panel A) and stroke volume (Panel B) of anesthetized mice (*n*=5-6 per experimental group and condition) measured using an intraventricular pressure-volume (PV) conductance catheter. mRNA expression of BNP and ANP at baseline and 24hrs after LPS injection (*n*=5-6 per experimental group and condition) a: significantly different from baseline within same experimental group (P<0.05). b: significantly different from WT within same experimental condition (P<0.05).

Figure 19: Effect of LPS on mitochondrial function in hearts from WT and Parkin$^{-/-}$ mice.

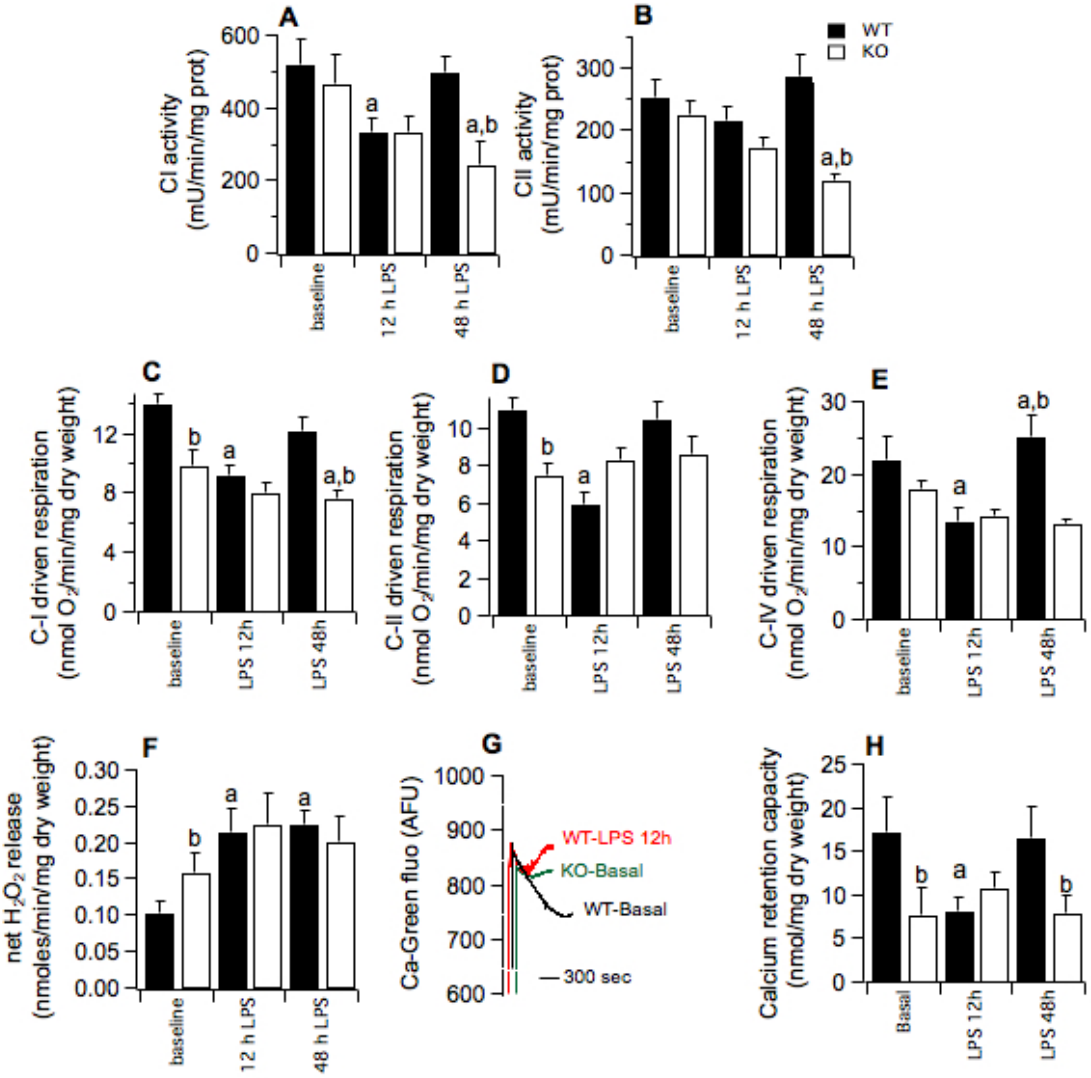

Figure 19: Effect of LPS on mitochondrial function in hearts from WT and Parkin$^{-/-}$ mice.
Panels A-B: Enzyme activities of complex I and complex II at baseline and after LPS injection. Panels C-E: Rates of respiration measured under phosphorylating conditions (2 mM ADP) in presence of substrates for complex I (Panel C, glutamate-malate 5:2.5 mM), complex II (Panel D, succinate 5 mM) and complex IV (Panel E, TMPD-ascorbate 0.9:9.0 mM)**.** Panel F: Net rate of H_2O_2 release by the respiratory chain in presence of glutamate and succinate (5:5mM). Panel G: Representative calcium-green 5N traces showing changes in fluorescence (AFU: arbitrary fluorescence units) as a result of mitochondrial Ca^{2+} uptake and PTP-induced Ca^{2+}release. For clarity, tracings shown for selected experimental conditions only. Panel H: Total amount of Ca^{2+} accumulated by mitochondria prior to PTP opening. All mitochondrial functions and enzyme activities measured in set of hearts. A total of 6-8 hearts used in each group and experimental condition. a: significantly different from baseline within same experimental group ($P<0.05$). b: significantly different from WT within same experimental condition ($P<0.05$).

3.3.3.3 Parkin deficiency does not impair mitophagy and enhances macro-autophagy in the heart:

To investigate the underlying mechanisms of myocardial abnormalities in Parkin$^{-/-}$ mice, we first assessed the effect of sepsis on Parkin cellular localization. As shown in Figure 22, Parkin recruitment to mitochondria was strongly upregulated after injection of LPS into WT mice, suggesting an important role for Parkin in mitochondrial QC during sepsis. We then determined whether the absence of Parkin impaired mitochondrial QC by altering the initial steps involved in the sequestration of mitochondria into autophagosomes. For this, mitochondrial fractions were probed with an antibody directed against polyubiquitinated proteins. As shown in Figure 21, polyubiquitinated protein levels in the mitochondrial fraction were increased at 12 hrs and returned toward baseline values 48 hrs after LPS injection. LPS also induced recruitment of the ubiquitin- and LC3-binding protein P62/SQSTM1 and LC3-II to the mitochondrial fraction, indicating that sepsis activated mitochondrial recycling through autophagic degradation. NIX and BNIP3, two proteins involved in triggering mitophagy, were also recruited to the mitochondrial fraction in response to LPS. However, no differences in the recruitment of any of these proteins were observed between WT and Parkin$^{-/-}$ hearts. Moreover, at baseline, the degree of ubiquitination of mitochondrial proteins, and the amount of LC3-II and BNIP3 were all significantly higher in *Parkin*$^{-/-}$ hearts compared to WT. Together, these observations suggested that targeting of mitochondria for degradation can still occur during sepsis in the absence of Parkin, likely through as yet unclear compensatory mechanisms. This was further confirmed by the presence of several autophagosomes containing only mitochondria in both WT and Parkin$^{-/-}$ hearts (Figure 20). Of note, although these morphological and signalling evidences of enhanced mitophagy were clearly visible in the heart following LPS administration, this did not result in significant changes in the whole mitochondrial biomass of the heart, as assessed by the activity of citrate synthase, a recognized mitochondrial marker (Figure 23).

Because TEM images suggested an increased abundance of autophagosome-like structures in the hearts of Parkin$^{-/-}$ mice, we investigated whether macro-autophagy was enhanced. As shown in Figure 22, mRNA levels for *Lc3b*, *Gabarap* and *Bnip3* were all higher in Parkin$^{-/-}$ hearts under baseline conditions, and myocardial autophagic flux, measured as the amount of LC3b-II that accumulated after autophagosome-lysosome fusion was blocked, was ~40% greater in Parkin$^{-/-}$ hearts compared to WT hearts. Furthermore, in response to LPS injection, myocardial autophagy flux increased 3.7-fold in WT hearts and 4.5-fold in Parkin$^{-/-}$ hearts. Comparison of TEM images obtained 48 hrs after LPS injection revealed the presence of relatively greater numbers of autophagosomes in Parkin$^{-/-}$ hearts compared to their WT counterparts (Figure 22). Several very large autophagosomes containing mitochondrial remnants, together with lipid droplets and damaged cellular material, were often seen in Parkin-/- hearts, but not in those of WT hearts (Figure 22). Together, these data indicate that enhanced macro-autophagy may represent an attempt to compensate for a lack of Parkin.

3.3.3.4 Parkin deficiency does not affect mitochondrial biogenesis signalling and OPA1 levels in response to sepsis:

In the heart, sepsis has been previously shown to induce mtDNA deletion and alter mitochondrial biogenesis signaling (13, 16, 18) Since Parkin has been recently implicated in the positive modulation of PGC1α expression (8) and the maintenance of mtDNA integrity (9), we investigated whether Parkin deficiency results in increased impairment of mitochondrial biogenesis signalling. As shown in Figure 23, baseline mRNA expressions of the major transcriptional factors and co-activators of mitochondrial biogenesis, as well as nuclear and mtDNA-encoded downstream target genes, were similar in WT and Parkin$^{-/-}$ hearts. Although expressions of *Pgc1*β, *Pgc1*α, and *Tfam* were depressed 24 hrs after LPS administration, this effect was no more pronounced in Parkin$^{-/-}$ hearts than in WT hearts. Furthermore, expressions of mtDNA encoded respiratory chain subunits were not significantly altered following

LPS treatment, suggesting that overt damage to mitochondrial DNA as a result of sepsis and/or Parkin deficiency is minimal.

Finally, Parkin was recently suggested to protect mitochondria by activating the NF-kB pro-survival pathway through linear ubiquitination of NF-kB essential modulator (NEMO), resulting in increased OPA1 expression, and maintenance of mitochondrial integrity (10). For this reason, OPA1 expression was measured in hearts of WT and Parkin$^{-/-}$ hearts. As shown in Figure 23, OPA1 expression was only slightly up regulated in response to the administration of LPS, and no differences were observed across genotypes.

Figure 20: Morphological evidence of mitophagy in hearts from WT and Parkin$^{-/-}$ mice in response to LPS.

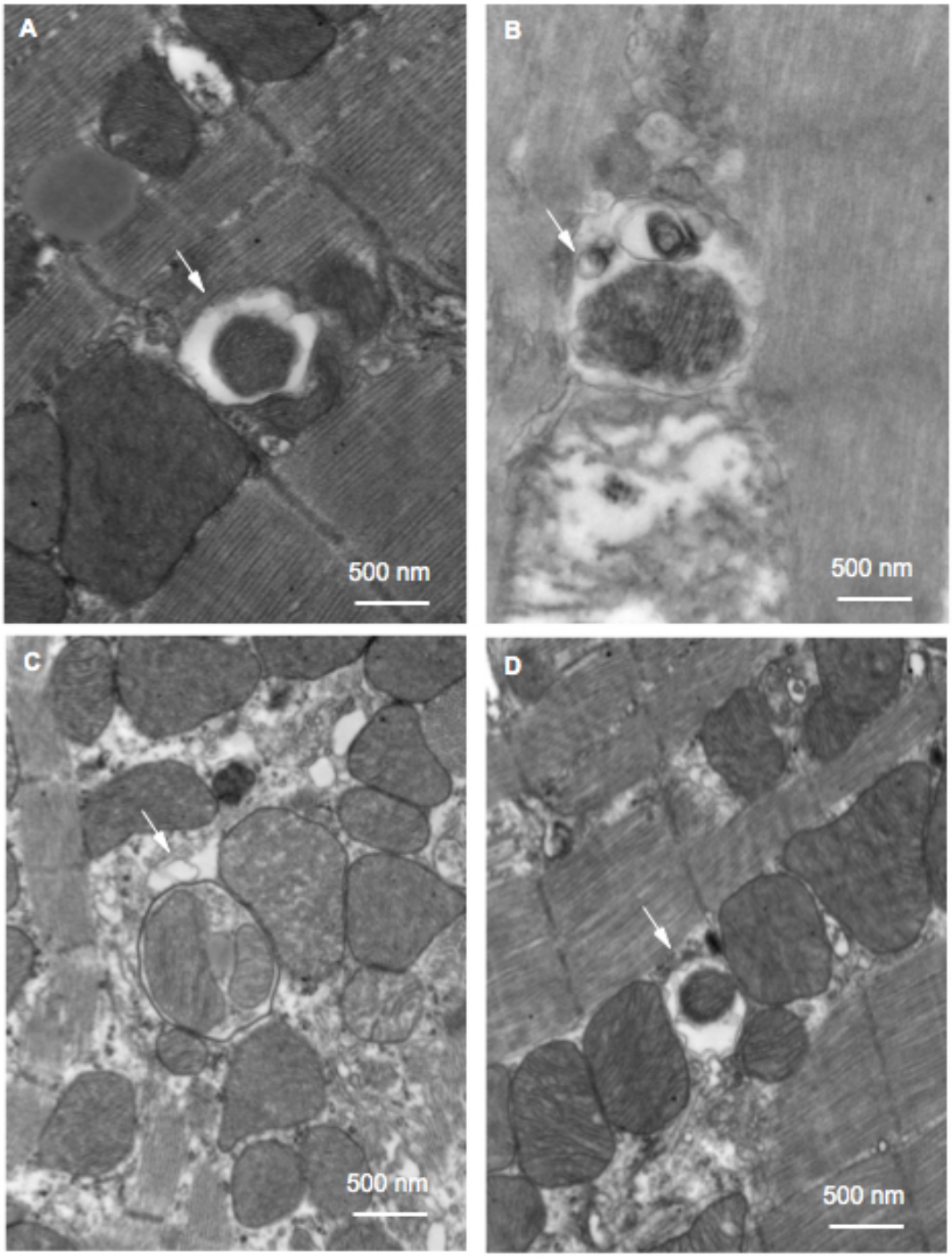

Figure 20: Morphological evidence of mitophagy in hearts from WT and Parkin$^{-/-}$ mice in response to LPS. Representative electron micrographs at 25000X of WT (Panels A-B) and Parkin$^{-/-}$ (Panel C-D) hearts showing autophagosomes containing only mitochondria (indicated by arrows).

Figure 21: Mitophagy signaling in cardiac mitochondria from WT and Parkin$^{-/-}$ mice

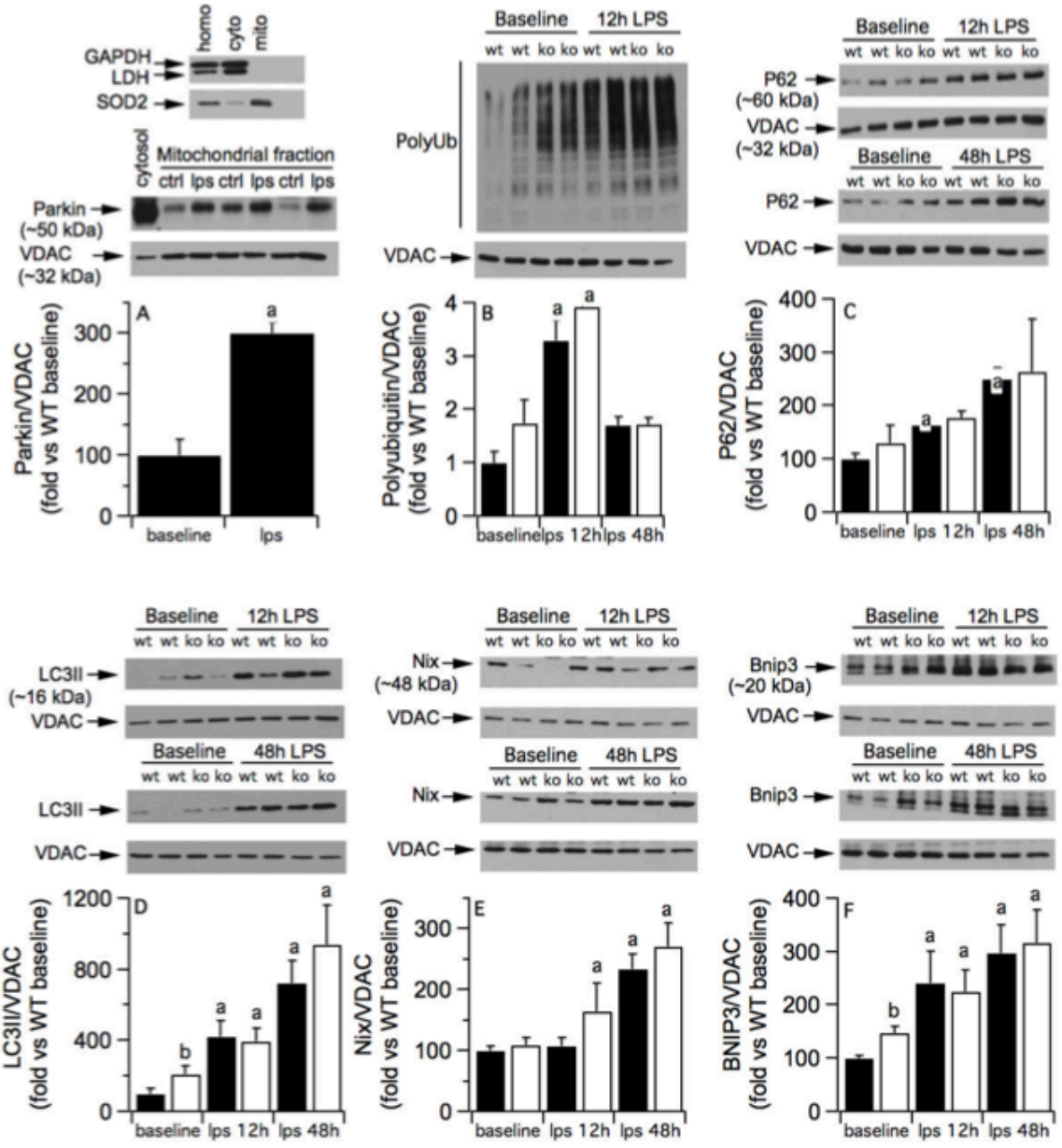

Figure 21: Mitophagy signaling in cardiac mitochondria from WT and Parkin$^{-/-}$ mice. Results of immunoblot experiments performed in mitochondrial fractions isolated from mouse hearts at baseline and 12-48 h after administration of LPS. *n*=6-10 per experimental group and condition. Representative blot and quantitative analysis shown for each. Protein content normalized to loading control VDAC and expressed as fold change relative to baseline values obtained from WT hearts. Panel A shows amount of Parkin in mitochondrial fraction 24h after injection of saline or LPS into WT hearts. An equal amount of cytosolic extract was loaded into the first well to show predominant localization of Parkin in cytosolic fraction. Other panels show time-dependent changes in content of polyubiquitinated proteins (Panel B), p62 (Panel C), Lc3b-II (Panel D), Nix (Panel E), and Bnip3 (Panel F). a: significantly different from baseline within same experimental group ($P<0.05$). b: significantly different from WT within same experimental condition ($P<0.05$).

Figure 22: Effects of LPS and Parkin-deficiency on autophagy.

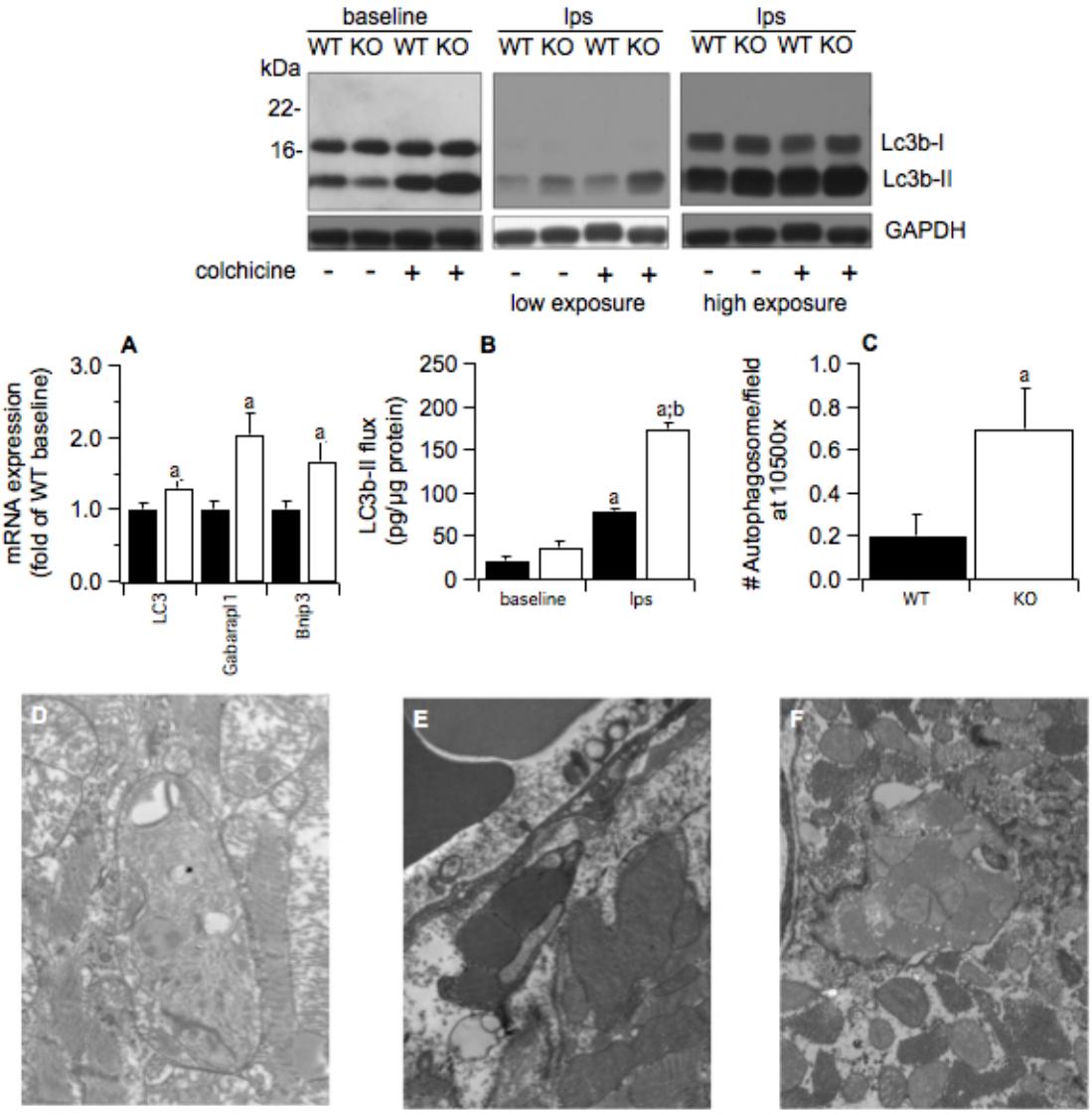

Figure 22: Effects of LPS and Parkin-deficiency on autophagy. Panel A: Baseline mRNA expression levels of selected autophagy-related genes in wild-type and Parkin $^{-/-}$ hearts (*n*=6per group). Panel B: Autophagy flux as measured by accumulation of Lc3b-II following *in vivo* colchicine-induced inhibition of autophagosome-lysosome fusion (*n*=6per group and condition). Representative Lc3b blots are shown at baseline and after LPS injection in both mouse strains in absence or presence of colchicine. Panel C: Number of autophagosomes per field of view in viable cardiomyocytes from WT and Parkin$^{-/-}$ hearts 48 hrs after LPS injection. Panels D-F: Electron micrographs showing presence of very large autophagosomes in Parkin$^{-/-}$hearts 48hrs after LPS injection. Autophagosomes of comparable size not found in WT hearts. a: significantly different from baseline within same experimental group (P<0.05). b: significantly different from WT within same experimental condition (P<0.05). Black bars: WT and white bars Parkin -/-.

Figure 23: Effects of LPS and Parkin-deficiency on mitochondrial biogenesis.

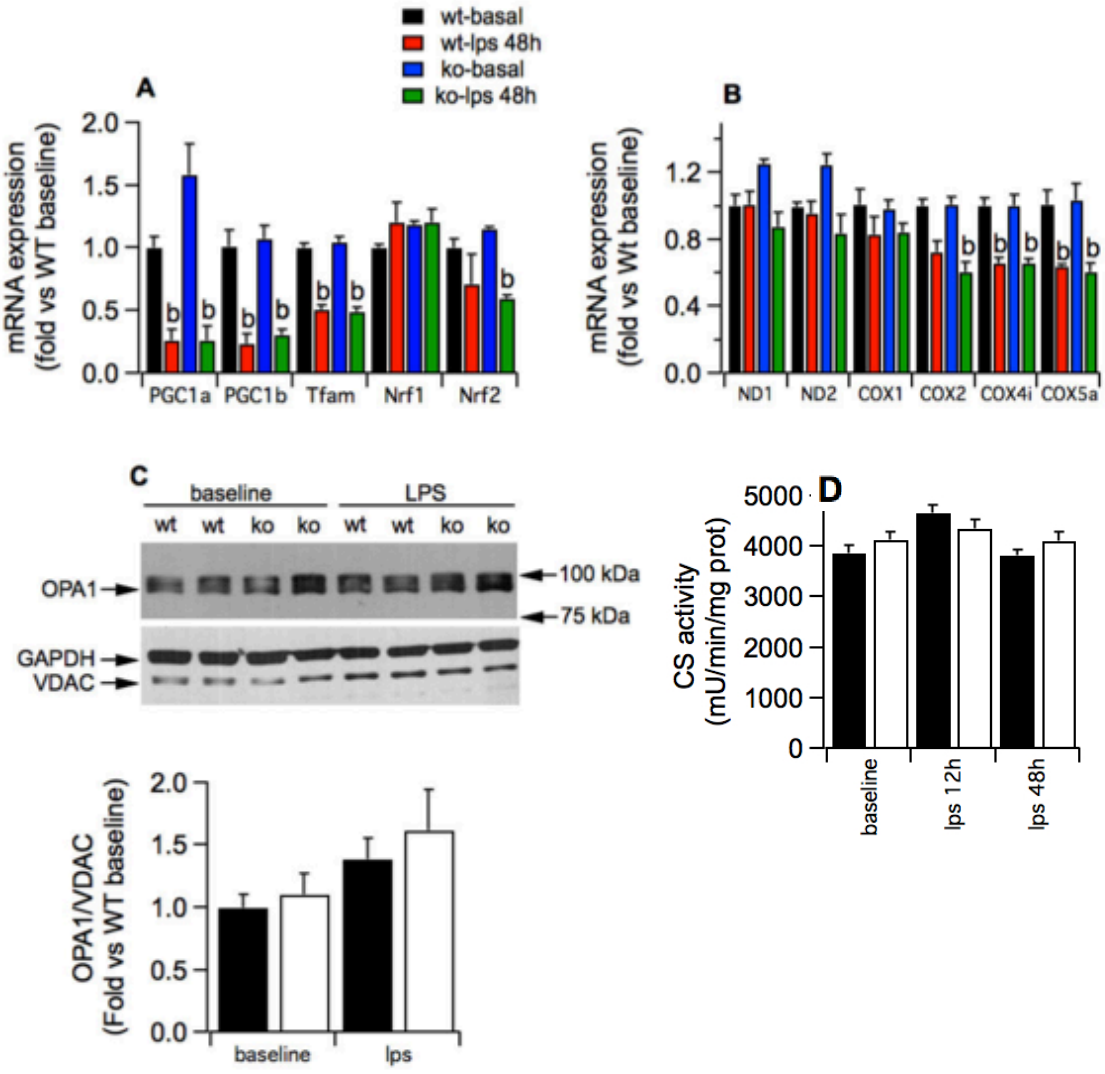

Figure 23: Effects of LPS and Parkin-deficiency on mitochondrial biogenesis. Panel A: mRNA expression levels of key transcriptional factors and co-activators of mitochondrial biogenesis WT and Parkin$^{-/-}$ hearts at baseline and 48hrs after LPS injection. Panel B: mRNA expression levels of complex I (*Nd*) and complex IV (*Cox*) subunits encoded in nuclear (*Cox4i*, *Cox5a*) or mtDNA (*Nd1*, *Nd2*, *Cox1*, *Cox2*). Panel C: Expression of OPA1 in hearts of WT and Parkin$^{-/-}$ mice at baseline and 24hrs after administration of LPS. Panel D: Whole tissue activity of mitochondrial marker citrate synthase (n=5-6 animals per group). Expression is normalized against the mitochondrial loading control VDAC. b: significantly different from WT within same experimental condition ($P<0.05$). Black bars: WT and white bars Parkin -/-.

3.3.4 DISCUSSION

Mitochondrial QC has been increasingly implicated as an important factor in the maintenance of cellular homeostasis under physiological and pathological conditions (1, 2, 4), although the role of this process in the heart remains largely undefined. In the present study, we report several novel observations relating to mitophagy and autophagy in the cardiac response to sepsis and on the role of the E3-ligase Parkin in mitochondrial QC in the heart. We show that mitochondrial dysfunction induced by LPS administration potently stimulates cardiac mitophagy. The effect of this, most likely, is to facilitate recovery from sepsis-induced mitochondrial and cardiac dysfunction. This observation is important since a strong association between the severity of mitochondrial abnormalities and increased mortality has been reported in patients with sepsis (21). We also demonstrate that although Parkin deficiency impairs mitochondrial and cardiac recovery following sepsis, autophagic clearance of damaged mitochondria is still possible, likely due to as yet unidentified compensatory mechanisms. We speculate that these mechanisms may include Parkin-independent mitophagy and upregulation of the process of autophagosome formation. Redundant compensatory mechanisms such as these, besides attesting to the importance of mitochondrial QC for heart survival under stress, likely explain why Parkin deficiency only results in mild alterations of mitochondrial function in the heart under baseline conditions.

3.3.4.1 Sepsis-induced mitochondrial dysfunction and QC:

Although several factors contribute to the pathogenesis of cardiac dysfunction during sepsis, mitochondrial dysfunction is commonly recognized as an important contributor (21-23). During the acute phase of sepsis, dysfunction occurs in response to LPS-mediated activation of toll-like receptor 4 (TLR4), which in turn, activates the inflammatory processes (24, 25). This study and previously published reports (15, 16, 26, 27) confirm that LPS injection triggers significant inhibition of mitochondrial respiration, attenuation of the activity of ETC complexes, a rise in the generation of reactive oxygen species and the development of oxidative stress

(Figure 21). While these changes primarily result in metabolic depression, a condition termed cytopathic hypoxia (22, 23) persistent inhibition of the respiratory chain and oxidative damage also results in increased vulnerability of mitochondria to irreversible permeability transition. This is indicated by the significant reduction in the threshold for Ca^{2+}-induced PTP opening that was observed in WT hearts 12 hrs after LPS injection (Figure 21) and the presence of swollen mitochondria in the hearts of LPS-treated mice (Figure 22 and 24).

Opening of the PTP is likely an important determinant of the severity and reversibility of cardiac dysfunction during sepsis. Indeed, if permeability transition remains circumscribed to a few mitochondria within a cardiomyocyte, its viability may not be compromised. However, spreading of permeability transition to a significant proportion of the mitochondrial network will clearly trigger activation of the internal caspase pathway (Caspase 9). The eventual activation of effector caspase 3, in addition to possibly causing apoptosis and necrosis (28) is apparently responsible for proteolytic digestion of the contractile apparatus (29) during septic myocardial depression.

While progressive attenuation of TLR4-mediated inflammatory responses following exposure to LPS is a determinant of recovery of cardiac and mitochondrial function (24), stimulation in host cells of pro-survival mechanisms that limit accumulation of life threatening mitochondrial dysfunctions are also likely to play a key role. In this regard, previous studies by our group (30) and others (31) have reported that activation of macro-autophagy occurs in myocytes in response to sepsis and is required to protect the heart and skeletal muscles from excessive damage [31]. In the present study, we also found evidence of increased autophagy in septic cardiac tissue, as indicated by increased autophagy flux and the presence of autophagosomes in the hearts of WT and Parkin$^{-/-}$ mice 24-48 hrs after LPS injection (Figure 22). While this process is stimulated by several signals that are not strictly related to mitochondrial dysfunction, damaged mitochondria can nevertheless be

sequestered by the autophagosomes and delivered to lysosomes along with other cellular constituents (32-33)

In addition to this process, we now demonstrate for the first time that mitophagy is potently induced during sepsis. This is evidenced by: 1) the presence in the septic heart of several double membrane autophagosomes containing only mitochondria (Figure 22); 2) increased abundance of LC3b-II and the polyubiquitin binding protein P62/SQSTM1 in the mitochondrial fraction; and 3) mitochondrial recruitment of many important proteins purported to be involved in triggering mitophagy, such as Parkin, NIX and BNIP3 (Figure 23). Our data thus provide evidence that in addition to macro-autophagy, the more selective process of mitophagy is also triggered during sepsis. This mechanism, by specifically promoting the removal of damaged organelles, might have contributed to the recovery of mitochondrial and cardiac function that was observed following administration of LPS. A recent study has suggested that mitophagy may also be important for ischemic preconditioning by allowing the removal of frail organelles that would otherwise trigger cell death (34). Activation of mitophagy has also been recently described in the heart following myocardial infarction, specifically in the border zone, in cardiomyocytes that were exposed to sub-lethal stress (35). Collectively, these studies provide growing support for the important role that mitophagy plays in the cardiac response to stress.

It should be noted that despite morphological and signalling evidence of increased mitophagy in the heart of LPS-treated mice, whole tissue activity/content of CS and VDAC did not significantly differ from that observed in vehicle-treated mice. One plausible explanation is that these indices of tissue mitochondrial biomass are not sensitive enough to detect changes in mitophagy, which arguably affects a small proportion of the total mitochondrial pool of the heart. This condition differs drastically from cell culture studies in which 100% of the mitochondrial pool within cells can disappear 24h after depolarization with CCCP (36).

Upregulation of the transcriptional co-activator PGC1 α has been reported in animal models of sepsis (16, 27), and has led to the suggestion that stimulation of mitochondrial biogenesis, and perhaps of the expression of several anti-oxidant genes regulated by PGCα, constitutes another compensatory response to mitochondrial dysfunction (22). Support in favour of this hypothesis has been obtained in a study using human tissue that showed that, following admission to ICU, survival from critical illness is associated with early activation of mitochondrial biogenesis (22). Furthermore, there are several reports of significant reductions of key regulators of mitochondrial biogenesis in response to LPS (25, 30). In line with these latter results, our data show that mRNA expressions of *Pgc1*α, *Pgc1*β and *Tfam* are severely attenuated in the hearts of WT mice 24 hrs after LPS injection (Figure 25), suggesting that in our model of sepsis, enhanced mitochondrial biogenesis signaling does not play a major role in the recovery of mitochondrial functions. In fact, recovery of mitochondrial function following sepsis is more likely to depend on a complex balance between prevailing levels of mitochondrial stress, biogenesis signaling and mitochondrial QC, rather than on a single factor. Future studies are required to shed further light on this important interaction.

3.3.4.2 Mechanisms of autophagic mitochondrial clearance during sepsis:

Several studies have shown that the Pink1-Parkin pathway plays a central role in the regulation of mitophagy (7, 36). According to the prevailing model, derived largely from cell culture experiments, loss of membrane potential (ψ) in defective mitochondria leads to selective recruitment of Parkin and subsequent ubiquitination of outer membrane proteins such as MFN2 and VDAC (5, 37). This process, which requires the mitochondrial kinase PINK1, promotes, through as yet unknown mechanisms, the incorporation of defective organelles into double membrane autophagosomes that are delivered to lysosomes for recycling. While this model has been firmly established under conditions where mitochondrial ψ has been artificially depolarized with the agent CCCP, the role of the Parkin pathway and how it integrates with other mechanisms of mitophagy in the *in vivo* heart remains largely unknown.

In the present study, we observed that Parkin was potently recruited to mitochondria 24 hrs after administration of LPS to WT mice. Loss of ψ in response to sepsis might have occurred as a result of sudden opening of the PTP, which is a common physiological cause of depolarization. This would be consistent with the presence of swollen mitochondria in the hearts of LPS-treated mice (Figure 22 and 24) and the significant increase in susceptibility to Ca^{2+}-induced PTP opening observed in permeabilized fibers 12 hrs after LPS injection (Figure 21). It is also consistent with previous cell culture studies that have shown that forced induction of PTP opening in subsets of mitochondria directly triggers their autophagic removal (38, 39). In addition to PTP opening, sepsis-induced inhibition of ETC complexes (Figure 21) might also lower ψ and thus promote Parkin recruitment. However, it is currently unclear as to whether partial depolarization is a sufficient trigger for Parkin recruitment. Altogether though, our data support the notion that in normal mice sepsis-induced mitochondrial dysfunction triggers Parkin recruitment and that this likely contributes to the activation of mitophagy.

Another important observation in our study is that although Parkin translocates to mitochondria in response to sepsis, its ablation does not overtly compromise the activation of mitophagy. Indeed, autophagosomes containing mitochondria alone were clearly visible in the hearts of Parkin$^{-/-}$ mice after LPS injection (Figure 23). Moreover, immunoblot analysis of mitochondrial fractions purified from WT and Parkin$^{-/-}$ hearts showed no differences in recruitment of the autophagosome membrane marker LC3-II to mitochondria (Figure 23). In fact, at baseline, the presence of LC3-II to mitochondria was even higher in Parkin$^{-/-}$ hearts than in WT. These data thus suggest that multiple mechanisms are involved in triggering mitophagy in the septic heart and that one or several of these mechanisms can compensate for the ablation of Parkin at the germline level. In this regard, we observed that at baseline, the degree of ubiquitination of mitochondrial proteins was greater in Parkin$^{-/-}$ hearts than in WT hearts, while in response to LPS, ubiquitination of mitochondrial proteins was similar across genotypes. Therefore, one likely mechanism that may compensate for the lack of Parkin may involve

alternate E3 ligases, as previously suggested by Ding *et al.* (40). Candidates maybe RNF-185 and GP78, which in cell culture models have recently been shown to regulate mitophagy though ubiquitin-dependent mitochondrial outer membrane interactions (41, 42).

Mitophagy can also be achieved via an ubiquitin-independent pathway involving direct binding of ATG8 family proteins to autophagy receptors on the mitochondria. In yeast, ubiquitin-independent mitophagy is regulated by a single pathway that requires the mitochondrial autophagy receptor ATG32 (43,44). ATG32 is a mitochondrial outer membrane protein that interacts directly with ATG8 on its LC3-interacting region (43, 44). In mammalian cells, NIX and BNIP3 have been identified as mitochondrial autophagy receptors (45). Indeed, both proteins target mitochondria for autophagy by directly binding to the ATG8 homologue GABARAP on the autophagosome via their conserved LC3-interacting region motifs (43, 44). In the present study, we found that in response to LPS administration NIX and BNIP3 are recruited to mitochondria in both mouse strains in a time-dependent manner (Figure 23). Moreover, at baseline, BNIP3 levels in the mitochondrial fraction (Figure 23) and the transcript levels of *Gabarap* (Figure 24) are greater in Parkin$^{-/-}$ mouse hearts. Therefore, recruitment of autophagosomes to mitochondria through direct interactions between NIX/BNIP3, GABARAP and LC3b-II may compensate for the absence of Parkin-dependent mitophagy in Parkin$^{-/-}$ mice. However, this compensation may only be partial, since mitochondrial NIX and BNIP3 also activate mitophagy by promoting the recruitment of Parkin (39, 45), which does not occur in Parkin$^{-/-}$ mice.

We also found that Parkin deficiency is associated with significant upregulation of macro- autophagy, which has not been reported previously. Indeed, baseline transcript levels of major autophagy-regulating genes are increased in Parkin$^{-/-}$ mice hearts as compared to those of their WT counterparts, and this is associated with increased autophagy flux, both at baseline and following LPS injection (Figure 24). Morphological evidence obtained from LPS-treated mouse

hearts shows a greater number of autophagosomes in Parkin$^{-/-}$ hearts compared to controls. Furthermore, very large autophagosomes containing mitochondrial remnants, together with lipid droplets and damaged cellular material, were often seen in the hearts of Parkin$^{-/-}$ mice, while none were observed in the hearts of WT mice (Figure 24). While the underlying mechanisms remain to be defined, these results suggest that macro-autophagy is enhanced as a result of Parkin ablation, which is likely to contribute to the clearance of damaged organelles, albeit in a less specific fashion.

One issue that currently remains unclear relates to the reasons why Parkin$^{-/-}$ hearts display baseline mitochondrial dysfunctions and impaired responses to sepsis despite the presence of these compensatory mechanisms. A plausible explanation is that compensation for a lack of Parkin-dependent mitophagy is only partial and/or is associated with deleterious "side effects". For instance mitochondrial NIX and BNIP3, in addition to directly regulating mitophagy (*i.e.* through direct binding to LC3b) (43, 44) and via Parkin-recruitment (40,46), are also known to trigger mitochondrial membrane permeabilization and cell death (reviewed in 47). In view of this dual role, recruitment of NIX and BNIP3 to mitochondria in the absence of Parkin may result in less effective stimulation of mitophagy and greater mitochondrial dysfunction, ultimately impairing cardiac recovery from sepsis. In addition, it is increasingly appreciated that excessive stimulation of autophagy can be damaging (1). This has been well demonstrated in skeletal muscle (48). Moreover, recent reports have indicated that excessive autophagy is involved in doxorubicin- induced cardiotoxicity (49-51). Therefore, overactivation of macro-autophagy in Parkin-deficient mice during sepsis may turn out to be detrimental to the heart. Finally, it should be acknowledged that in *Parkin*$^{-/-}$ mice, the presence mild mitochondrial dysfunctions at baseline may predispose the heart to sepsis-induced contractile dysfunction.

A growing number of experimental evidence suggest that Parkin has multiple functions in mitochondria beside mitophagy. Parkin deficiency has been reported to

repress PGC1α-mediated mitochondrial biogenesis, and impair mtDNA repair capacity following oxidative stress in the brain (8, 9). However, in the present study, the impact of sepsis on mitochondrial biogenesis signalling was similar in WT and Parkin$^{-/-}$ hearts. Furthermore, using long-range PCR, we were unable to detect any mtDNA deletions in our experimental conditions (data not shown), ruling out the contribution of mtDNA damage to impaired functional recovery of cardiac mitochondria in septic Parkin$^{-/-}$ mice. Finally, it has been recently suggested that Parkin can activate the NF-kB pro-survival pathway through linear ubiquitination of NF-kB essential modulator (NEMO), resulting in increased OPA1 expression and maintenance of mitochondrial integrity (10). Although in the present study, OPA1 expression was mildly increased in response to LPS injection, this response was not impaired in Parkin$^{-/-}$ hearts (Figure 25)

In summary, we demonstrate that during the early phase of sub-lethal sepsis, mitochondrial dysfunction potently induces selective clearance of damaged organelles through mitophagy, which likely promotes subsequent recovery of mitochondrial and cardiac function. This protective response occurs through mitochondrial recruitment of several proteins that are involved in priming damaged organelles for engulfment by the autophagy machinery, including Parkin, Nix and Bnip3. We also demonstrate that autophagic clearance of damaged mitochondria remains possible in Parkin-deficient mice through Parkin-independent mitophagy and upregulation of macro-autophagy. Nevertheless, Parkin deficiency is still associated with mild mitochondrial dysfunction under baseline conditions and, more importantly, with impaired recovery from sepsis-induced mitochondrial and cardiac dysfunction. This suggests that compensation for a lack of Parkin-dependent mitophagy is only partial. Together, these results support the notions that, in the heart, mitophagy is fundamentally important to the maintenance of mitochondrial function and recovery from sepsis and that redundancy exists in the regulation of these processes *in vivo.*

3.3.5 METHODS

3.3.5.1 Animal care:

Wild type (WT) and Parkin-deficient (Parkin$^{-/-}$) mice (obtained from INSERM-Sanofis-Adventis) (51) were bred and maintained at the Institut de Recherche en Immunologie et en Cancérologie, Université de Montréal (Montréal, QC). All procedures were approved by the animal ethics committees of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care. Twelve to sixteen-weeks-old male Mice were injected i.p. with a single dose of phosphate-buffered saline (PBS, baseline group) or *E. coli* lipopolysaccharide (LPS) (5 mg/kg serotype 055:B5; Sigma-Aldrich). Animals were euthanized by cervical dislocation 12, 24 or 48 hrs after injection, hearts were rapidly excised, weighed and used either immediately or flash frozen and stored at -80^{o}C for later use.

3.3.5.2 Cardiac function:

Mice were anaesthetized with 2% isoflurane, intubated and ventilated. A left thoracotomy was performed and the pericardium was dissected to expose the heart. A pressure-volume (PV) conductance catheter (SPR-839; Millar Instruments) was inserted through a 30-gauge apical stab through the left ventricle (LV) apex and connected to a MVPS Ultra® transducer system (Millar). To generate PV loops, PV signals were recorded at steady state and during transient reduction of cardiac preload, which was achieved by inferior vena cava occlusion. Pressure waveforms were recorded over 30 min at 1kHz using 16-channel data acquisition hardware (EMKA Technologies). Ventilation was momentarily turned off at different time intervals for at least 10 consecutive cardiac cycles to avoid respiratory fluctuation of cardiac signals. Real-time signals were processed with iox2 2.5.1.18 software (EMKA) and heart rate (HR), stroke volume (SV), cardiac output (CO) and ejection fraction (EF) were obtained.

3.3.5.3 Mitochondrial functional assays in permeabilized muscle fibers:

Dissections and permeabilization of fiber bundles with saponin were performed using the left ventricle, as previously described (52). Ghost fibers were prepared by incubating permeabilized bundles in a high KCl medium that allows extraction of myosin. Permeabilized myofibers and ghost fiber bundles were kept on ice until use. All mitochondrial functional parameters were determined at minimum in duplicate and expressed per mg of dry fiber weight.

3.3.5.4 Mitochondrial respiration:

Mitochondrial respiratory function was determined in permeabilized fibers as per Picard *et al*. (52) in solution B (in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA (100nM free Ca^{2+}), 6.56 $MgCl_2$ (1mM free Mg^{2+}), 20 taurine, 0.5 DTT, 50 K-methane sulfonate (160mM ionic strength), 20 imidazole, pH 7.1)) at 23°C under continuous stirring. Rates of O_2 consumption were determined following the sequential addition of: the complex I substrates glutamate-malate (5:2.5mM, V_{GM}); ADP (2mM, V_{ADP}); the complex I blocker amytal (2mM); the complex II substrate succinate (5mM, V_{succ}); the complex III blocker antimycin-A (8µM); and the complex IV substrates N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD)-ascorbate (0.9:9mM, V_{TMPD}).

3.3.5.5 Mitochondrial H_2O_2 release:

Net H_2O_2 release by respiring mitochondria was measured in permeabilized fibers incubated in buffer Z (in mM: 110 K-MES, 35 KCl, 1 EGTA, 5 K_2HPO4, 3 $MgCl_26H_2O$) and 0.5mg/ml BSA, 1.2U/ml horseradish peroxidase, pH 7.3, 37°C) and the fluorescent probe Amplex® red (20µM: excitation-emission: 563-587nm) as previously described (53). Baseline fluorescence readings were taken in the absence of any exogenous respiratory substrates. The following additions were then made sequentially: glutamate (5mM); succinate (5mM);ADP (10mM); and antimycin-A (8µM). Rates of H_2O_2 production were calculated from a standard curve established under the same experimental conditions.

3.3.5.6 Calcium retention capacity:

Calcium retention capacity (CRC) was measured in ghost fibers in CRC buffer (in mM: 250 sucrose, 10 MOPS, 0.005 EGTA, 10 Pi-Tris, pH 7.3) supplemented with glutamate-malate (5:2.5mM) and 0.5nM oligomycin, as per Picard *et al*. (52). Following the addition of fibers and respiratory substrates, a single pulse of 20nmoles of Ca^{2+} was added. CRC was defined as the total amount of Ca^{2+} accumulated by mitochondria prior to PTP opening-induced Ca^{2+} release. Ca^{2+} concentration was calculated from a standard curve relating $[Ca^{2+}]$ to the fluorescence of Ca-Green 5N (excitation-emission: 505-535 nm).

3.3.5.7 Enzyme activity:

Activities of complex I (CI, NADH-CoQ reductase), complex II (CII, succinate dehydrogenase), complex IV (CIV, cytochrome oxidase complex), and citrate synthase (CS) were measured spectrophotometrically with a plate reader using standard coupled enzyme assays as per Marcil *et al*. (54) with minor modifications (55). Activities were expressed in mU/min/mg of wet muscle weight.

3.3.5.8 Preparation of isolated mitochondria:

Cardiac mitochondria were isolated using standard procedures. Briefly, hearts were minced in 4ml of IB-1 solution (in mM: 225 mannitol, 75 sucrose, 0.5% BSA, 0.5 EGTA, 30 Tris–HCl, pH 7.4) with a glass-Teflon® homogenizer. Homogenate was centrifuged at 800 g for 5min. Supernatant was centrifuged at 9000g for 10min. The pellet obtained was re-suspended in 2ml of IB-2 buffer (in mM: 225 mannitol, 75 sucrose, 0.5% BSA, 30 Tris–HCl, pH 7.4) and centrifuged at 10000g for 10min. The pellet was then re-suspended in 2ml of IB3 buffer (in mM: 225 mannitol, 75 sucrose, 30 Tris–HCl, pH 7.4) and centrifuged at 10000 g for 10min. The final pellet was re-suspended in 50µL of RIPA buffer with anti-protease (1mM PMSF, 1mM Na_3VO_4, 2µg/ml apoptinin, and 5µg/ml leupeptin), 1% Triton™ X-100 and anti-deubiquitinase (2mM n-ethylmaleimide) (56).

3.3.5.9 Transmission electron microscopy:

Samples of mouse left ventricular papillary muscle (five hearts per experimental group) were prepared for transmission electron microscopy as previously described (57). Ultrathin (60–65nm) sections were contrasted with lead citrate and imaged using a Philips Tecnai™ 12 Transmission Electron Microscope (TEM) at magnifications raging from 2700 to 34000X. For quantitative analysis of mitochondrial morphology, five adjacent fields per myocyte were taken at 4600X in five different hearts per experimental group. All mitochondria within each field were then selected as the region of interest using Image J software (NIH, Bethesda, MD). The average area and size distribution of a single mitochondrion, as well as total mitochondrial surface relative to myocyte surface area, was then measured. This analysis was performed on five different hearts per group for a total of 688-721 individual mitochondria. For quantification of autophagosomes in hearts following LPS injection, viable myocytes were first identified at 3600X magnification. Thirty adjacent fields per myocyte were then taken at 10500X and the number of autophagosomes per field was determined in 5 myocytes per hearts for a total of five hearts per experimental group.

3.3.5.10 Immunoblotting:

Following extraction from heart tissue or from isolated cardiac mitochondria, protein determinations were performed using the bicinchoninic acid method (Pierce Protein), with bovine serum albumin as a standard. Proteins were then separated on 10% (Parkin, p62, Nix (Bnip3L), Bnip3, Pgc1α, and Ubiquitin) or 15% (Lc3b) SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes. After 1hr of blocking in Tris-buffered saline with 0.1% TWEEN® 20 (TBS-T) supplemented with 5% non-fat milk, membranes were incubated overnight at 4°C with primary antibody diluted in TBS-T supplemented with 5% non-fat milk or 5% bovine serum albumin (BSA), according to the antibody (PARKIN (Sigma-Aldrich P6248, produced in mouse); P62 (Abnova H00008878-M01, produced in mouse); LC3b (Cell Signaling 3868S, produced in rabbit); NIX (Invitrogen 39-3300,

produced in mouse); BNIP3 (Cell Signaling 3769, produced in rabbit); GAPDH (Cell Signaling 5174, produced in rabbit); VDAC (Cell Signaling 4866S, produced in rabbit); SOD2 (Abcam #ab16956); OPA1 (BD Transduction Laboratories, #612606); LDH (Santa Cruz # sc-33781). After washing, membranes were incubated for 1 hr at room temperature with a secondary antibody diluted in TBS-T supplemented with 5% non-fat milk (anti-mouse IgG-peroxidase antibody (Cell Signaling 7076)); anti-rabbit IgG-peroxidase antibody (Sigma-Aldrich A0545)). Protein detection was performed using a chemiluminescent substrate (Amersham Biosciences Corp) with film exposures ranging from 30 sec to 5 min. Following film scanning, bands were quantified using Image J software.

3.3.5.11 Autophagy flux:

WT and Parkin$^{-/-}$ mice were injected with *phosphate buffered saline or* colchicine, a lysosomal inhibitor that prevents degradation of autophagosome*s (58).* Autophagic flux was measured at *baseline* (control) and in response to LPS injection. To measure baseline autophagic flux, mice received two i.p. injections of PBS (*control* group) or colchicine (colchicine group, 0.4 mg/kg/day). Twenty-four hrs after the second PBS or colchicine injection, animals were euthanized, hearts were excised and tissue samples were prepared for immunoblotting to detect Lc3b proteins (Sigma-Aldrich). To measure LPS-induced autophagic flux, mice received two i.p. injections of PBS (control group) or colchicine (colchicine group, 0.4 mg/kg/day). Twenty-four hrs after the second PBS or colchicine injection, animals received a single *E. coli* LPS injection (5mg/kg) and were euthanized 12 hrs later. Hearts were excised and tissue samples were prepared for immunoblotting to detect Lc3b proteins. A standard curve consisting of purified Lc3b was run alongside experimental gels (range: 0.4 to 1.2ng protein per lane) to facilitate quantification of Lc3b protein levels (Lc3b-I and Lc3b-II).Lc3b protein density in a given sample was converted to Lc3b protein quantity by extrapolation from the standard curve using regression analysis tools. Values were then normalized to mg of total muscle protein loaded per lane. Since Lc3b-II binds tightly to autophagosomal membranes and

serves as an autophagic marker protein, the difference in Lc3b-II levels in the presence and absence of colchicine represents autophagic flux.

3.3.5.12 Quantitative real-time PCR:

Frozen ventricular tissue (50mg) was homogenized in 1ml of TRIzol® reagent (Invitrogen) and RNA was isolated according to the kit manufacturer's instructions. RNA concentration and integrity was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies Canada) using the RNA integrity number (RIN) software algorithm. Samples with a RIN score of 9 and above were used for analysis. Reverse transcription was performed in a standard fashion using a High Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814, Applied Biosystems) supplemented with DNase treatment (Cat. No. AM2222, Ambion). TaqMan® assays were designed (primer sequences and probes in supplementary data) for the following genes using the Roche Universal Probe Library: Lc3, Gabarap, Bnip3, Pgc1α, Pgc1β, Tfam, Nrf1, Nrf2, Cox1, Cox2, Cox4i, Cox5a, Nd1, and Nd2. mRNA expressions were detected using real-time PCR with an ABI PRISM® 7000 sequence detector (Applied Biosystems) and analyzed using SDS 2.2.2 software. In all assays, 1.5 µl of reverse-transcriptase reagent was added to 5µl of TaqMan® Fast qPCR Master Mix (Applied Biosystems), 0.05 µl of primers, 1 µl of UPL probes and 2.5µl H_2O. The thermal profile used was as follows: 95°C for 3 min; 40 cycles each of 95°C for 5 sec; 60°C for 30 sec. All real-time PCR experiments were performed in triplicate. Relative mRNA quantifications of target genes were determined using the threshold cycle ($\Delta\Delta C_T$) method using the housekeeping genes Gapdh and Hprt. Data were expressed as fold change relative to control WT mice.

3.3.5.13 Statistical analysis:

Results are expressed as means ± SEM. Statistical differences were analyzed using two-way ANOVA (SPSS package). Tukey post-hoc tests were used to identify significant differences between means ($P<0.05$).

Figure 24: Normal baseline cardiac histology in hearts from WT and Parkin$^{-/-}$ mice.

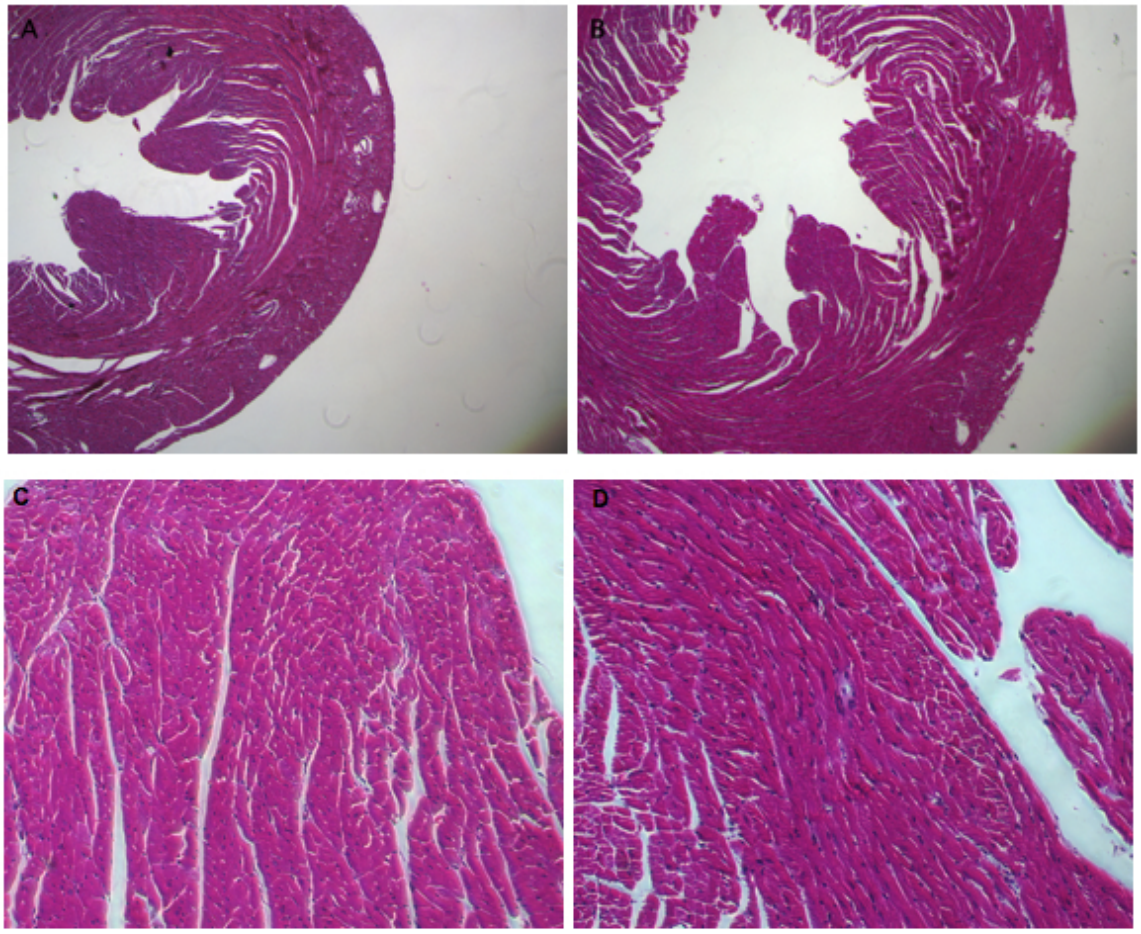

Figure 24: Normal baseline cardiac histology in hearts from WT and Parkin$^{-/-}$ mice. Representative left ventricular cross-sections stained with hematoxylin and eosin from WT (A, C) and Parkin$^{-/-}$ (B, D) hearts at 40X (A,B) and 200X (C,D).

Figure 25: Normal baseline cardiac ultrastructure in hearts from WT and Parkin$^{-/-}$ mice.

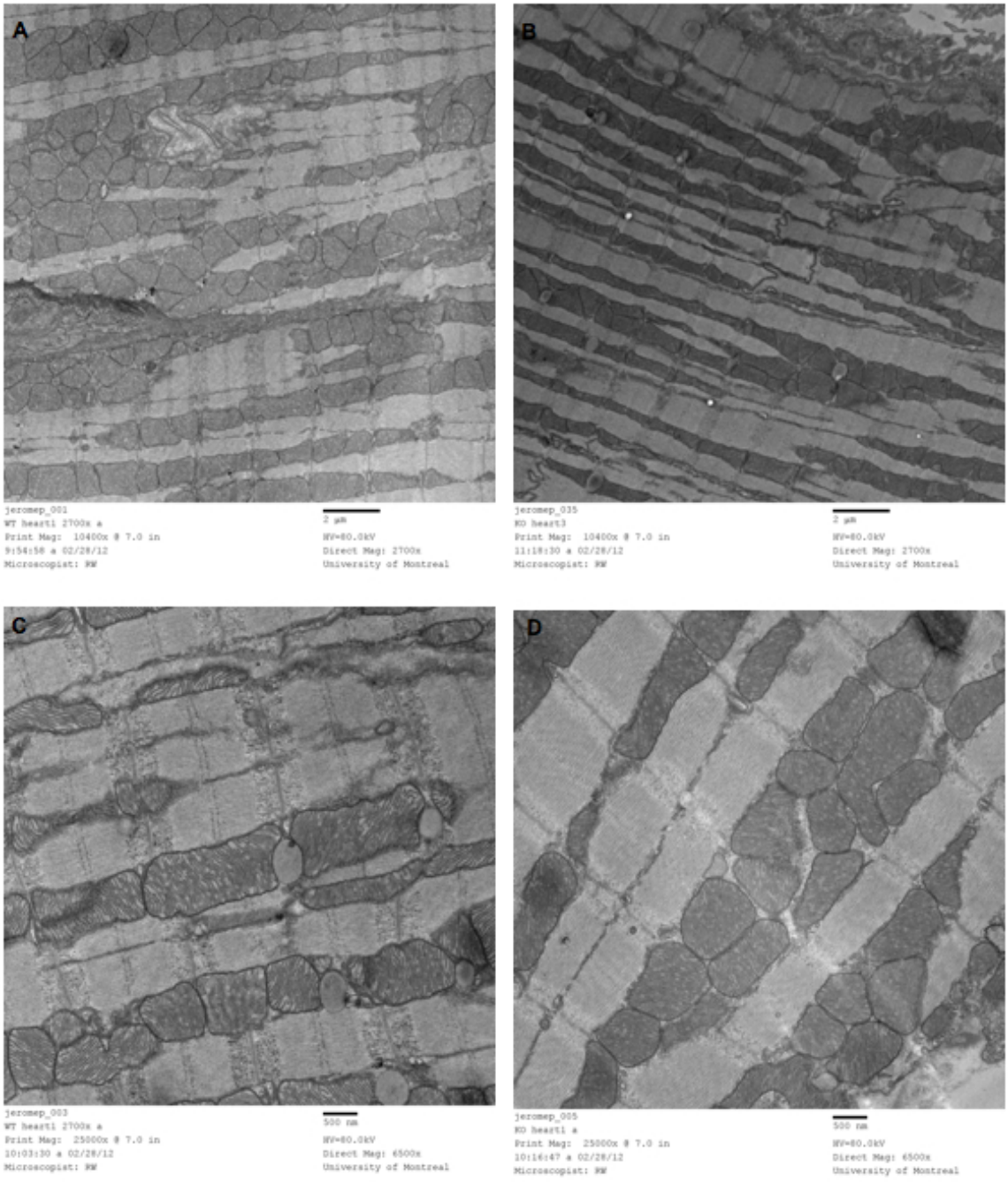

Figure 25: Normal baseline cardiac ultrastructure in hearts from WT and Parkin$^{-/-}$ mice. Representative electron micrographs of WT (A,C) and Parkin$^{-/-}$ (B,D) hearts at 2700X (A,B) and 6500 X (C,D).

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4 Conclusion

In the first study, "*Cyclophilin-D is dispensable for atrophy and mitochondrial apoptotic signaling in denervated muscle*", we show that CypD is not an obligatory to induce apoptotic signaling and atrophy in a model of muscle inactivity. Although the genetic ablation of this important PTP modulator did improve the resistance to a calcium overload in vitro, it did not prevent the release of pro-apoptotic factors normally sequestered in the mitochondria of hindlimb skeletal muscles upon surgical transection of the sciatic nerve, nor did it attenuate atrophy. These results were somewhat surprising considering that other severe conditions with similar pathological features have shown that inhibition (genetic or pharmacological) of CypD attenuates 1) the detrimental effects of ischemia-reperfusion on cardiac muscle (Baines, 2005, Nature) and 2) the myocellular damage associated with dystrophic disorders (Palma, 2009, Hum Mol Genet / Angelin, 2007, Proc Natl Acad Sci).

When interpreting the results, it is important to consider that PTP opening in the absence of CypD is only delayed, not completely prevented. Indeed, the absence of CypD did not completely prevent PTP opening in vitro, it decreased sensitivity to an acute stress. Accordingly, there is now a consensus around CypD being a critical determinant of sensitivity but not being obligatory for permeability transition (Bernardi, 2013, Front Physiol) There was no such agreement at the time of this first study. Indeed, the work of Halestrap and colleagues was showing compelling evidence of CypD, VDAC and ANT being important components of the PTP. The results obtained here are in line The model of Giorgio and Bernardi in which the PTP is formed by a dimer of ATP synthase that can be regulated by CypD is in line with the results of the first study. Nonetheless, it is still possible that the absence of CypD may be able to prevent PTP opening in the face of an acute stress such as ischemia-reperfusion but would be unable to prevent PTP opening during a prolonged exposure (14 days) to stress, such as during denervation. In a long-term setting, the overwhelming forces acting on the PTP may eventually result in induction of apoptosis and could explain why we did not observe any differences in the cytosolic

content of mitochondrial pro-apoptotic factors 14 days after denervation. Perhaps, we would have been able to detect the heightened resistance to PTP opening in the first hours/days after denervation. Notwithstanding of the true molecular identity of the PTP, the role of apoptosis in mediating atrophy in response to denervation appears to be subdued at best. Siu and Alway observed only a slight attenuation of atrophy in an apoptosis-resistant mouse model (i.e. *Bax*$^{-/-}$ mice) (Siu, 2006, J Physiol). Additionally, proteasomal and autophagolysosomal degradation are suggested to be the dominant effectors in disuse-induced atrophy (Paul, 2011, Autophagy), with apoptosis probably serving mostly to maintain an appropriate myonuclear domain size (Allen, 1999, Muscle Nerve).

Despite the lack of CypD we also observed a sensitization to PTP opening following the loss of innervation. So far, it was thought that a large part of the sensitization to pore opening after denervation was due to CypD (Csukly, 2006, J Physiol). Obviously, there are other pathways that may contribute to this process in the absence of CypD. This phenomenon certainly warrants further investigation as it remains unclear what are the forces that may be involved in this sensitization process. Possibly, oxidative stress may exert a role in sensitizing PTP opening in denervation. Precisely, arachidonic acid metabolites formed from the increased enzymatic activity of phospholipase A_2 (PLA_2) upon denervation are thought to induce important mitochondrial damage that would facilitate permeabilization (Jezek, 2010, Physiol Res / Bhattacharya, 2009, J Biol Chem). In fact, results unpublished results from our laboratory revealed that the augmentation in ROS production during denervation is mostly related to the increased activity of mitochondrial PLA_2.

Data not shown in the first study also indicated that AIF release from mitochondria was different from what was observed with cytochrome c and SMAC/Diablo. Accordingly, a 2008 paper shows that different pro-apoptotic factors are released over different time-courses, suggesting that different factors control the release of pro-apoptotic molecules rather than a simple general release of all

mitochondrial content (Munoz-Pinedo, 2006, Proc Natl Acad Sci). Siu and Alway also showed results that would support the idea of differential controls of the various mitochondria residing pro-apoptotic factors (Siu, 2006, apoptosis). Questions regarding this possibility remain unanswered and certainly deserve further investigation.

The role of mitochondrial permeabilisation in denervation-induced atrophy remains questionable but so is the role of apoptosis altogether. Indeed, Siu and Always reported only prevented 10% of the normal atrophy levels after denervation in apoptosis-null animals. Apoptosis however may have a role in maintaining somewhat optimal function throughout the atrophy process. Indeed, the regulation myonuclear domains may be a critical role of apoptosis for long-term muscular functions although it is dispensable for short term muscle degradation.

In the second study, "*PGC1α gene transfer during muscle regeneration restores mitochondrial biomass and improves mitochondrial calcium handling in post-necrotic mdx skeletal muscle*", we show that duchenne muscular dystrophy is inherently linked with a lower mitochondrial biomass as well as mitochondrial dysfunctions. We also show PGC1α transfection ameliorates the histological appearance of the diseased muscle meanwhile improving mitochondrial biomass and function. In particular, exogenous PGC1α overexpression lowered mitochondrial apoptotic susceptibility, and improved Ca^{2+} buffering capacity, resulting in lower activities of calpain and caspases (3 & 9). Other studies have found a beneficial effect of PGC1α in DMD (Handschin, 2007, Genes Dev / Selsby, 2012, Plos One). The study by Handschin and colleagues indicate that PGC1α overexpression improves muscular dystrophy by improving neuromuscular junction gene expression; whereas Selsby and colleagues, while corroborating the results obtained by Handschin et al., additionnally postulate that the more oxidative profile established by PGC1α overexpression also has beneficial effects on muscular dystrophy. With PGC1α normally associated with better mitochondrial functions,

we decided to investigate whether some of the beneficial effects of PGC1α may be mediated by such improvements. Additionally, our study took a more clinically-relevant approach to address the potential therapeutic effects of PGC1. Indeed, previous studies have induced PGC1 overexpression from the germline (Handschin, 2007, Genes Dev) or, prior to the first clinical signs of disease (Selsby, 2012, Plos One) whereas we implemented it as a therapy after the onset of the disease. Certainly, the ultimate therapy for muscular dystrophy would be gene therapy. Alternatively, treatments aimed at improving membrane stability could be beneficial. Mice harbouring a dystrophin mutation tend to see a more benign phenotype than humans. It has been proposed that this is the result of compensatory increase in utrophin, which is only observed in the rodents. Efforts are currently being deployed in developping a therapy that would induce utrophin overexpression (Miura, 2006, Trend Mol Med) as a therapy for DMD. Interestingly, PGC1α, in addition to improving the metabolic profile, the neuromuscular junction gene program, the antioxidant buffering capacity and all of the mitochondrial functions, it is known to promote utrophin expression (Angus, 2005, Am J Physiol). By targeting various aspects of the disease, PGC1α is becoming a viable alternative to gene therapy.

In the third study, "*Protective role of Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction*", we show the importance of mitochondrial quality control in maintaining healthy cardiac mitochondrial functions especially during the recovery of sepsis-induced myocardial depression. Organellar quality control at the mitochondrial level is a topic of interest, which has only just recently emerged. Different levels of what appears to be a hierarchical network of interacting pathways regulating mitochondrial quality control have been characterized but their integration into a comprehensive model has not yet been detailed. This study thus contributes to advance our understanding of the integration of mitophagy with other physiological processes such as mitochondrial functions and biogenesis in quality control.

Despite indications that Parkin is not necessary to conduct mitophagy and that there exists alternate mitophagic pathways, it is not clear how the absence of Parkin translates into a poor recovery of mitochondrial and cardiac functions in the septic heart. This may suggest that Parkin has other vital roles in the cell. In addition to being touted as an important regulator of mitophagy, Parkin has been suggested to interact directly with mtDNA to maintain its integrity (Rothfuss, 2009, Hum Mol Genet), to facilitate lipid oxidation by stabilizing CD36 via ubiquitination (Kim, 2011, J Clin Invest) and to exert a transcriptional control over PGC1α (Pacelli, 2011, Biochim Biophys Acta). It would not be surprising to uncover additional roles for this protein as research surrounding the role of this protein beyond its implication in Parkinson have really just started in 2003 with the observation of severe myopathies with mitochondrial involvements in drosophila that are devoid of the protein (Greene, 2003, Proc Natl Acad Sci) but only accelerated in 2008 after the team of Youle first described the role of the protein in targeting mitochondria for autophagy (Narendra, 2008, J Cell Biol).

The third article also highlights the existence of alternate mitophagy pathways ans possibly other E3 ligase that may correct some of the imbalances caused by the absence of Parkin. These alternate pathways remain poorly understood and their redundancy with other autophagic/quality control pathways is relatively unscathed. One of these would be the formation of mitochondria-derived vesicles. Recent data actually indicates that Parkin is necessary in forming vesicles that contain damaged mitochondrial content which are then shipped to the lysosome for degradation (Soubannier, 2012, Curr Biol). This would constitute a novel quality control mechanism and could explain how Parkin is necessary to maintain optimal mitochondrial function.

Overall, this thesis clearly demonstrates an involvement of mitochondrial dysfunction in the pathogenesis of a wide range of myopathies. The chicken and egg

conundrum remains well and alive in determining whether these dysfunctions appear as a result of the disease or vice versa. Nonetheless, treatments aimed at improving mitochondrial functions seem to have beneficial effects in most myopathies making the organelle a preferred therapeutic target. Very few studies have examined mitochondrial functions within the scope of an integrated mitochondrial life cycle, such as described in figure 28. Although the third study of this thesis is a better attempt at interpreting the data with an understanding of this life cycle, future studies need to address their data in a manner that is consistent with it if we are to build a comprehensive model of how mitochondrial function are integrated within muscle physiopathology.

Figure 26: Mitochondria life cycle

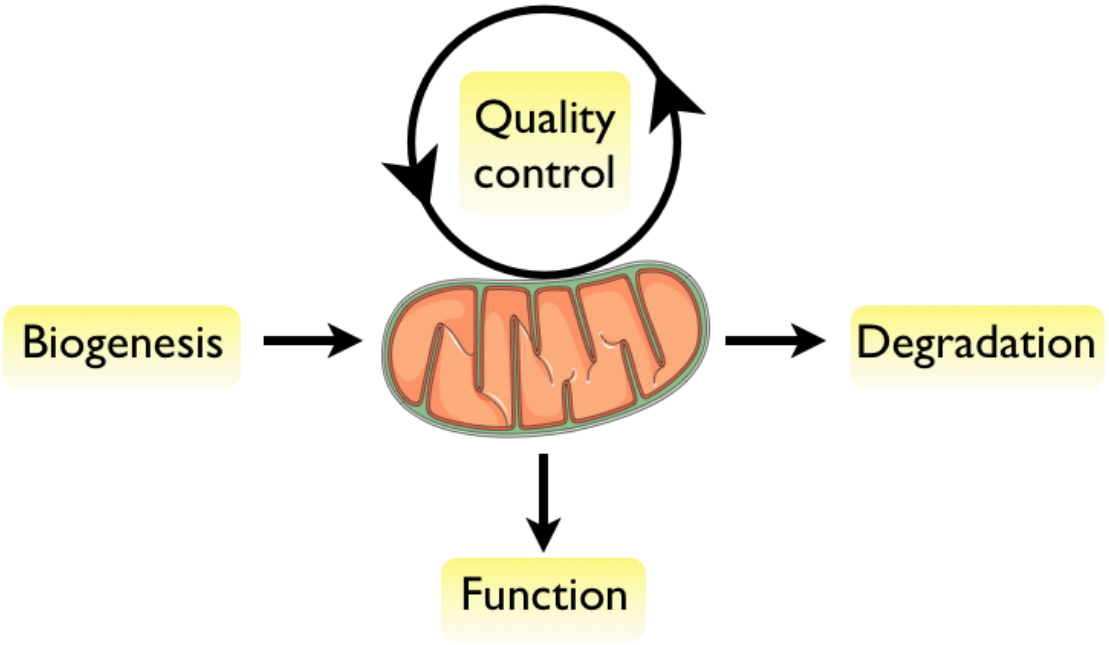

Figure 26: Mitochondria life cycle

Mitochondrial life cycle is dictated by the formation of new organelles (biogenesis) and their destruction (mitophagy). During the lifetime of a mitochondrion, functional capacity will be maintained by various levels of quality control.

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