From the Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

Mitochondria, contractility and Ca^{2+} handling

Cardiac and skeletal muscle adaptations in health and disease

Daniel C Andersson

M.D.

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For Maria and my parents

"Science is an essentially anarchic enterprise: theoretical anarchism is more humanitarian and more likely to encourage progress than its law-and-order alternatives."

Paul Feyerabend (1975), Against Method

ABSTRACT

Contractility is a fundamental feature of skeletal and cardiac muscles. An indispensable step in the cellular signal for contraction is a transient elevation in cytoplasmic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$). Both the production of contractile force and Ca^{2+} handling processes are highly energy demanding. Mitochondrial ATP production from the respiratory chain is thus of pivotal importance for muscle cell function. Moreover, the mitochondria are also directly involved in Ca^{2+} signaling and are the foremost source of reactive oxygen species (ROS). Mutations in the genomes encoding for the mitochondrial respiratory chain, either the nuclear or the mitochondrial (mtDNA) genome, can give rise to primary mitochondrial diseases. Mitochondrial dysfunction is also implicated in other diseases, such as heart failure, obesity and diabetes.

Paper I and II investigate two mouse models of primary mitochondrial myopathy (*Tfam* KO) and cardiomyopathy (*Mterf3* KO). Furthermore, the obese, pre-diabetic *ob/ob* mouse was studied in papers III and IV. Skeletal and cardiac muscle cells were studied primarily with respect to contractility, $[Ca^{2+}]_i$ and mitochondrial ROS production.

Skeletal muscle fibers of myopathy *Tfam* KO mice produce less force compared to control mice. This was explained by reduced tetanic $[Ca^{2+}]_i$, decreased SR Ca^{2+} release and reduced SR Ca^{2+} storage via calsequestrin 2. Moreover, *Tfam* KO but not control fibers, displayed a markedly increased mitochondrial $[Ca^{2+}]$ during fatigue, partly through a cyclosporin A-sensitive mechanism. Elevated $[Ca^{2+}]$ in the mitochondria can trigger cellular damage. Thus, reducing mitochondrial Ca^{2+} with cyclosporin A may provide one way for treatment of mitochondrial myopathy.

Mterf3 KO mice develop hypertrophic cardiomyopathy and die suddenly. Cardiomyocytes from these mice display elevated SR Ca^{2+} cycling, increased SR Ca^{2+} load and aberrant pro-arrhythmic Ca^{2+} releases compatible with that seen in sympathetic stimulation. In support of this, electrocardiography revealed signs of elevated catecholaminergic drive. Moreover, in the moribund stage *Mterf3* KO mice develop terminal AV-block and bradycardia.

Acutely exposing WT cardiomyocytes to an excess of the saturated fatty acid palmitate caused dissipation of the mitochondrial membrane potential and a large increase in mitochondrial ROS production. In turn, this ROS increase impaired the cellular Ca^{2+} cycling and contractility. However in *ob/ob* cardiomyocytes, palmitate did not cause increased ROS production and the function of *ob/ob* cardiomyocytes was in fact improved by palmitate. This suggests that the *ob/ob* heart has adapted to a high fat environment and metabolizes fatty acids without the producing large amounts of mitochondrial ROS.

In WT cardiomyocytes, application of the β-adrenergic agonist isoproterenol (ISO) stimulated mitochondrial ROS production. Concomitant application of the ROS scavenger *N*-acetylcysteine (NAC) diminished the inotropic effect of ISO on cardiomyocyte $[Ca^{2+}]_i$ transients and contractility. On the other hand, *ob/ob* cardiomyocytes failed to increase ROS production when exposed to ISO and NAC did not alter the effect of ISO on $[Ca^{2+}]_i$ transients. Hence, mitochondrial ROS is integrated in, but not essential to, the inotropic mechanism of β-adrenergic stimulation.

In all the studied disease models, neither an increase in mitochondrial ROS production nor signs of oxidative damage were found.

In conclusion, dysfunctional mitochondria cause long-term adaptive/maladaptive changes in Ca^{2+} handling as seen in the *Tfam* and *Mterf3* KO mice. Mitochondrial functions can influence cellular Ca^{2+} handling also in the short term. This is evident by the effects of palmitate and ISO-stimulated mitochondrial ROS production.

LIST OF PUBLICATIONS

The thesis is based on the following papers, referred to in the text by their Roman numerals

I. AYDIN J, **ANDERSSON DC**, HÄNNINEN S, WREDENBERG A, TAVI P, PARK CB, LARSSON NG, BRUTON JD, WESTERBLAD H. (2009) Increased mitochondrial Ca^{2+} and decreased sarcoplasmic reticulum Ca^{2+} in mitochondrial myopathy. *Hum Mol Genet* **18**, 278-88.

II. **ANDERSSON DC**, FAUCONNIER J, PARK CB, ZHANG SJ, THIREAU J, WIBOM R, LARSSON NG, WESTERBLAD H. Sympathetic overdrive and enhanced cardiomyocyte Ca^{2+} cycling precede terminal atrio-ventricular conduction block in mitochondrial cardiomyopathy *MTERF3* KO mice. Manuscript.

III. FAUCONNIER J, **ANDERSSON DC**, ZHANG SJ, LANNER JT, WIBOM R, KATZ A, BRUTON JD, WESTERBLAD H. (2007) Effects of palmitate on Ca^{2+} handling in adult control and *ob/ob* cardiomyocytes: impact of mitochondrial reactive oxygen species. *Diabetes* **56**, 1136-42.

IV. **ANDERSSON DC**, FAUCONNIER J, YAMADA T, WIBOM R, KATZ A, WESTERBLAD H. Mitochondrial production of reactive oxygen species contributes to the beta-adrenergic-induced cardiac inotropy in wild-type but not in *ob/ob* mice**.** Submitted, 2009.

CONTENTS

LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Cytoplasmic free $[Ca^{2+}]$
$[Ca^{2+}]_{mit}$	Mitochondrial $[Ca^{2+}]$
$\Delta\Psi_m$	Mitochondrial membrane potential
τ	Decay time constant (Tau)
ADP	Adenosine diphosphate
ANP	Atrial natriuretic peptide
ATP	Adenosine-5'-triphosphate
CSA	Cyclosporine A
ECG	Electrocardiography
DNA	Deoxyribonucleic acid
E-C coupling	Excitation-contraction coupling
FDB	Flexor digitorum brevis
H_2O_2	Hydrogen peroxide
ISO	Isoproterenol
KO	Knock-out
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
Mterf3	Mitochondrial transcription termination factor 3
NAC	*N*-acetylcysteine
NCX	Na^+/Ca^{2+} exchanger
O_2^{*-}	Superoxide ion

ob/ob	Leptin deficient mouse model of the metabolic syndrome
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SR	Sarcoplasmatic reticulum
SERCA	Sarcoplasmatic reticulum Ca^{2+}-ATPase
SDNN	Standard deviation of all normal R-R intervals
Tfam	Mitochondrial transcription factor A
TMRE	Tetramethylrhodamine ethyl ester
WT	Wild-type

1 INTRODUCTION

A central theme in physiology is to study the biophysical processes of living matter. Physiology focuses on understanding the dynamics of the biological phenomena rather than to merely characterize morphological features. It is therefore common in this discipline to find descriptions that imply a time dependency, e.g. flow, feed-back control and adaptation. The biophysical properties that are studied span phenomena like ion fluxes, transformations of molecular complexes, cellular contractions and locomotion of the organism. Thus, physiology has classically covered processes occurring on different levels, ranging from molecules up to the whole organism. In this way, e.g. ion fluxes found on underlying levels are used to explain the functions of cells, organs all the way up to the entire organism.

Studying cardiac and skeletal muscle contractility by examination of cellular and sub-cellular phenomena will thus increase our knowledge of heart function and locomotion of the human body. In many instances, pathology can be thought of as a distorted, maladapted state of physiological mechanisms. Therefore, acquiring new insights in physiology does not only provide the physiologist with a moment of joy, but also contributes to the understanding of disease processes.

2 BACKGROUND

2.1 EXCITATION-CONTRACTION COUPLING

Contractility is an essential property of all types of muscles. This feature enables the heart to produce the power necessary for its pump function and the skeletal muscles to allow movement. Execution of muscle contraction at the cellular level occurs through a process referred to as excitation-contraction coupling, or E-C coupling. This process starts with an action potential in the cell membrane (excitation). Then, a series of steps occur that couple the initial excitation to commencement of contractile work. Central to the E-C coupling process is an increase in cytoplasmic free [Ca^{2+}] ([$Ca^{2+}]_i$). Cellular Ca^{2+} handling is a highly controlled process that involves ion exchange systems, ion pumps and specialized compartments for Ca^{2+} storage within the cell. The general principles of E-C coupling in cardiac and skeletal muscle are similar. However, there are some important differences which will be explained below.

2.1.1 Skeletal muscle E-C coupling

Activation of skeletal muscle cells (fibers) starts with an acetylcholine-mediated depolarization of the plasma membrane (sarcolemma) at the neuromuscular junction. This leads to the opening of voltage gated Na^+-channels which initiate an action potential (AP). The AP propagates along the surface sarcolemma, but it also travels deep into the muscle cell via narrow invaginations of the muscle cell membrane called the transverse tubule system (t-tubules). In the T-tubules, AP causes activation of voltage sensing L-type Ca^{2+} channels (dihydropyridine receptors, DHPRs). However, in skeletal muscle the L-type Ca^{2+} channel is unable to conduct any Ca^{2+} current (this stands in contrast to the cardiac L-type Ca^{2+} channel). Instead, upon AP activation the L-type channel interacts directly with a juxtaposed ryanodine receptor (RyR), the sarcoplasmic reticulum (SR) Ca^{2+} release channel. At rest, the SR lumen contains ~1-2 mM Ca^{2+} whereas $[Ca^{2+}]_i$ is only ~50nM. Thus when activated, the RyR allow Ca^{2+} to be released of from the SR into the cytoplasm. The increase in $[Ca^{2+}]_i$ permits Ca^{2+} binding to troponin C, a myofilament regulatory protein which in turn facilitates interaction of myosin and actin filaments and contraction can start. For relaxation to occur, $[Ca^{2+}]_i$ must be reduced to the resting level. This is done by pumping Ca^{2+} back

into the SR, a highly energy-requiring process dependent on the SR Ca^{2+}-ATPase (SERCA). Thus in skeletal muscle, cellular Ca^{2+} is cycled between the SR and cytoplasm with practically no exchange with the extracellular environment (Dulhunty, 1992; Stephenson *et al.*, 1995; Allen *et al.*, 2008) (figure 1).

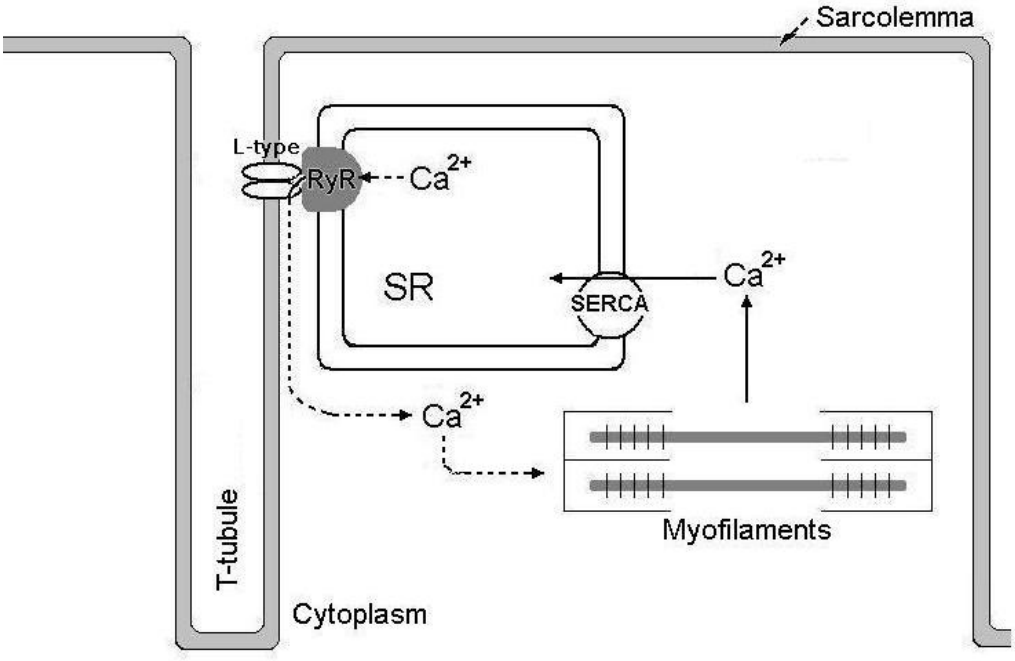

Figure 1. Schematic drawing of cytoplasmic Ca^{2+} handling in a skeletal muscle cell. L-type: L-type Ca^{2+} channel (DHPR), RyR: Ryanodine receptor, SERCA: SR Ca^{2+}-ATPase, SR: sarcoplasmic reticulum. (Picture by Armiento, M.)

2.1.2 Cardiac E-C coupling

The electrical activation of the heart cells is not initiated by the release of synaptic neurotransmitters as in the skeletal muscle. Instead, the AP generation in the heart occurs in specialized pacemaker cells in the sino-atrial (sinus) node, from which a depolarization wave propagates from cell to cell via gap junctions (intercalated discs), thereby electrically linking the cardiomyocytes. It is also worth noting that the duration of the cardiac AP is considerably longer than that of the skeletal muscle. Action potentials in the human ventricular cardiomyocyte have a duration of ~200-300 ms, compared to only 2-3 ms in skeletal muscle cells.

E-C coupling in the cardiomyocyte is also slightly different from that of the skeletal muscle cell. As in the skeletal muscle, the AP propagates over the plasma membrane and into the cell via t-tubules. However, voltage activation of the cardiac L-type Ca^{2+} channels allows a Ca^{2+} current ($I_{Ca,L}$) to be conducted over the sarcolemma. The

transsarcolemmal Ca^{2+} influx occurs down a ~10,000 fold concentration gradient, from ~1 mM to ~100 nM Ca^{2+} in the extra- and intracellular spaces, respectively. The $I_{Ca,L}$ is an important factor behind the relatively long AP duration in cardiac cells. As in skeletal muscles, a narrow junction between the t-tubules and SR places the cardiac L-type Ca^{2+} channels in close proximity to RyRs. However, cardiac SR Ca^{2+} release is not triggered by a physical interaction between the L-type channel and the RyR. Instead, the transsarcolemmal Ca^{2+}-influx triggers RyR opening, which then permits releases of Ca^{2+} from the SR. This process is called the Ca^{2+}-induced Ca^{2+} release. The Ca^{2+} released from the SR contributes substantially more to the total increase in $[Ca^{2+}]_i$ than the L-type Ca^{2+} influx. In humans, ~70% (mice ~90%) of the total increase in $[Ca^{2+}]_i$ can be attributed to SR Ca^{2+} release (Bers, 2002). Similar to skeletal muscle, the increased $[Ca^{2+}]_i$ activates the cardiomyocyte myofilaments and contraction occurs (systole). To allow relaxation of the heart (diastole), $[Ca^{2+}]_i$ is reduced mainly by uptake to the SR via the cardiac SR Ca^{2+}-ATPase (SERCA2a). However in the steady state condition, an efflux of Ca^{2+} over the sarcolemma must also be present to balance the L-type Ca^{2+} influx (otherwise there would be a net build-up of total cellular Ca^{2+}). This efflux is mainly effectuated by the Na/Ca exchanger (NCX) that removes ~30% (~10% in mice) of the systolic $[Ca^{2+}]_i$ (Bers, 2001) (figure 2).

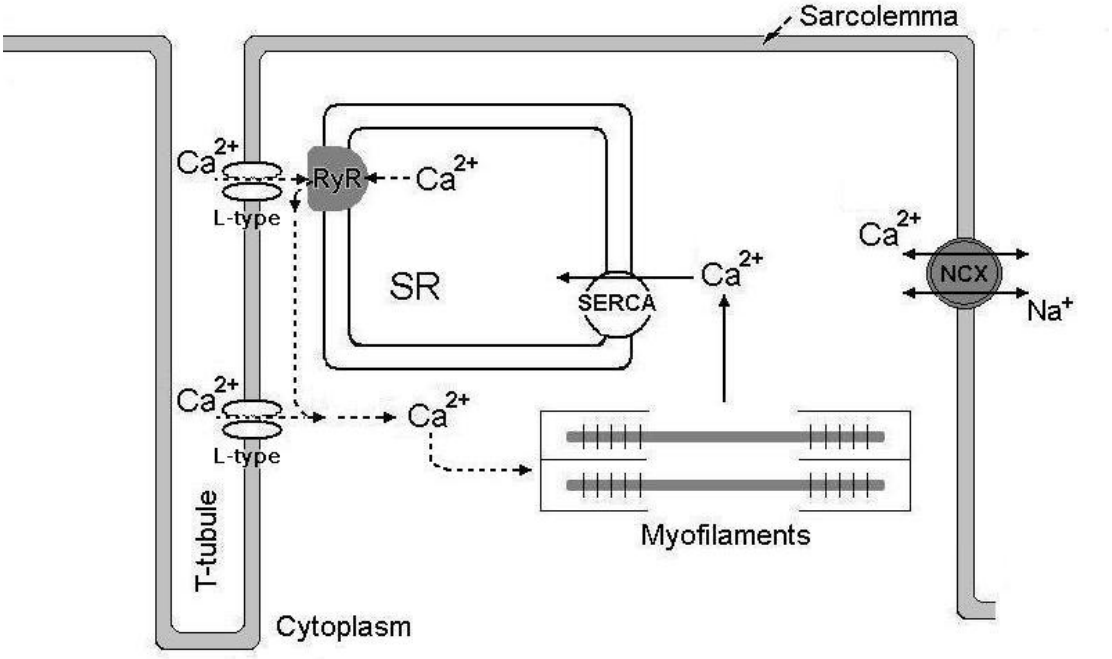

Figure 2. Schematic drawing of cardiomyocyte Ca^{2+} handling. L-type: L-type Ca^{2+} channel (DHPR), NCX: Na^+/Ca^{2+}-exchanger, RyR: ryanodine receptor, SERCA: SR Ca^{2+}-ATPase, SR: sarcoplasmic reticulum. (Picture by Armiento, M.)

The magnitude of cardiomyocyte contractility is in large part decided by the $[Ca^{2+}]_i$. Increased contractility is thus dominated by mechanisms leading to increased $[Ca^{2+}]_i$. The cardiac Ca^{2+} handling can be influenced by several factors, e.g. redox modifications, $[ADP][P_i]/[ATP]$ and phosphorylation (Zima & Blatter, 2006; Xiang & Kentish, 1995; Bers, 2002). An important stimulus that influences Ca^{2+} handling is mediated by catecholamines from the sympathetic adrenergic system. In the heart, catecholamines bind to the β-adrenergic receptors (β-receptor). Activation of these receptors cause increases in heart rate (chronotropy), relaxation speed (lusitropy) and contractility (inotropy). The β-receptor-induced increase in relaxation speed and contractility are considered to be mainly the effect of cAMP-dependent protein kinase (PKA)-mediated phosphorylation of proteins involved in cardiomyocyte Ca^{2+} handling (Bers, 2002). Three important Ca^{2+} handling proteins that are targeted by PKA-mediated phosphorylation are phospholamban, RyR and the L-type Ca^{2+} channel. Phosphorylation of phospholamban leads to increased activity of SERCA2a, which accelerates SR Ca^{2+} uptake (Maclennan & Kranias, 2003). Phosphorylation of the RyR increases the channel open probability and has also been suggested to enhance SR Ca^{2+} release (Zalk *et al.*, 2007). Moreover, Ca^{2+} influx is increased by PKA-mediated phosphorylation of the L-type Ca^{2+}channel (Tsien *et al.*, 1986; Bers, 2008). This increase in L-type Ca^{2+} entry will further amplify the release of SR Ca^{2+} via the Ca^{2+}-induced Ca^{2+} release mechanism (Bassani *et al.*, 1995). β-adrenergic signaling is therefore of central importance in regulating heart function and cardiovascular homeostasis. However, chronically sustained adrenergic stress can be deleterious for cardiac function and is associated with development of heart failure and cardiac arrhythmias (Clark & Cleland, 2000; Marx *et al.*, 2000; Wehrens *et al.*, 2004; Curran *et al.*, 2007).

2.2 THE MITOCHONDRION

2.2.1 Structure and origin

The mitochondria are double-membraned, DNA containing organelles found within most cells of the multi-cellular organisms, including humans and mice (Andersson *et al.*, 2003). Two layers of lipid membrane, the outer and inner membranes, separate the mitochondrial matrix from the cytoplasm. The permeability of the outer membrane is

relatively high, allowing passage of molecules up to 10 kD in size. However, the inner membrane works as a barrier over which fluxes of molecules and ions can be firmly controlled. Invaginations of the inner membrane create a large surface to volume ratio and facilitate exchange of cellular matter between the cytoplasm and the mitochondrial matrix. The amount of mitochondria within cells varies depending on the tissue type. In fast- and slow-twitch skeletal muscle ~2% and 5% respectively of the total of the cell volume is occupied by mitochondria (Eisenberg & Kuda, 1975), whereas in cardiac ventricles they account for ~35% of the volume (Barth *et al.*, 1992). The great difference in mitochondrial density reflects the varying demand for oxidative energy production in the respective tissue type. The citric acid cycle and β-oxidation of fatty acids occur within the mitochondria and feed the electron transport chain with metabolites (Bartlett & Eaton, 2004). Oxidative ATP production in the electron transport chain is the most recognized function of the mitochondrion (Saraste, 1999), thus the popular reference to mitochondria as the power plants of the cell (Wallace, 1997). However, the mitochondria are involved in other cellular functions, such as cell death (apoptosis and necrosis) (Ott *et al.*, 2007), production of reactive oxygen species (Turrens, 2003) and Ca^{2+} signaling (Brookes *et al.*, 2004).

In many cell types, the mitochondria form a dynamic network, rather than isolated organelles, with the ability to fuse (fusion) and divide (fission) (Yaffe, 2003; Okamoto & Shaw, 2005; Duvezin-Caubet *et al.*, 2006). This enables the mitochondria to redistribute within the cell and change its morphology on demand (Yaffe, 2003). Fusion of mitochondria has been suggested to provide a way to reduce local effects of mtDNA mutations within the cell, via mixing wild-type and mutated mtDNA among the mitochondria (Rube & van der Bliek, 2004). In contrast to many other cell types, the cellular localizations of the mitochondria in skeletal and cardiac muscle cells, appear to be more fixed in relation to the intracellular structures (e.g. SR, myofibrils and the sarcolemma) (Vendelin *et al.*, 2005). Nevertheless, the balance between fusion and fission is believed to be crucial to adaptive mitochondrial biogenesis in skeletal muscle in response to exercise (Hood *et al.*, 2006).

According to the so called endosymbiosis theory, mitochondria are thought to have evolved from an early respiring bacteria living in symbiosis inside a proto-eukaryotic cell (Gray *et al.*, 1999). This symbiosis supplied the early eukaryotic cell with a system

for oxidative energy metabolism, forming an aerobic eukaryote cell with a new efficient system for energy turnover (Karlberg & Andersson, 2003). The advent of mitochondria is thought to have occurred ~2,300 million years ago (Karlberg & Andersson, 2003), at the same time as atmospheric oxygen tension rose (Farquhar *et al.*, 2000). It is thus believed that the timely evolution of oxidative metabolism under the increased oxygen tension provided a great survival benefit for the eukaryotic cell (Gray *et al.*, 1999; Andersson *et al.*, 2003). The strongest evidence for the endosymbiotic theory comes from the fact that the mitochondrion has its own circular genome resembling the type found in bacteria (Gray & Doolittle, 1982). From mitochondrial DNA sequencing, the ancestors of mitochondria have been traced to the α-proteobacteria class (Yang *et al.*, 1985). Among bacteria now existing, the one most closely related to mitochondria is *Rickettsia prowazekii* (Andersson *et al.*, 2003).

2.2.2 Mitochondrial DNA transcription

A unique feature of the mitochondrion is that it carries a genome of its own, the mitochondrial DNA (mtDNA). The inheritance of mtDNA is maternal due to ubiquitination-mediated degradation of sperm mitochondria inside the fertilized oocyte (Sutovsky *et al.*, 2000; Sutovsky *et al.*, 1999). The mitochondrial genome is polyploid and in a somatic cell 1000–10,000 mtDNA copies can be found (Smeitink *et al.*, 2001). The human (and mouse) mtDNA is double-stranded, circular and ~16,500 base pair in size. It encodes for 13 proteins, 22 tRNA and 2 rRNA. All the 13 encoded proteins are part of the respiratory chain (Falkenberg *et al.*, 2007). Given that the respiratory chain contains ~90 proteins in total, this implies that a majority of the proteins are encoded by the nucleus. Furthermore, if one takes into account that the mitochondrion is estimated to be composed of a total of ~1500 proteins (Calvo *et al.*, 2006), this gives an even smaller contribution of the mtDNA in the protein build-up of the organelle. However, the 13 protein products of mammalian mtDNA are critical for the function of oxidative phosphorylation and thus the viability of the organism (Larsson *et al.*, 1998; Wredenberg *et al.*, 2002; Park *et al.*, 2007).

Transcription of mtDNA is under the control of a set of regulatory proteins and transcription factors. All of the proteins involved in the mtDNA transcription machinery are encoded in the nucleus and imported into the mitochondria. To

reconstitute mammalian mtDNA transcription *in vitro*, the presence of the following proteins have been shown to be necessary: mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (Tfam), mitochondrial transcription factor B1 or B2 (TFB1M, TFB2M) (Falkenberg *et al.*, 2007; Falkenberg *et al.*, 2002).

Tfam was the first described mitochondrial transcription factor (Fisher & Clayton, 1988). It belongs to the high mobility group proteins and has the ability to bind mtDNA. Upon binding to mtDNA, Tfam activates mtDNA transcription (Dairaghi *et al.*, 1995). Moreover, it has been proposed that Tfam can facilitate transcription also by changing the structure of mtDNA, such as unwinding of promoter regions (Fisher *et al.*, 1992; Falkenberg *et al.*, 2007). Global ablation of the *Tfam* gene in mouse causes mtDNA depletion, respiratory chain dysfunction and is embryonically lethal (Larsson *et al.*, 1998). Several tissue specific *Tfam* gene knock-out (*Tfam* KO) mice that survive embryogenesis have been constructed, including *Tfam* KO in skeletal and cardiac muscle. These mice develop respiratory chain failure and they display impaired functions in the respective tissue (Wang *et al.*, 1999; Wredenberg *et al.*, 2002; Ekstrand *et al.*, 2007; Sorensen *et al.*, 2001; Silva *et al.*, 2000).

Tfam is not an ideal regulator for rapid adjustments of mtDNA transcription, due to its abundance in the mitochondria and that it fully coats the mtDNA (Park *et al.*, 2007). A more suitable candidate for adjusting transcription is the recently discovered protein mitochondrial transcription termination factor 3 (MTERF3) (Linder *et al.*, 2005; Park *et al.*, 2007). *Mterf3* interacts with the mtDNA promoter region, where it suppresses the initiation of mitochondrial transcription. This type of negative regulation of mtDNA transcription has been proposed to constitute a fine-tuning mechanism of mitochondrial gene expression, in response to physiological demands (Park *et al.*, 2007). The importance of MTERF3 as a regulatory protein is underscored by the fact that homozygote *Mterf3* gene knock-out mice die at an embryonic stage and that muscle specific *Mterf3* KO develop cardiac hypertrophy and die prematurely (Park *et al.*, 2007)(Paper II).

2.2.3 The mitochondrial respiratory chain

Mitochondria are the foremost energy producers in the cell. This is carried out by the respiratory chain, or electron transport chain, which is the site for oxidative phosphorylation (OXPHOS). The respiratory chain is composed of five large protein complexes, denoted complex I-V, that are integrated in the inner mitochondrial membrane. The complexes I, III, IV and V are built up by proteins both imported from the cytoplasm and derived from the mitochondria via translation of mtRNA. Complex II is thus the only complex that is composed solely of proteins encoded by the nuclear genes (Falkenberg *et al.*, 2007).

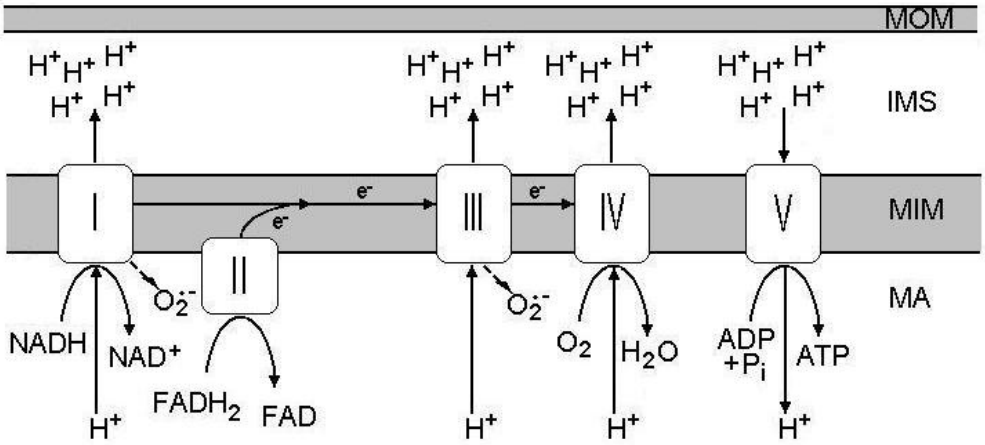

Figure 3. The mitochondrial respiratory chain. The respiratory chain complexes are indicated by roman numerals. e^-: electrons, MOM: mitochondrial outer membrane, IMS: inter-membrane space, MIM: mitochondrial inner membrane, MA: mitochondrial matrix. (Picture by Armiento, M.)

The metabolism of carbohydrates and fatty acids through the citric acid cycle and β-oxidation transfers energy to NAD^+ and FAD by reducing them to NADH and $FADH_2$ correspondingly. NADH and $FADH_2$ subsequently donate electrons at complex I and II, respectively, whereafter the electrons are transferred to complex III and then to complex IV. Furthermore, the electron flow drives the translocation of protons (H^+) from the mitochondrial matrix over the inner membrane, which leads to the build-up of an H^+ gradient across the inner mitochondrial membrane (Saraste, 1999). This proton pumping is carried out at complex I, III and IV but not at complex II, which only serves as a port for electron feeding (Hagerhall, 1997). The respiratory chain got its name from the fact that it consumes oxygen (O_2). This takes place at complex IV where O_2 is reduced to H_2O in a process driven by the electron transfer (Saraste, 1999). By allowing H^+ to pass through complex V, the ATP synthase (F_0F_1 ATPase), the proton gradient

across the inner mitochondrial membrane can be utilized to drive the synthesis of ATP from ADP + P_i (Boyer, 1997). In this fashion, the mitochondrial respiratory chain provides a way to efficiently convert energy substrates, such as carbohydrates and fatty acids, to ATP within the cell (figure 3).

2.3 MITOCHONDRIAL CA^{2+} HANDLING

Cellular Ca^{2+} handling is not a process that takes place only between the cytoplasm, the SR and the extracellular space. The mitochondrion has long been recognized to have an ability to sequester Ca^{2+} (DELUCA & Engstrom, 1961; VASINGTON & MURPHY, 1962). Several mitochondrial dehydrogenases, linked to the citric acid cycle, are known to be sensitized by Ca^{2+} (McCormack *et al.*, 1990). Moreover, it has been shown in both skeletal and cardiac muscle that the respiratory chain could be directly stimulated by Ca^{2+} (Kavanagh *et al.*, 2000; Territo *et al.*, 2000; Balaban *et al.*, 2003). Elevating $[Ca^{2+}]$ in the mitochondrial matrix is thus thought to provide a mechanism to stimulate ATP production.

Uptake of Ca^{2+} in the mitochondria is dependent on the electrochemical gradient for Ca^{2+} across the inner mitochondrial membrane (Bernardi, 1999). The primary route whereby mitochondria take up Ca^{2+} is through the mitochondrial calcium uniporter (MCU) (Bernardi, 1999). The molecular structure of the MCU has not yet been defined. However, there is evidence indicating that the MCU has the characteristics of an ion channel (Kirichok *et al.*, 2004). Extrusion of mitochondrial Ca^{2+} occurs mainly in exchange with Na^+ via a mitochondrial Na^+/Ca^{2+} exchanger (Murphy & Eisner, 2009). Moreover, transient opening of the mitochondrial permeability transition pore (MPTP) (Crompton *et al.*, 1987; Halestrap, 2009), a large inducible pore in the inner mitochondrial membrane, may also permit Ca^{2+} fluxes (Murgia *et al.*, 2009).

In contracting cardiomyocytes, Ca^{2+} is taken up in the mitochondria as a consequence of the transient increases in systolic $[Ca^{2+}]_i$ (Dedkova & Blatter, 2008). However, the kinetics and mode of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_{mit}$) dynamics in cardiac contraction is strongly debated (O'Rourke & Blatter, 2008). In short, the controversy revolves around whether $[Ca^{2+}]_{mit}$ rapidly responds to a $[Ca^{2+}]_i$ transient on a beat-to-beat basis or if the increased $[Ca^{2+}]_{mit}$ is a slow integration of $[Ca^{2+}]_i$ transients (O'Rourke & Blatter,

2008). Nonetheless, the Ca^{2+} sensitive ATP-generation of the mitochondrial matrix would, at least over time, be stimulated by an increased amplitude and frequency of $[Ca^{2+}]_i$ transients. In a recent review article, it was suggested that ~80% of the increased cardiac energy expenditure during exercise (due to increased heart rate and contractility) is under influence of cellular Ca^{2+} signaling (Duncker & Bache, 2008). Based on this it was argued that $[Ca^{2+}]_{mit}$ is an effective feed-forward signal for matching energetic supply and demand in the beating heart (Balaban, 2009).

Also in skeletal muscle, uptake of Ca^{2+} in the mitochondria can occur during contraction. In oxidative slow-twitch soleus muscle fibers from mouse, increased $[Ca^{2+}]_{mit}$ upon stimulation has been shown (Bruton *et al.*, 2003). However, contraction did not increase $[Ca^{2+}]_{mit}$ in mouse fast-twitch FDB muscle fibers (Lännergren *et al.*, 2001). The fact that some types of muscle fibers take up Ca^{2+} into the mitochondria during contraction while others do not suggests that $[Ca^{2+}]_{mit}$ in skeletal muscle is highly regulated with respect to the local environment and metabolic demands of the particular muscle cell.

2.4 MITOCHONDRIAL REACTIVE OXYGEN SPECIES

2.4.1 Formation and scavenging of reactive oxygen species

Reactive oxygen species (ROS) is a term encompassing an array of bio-reactive compounds that are derived from molecular oxygen (O_2). Endogenously produced ROS commonly include: superoxide (O_2^{*-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{-*}) (Murphy, 2009; Moylan & Reid, 2007). The principal precursor of most ROS is O_2^{*-}. It has been estimated that approximately 90% of the formation of O_2^{*-} occurs within the mitochondria, in the electron transfer process of the respiratory chain (Balaban *et al.*, 2005). More specifically, complex I and III are recognized to be the sites where the lion's share of cellular O_2^{*-} formation takes place (Murphy, 2009; Balaban *et al.*, 2005). Early accounts of the magnitude of ROS production in the mitochondria suggested that 1-2% of the total O_2 consumption was transformed into ROS (Chance *et al.*, 1979). However, these numbers arose mainly from extrapolations of *in vitro* based studies and are considered to be an inflated number. More recent estimates of ROS production *in vivo* come to a figure amounting to 0.1-0.2% of the O_2

consumption (Murphy, 2009). Although the mitochondria are supposed to be the dominating production site, ROS can also be produced in the cytoplasm, e.g. via NADPH oxidase and xanthine oxidase catalyzed reactions (Droge, 2002).

O_2^{*-} is highly reactive to biological matter and thus a short lived substance in the intracellular environment. In order to reduce unwanted reactions within the cell, O_2^{*-} is dismutated into the less reactive H_2O_2 and O_2 in a reaction catalyzed by a group of enzymes called superoxide dismutases (SOD). Three forms of SOD exist, with different cellular locations: cytoplasmic SOD1 (CuZnSOD), mitochondrial SOD2 (MnSOD) and extracellular SOD3 (Moylan & Reid, 2007). SOD catalyzes the dismutation reaction (I) described below:

$$2O_2^{*-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad Reaction\ I$$

Although H_2O_2 is less reactive than O_2^{*-}, and therefore can diffuse more freely within the cell, it is still a substance with a strong oxidative ability that can influence cellular functions (Starkov, 2008). H_2O_2 can be metabolized in the presence of reduced glutathione (GSH) and the enzyme glutathione peroxidase, which results in the formation of water and oxidized glutathione (GSSG) (Turrens, 2003). To a lesser extent, catalase can also deactivate H_2O_2 that transforms into water and oxygen (Radi *et al.*, 1991). Moreover, H_2O_2 can react with cellular transition-metals such as ferrous iron(II) and via the non-enzymatic Fenton reaction form OH^{*-}, a substance with a very high biological reactivity and capacity to cause damage within the cell (Starkov, 2008; Moylan & Reid, 2007). ROS-induced damage to cell components, e.g. proteins, lipids and nucleic acids, are generally referred to as oxidative stress (Moylan & Reid, 2007). In this respect, several markers of oxidative stress exist. One such marker is to measure the extent of lipid oxidation products (Aldini *et al.*, 2007).

2.4.2 ROS in pathology and physiology

ROS has the reputation as being a causative agent of cellular damage. In fact, cell death can be mediated by the mitochondria through mechanisms that involve ROS formation (Orrenius *et al.*, 2007). The disease processes that have been attributed to increased

ROS production or oxidative stress are vast, with neurodegeneration, cardiac disease, obesity, type 2 diabetes, inflammatory diseases, cancer, to mention a few (McCord, 2000; Lin & Beal, 2006; Esposito *et al.*, 1999; Houstis *et al.*, 2006; Storz, 2005). Also, biological aging has been hypothesized to be caused by an interplay between dysfunctional mitochondria and oxidative stress (Harman, 1956; Wallace, 1999). However, the notion of increased ROS in ageing has recently been challenged. Consecutively, two mouse models of ageing, where accumulation of mtDNA mutations led to respiratory chain failure, both lack evidence of oxidative stress (Trifunovic *et al.*, 2005; Kujoth *et al.*, 2005).

Apart from being mediators in various pathologies, ROS are also important in normal cellular signaling. ROS have been found to be a modulator of an array of molecules important to cellular signaling such as NF-κB, the insulin receptor and mitogen-activated protein kinases (Droge, 2002). In this context, Ca^{2+} handling in cardiac cells can also be altered by changes in ROS, where ROS activates the RyR and inhibits SR Ca^{2+} uptake (Zima & Blatter, 2006). Moreover, the cardiac L-type Ca^{2+} current has been shown to be redox sensitive (Sims & Harvey, 2004; Hool, 2008). Thus, ROS-mediated signaling can influence cardiac E-C coupling. Also in skeletal muscle, E-C coupling has been shown to be influenced by ROS (Andrade *et al.*, 1998; Andrade *et al.*, 2001). In these studies, ROS at low concentration could enhance muscle function, but the function was impaired with higher concentrations or prolonged exposure. It was thus concluded that the effect of ROS followed a biphasic pattern. Similarly, McCord has put forward the notion of a bell-shaped dose-response relationship in the context of ROS effects (McCord, 2008). He based this on the results that an increase in lipid peroxidation was found at both very low and very high activity of O_2^{*-} scavenging (McCord, 2008). These notions imply the existence of a certain optimum level of ROS, which emphasizes the role of ROS in both the physiological and pathological setting.

2.5 MITOCHONDRIAL DISEASE

Primary mitochondrial diseases are a heterogenic group of genetic disorders where the respiratory chain is affected (Schapira, 2006). Due to the dependence of the respiratory chain on both the mtDNA and nuclear DNA, a mutation in either of the two genomes

could cause mitochondrial dysfunction. These diseases can be inherited but also occur spontaneously. Transmission of mtDNA mutations to the offspring is strictly maternal, due to the inheritance pattern of mtDNA. Any organ could be affected in mitochondrial diseases, and this contributes to the great variability of symptoms found in this disease category. However, tissues with high energy demand, such as the brain, endocrine pancreas, skeletal and cardiac muscle, are more frequently affected. Moreover, post-mitotic tissues, e.g. heart and skeletal muscle, with negligible cell division are more sensitive to the occurrence of respiratory chain defects (Larsson & Oldfors, 2001).

Respiratory chain defects in skeletal muscle can cause mitochondrial myopathy. Symptoms include progressive muscle weakness, fatigue intolerance and muscle atrophy (Munnich & Rustin, 2001). The muscle symptoms can be isolated or come with symptoms from other organ systems, e.g. nervous system. Lactate levels can be elevated, especially during exercise, and are thus used as a diagnostic tool. Also reduced phosphocreatine levels can be used as a disease marker. A hallmark of mitochondrial myopathy is the presence of ragged-red fibers on histological muscle biopsy examination. The ragged-red fibers represent the proliferation of mitochondria with an abnormal ultrastructure that is seen as an adaptation to respiratory chain failure (Larsson & Clayton, 1995; Larsson *et al.*, 1998).

Mitochondrial cardiomyopathy is considered one of the more common forms of cardiomyopathy in children (Marin-Garcia & Goldenthal, 1997). However, late onset forms of mitochondrial cardiomyopathy also exist (Munnich & Rustin, 2001). Usually, the cardiomyopathy phenotype is characterized by left or bi-ventricular hypertrophy. Dilated cardiomyopathy with increased ventricular size can, however, also be present. As with the skeletal muscle, mitochondrial cardiomyopaties are commonly found in association with neurological manifestations such as encephalopathy and epilepsy (Marin-Garcia & Goldenthal, 1997).

2.6 THE METABOLIC SYNDROME AND CARDIAC DISEASE

The coexistence of obesity, hyperlipidemia, and insulin resistance are categorized as the metabolic syndrome (Reaven, 1988). This disease complex is highly prevalent and associated with increased risk of developing type 2 diabetes and cardiovascular morbidities (Kim & Reaven, 2004). An array of pathogenic signals is found in the

metabolic syndrome, which interacts in the aggravation of the disease process. For example, inflammatory stress signaling as well as catecholaminergic stress signals emanating from the sympathetic system are increased in the metabolic syndrome (Wellen & Hotamisligil, 2005; Tentolouris *et al.*, 2006; Nicolson, 2007). Additionally, increased ROS production has been associated with this condition (Houstis *et al.*, 2006).

Development of cardiac dysfunction is particularly common in the metabolic syndrome (Bonora, 2006). This can be manifested as an increased risk of heart failure (Abel *et al.*, 2008; Kenchaiah *et al.*, 2002; Harmancey *et al.*, 2008b). In association with this, mitochondrial respiratory chain dysfunction has been suggested as a pathological feature of cardiac failure in obesity and the metabolic syndrome (Bugger & Abel, 2008). Commonly used animal models of the metabolic syndrome (*ob/ob* and *db/db* mice), indeed exhibit defects in mitochondrial oxidative capacity (Abel *et al.*, 2008). Interestingly, cardiac contractile dysfunction is seen in both of these models.

3 AIMS

In this thesis, the function of skeletal and cardiac muscle cells was investigated in mouse models of: (1) mitochondrial myopathy, (2) mitochondrial cardiomyopathy and (3) the metabolic syndrome. Both primary mitochondrial diseases and the metabolic syndrome are linked to disorders in contractility. Therefore, one major aim was to reveal changes in contractility and cellular Ca^{2+} handling either in skeletal muscle cells (paper I) or in cardiomyocytes (paper II-IV). Mitochondrial diseases as well as the metabolic syndrome are believed to be disorders with tissue damage due to increased levels of ROS. Thus, an additional aim of this thesis was to elucidate the presence of oxidative stress in cardiac and skeletal muscle tissue.

The specific aims were:

- To explore the mechanisms of contractile dysfunction in the *Tfam* KO mitochondrial myopathy model.

- To characterize changes in heart function and cardiomyocyte Ca^{2+} handling of MTERF3 KO mice which display mitochondrial cardiomyopathy and sudden death.

- To investigate effects of elevated saturated fatty acid concentration on cardiomyocyte Ca^{2+} handling and mitochondrial ROS production in lean WT and obese *ob/ob* mice.

- To study the role of ROS production in adrenergic stress signaling in the hearts of WT and *ob/ob* mice.

4 METHODOLOGY

4.1 MOUSE MODELS (PAPERS I-IV)

4.1.1 General aspects

All four papers of this thesis involve the use of mice for experimental purposes. The mice were kept at room temperature with a 12 h:12 h light:dark cycle. Food and water were supplied *ad libitum*. The animals were sacrificed by rapid cervical dislocation after which isolation of tissue took place. The local Stockholm North ethical committee approved all procedures.

4.1.2 Mouse strains used

Genetically modified mice were utilized in all the papers (I-IV) of this thesis. The types of mice were:

- *ob/ob*
- *Tfam* KO
- *Mterf3* KO

4.1.2.1 ob/ob

This mouse strain carries a deficiency in the gene for the hormone leptin. Leptin regulates satiety and due to the defect in this hormone *ob/ob* mice become hyperphagic and develop obesity, insulin resistance and hyperlipidemia. Thus, *ob/ob* has become a standard mouse model for studying obesity and the metabolic syndrome (Lindstrom, 2007). The *ob/ob* mouse is bred on a C57Bl/6 mouse background. Therefore, C57Bl/6 mice were used as wild type (WT) controls in all experiments involving *ob/ob* mice.

4.1.2.2 Tfam KO

These mice were created as a model for mitochondrial myopathy (Wredenberg *et al.*, 2002). The mitochondrial transcription factor A (*Tfam*) gene was knocked out using the *LoxP-Cre* recombinase system (Ekstrand & Larsson, 2002). *LoxP* is a 34 base pair sequence that is inserted in the mouse genom such that two *LoxP* segments flank the

Tfam gene. The *LoxP* sites are recognized by the *Cre* recombinase and causes recombination between these sites, thereby making the *Tfam* gene nonfunctional. By expressing *Cre* recombinase under the control of the myosin light-chain 1f (Mlc1f) gene that is active predominatly in fast-twitch type II muscle fibres (Bothe *et al.*, 2000), skeletal muscle (fast twitch) specific ablation of the *Tfam* gene was induced (Wredenberg *et al.*, 2002). In this thesis mice with the genotype $Tfam^{LoxP/LoxP}$, +/*MLC1f- Cre* are referred to as Tfam KO; Tfam KO littermates of the genotype $Tfam^{LoxP/LoxP}$ were used as controls. The *Tfam* KO mice develops progressive respiratory chain failure, myopathy and die prematurely around 5 month of age (Wredenberg *et al.*, 2002).

4.1.2.3 Mterf3 KO

Mice with cardiac and skeletal muscle specific deficiency of the mitochondrial transcription termination factor 3 (*Mterf3*) gene was generated as described elsewere (Park *et al.*, 2007). The method used to generate these mice were similar to that of the *Tfam* KO, e.g. utilizing the *LoxP-Cre* system. The *Cre* recombinase was linked to the expression of the skeletal and heart muscle creatinkinase isoform (ckmm). Thus, the genotype $Mterf3^{LoxP/LoxP}$, *ckmm-Cre* is referred to as *Mterf3* KO and the littermates with the genotype $Mterf3^{LoxP/LoxP}$ were used as controls and referred to as WT.

4.2 TISSUE ISOLATION

4.2.1 Skeletal muscle (Study I)

Whole flexor digitorum brevis (FDB) muscles were isolated by dissection. The FDB muscle was either frozen for biochemical analysis or used for single fiber experiments prepared by either: (1) microscope-led mechanical microdissection or (2) enzymatic dissociation.

4.2.1.1 Microdissection

Single fibers from FDB muscle were prepared through microscope-led dissection using a pair of microsurgical forceps and iris scissors (Lännergren & Westerblad, 1987). During the later stage of dissection, remaining fibers were probed for viability by focal electrical stimulation, causing contraction in the healthy cells. The probing procedure

guided the selection of which fiber that would finally be isolated. After selecting one muscle fiber, the other fibers were pinched, thus leaving only one viable cell. Platinum-foil micro-clips were attached to the tendons of the single fiber and it was thereafter mounted in the experimental chamber. One end of the muscle fiber was attached to semiconductor force transducer (Akers AE 801) and the other end was attached to an variable holder, allowing the fiber to be stretched to the optimal length (Lännergren & Westerblad, 1987). Moreover, the fiber was continuously perfused with Tyrode solution of the following composition (mM): NaCl 121, KCl 5, $MgCl_2$ 0.5, Na_2PO_4 0.4, $CaCl_2$ 1.8, EDTA 0.1, $NaHCO_3$ 24, glucose 5.5, and fetal calf serum (0.2%, Gibco). The Tyrode solution was continuously bubbled with O_2 (95%) and CO_2 (5%) giving a pH of 7.4. The experiments were performed at room temperature (~24°C).

4.2.1.2 Enzymatic dissection

The method used to obtain single muscle fibers by enzymatic digestion of FDB muscles followed a protocol taken from the literature (Liu *et al.*, 1997). In short, the FDB muscle was placed in a solution of Dulbecco's modified eagles medium (DMEM) containing fetal bovine serum (10%, Gibco) and collagenase type I (0.3%, Sigma-Aldrich). After ~2 hours in an incubator (37°C, 95% O_2/5% CO_2), the muscle was transferred to a fresh solution without collagenase and was gently pipetted with a 1 ml pipette until the muscle fibers were fully dissociated (~10 times). The suspension of muscle fibers was aliquoted (~0.3 ml; ~50-100 cells) onto laminin coated glass-bottom dishes. After allowing the fibers to attach to the glass-bottom for ~10 min, the dish was filled with an abundance of DMEM (~2 ml) and kept in room temperature until use (within 12 hours).

4.2.2 Heart muscle (Study II, III and IV)

4.2.2.1 Cardiomyocyte isolation

Isolation of cardiomyocytes were done according to protocols of the Alliance for Cellular Signalling (AfCS Procedure Protocol ID PP00000 125) (Sambrano *et al.*, 2002) Briefly, ~10 min prior to sacrificing the mouse was intraperitoneally injected with 0.5 ml of heparin dissolved in phosphate buffered saline (PBS; 100 U/ml). The mouse was then killed, had its thoracic cavity opened followed by a swift excision of the intact heart with ~2 mm of the ascending aorta still attached to the ventricle. The

aorta was cannulated with a syringe attached to a perfusion system. Perfusion of the heart was commenced within 1 minute from the excision. Initially the heart was perfused for ~5 min with a solution (perfusion buffer) containing (mM): NaCl 113, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$-$7H_2O$ 1.2, phenol red 0.032, $NaHCO_3$ 12, $KHCO_3$ 10, HEPES 10, taurine 30, 2,3-butanedione monoxime (BDM) 10, Glucose 5.5. The perfusate was then changed to a digestion buffer based on the perfusion buffer but with addition of Liberase blendzyme 1 (0.25 mg/ml, Roch) and $CaCl_2$ (12.5 µM). This step was proceeded until the heart became pale and swollen (~10-20 min). At this step the heart was taken down from the perfusion set-up, the atria were removed and the ventricular tissue was gently teared into pieces. The ventricular pieces were gently pipetted several times with digestion buffer until a suspension of cells was achieved. To stop the digestion process, perfusion buffer containing 10% bovine calf serum (stopping buffer) was added. Debris was allowed to sediment and the supernatant (containing isolated cardiomyocytes) was transferred to a new tube and re-suspended with new stopping buffer. The Ca^{2+} concentration was then increased in increments (62µM, 112 µM, 212 µM, 500 µM, 1 mM (final concentration)), waiting 4 min between each step.

4.2.2.2 Preparing cardiomyocytes for experiment

Cardiomyocytes were loaded with fluorescent indicators (see below) and ~0.3 ml of cell suspension (~100-200 cardiomyocytes) was placed on laminin-coated coverslips that made up the bottom of the perfusion chamber. Cells were allowed to attach to the coverslip for ~5 min before the start of experiments. The cells were then superfused with Tyrode solution (mM): NaCl 121, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 24, EDTA 0.1, and glucose 5.5. The Tyrode solution was bubbled with 5% CO_2/95% O_2, which gives a pH of 7.4, and experiments were performed at room temperature (~24°C). Cells were continuously stimulated at 0.5 Hz, 1 Hz or 3 Hz with 1-ms current pulses delivered via two platinum electrodes, one on each side of the perfusion chamber. Measurements were only performed on rod-shaped cells that displayed a uniform contraction in response to each stimulation pulse and showed no spontaneous contractile activity.

4.3 CONFOCAL MICROSCOPY (PAPER I-IV)

Confocal microscopy was performed using a Bio-Rad MRC 1024 unit attached to a Nikon Diaphot inverted microscope with a Nikon Plan Apo 20x, 40x or 60x objective (for the 40x and 60x objectives oil immersion was used) with a numerical aperture (NA) of 0.75, 1.3 and 1.4 respectively.

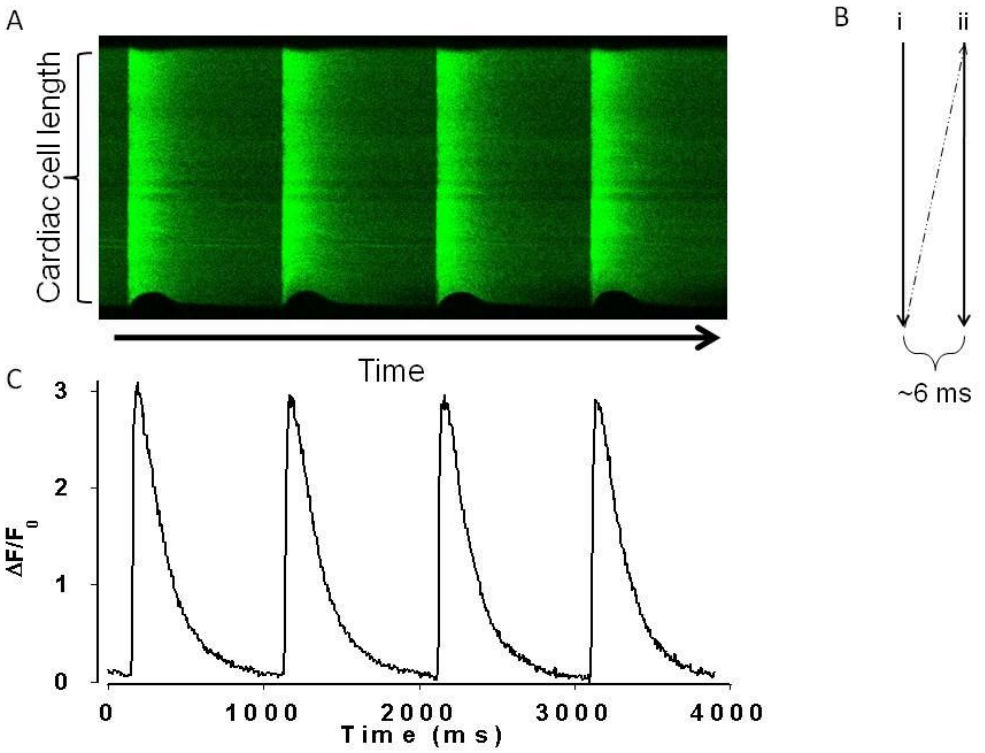

Figure 4. Confocal line scan. *A* shows a typical confocal line scan. The x-axis represents the cardiac cell length (typically ~100 µm) and the y-axis corresponds to time. The scheme in *B* displays how the light beam (solid line) scans along the length of the specimen (i), jumps back to the starting position (dotted line) and scans again (ii), this process is repeated typically 1500 times for a full line scan measurement. The temporal scanning resolution of this system is ~6 ms. *C*: Cytoplasmic Ca^{2+} transients, represented by the fluorescence intensity (an average over the cell length in *A*) plotted as a function of time.

4.3.1 Cytoplasmic [Ca^{2+}] and contractility measurements in cardiomyocytes (paper II-IV)

Cytoplasmic free [Ca^{2+}] ($[Ca^{2+}]_i$) in cardiomyocytes was measured using the fluorescent Ca^{2+} indicator fluo-3 (5µM, 20 min incubation; Invitrogen/Molecular Probes) (Fauconnier *et al.*, 2007). Confocal line scans along the long axis of the cardiomyocyte were obtained using an excitation wavelength of 488 nm and measuring the emitted light at 515 nm (figure 4). Images were analyzed off line with ImageJ (National Institutes of Health; available at: http://rsb.info.nih.gov/ij). To enable

comparisons between cells, changes in the fluo-3 fluorescence signal (ΔF) were divided by the fluorescence immediately before a stimulation pulse was given under control conditions (F_0). The decay of the Ca^{2+} transients was assessed by measuring the decay time constant (τ) of the exponential part of the decay phase using a single exponential fit (Diaz *et al.*, 2001). Analysis of the Ca^{2+} transients was done using the software Origin (version 7.5; OriginLab).

Shortening of the cardiomyocyte was used as a measure of contractility in papers II-IV (Fauconnier *et al.*, 2007). Cell shortening was determined simultaneously with the Ca^{2+} measurements by following the edges of the fluorescence images of the cells during the contractions. The cell length was measured in the relaxed state and the maximally contracted state. Fractional shortening (FS%) was defined as:

$$FS\% = \frac{(\text{relaxed cell length} - \text{contracted cell length})}{\text{relaxed cell length}} \times 100$$

In paper II and IV measurements of Ca^{2+} transients and cardiomyocyte shortening were also performed after exposure to the β-adrenergic agonist isoproterenol (ISO; 100nM). These experiments were done within 4-15 minutes after application of the drug.

In paper II the presence of spontaneous (i.e. not caused by electrical stimulation) Ca^{2+} events during line scanning was studied. A spontaneous Ca^{2+} event was defined as either: 1) the presence of an aberrant Ca^{2+} transient; 2) a localized transient increase in fluorescence that is larger than 2 standard deviations of the surrounding fluorescence at the same time point. Line scans that displayed spontaneous Ca^{2+} events was not included in the measurements of Ca^{2+} transient amplitude and decay, or cell shortening.

4.3.2 Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) (paper I-III)

In study I-III mitochondrial membrane potential ($\Delta\Psi_m$) was measured using tetramethylrhodamine ethyl ester (TMRE; Invitrogen/Molecular Probes). (Duchen *et al.*, 1998; Fauconnier *et al.*, 2007; Aydin *et al.*, 2009)Isolated fully viable myocytes were loaded with TMRE (0.1-1 μM) for 15 min at room temperature, followed by washout. Confocal images of TMRE fluorescence were obtained by excitation at 568

nm while measuring the emitted light at 585 nm. Skeletal muscle cells were fatigued according to the protocol described elsewhere (Aydin *et al.*, 2009), and images were obtained before and immediately after the 50 fatiguing tetani. Cardiomyocytes were continuously stimulated at 1 Hz with images taken at regular intervals as described in the respective paper (II and III). At the end of the experiment, myocytes were exposed to the mitochondrial uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1-4 µmol/l), which depolarizes the mitochondria thereby allowing the dynamic range of the dye to be determined. Measurements were done in the same cell regions at each time point. Fluorescence signals obtained from mitochondria-rich areas were divided by fluorescence from an adjacent cytosolic region.

4.3.3 Mitochondrial [Ca^{2+}] measurement (paper I)

The fluorescent Ca^{2+} indicator Rhod-2 AM (Invitrogen/Molecular Probes) was used to measure mitochondrial Ca^{2+} ($[Ca^{2+}]_{mit}$). Rhod-2 AM permeates the plasma membrane and this positively charged indicator accumulates in the negatively charged mitochondrial matrix and can thus be used to measure changes in $[Ca^{2+}]_{mit}$. To load the muscle cells with the indicator, isolated FDB fibers were incubated in 5 µM Rhod-2 AM for 90–120 min at room temperature and then washed in indicator-free medium (Bruton *et al.*, 2003). To excite the indicator, a wavelength of 568 nm was used. Emitted light was collected through a 585 nm long-pass filter. The data were normalized as F/F_0, F being the fluorescence intensity at any given time point and F_0 is the value at the start of the experiment. Changes in $[Ca^{2+}]_{mit}$ were measured during fatigue induced by 50 repeated tetani. During the fatigue run, stimulation was stopped for ~6 s at regular intervals and the confocal images were obtained in these pauses (Bruton *et al.*, 2003). The recovery of $[Ca^{2+}]_{mit}$ after fatigue was followed by obtaining images at regular intervals for 10 min.

4.3.4 Measurement of mitochondrial superoxide (paper I-IV)

Changes in mitochondrial superoxide O_2^{*-} production were monitored using the fluorescent indicator MitoSOX Red (Invitrogen/Molecular Probes) (Martins *et al.*, 2008). Muscle cells were incubated with MitoSOX Red (5 µM) for ~30 min at room temperature and experiments started after 5 min of washing. MitoSOX Red was excited with 488 nm light and emitted light was collected through a 585 nm long-pass filter.

Single dissected FDB fibers were used to monitor changes in mitochondrial $O_2^{\bullet-}$ production during fatigue induced by 50 repeated tetani (as described above) with regular pauses to obtain confocal images. Cardiomyocytes were paced at 1 Hz with pauses to obtain confocal images.

4.4 FORCE AND $[CA^{2+}]_I$ MEASUREMENTS IN SINGLE SKELETAL MUSCLE FIBERS (PAPER I)

4.4.1 Force measurement

Tetanic stimulation was achieved by supramaximum current pulses delivered via platinum plate electrodes lying parallel to the fiber, each with a duration of 0.5 ms. Force was measured as the mean over 100 ms where it was maximal and expressed relative to the cross-sectional area. To establish the force-frequency relationship the following stimulation frequencies were used: 20, 30, 40, 50, 70 and 100 Hz. In the fatigue protocol, pulse-trains of 300 ms duration and 70 Hz stimulation frequency were given at 2 s interval. The fatigue protocol consisted of 50 pulse-trains.

4.4.2 $[Ca^{2+}]_i$ measurement

Mechanically dissected single FDB fibers (described above) were connected to a force transducer and injected with the fluorescent Ca^{2+} indicator indo-1. This allowed for simultaneous recording of force and cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$. Indo-1 was excited with UV-light at 360 ± 5 nm. Upon Ca^{2+}-binding to the indicator, a wavelength shift of the emitted light takes place. Fluorescence emission was collected at the wavelengths 405 ± 5 nm and 495 ± 5 nm. After calibration at known Ca^{2+} concentrations, the ratio of the fluorescence intensity between the emission wavelengths 405 and 495 nm (R) can be used to calculate $[Ca^{2+}]_i$. The following equation (equation 1) was used for conversion of the measured R value to $[Ca^{2+}]_i$:

$$[Ca^{2+}]_i = K_D \beta \frac{(R - R_{min})}{(R_{max} - R)} \qquad \text{(equation 1)}$$

K_D is the dissociation constant of Indo-1, β is the ratio of the 495 nm emission at very low and saturating $[Ca^{2+}]_i$, R_{min} is the ratio of 405 and 495 nm at very low $[Ca^{2+}]_i$, R_{max} is the ratio of 405 and 495 nm at saturating $[Ca^{2+}]_i$. K_D, β, R_{min} and R_{max} values were

established from intracellular calibrations as described previously (Andrade *et al.*, 1998).

By plotting force against $[Ca^{2+}]_i$ for the stimulation frequencies 20-100 Hz, a force-$[Ca^{2+}]_i$ curve could be produced. Force-$[Ca^{2+}]_i$ data were fitted to a Hill equation (equation 2) to enable quantitative comparison of the force-$[Ca^{2+}]_i$ properties between WT and Tfam KO (Aydin *et al.*, 2009).

$$P = P_{\max} \frac{[Ca^{2+}]_i^N}{Ca_{50}^{2+} + [Ca^{2+}]_i^N} \qquad \text{(equation 2)}$$

where P is force per cross-sectional area, P_{max} is the force at full Ca^{2+} activation, Ca_{50}^{2+} is the $[Ca^{2+}]_i$ at 50% of P_{max}, and N is the coefficient (Hill coefficient) that describes the steepness of the curve.

4.5 PATCH CLAMP AND $I_{CA,L}$ MEASUREMENT IN CARDIOMYOCYTES (PAPER IV)

Cardiomyocytes were isolated as described above. On these, whole-cell patch-clamp experiments were performed. In brief, cardiomyocytes were patched using a glass patch pipette with 2-3 MΩ resistance. To allow for whole-cell measurement, the membrane patch was ruptured by suction. The measured currents were normalized to cell membrane capacitance and expressed as current densities (pA/pF). To record L-type Ca^{2+} currents ($I_{Ca,L}$), the following pipette solution was used (mM): CsCl 140, HEPES 10, ATP(Mg) 3, GTP(Na) 0.4, EGTA 10; pH adjusted to 7.2 with CsOH. The bath solution contained (mM): TEA-Cl 135, $MgCl_2$ 2, Glucose 10, HEPES 10, $CaCl_2$ 1.8; pH adjusted to 7.4 with TEAOH. In some experiments the β-receptor agonist isoproterenol (ISO; 100nM) and/or the general antioxidant N-acetylcysteine (NAC; 5mM) were added to the bath. To trigger $I_{Ca,L}$, test pulses ranging from -60 mV to +60 mV were applied from a holding potential of -80 mV. Data acquisition and analyses were performed using pCLAMP (version 6, Axon Instruments).

4.6 WESTERN BLOT (PAPER I, III AND IV)

Protein expression in both cardiac and skeletal muscle was assessed by western blotting. Muscles were homogenized in ice-cold lysis buffer of the following composition (mM): HEPES 20, NaCl 150, EDTA 5, KF 25, Na_3VO_4 1, protease inhibitor coctail (Roche), glycerol (20% v/v), Triton X-100 (0.5% v/v), yielding pH 7.6. The homogenate was then centrifuged. The supernatant protein content was determined using Bradford assay (Bio-Rad). Samples were diluted 1:1 with Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol and heated at 95°C for 10 min. Twenty micrograms of supernatant protein was separated by electrophoresis on NuPAGE Novex 4–12% Bis–Tris gels (Invitrogen) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in 5% (w/v) non-fat milk dissolved in tris-buffered saline tween-20 (TBS-T), followed by overnight incubation at 4 °C with a primary antibody. Membranes were then washed and incubated for 1.5 h at room temperature with the appropriate secondary antibody. Immunoreactive bands were visualized using enhanced chemiluminescence (Super Signal; Pierce, Rockford, IL, USA). Band densities were analysed with ImageJ (NIH, USA; http://rsb.info.nih.gov/j/). The antibodies that were used are described in the method sections of the papers.

4.7 ANALYSIS OF mRNA EXPRESSION (PAPER I AND II)

4.7.1 Quantitative PCR (paper I)

Total RNA from control and *Tfam* KO mouse EDL muscles were isolated using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas), and quantitative PCR reactions were performed with the ABI 7700 Sequence Detection System (Applied Biosystems, USA) using TaqMan chemistry. The results from the genes of interest were normalized to 18S rRNA that was quantified from the same sample. The primer and fluorogenic probe sequences are described in paper I.

4.7.2 Northern blot (paper I and II)

RNA from heart was isolated with the Trizol Reagent (GIBCO/BRL, Life Technology). DNA probes specific to the genes of interest were labeled with [α-^{32}P]-dCTP by using

random primer extension method. Phosphorimager analysis of northern blots was used to assess levels of transcripts in comparison to the nuclear 18S rRNA levels as described previously (Sorensen *et al.*, 2001).

4.8 TELEMETRY MEASUREMENTS

Electrocardiography (ECG), core body temperature and locomotor activity recordings were performed on conscious, freely moving individually caged mice (Johansson & Thoren, 1997). The telemetry transmitter-receiver system (TA10ETA-F20; RPC-1, DSI, USA) was connected to a data acquisition system (DSI, USA). Data were collected continuously (24 hours/day) at a sampling rate of 1000Hz for up to 14 days. Transmitters were implanted in the abdominally cavity during isoflurane anesthesia (~20 min). Two ECG electrodes were placed subcutaneously, one at the right shoulder (negative) and the other in the lower left chest region (positive) approximating the Einthoven lead II (Kramer *et al.*, 1993).

4.8.1 Analysis of ECG recordings

ECG recordings were analyzed off line using the software ECG-auto (EMKA Technologies, v1.5.12.22). A detailed description of the analysis procedure is described elsewhere (Thireau *et al.*, 2008a; Thireau *et al.*, 2008b). In brief, ECG signals were digitally filtered between 0.1 and 1000 Hz and then analyzed. R-R, P-R, QRS and Q-T intervals were calculated from 3 days prior to the endpoint of the *Mterf3* KO and compared to results obtained in age matched WT mice. On the same records, heart rate variability (HRV) was calculated from the R-R intervals. The standard deviation of all normal R-R intervals (SDNN) was used as the measure for HRV. Power spectral analysis of the HRV was also performed. One ECG period of 3 min was selected every 2 h during a period when the locomotor activity was low. The power spectrum was produced using the fast Fourier transform. The frequency range was divided into a low-frequency range (LF: 0.15-1.5 Hz) and a high-frequency range (HF: 1.5-5 Hz) (Thireau *et al.*, 2008a).

5 RESULTS AND DISCUSSION

5.1 GENERAL BACKGROUND PAPER I AND II

In paper I and II, two models of primary mitochondrial disease, the *Tfam* KO and the *Mterf3* KO, were studied. Both models were created by a tissue specific knockout of the genes for mitochondrial transcription regulators *Tfam* (mitochondrial transcriptionfactor A) or *Mterf3* (mitochondrial transcription termination factor 3), utilizing the cre-lox recombinase system (Ekstrand & Larsson, 2002). The *Tfam* KO was specifically targeted to fast twitch skeletal muscle, whereas the *Mterf3* KO affected striated muscle in general, i.e. both skeletal and cardiac muscle. Global ablation of either *Tfam* or *Mterf3* was embryonically lethal, thus inferring a rational for creating tissue specific knockouts. Lack of either Tfam or MTERF3 causes progressive respiratory chain failure. By targeting skeletal and cardiac muscle it is possible to create specific models of mitochondrial myopathy and cardiomyopathy, two common features of mitochondrial disease (Wredenberg *et al.*, 2002; Park *et al.*, 2007). As mitochondrial dysfunction is implicated in the pathogenesis of other forms of skeletal muscle disease (Marzetti & Leeuwenburgh, 2006; Bernardi & Bonaldo, 2008; Oldfors *et al.*, 2006), models of muscle specific mitochondrial dysfunction shed light on the impact of defective mitochondria in a more general sense. Similarly, cardiac disease, e.g. heart failure and ischemia reperfusion damage, are believed to involve disturbed mitochondrial function (Marin-Garcia & Goldenthal, 2008; Murphy & Steenbergen, 2008).

5.2 PAPER I

5.2.1 Background

Genetic mutations that affect mitochondrial function often cause skeletal muscle dysfunction (Larsson & Oldfors, 2001). In paper I, skeletal muscle specific *Tfam* KO mice were used. These mice display important hallmarks of mitochondrial myopathy, such as ragged-red muscle fibers, accumulation of abnormally appearing mitochondria, progressively deteriorating respiratory chain function and reduced phosphocreatine concentration (Wredenberg *et al.*, 2002; Wredenberg *et al.*, 2006). Moreover, whole

muscles from *Tfam* KO are weaker than the control muscle (Wredenberg *et al.*, 2002). The aim of this study was to investigate whether changes in cellular Ca^{2+} handling was part of the mechanism of muscle weakness in *Tfam* KO. Moreover, the occurrence of oxidative stress in *Tfam* KO muscles was scrutinized.

5.2.2 Decreased force, tetanic $[Ca^{2+}]_i$ and expression of calsequestrin-1 in *Tfam* KO skeletal muscle

Tfam KO fibers displayed significantly lower tetanic $[Ca^{2+}]_i$ than control fibers at all stimulation frequencies. Also, force was significantly lower at 40 to 100 Hz ($P < 0.05$) in the *Tfam* KO. Tetanic force in the presence of caffeine, that can be used to assess sarcoplasmic reticulum (SR) Ca^{2+} load and the force produced at saturating $[Ca^{2+}]_i$ (Allen & Westerblad, 1995), was significantly lower in *Tfam* KO than in control cells. The force – $[Ca^{2+}]_i$ relationships of *Tfam* KO and control fibers were not different with the exception of a slightly reduced F_{max} in the *Tfam* KO. These results support a view of decreased force production in the muscle fibers that is predominantly due to decreased SR Ca^{2+} release, but not to impaired myofibrillar function. Reduced SR Ca^{2+} release could be caused by lower Ca^{2+} storage capacity in the SR due to a reduction in SR luminal Ca^{2+} buffering. To investigate this, expression of the skeletal muscle SR Ca^{2+} buffering protein calsequestrin 1 was measured (Beard *et al.*, 2004). Indeed, a reduction in calsequestrin 1 mRNA and protein expression was found in the *Tfam* KO. In line with this, fast-twitch muscle cells of mice lacking CASQ1 show markedly smaller $[Ca^{2+}]_i$ increases in response to electrical stimulation and caffeine application (Paolini *et al.*, 2007).

Mitochondrial myopathy patients typically display increased fatigability (Tarnopolsky & Raha, 2005). Surprisingly, *Tfam* KO and control muscle fibers displayed similar fatigue resistance. However, the fatigue experience in an exercising individual begins when the physical activity cannot be sustained as intended or when it is accompanied by the sensation of excessive effort and distress. During exercise *in vivo*, the muscle weakness observed in *Tfam* KO would therefore lead to early fatigue development because muscles always have to work at a higher fraction of their maximal capacity (Allen *et al.*, 2008).

5.2.3 No signs of oxidative stress or increased ROS production in *Tfam* KO muscle

During fatiguing stimulation neither *Tfam* KO nor control muscle fibers showed an increase in mitochondrial ROS production when measured as MitoSOX Red (Martins *et al.*, 2008). To assess the rate of mitochondrial $O_2^{\bullet-}$ production, muscle fibers were exposed to 100 µM H_2O_2, which would increase the mitochondrial [$O_2^{\bullet-}$] by inducing product inhibition of SOD2 and thereby inhibit the conversion of $O_2^{\bullet-}$ to H_2O_2 (McAdam *et al.*, 1977; Hearn *et al.*, 1999). Application of H_2O_2 resulted in a clear increase in the MitoSOX Red signal and the amplitude of this H_2O_2-induced increase was significantly smaller in *Tfam* KO than in WT muscle cells. This indicates that, if anything, mitochondrial $O_2^{\bullet-}$ production in *Tfam* KO is lower than in WT. Furthermore, the expression SOD2 protein has been reported to be increased by oxidative stress. No difference in SOD2 expression or activity was found between *Tfam* KO and control muscles. Moreover, oxidative stress is associated with the formation of reactive carbonyl species such as 4-hydroxy-trans-2-nonenal (HNE) and malondialdehyde (MDA) (Aldini *et al.*, 2007). These compounds form adducts to proteins and can be detected by western blot. No difference in HNE or MDA protein adducts was found in *Tfam* KO compared to control. Thus, on the basis of multiple methods, no signs increased ROS-induced damage could be found as part of a pathological phenotype in *Tfam* KO mitochondrial myopathy.

5.2.4 Increased mitochondrial [Ca^{2+}] but no change of $\Delta\Psi_m$ in fatiguing *Tfam* KO fibers

Control fibers showed no change in $[Ca^{2+}]_{mit}$, during the 50 fatiguing tetani or recovery, which is consistent with previous measurements in wild-type FDB fibers (Lännergren *et al.*, 2001; Lännergren *et al.*, 1999). In contrast, $[Ca^{2+}]_{mit}$ displayed a 3–4-fold increase during fatigue in *Tfam* KO cells (figure 5). Moreover, the $[Ca^{2+}]_{mit}$ recovered slowly in 4 month old *Tfam* KO mice and even 10 min after the end of fatiguing stimulation, $[Ca^{2+}]_{mit}$ was increased above the pre-fatigue value by ~60%. Interestingly, *Tfam* KO fibers from 2 month old mice (that lack overt myopathy symptoms) showed a much faster recovery of $[Ca^{2+}]_{mit}$ after fatigue compared to the terminally ill 4 month old *Tfam* KO mice. Application of the cyclophilin D-binding inhibitor cyclosporin A (CSA) resulted in a ~40% smaller contraction-induced increase in $[Ca^{2+}]_{mit}$ compared to the non-treated *Tfam* KO fibers (figure 5). CSA had no effect in control fibers. Excessive

increase in $[Ca^{2+}]_{mit}$ can be associated with decreased mitochondrial membrane potential ($\Delta\Psi_m$). In *Tfam* KO fibers, however, after 50 fatiguing tetani the TMRE fluorescence signal (which was used to measure $\Delta\Psi_m$) was 99 ± 3% (n = 7) of the pre-fatigue value. Thus, the mitochondria in *Tfam* KO muscles retained their membrane potential during the fatigue.

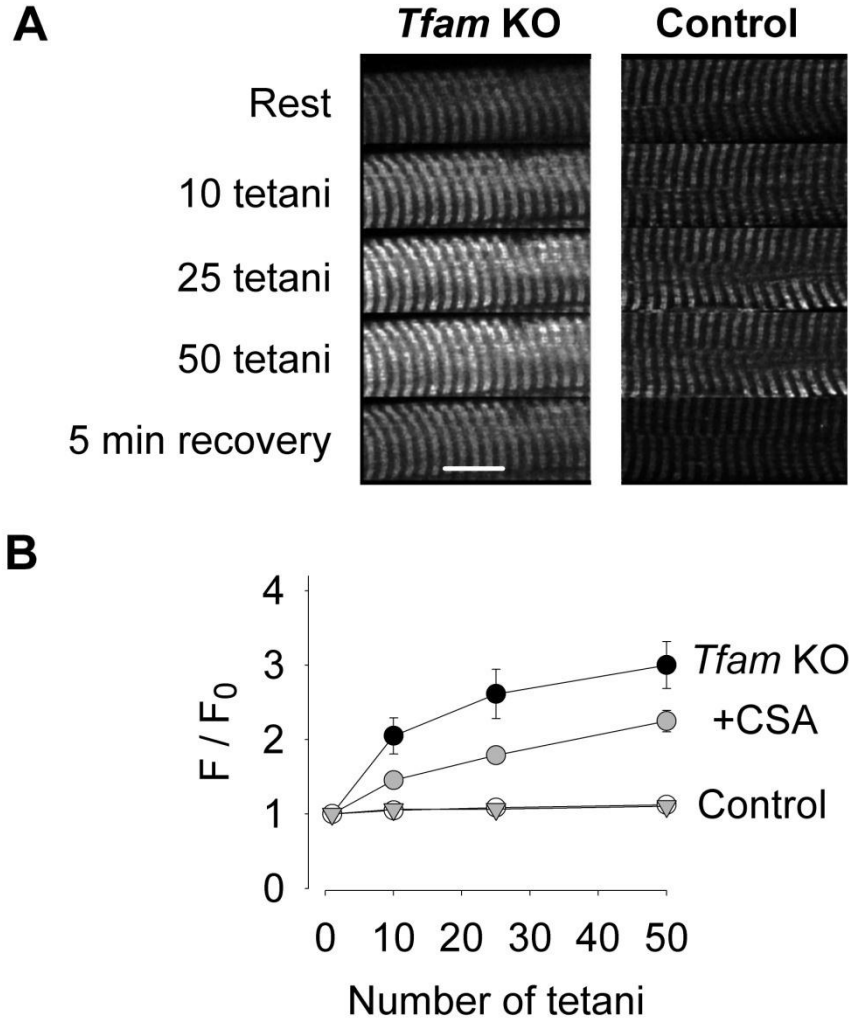

Figure 5. $[Ca^{2+}]_{mit}$ increases during fatigue in *Tfam* KO muscle fibres. Original confocal images of Rhod-2 loaded FDB fibers measuring $[Ca^{2+}]_{mit}$ in response to fatigue (A). The graph in B shows mean (±SEM) $[Ca^{2+}]_{mit}$ of control (white circles [partly concealed by the grey triangles]), *Tfam* KO (black circles), control + CSA (grey triangle), *Tfam* KO +CSA (grey circle) during fatigue and recovery.

Mitochondrial respiration is stimulated by an increase in $[Ca^{2+}]_{mit}$ (McCormack & Denton, 1993; Jouaville *et al.*, 1999; Rutter *et al.*, 1996) and hence the observed increase in $[Ca^{2+}]_{mit}$ can be seen as an adaptive response to the respiratory chain failure, particularly in the early stage of disease. Muscle cells of terminally ill *Tfam* KO mice, not only showed an increase in $[Ca^{2+}]_{mit}$ during the period with contractions, but also a

sustained $[Ca^{2+}]_{mit}$ increase 10 min after the end of stimulation. Prolonged increases in $[Ca^{2+}]_{mit}$ are generally considered as having deleterious effects on cell function (Brookes *et al.*, 2004; Duchen, 2000). It is therefore of therapeutic promise that CSA treatment decreased the stimulation-induced increase of $[Ca^{2+}]_{mit}$ in *Tfam* KO. The action of CSA is generally ascribed to its ability to inhibit the opening of mitochondrial permeability transition pores (MPTP) in stressed and Ca^{2+}-overloaded mitochondria and, in this way, prevent mitochondrial Ca^{2+} release and depolarization leading to pathological cell damage and death (Brookes *et al.*, 2004; Duchen, 2000). Our results show a novel action of CSA: it significantly inhibited the contraction-induced increase in $[Ca^{2+}]_{mit}$ in *Tfam* KO fibers. In adult mammalian skeletal muscles, $[Ca^{2+}]_{mit}$ is tightly controlled and when $[Ca^{2+}]_i$ increases (e.g. during contraction), the electrochemical gradient is in the direction of Ca^{2+} flowing into the mitochondria (Bruton *et al.*, 2003; Lännergren *et al.*, 2001). This can explain the increase in $[Ca^{2+}]_{mit}$ during fatigue in *Tfam* KO cells. Thus, Ca^{2+} can pass the mitochondrial membrane in *Tfam* KO fibres and this occurs, at least partially, via a CSA/cyclophilin D-dependent pathway. Interestingly, a few recent studies on other types of myopathies with mitochondrial engagement have shown beneficial effects of CSA treatment (Millay *et al.*, 2008; Angelin *et al.*, 2007; Merlini *et al.*, 2008).

5.2.5 Conclusion

Tfam KO muscle cells experience a progressive deterioration of mitochondrial respiratory chain function with a threatening collapse in the cellular ATP supply. To counteract this, the cells adapt by triggering measures to enhance ATP production and reduce energy expenditure. Increased mitochondrial mass improves ATP production capacity (Wredenberg *et al.*, 2002), and increased $[Ca^{2+}]_{mit}$ can stimulate ATP production in the mitochondria. Decreased SR Ca^{2+} release may reduce energy expenditure through decreased ATP consumption of both cross-bridge cycling and SR Ca^{2+} pumping. However, these adaptations come with a price: Long-term increase in $[Ca^{2+}]_{mit}$ can trigger mechanisms that lead to cellular damage and reduced SR Ca^{2+} release causes muscle weakness and locomotor problems.

5.3 PAPER II

5.3.1 Background

In paper II another model of primary mitochondrial dysfunction, the *Mterf3* KO, was studied. MTERF3 ablation causes cardiomyopathy with severe respiratory chain dysfunction, cardiac hypertrophy and sudden premature death (Park *et al.*, 2007). The major aims of the study were to elucidate if the mitochondrial cardiomyopathy model *Mterf3* KO: (1) displays changes in cardiomyocyte Ca^{2+} handling and electrical propagation in the heart that could underlie the sudden death and (2) shows signs of oxidative stress in the heart.

5.3.2 No evidence of altered $\Delta\Psi_m$ or oxidative stress in *Mterf3* KO hearts

Changes in the expression of SOD2 can be altered under conditions of oxidative stress (St Pierre *et al.*, 2006). The expression was, however, similar between *Mterf3* KO and WT hearts. Moreover, mitochondrial O_2^{*-} production (measured with MitoSOX Red) was not changed during 1 Hz stimulation period in either WT or *Mterf3* KO. To assess the mitochondrial capacity of O_2^{*-} production cardiomyocytes were exposed to 1 mM of H_2O_2, which would induce product inhibition of the SOD2 and thereby restrain the conversion of O_2^{*-} to H_2O_2 (se paper I) (Hearn *et al.*, 1999; McAdam *et al.*, 1977). This resulted in large increases in mitochondrial O_2^{*-} accumulation in the WT cardiomyocytes, whereas this increase was considerably smaller in the *Mterf3* KO. A decrease in the activity of the mitochondrial enzyme aconitase is used as a sign of increased ROS production (Gardner, 2002). Interestingly the opposite was observed, with an *increased* aconitase activity in *Mterf3* KO hearts compared to WT. Mitochondrial membrane potential ($\Delta\Psi_m$; measured with TMRE) was not different between *Mterf3* KO and WT. Taken together, no evidence of increased ROS production and oxidative stress could be found in the hearts from mitochondrial cardiomyopathy *Mterf3* KO mice. Conversely, there were signs of reduced ROS production in the mitochondria.

5.3.3 *Mterf3* KO displays changes in gene expression typical of heart failure

Elevated levels of atrial natriuretic peptide (ANP) is an established indicator of heart failure (Saito *et al.*, 1989; Scheuermann-Freestone *et al.*, 2001). Moreover, decreased expression of the cardiac SR Ca^{2+}-ATPase (SERCA2a) and its regulatory protein phospholamban (PLB) as well as increased expression of Na^{+}-Ca^{2+} exchanger (NCX) is typically found in failing hearts (Hasenfuss & Pieske, 2002). In *Mterf3* KO hearts, increased mRNA expression of cardiac ANP was found. Furthermore, mRNA expression of SERCA2a and PLB was decreased, whereas NCX mRNA expression was increased in *Mterf3* KO hearts. Thus, these results are consistent with results from previous studies on cardiomyopathies in both animals and humans (Hasenfuss & Pieske, 2002).

5.3.4 Enhanced cardiomyocyte SR Ca^{2+} cycling and aberrant Ca^{2+} releases in *Mterf3* KO

End-stage heart failure cardiomyocytes typically exhibit decreased SR Ca^{2+} cycling properties with reduced SR Ca^{2+} release during systole and slowed SR Ca^{2+} uptake during diastole (Shannon & Bers, 2004; Lehnart *et al.*, 2004). Surprisingly, SR Ca^{2+} cycling was increased in *Mterf3* KO, with increased $[Ca^{2+}]_i$ transient amplitudes, faster SR Ca^{2+} uptake and increased SR Ca^{2+} load (figure 6). Moreover, as a consequence of the increased Ca^{2+} transients, contractility was also enhanced in *Mterf3* KO cardiomyocytes. These findings are in line with what is seen during β-adrenergic stimulation (Bers, 2002). Accordingly, the Ca^{2+} transient properties were similar in *Mterf3* KO and WT when the β-adrenergic agonist isoproterenol (ISO) was applied. Enhanced SR Ca^{2+} load and cycling is beneficial for improving the contractile performance (Bers, 2002). However, elevated SR Ca^{2+} load can trigger spontaneous Ca^{2+} release events through increased opening of the SR Ca^{2+} release channels (Venetucci *et al.*, 2008; Yuan *et al.*, 2007). *Mterf3* KO cardiomyocytes displayed a high prevalence of spontaneous Ca^{2+} release events, particularly when treated with β-receptor agonists (figure 6). Spontaneous Ca^{2+} releases can lead to triggered arrhythmias and could thereby pose a risk of offsetting the benefits of increased contractility under conditions of elevated SR Ca^{2+} load (Yuan *et al.*, 2007; Satoh *et al.*, 1997; Diaz *et al.*, 2005).

Increased Ca^{2+} transients and contractility in an early phase following cardiac infarction have been described previously (Mork *et al.*, 2007). It was suggested that increases in L-type Ca^{2+} currents, SR Ca^{2+} cycling and contractility constituted a compensatory effort to sustain global heart function after the loss of cardiac tissue (Mork *et al.*, 2007). Although L-type Ca^{2+} currents were not measured in *Mterf3* KO cardiomyocytes, the resemblance in altered SR Ca^{2+} cycling is striking. Thus, it is likely that these compensatory phenomena on the cellular level are mediated by an increased activity of the sympathetic adrenergic system as this constitutes the key signal by which the organism tries to compensate for impaired cardiac function.

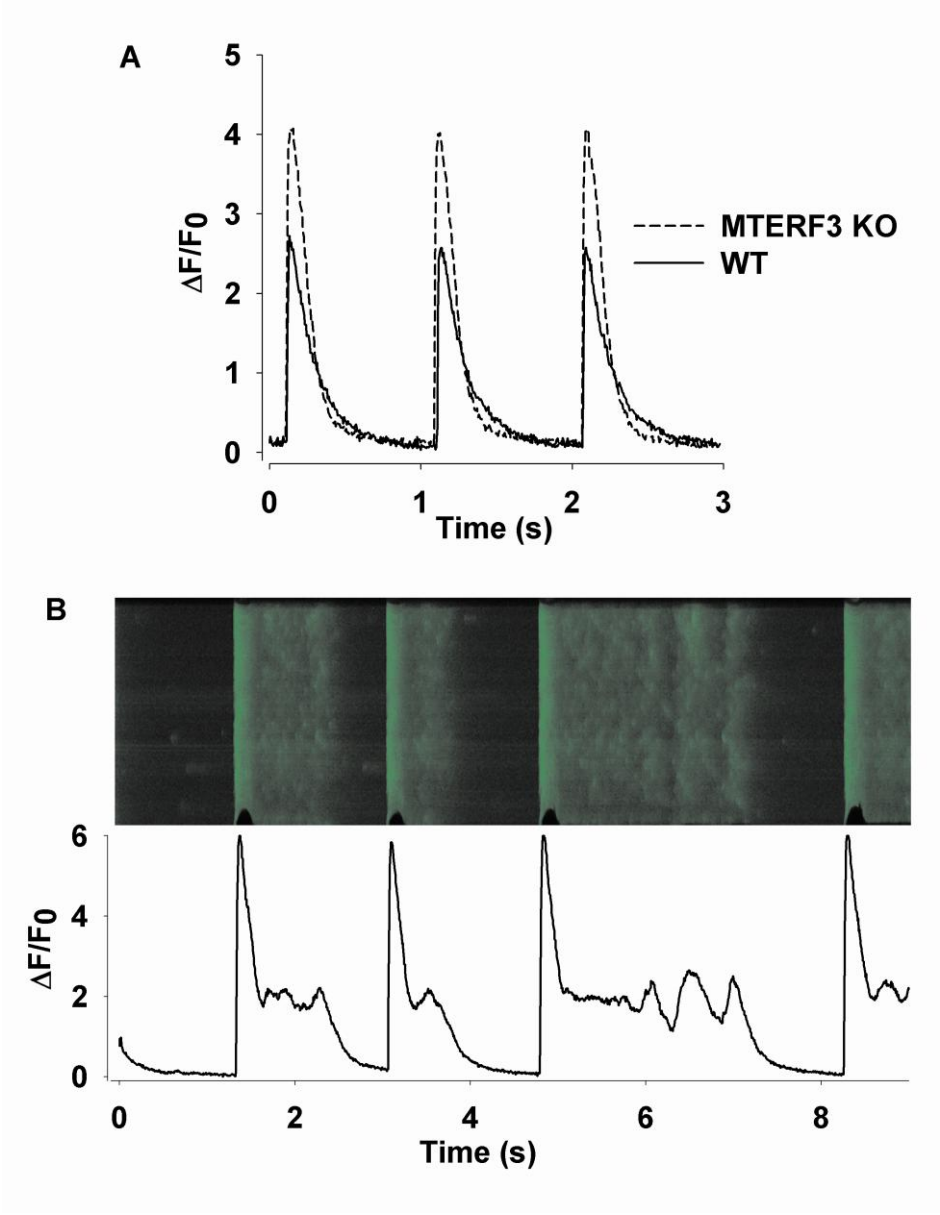

Figure 6. Increased SR Ca^{2+} cycling and arrhythmogenic Ca^{2+} releases in *Mterf3* KO mice. Typical examples of cardiomyocyte Ca^{2+} transients in WT and MTERF 3 KO are displayed in A. B shows a *Mterf3* KO cardiomyocyte with aberrant Ca^{2+} releases (upper figure: line scan; lower figure: the corresponding fluorescence intensity plot).

5.3.5 *Mterf3* KO displays decreased heart rate variability consistent with increased adrenergic load

To look for signs of enhanced sympathetic-adrenergic drive *in vivo* and the cause of the sudden death, long-term telemetric ECG recordings were performed on *Mterf3* KO and WT mice (Thireau *et al.*, 2008b; van de Borne *et al.*, 1997). Heart rate variability (SDNN), >24 h prior to death, was significantly lower in *Mterf3* KO than in WT mice (*Mterf3* KO: 8.3±0.3 ms, WT: 16.2±0.5 ms; $p<0.05$). Moreover, frequency domain analysis of the heart rate variability revealed a collapsed low frequency (LF) power spectrum and a low LF/HF ratio in *Mterf3* KO, which is seen on increased adrenergic drive in severe heart failure (Kienzle *et al.*, 1992; van de Borne *et al.*, 1997). These data are, thus, consistent with a picture of chronic adrenergic stress in *Mterf3* KO. (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; La Rovere *et al.*, 2003) This fits with the notion that enhanced SR Ca^{2+} cycling in *Mterf3* KO cardiomyocytes is a consequence of increased adrenergic signaling.

In the last moribund 24 h of the *Mterf3* KO, P-Q time increased and heart rate decreased progressively, ending with complete atrio-ventricular (AV) block. The role of mitochondria in the cellular mechanisms underlying AV-block is elusive. However, the presence of AV-block in experimental models of mitochondrial cardiomyopathy, such as the *Mterf3* KO and the *Tfam* KO (Wang *et al.*, 1999) as well as in patients with mitochondrial disease (Larsson & Oldfors, 2001; Anan *et al.*, 1995), suggests a role of the mitochondria in the cellular mechanisms of AV-block.

Concomitant with the progression into AV block, there was a decrease in core body temperature from ~35°C to 25°C and reduction in locomotor activity by ~75% in *Mterf3* KO mice. Short-term reductions core body temperature and metabolic rate, torpor, occur normally in the mouse and allows for preservation of metabolic fuels (Gavrilova *et al.*, 1999). Such hibernation-like events can be triggered by acute food deprivation and has been shown to be mediated by the sympathetic nervous system (Swoap & Weinshenker, 2008). Similar to *Mterf3* KO mice, hypothermia was seen in another mouse model with mitochondrial dysfunction (Zhou *et al.*, 2008). In a study on djungarian hamsters, daily torpor was associated with ECG changes resembling those seen in the moribund *Mterf3* KO mice, e.g. prolonged P-Q interval and bradychardia

(Mertens *et al.*, 2008). Intriguingly, there appears to be a link between defective mitochondrial function and the development of a torpor-like state with atrio-ventricular conduction defects.

5.3.6 Conclusion

Taken together, paper II show that mitochondrial dysfunction can lead to cardiomyopathy without signs of oxidative stress but comprising a complex arrhythmogenic phenotype with an increase in cardiomyocyte Ca^{2+} cycling and contractility.

5.4 GENERAL DISCUSSION PAPER I AND II

Both paper I and II investigate genetic mouse models of mitochondrial disease, with progressively deteriorating respiratory chain function in striated muscle tissue. This enables some parallels to be drawn.

In neither of the models any evidence of increased ROS production could be found. This contrast the general belief that increased ROS production and oxidative stress are important factors underlying the pathological changes in primary mitochondrial diseases (Esposito *et al.*, 1999; Tarnopolsky & Raha, 2005). However, these results agree with what has been found in two other genetic mouse models with primary mitochondrial respiratory chain dysfunction, both of which have failed to show an increase in ROS production or ROS induced cell damage (Kujoth *et al.*, 2005; Trifunovic *et al.*, 2005). Moreover in a *C. elegance* model, reduced activity of the electron transport chain was coupled with less oxidative stress-induced damage (Feng *et al.*, 2001). Based on the results in paper I and II, it can even be argued that respiratory chain dysfunction is associated with decreased mitochondrial ROS production.

Excitation-contraction coupling in skeletal and cardiac muscle critically depends on Ca^{2+} handling. Both the *Tfam* KO and *Mterf3* KO models display changes in the Ca^{2+} handling. These changes are likely part of adaptation to the perturbed mitochondrial function. The priority of the adaptation in *Tfam* KO muscle seems to be the upholding of energy homeostasis, whereas in the *Mterf3* KO sustained contractility is the goal.

These two adaptational events have to some extent different mechanisms. In *Tfam* KO, the skeletal muscle cell itself is likely in control of the adaptation by reducing calsequestrin 1, SR Ca^{2+} release and force production in order to reduce energy expenditure. Control of Ca^{2+} handling in *Mterf3* KO mice is mostly governed by the demands of cardiac output from the superimposed organism level and is effectuated by events downstream of the sympathetic nervous system. This enables the organism to provisionally override the cardiomyocytes to increase contractility even under conditions of scarce cellular energy supply, such as in respiratory chain dysfunction. The adaptations in *Tfam* KO and *Mterf3* KO may be functional from the point of view of sustaining energy homeostasis and cardiac output, respectively. However, both adaptations come with the cost of also causing impaired function, as showed by the pathological phenotypes of the *Tfam* KO (locomotor dysfunction) and *Mterf3* KO (increased SR Ca^{2+} load and arrhythmogenisis).

5.5 GENERAL BACKGROUND PAPER III AND IV

In papers III and IV changes in cardiomyocyte Ca^{2+} handling and mitochondrial function were studied under two stress conditions: (1) High concentration of the saturated fatty acid palmitate was used to mimic hyperlipidemia; (2) the β-adrenergic agonist isoproterenol (ISO) was used to study adrenergic stress signaling and inotropy. Moreover, elevated levels of both fatty acids and catecholamines are found in patients with the metabolic syndrome (Tentolouris *et al.*, 2006). To compare the effect of the palmitate and ISO in a metabolic-syndrome-like condition, cardiomyocytes from the obese, dyslipidemic, insulin resistant *ob/ob* mouse was used (Mazumder *et al.*, 2004).

5.6 PAPER III

5.6.1 Background

The effect of palmitate on cardiomyocyte Ca^{2+} homeostasis and mitochondrial function, in particular $\Delta\Psi_m$ and ROS production, was studied in paper III. Large increases in ROS production can interfere with the function of proteins associated with Ca^{2+} handling and is thus implicated in contractile disturbances of the heart. Cardiac

utilization of fatty acids as an energy source is enhanced in humans as well as in animal models with obesity, hyperlipidemia and insulin resistance, such as the *ob/ob* mouse (Carley & Severson, 2005). Moreover, the *ob/ob* mouse has been shown to display noticeably different metabolic responses to palmitate exposure compared to WT (Mazumder *et al.*, 2004) in a way that suggests that it has adapted to a high fat environment. Therefore, the effects of acute palmitate exposure were compared between cardiomyocytes from lean WT and obese *ob/ob* mice.

5.6.2 Palmitate causes dissipation of $\Delta\Psi_m$ and a ROS-mediated impairment of SR Ca^{2+}handling and contractility in WT cardiomyocytes

Changes in cardiomyocyte $\Delta\Psi_m$ (measured with TMRE) were measured during exposure to palmitate. In WT cells, a considerable decrease in $\Delta\Psi_m$ was seen already 5 min after palmitate (1.2 mM) exposure. In the next series of experiments, the effect of palmitate on mitochondrial O_2^{*-} production, using MitoSOX Red, was measured. Palmitate application caused a large increase in the MitoSOX Red signal in WT cardiomyocytes, amounting a ~100% increase from baseline level to the end of the experiment (figure 7). Palmitate has previously been shown to promote proapoptotic signaling in a variety of cell types, including cardiomyocytes. These signaling events were coupled with $\Delta\Psi_m$ dissipation and increased mitochondrial ROS production (Sparagna *et al.*, 2000; Listenberger *et al.*, 2001; Listenberger & Schaffer, 2002; Miller *et al.*, 2005). Interestingly, the function of respiratory chain complex I and III, the main sites of O_2^{*-} production, have been shown to be modified by palmitate (Sparagna *et al.*, 2000; Loskovich *et al.*, 2005). This raises the possibility that the observed increase in ROS production is due to a direct effect of palmitate on the O_2^{*-} production site in the mitochondria.

Large increases in ROS production may perturb cellular Ca^{2+} handling and interfere with cardiac contractility (Kourie, 1998; Kawakami & Okabe, 1998; Kaplan *et al.*, 2003). Therefore, cytoplasmic Ca^{2+} transients and contractility were assessed in WT cardiomyocytes after adding the ROS-inducing palmitate. Application of palmitate significantly decreased the Ca^{2+} transient amplitude by ~20% and increased the half-width as well as the decay time constant. Furthermore, SR Ca^{2+} load was assessed by measuring the amplitude of the caffeine-induced Ca^{2+} transient. Palmitate decreased the

caffeine-induced Ca^{2+} transient by ~25%. Fractional shortening was decreased by ~20% in the presence of palmitate. In short, palmitate impaired cardiac Ca^{2+} cycling and contractility in WT mice.

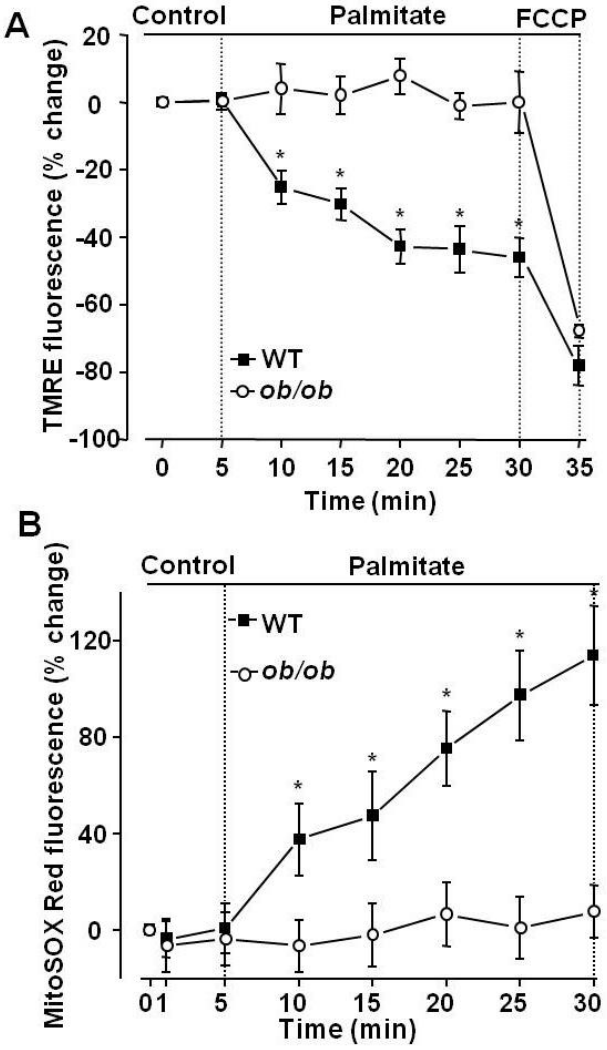

Figure 7. Palmitate dissipates $\Delta\Psi_m$ and increases mitochondrial ROS production in WT but not *ob/ob* cardiomyocytes. *A:* $\Delta\Psi_m$ (measured with TMRE fluorescence) recorded under control conditions, during 25 min with palmitate, and finally after 5 min in 1 µM $\Delta\Psi_m$ depolarizing agent FCCP. *B*: Average increase in mitochondrial ROS (measured with MitoSOX Red) upon palmitate exposure to cardiomyocytes. WT (black circles) and *ob/ob* (white circles). Data are average ± SEM, *Statistical difference between WT and *ob/ob* ($P < 0.05$).

To test whether the palmitate-induced changes in WT cardiomyocyte Ca^{2+} handling and contractility was an effect of increased ROS production, cytosolic Ca^{2+} transients and cell shortening were measured in the presence of the antioxidants *N*-acetylcysteine (NAC) or ebselen (Cotgreave *et al.*, 1987). When palmitate was applied together with an antioxidant, the palmitate-induced impairment of cardiomyocyte Ca^{2+} transients, SR Ca^{2+} content and cell shortening was abolished.

Taken together, these results suggest that the palmitate-induced impairment of WT cardiac function is due to increased ROS production. These findings are in line with the notion that ROS production can modulate Ca^{2+} handling and contraction of cardiac cells (Zima & Blatter, 2006; Schrier & Hess, 1988). Greatly increased ROS production has been shown to impair cellular Ca^{2+} handling by reducing the L-type Ca^{2+} current amplitude, increasing the leak through the SR Ca^{2+} release channels, increasing the sarcolemmal Na^{+}/Ca^{2+} exchange activity, slowing SR Ca^{2+} reuptake, and eventually leading to reduced SR Ca^{2+} content (Kourie, 1998; Kawakami & Okabe, 1998; Kaplan *et al.*, 2003; Goldhaber & Qayyum, 2000).

5.6.3 No increased ROS production and improved cardiomyocyte function with palmitate in *ob/ob* mice

In contrast to WT mice, superfusion of *ob/ob* cardiomyocytes with palmitate containing solution did not induce any noticeable changes in either $\Delta\Psi_m$ or in the MitoSOX Red signal. This indicates that mitochondrial ROS production and dissipation of membrane potential not induced by palmitate in the *ob/ob* heart. It could be argued, however, that the level of oxidative stress was elevated already in the basal state of the *ob/ob* heart. Therefore, *ob/ob* and WT hearts were compared by measuring aconitase activity and SOD2 protein expression, both of which have been shown to be altered under oxidative stress (Gardner, 2002; St Pierre *et al.*, 2006). Interestingly, neither aconitase activity nor SOD2 expression differed between *ob/ob* and WT, which speaks against oxidative stress in the former.

Without palmitate (the control condition), cardiomyocytes from *ob/ob* mice display cardiac dysfunction with smaller and slower Ca^{2+} transients compared to WT cells,(Fauconnier *et al.*, 2005). However, the function of *ob/ob* cardiomyocytes was improved by palmitate, with cells displaying faster Ca^{2+} transients and enhanced shortening. Moreover, application of antioxidants did not alter the palmitate-induced increases in Ca^{2+} transient kinetics and cell shortening in *ob*/*ob* cardiomyocytes.

Several studies have reported an increased preference for fatty acid oxidation and a reduction of glycolysis in obesity and type 2 diabetes (Mazumder *et al.*, 2004; Carley & Severson, 2005; Coort *et al.*, 2004). A faster ATP production with palmitate than with

glucose in *ob*/*ob* cardiomyocytes could be beneficial for ATPase dependent activities such as SR Ca^{2+} cycling and contraction. Accordingly, palmitate exposure improved the rate of SR Ca^{2+} uptake and cell shortening in *ob/ob* cardiomyocytes.

5.6.4 Conclusion

The ROS-dependent detrimental effects of palmitate on WT heart cells highlights the role of ROS in the pathogenic mechanisms leading to obesity-related cardiac diseases. High serum free fatty acid levels are an important contributor to the pathological adaptations in diabetes and are considered to involve overproduction of mitochondrial ROS (Unger & Orci, 2001; Listenberger & Schaffer, 2002). Hearts from the hyperlipidemic *ob/ob* mouse (Mazumder *et al.*, 2004) lack signs of increased mitochondrial ROS and display improved cardiomyocyte function when supplied with palmitate, which shows that an adaptation to a high-fat environment has occurred in these animals. In the heart, metabolic adaptability allows the preferred substrate to be switched depending on environmental and physiological or pathological conditions (Stanley *et al.*, 2005). However, there is always some latency before adaptation to a new setting fully occurs. Thus, in the transition between different substrate conditions, e.g. from a low fat to high fat environment, cellular functions may be disturbed before an adaptation has emerged. This phenomenon is reflected in the different responses to palmitate seen in cardiomyocytes from the lean WT and obese *ob/ob* mouse. Thus, in an early phase of the metabolic syndrome elevated levels of saturated fatty acids, trigger mitochondrial ROS production in cardiomyocytes. The increased ROS production constitutes a serious challenge to cardiomyocyte function and may result in severe pathological changes and even apoptosis. Alternatively, it results in major mitochondrial adaptations, which include reduced ATP production rate(Boudina *et al.*, 2005), a change in substrate utilization towards a preference for fatty acid oxidation(Mazumder *et al.*, 2004; Buchanan *et al.*, 2005; Carley & Severson, 2005), and decreased ROS production. The present results show that cardiomyocytes of *ob/ob* mice follow the latter pathway reaching a state where elevated levels of saturated fatty acids no longer results in increased ROS production.

5.7 PAPER IV

5.7.1 Background

Catecholaminergic stress signaling via β-adrenergic receptors induces inotropy at the level of the cardiomyocyte. The inotropic effects of β-adrenergic stimulation are considered to be largely mediated through cAMP-dependent protein kinase (PKA)-dependent phosphorylation of proteins involved in cardiomyocyte Ca^{2+} handling (Bers, 2002). Obesity and hyperlipidemia are associated with increased stress signaling, including those originating from the sympathetic-adrenergic system (Wellen & Hotamisligil, 2005; Tentolouris *et al.*, 2006; Nicolson, 2007). In paper IV the role of mitochondrial ROS production in the inotropic mechanism of β-adrenergic stimulation of cardiomyocytes was investigated. In parallel, obese and hyperlipidemic *ob/ob* mice were used to study β-adrenergic stimulation and ROS in cardiomyocytes that are chronically exposed to stress signaling.

5.7.2 Mitochondrial ROS production contributes to β-adrenergic-induced inotropy in WT cardiomyocytes

Application of β-adrenergic agonist isoproterenol (ISO) to paced WT cardiomyocytes resulted in a ~20% increase in mitochondrial ROS production (measured with MitoSOX Red). In accordance with these results, a recent study on permeabilized rat cardiomyocytes showed that addition of activated PKA results in a fast and reversible increase in mitochondrial ROS production (Nagasaka *et al.*, 2007). Additionally, prolonged (24 hours) β-adrenergic stimulation has been shown to induce apoptosis in adult rat cardiomyocytes (Remondino *et al.*, 2003). This apoptosis was stopped by SOD/catalase mimetics and by overexpression of catalase, indicating that the apoptotic signaling induced by β-adrenergic stimulation involves increased ROS production. Moreover, PKA as well as PKA anchoring proteins are associated with the mitochondria (Papa *et al.*, 2002), which suggests the possibility of a direct link between adrenergic signaling and mitochondrial function.

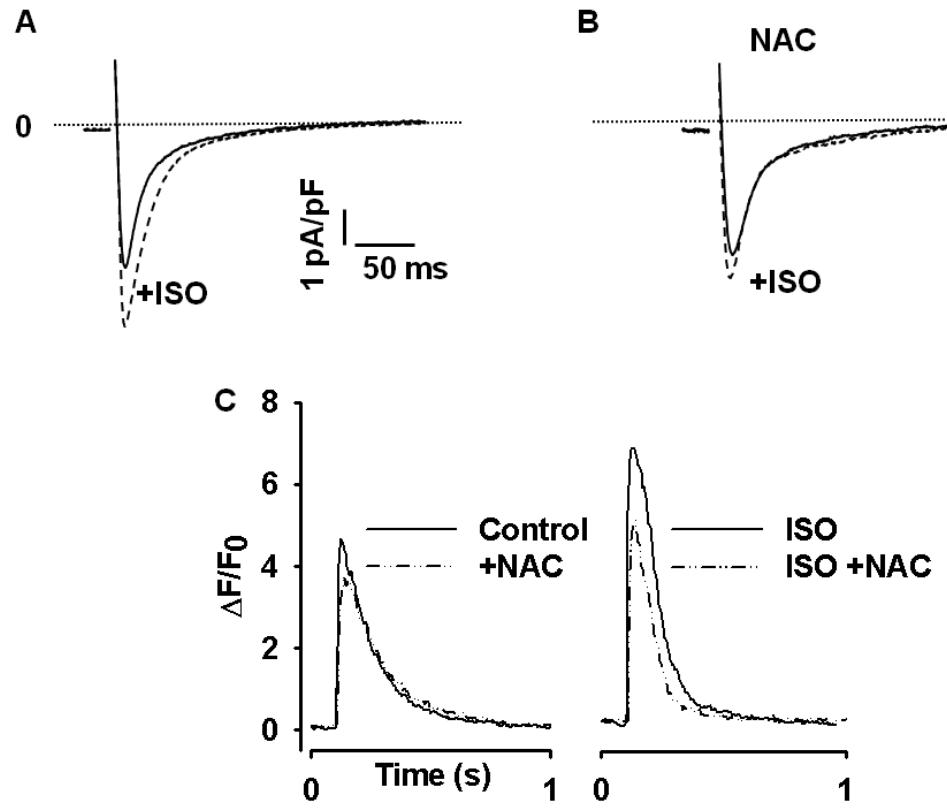

Figure 8. ROS contributes to the β-adrenergic inotropy. Representative L-type Ca^{2+}currents (A and B) and $[Ca^{2+}]_i$ transients (C) from WT cardiomyocytes in the presence of ISO (100 nM) and/or NAC (5 mM) as indicated.

ROS and the redox state are known to modify cardiac Ca^{2+} handling at multiple sites, including the L-type Ca^{2+} channel and SR Ca^{2+} release channel(Zima & Blatter, 2006; Sims & Harvey, 2004; Hool, 2008). This notion and the fact that β-adrenergic stimulation enhances cardiac Ca^{2+} cycling led to a hypothesis that ROS were involved in the ISO-mediated inotropy. To investigate this, ISO-induced changes in cardiomyocyte Ca^{2+} handling were studied in the absence and presence of the general antioxidant *N*-acetylcysteine (NAC). The WT cardiomyocyte displayed larger and faster Ca^{2+} transients with increased cell shortening upon ISO application. However in presence of NAC, the ISO-induced increase in Ca^{2+} transient amplitude (figure 8) and cell shortening was lessened. The rate of Ca^{2+} transient decay was not significantly affected by NAC either in the absence or presence of ISO. The increased $[Ca^{2+}]_i$ with adrenergic stimulation occurs in part through an amplified sarcolemmal L-type Ca^{2+} current. This current can be modified by redox changes (Sims & Harvey, 2004; Hool, 2008). Measurements of L-type Ca^{2+} current using whole cell patch clamp were performed in the presence and absence of ISO and NAC. Intriguingly, the ISO-induced increase in L-type Ca^{2+} current density was diminished in the presence of NAC,

whereas NAC by itself did not significantly affect the basal L-type Ca^{2+} current (figure 8). These data support a mechanism whereby moderately increased ROS, induced by β-adrenergic stimulation, facilitate Ca^{2+} entry through L-type Ca^{2+} channels. An increase in L-type Ca^{2+} entry does not only in itself increase cytosolic Ca^{2+}, but it also enhances SR Ca^{2+} release by increasing the gain of the Ca^{2+}-induced Ca^{2+} release mechanism (Bassani *et al.*, 1995).

5.7.3 No signs oxidative stress and absence of mitochondrial ROS production in the ISO-stimulated *ob/ob* heart

In paper III it was shown that WT cardiomyocytes exposed to the saturated fatty acid palmitate displayed a large increase in mitochondrial ROS production and that such an effect was absent in *ob/ob* cells. Interestingly, application of ISO to *ob/ob* cardiomyocytes did not significantly increase mitochondrial ROS, which was in contrast to the WT cells. When exposed to ISO, the *ob/ob* cardiomyocytes displayed enhanced SR Ca^{2+} cycling with increased Ca^{2+} transient amplitudes and decay kinetics. Furthermore, shortening of *ob/ob* cardiomyocytes was increased with ISO. In contrast to WT cardiomyocytes, NAC did not alter the effect of ISO on Ca^{2+} transient amplitude, rate of decay or cell shortening in *ob/ob* cardiomyocytes (figure 7).

The diminished ISO-induced increase in ROS production in *ob/ob* hearts might be due to them being exposed to oxidative stress already in the basal state. To investigate this possibility, MDA protein modifications (Aldini *et al.*, 2007) were compared in *ob/ob* and WT hearts. The results showed no difference in MDA protein adducts between the two groups of non-stimulated hearts, indicating that the basal oxidative load is not greater in *ob/ob* hearts. Moreover, the activity SOD was measured in WT and *ob/ob* hearts and no difference was found between the groups. These results corroborate those from paper III where no indications of increased basal oxidative stress were found in the *ob/ob* heart.

The lack of stress-induced ROS increase in *ob/ob* cardiomyocytes suggests that the capacity for mitochondrial ROS production is reduced in *ob/ob* heart. To assess the mitochondrial capacity of O_2^{*-} production, MitoSOX Red loaded cardiomyocytes were exposed to an abundance of H_2O_2, which would induce product inhibition of the SOD2 and thereby restrain the conversion of O_2^{*-} to H_2O_2 (Hearn *et al.*, 1999; McAdam *et al.*,

1977). The accumulated O_2^{*-} would then increase MitoSOX Red fluorescence (Aydin *et al.*, 2009). The H_2O_2 application resulted in a very large increase in the fluorescence signal from WT cardiomyocytes. In the *ob/ob* cells, however, the H_2O_2-induced increase in fluorescence was markedly smaller. In a control experiment, it was tested whether the H_2O_2-induced increase in fluorescence depended on mitochondrial respiratory chain function. WT cardiomyocytes loaded with MitoSOX Red were treated with rotenone, an inhibitor of the complex I in the electron transport chain. Next, the cells were exposed to H_2O_2. The rotenone treatment resulted in a clearly diminished H_2O_2-induced fluorescence increase. These results showed that, following H_2O_2 exposure, the increase in the O_2^{*-} sensitive MitoSOX Red fluorescence depends on mitochondrial respiration. The smaller increase in fluorescence from *ob/ob* compared to WT cardiomyocytes would then reflect a reduced rate of mitochondrial O_2^{*-} production.

5.7.4 Conclusion

WT hearts display a marked increases mitochondrial ROS production on acute β-adrenergic stimulation. Furthermore, this increase in ROS plays an important role in the β-adrenergic inotropic effect, because the ISO-induced increase in L-type Ca^{2+} current, cytoplasmic Ca^{2+} transient amplitude and contractility was diminished in the presence of the antioxidant NAC. One mechanism by which ROS-mediated increase in cytoplasmic [Ca^{2+}] and inotropy occurs is likely through the increase in L-type Ca^{2+} current (Bassani *et al.*, 1995). Though, it cannot be excluded that a concomitant ROS-mediated effect on the RyR contributes to the stimulatory effect of ISO, since other studies have shown that SR Ca^{2+} release is facilitated by oxidative modifications of the RyR (Zissimopoulos & Lai, 2006; Kawakami & Okabe, 1998). The ROS effect could also be mediated through potentiation of the kinases and/or inhibition of the phosphatases that are usually attributed to altered Ca^{2+} handling. In this respect, it is interesting to note that increase in PKCδ dependent phosphorylation of cardiac troponin I (TnI) can be stimulated by H_2O_2 (Sumandea *et al.*, 2008).

Cardiomyocytes from *ob/ob* mice produce little ROS in response to β-adrenergic stimulation, but they still display an inotropic response. The lack of β-adrenergic-induced ROS production can be explained by the general down-regulation of mitochondrial ROS production that is found in the *ob/ob* heart. NAC had no effect on ISO-mediated inotropy in *ob/ob* cardiomyocytes. The inotropic response in *ob/ob*

cardiomyocytes is most likely effectuated by a maintained PKA-mediated enhancement of Ca^{2+}-cycling.

5.8 GENERAL DISCUSSION PAPER III AND IV

Paper III and IV both study the effects stressor-induced ROS production on cardiomyocyte Ca^{2+} handling. Application of palmitate in paper III resulted in a large increase in ROS production in WT cardiomyocytes, which was accompanied by decreased Ca^{2+} transient amplitude and contractility. In paper IV, on the other hand, β-adrenergic stimulation induced an increase in mitochondrial ROS production but this ROS had a positive effect on Ca^{2+} handling. The difference can be explained by the fact that the ROS production was much larger with palmitate than with ISO; the increase in MitoSOX Red fluorescence was ~100% with palmitate and ~20% with ISO. This constitutes an example of the biphasic effect of ROS where an acute limited increase in ROS acts as an integral part in the normal physiological signaling, whereas a large prolonged increase has adverse effects and might contribute to pathological changes (Zima & Blatter, 2006; Goldstein *et al.*, 2005; Andrade *et al.*, 1998; Rojas *et al.*, 2006; McCord, 2008) (figure 9).

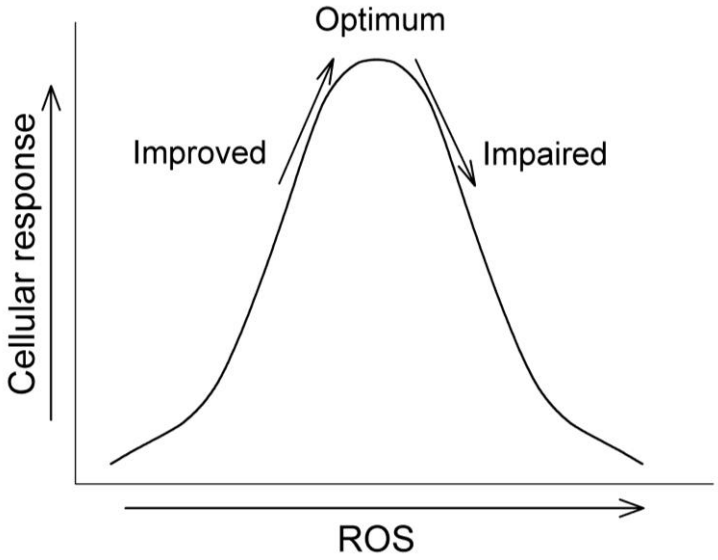

Figure 9. A tentative graph displaying the biphasic cellular response (e.g. $[Ca^{2+}]_i$ transients) to increased ROS.

The effects of stress signaling on ROS production in *ob/ob* mice were quite different from that in the WT. In contrast to WT, neither palmitate nor ISO exposure triggered any significant increases in mitochondrial ROS in the *ob/ob* cardiomyocyte. This was

associated with a decreased basal rate of mitochondrial $O_2^{\bullet-}$ production. Moreover, no signs of oxidative stress in the basal state were found in the *ob/ob* compared to WT heart, as judged from similar levels of MDA protein adducts, aconitase activity and SOD2 expression. As part of the pathological mechanisms behind obesity and insulin resistance, oxidative stress-induced changes have been suggested (Nicolson, 2007). The findings in paper III and IV do not exclude that oxidative stress, in an early phase, is part of the adaptive process in the *ob/ob* mouse (Harmancey *et al.*, 2008a). Indeed, such a scenario is supported by the finding that WT cardiomyocytes that were exposed to palmitate, at a concentration commonly found in *ob/ob* serum, displayed a large increase in mitochondrial ROS production.

Thus, in an early phase of the metabolic syndrome two types of systemic stress signals, increased β-adrenergic stimulation (Tentolouris *et al.*, 2006) and elevated levels of saturated fatty acids, trigger mitochondrial ROS production in cardiomyocytes. As the obesity disease progresses, it results in mitochondrial adaptations changes in substrate utilization towards a preference for fatty acid oxidation(Mazumder *et al.*, 2004; Buchanan *et al.*, 2005; Carley & Severson, 2005), and decreased ROS production.

6 PERSPECTIVES

In this thesis the interplay between the mitochondrial function and Ca^{2+} handling in cardiac and skeletal muscle was studied. The mitochondria can affect Ca^{2+} signaling through different modes. Acutely, increased mitochondrial ROS production following an exogenous stressor (e.g. palmitate or ISO), can modulate Ca^{2+} handling. In this way, the mitochondria have a hub-like function where diverse stress signals are received, modulated, transmitted and then effectuated throughout the cell. This gives a picture of the mitochondria as an important intracellular integrator of environmental stimuli.

When the exposure to stress signals is prolonged, long-term adaptations in the mitochondria as well as in cytoplasmic Ca^{2+} handling occur. Direct perturbation of the mitochondrial function by disruption of the respiratory chain (such as in the *Tfam* and *Mterf3* KO) evoke a complex adaptive reaction in the cell with changes in gene expression. The cellular Ca^{2+} handling machinery is an important target in these types of adaptations. Interestingly, it is the adaptations to respiratory chain dysfunction that cause the major pathological features, e.g. contractile disturbances in skeletal muscle and heart. This is in contrast to the idea that decreased ATP or increased ROS are the major causes of the pathological manifestations in mitochondrial disease (Esposito *et al.*, 1999; Tarnopolsky & Raha, 2005).

Mitochondrial dysfunction, either via a direct respiratory chain disruption (*Tfam* and *Mterf3* KO) or secondary to the complex adaptations of the metabolic syndrome (*ob/ob* mouse), does not lead to oxidative stress. Conversely, long-term mitochondrial dysfunction seems to be followed by a reduction in ROS production. Increased ROS production could nonetheless be important at the early disease stage, but this ROS-increase fades away as the disease progresses.

Antioxidants has been suggested as treatment of both primary mitochondrial diseases and in the metabolic syndrome (Tarnopolsky, 2008; Nicolson, 2007). In the light of this thesis, antioxidant treatment might be useful in the initial phase of disease, when clinically relevant symptoms are unlikely to be observed. However, given that most

patients in the clinical setting have reached a late stage of the disease, the potential of antioxidant treatment is most likely ineffective.

In the hyperlipidemia adapted *ob/ob* mouse, contractile function was improved when cardiac cells were supplied with an abundance of fatty acids. In this situation, acutely devoiding the obesity adapted cardiac cells of their preferred energy substrate has detrimental consequences. Based on this reasoning, intravenous supplementation with fat emulsion could be part of the emergency regime to treat acute decompensated heart failure in obese cardiomyopathy patients.

In primary mitochondrial diseases, it appears that treatment should be focused on the adaptive mechanisms, e.g. adrenergic stress signaling in the heart and increased $[Ca^{2+}]_{mit}$ in skeletal muscles. In fact, there are reports on improved heart function after treatment with β-receptor blockers in patients with mitochondrial cardiomyopathy (Finsterer *et al.*, 2006; Kosutic & Zamurovic, 2005). Cyclophilin D-binding substances like cyclosporin A could provide with a treatment for mitochondrial myopathy. Interestingly, this type of treatment has recently shown promise in patients with myopathies with mitochondrial involvement (Merlini *et al.*, 2008). At the same time as pharmacological treatment against a maladaptive response has clinical potential, there is also a risk of aggravating the disease condition by simultaneously undermining the functional adaptive strives.

A treatment that might both suppress dysfunctional and promote functional adaptations is physical exercise. Exercise training has important beneficial effects in the metabolic syndrome and improves both heart and skeletal muscle function in heart failure patients (Sorrentino, 2005; Coats, 2001; Wolfel, 2005; Piepoli *et al.*, 2001). The patients' experience of muscle fatigue could be caused by muscle weakness or reduced intrinsic fatigue resistance of the muscle. If muscle weakness is the cause of fatigue (as in the *Tfam* KO model), then resistance training should be the primary exercise regimen. In patients that display reduced intrinsic fatigue resistance in the muscle, endurance training would be the suitable treatment. Thus, in order to optimize the clinical effect of exercise training in myopathy patients, the underlying cause of muscle fatigue must be considered.

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