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Skeletal muscle toxicity and statins: role of mitochondrial adaptations

François Singh

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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Clinical Pharmacology and Toxicology, Basel, Switzerland

**EA3072 Mitochondrie, Stress Oxydant et Protection Musculaire,
Strasbourg, France**

THÈSE

Présentée par :

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soutenue le : **19 septembre 2016**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline: Science du Vivant

Spécialité : Aspects moléculaires et cellulaires de la Biologie

Skeletal muscle toxicity and statins: role of mitochondrial adaptations

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ABBREVIATIONS

¹AgO₂	singlet oxygen	IC₅₀	half-maximal inhibitory concentration
ADP	adenosine diphosphate	IMF	intermyofibrillar
AICAR	5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside	LSP	light strand promoter
AMP	adenosine monophosphate	MAC	mitochondrial apoptosis-induced channel
AMPK	AMP-dependent protein kinase	MEF2	myocyte enhancer factor-2
ANT	adenine nucleotide translocator	MFN	mitofusin
ATP	adenosine triphosphate	MHC	myosin heavy chain
Bcl-2	B-cell lymphoma 2	MOMP	mitochondrial outer membrane permeabilisation
CARD	caspase-activation recruitment domain	mtDNA	mitochondrial DNA
CK	creatine kinase	MuRF-1	muscle RING-finger protein-1
CO₂	carbon dioxide	NAD	nicotinamide adenine dinucleotide
CoA	coenzyme A	Nox	NADPH oxidase
CoQ₁₀	Coenzyme Q ₁₀	NRF	nuclear respiratory factor
CPT	carnitine palmitoyltransferase	O₂	dioxygen
CREB	C-AMP response element-binding protein	O²⁻	oxide ion
CVD	cardiovascular disease	O₂^{•-}	superoxide
DNA	deoxyribonucleic acid	O₂²⁻	peroxide ion
DRP1	Dynamamin related GTPase 1	O₃	ozone
ER	endoplasmic reticulum	OPA1	optic atrophy 1
ERRα	estrogen-related receptor α	OXPHOS	oxidative phosphorylation
ETC	electron transfer chain	PERC	PGC-1 related estrogen receptor coactivator
FAD	flavin adenine dinucleotide	PGC-1	peroxisome proliferator-activated receptor-γ-coactivator-1
FoxO3	forkhead box O3	PRC	PGC-1 related coactivator
GPx	glutathione peroxidase	Prx	peroxiredoxine
GSH	reduced glutathione	RNA	ribonucleic acid
GSSG	oxidised glutathione	ROS	reactive oxygen species
H₂O	water	rRNA	ribosomic RNA
H₂O₂	hydrogen peroxide	SAMS	statin-associated muscle symptoms
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A	SINAM	statin-induced necrotising autoimmune myopathy
HOCl	hypochlorous acid	SOD	superoxide dismutase
HSP	heavy strand promoter	SRM	statin-related myotoxicity

SKELETAL MUSCLE TOXICITY AND STATINS: ROLE OF MITOCHONDRIAL ADAPTATIONS

SS	subsarcolemal	tRNA	transfer RNA
TFAm	mitochondrial transcription factor A	UCP	uncoupling protein
TFB1M	mitochondrial transcription factor B1	VDAC	voltage dependant anion channel
TFB2M	mitochondrial transcription factor B2	XDH	xanthine dehydrogenase
TIM	transporter of the inner membrane	XO	xanthine oxidase
TOM	transporter of the outer membrane	$\Delta\Psi_m$	mitochondrial membrane potential
TPP	triphenylphosphonium cation		

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— PART I —

INTRODUCTION

Sola dosis facit venenum

— Philippus Theophrastus Aureolus Bombastus von Hohenheim —

A. MITOCHONDRIA

1) A brief History of sacrosomes, bioblasts, and mitochondria

Mitochondria are intracellular organelles, which have been known for over a century. During the last six decades, these organelles have become an important subject of research in diverse biological fields, such as cytology, biochemistry, physiology, molecular biology, and evolutionary biology (Ernster & Schatz 1981).

These organelles were first described by Albert von Kölliker, a Swiss anatomist and physiologist, who described these structures as “sacrosomes” in 1856 (van der Giezen 2011). In 1886, Richard Altmann, a German histologist and pathologist, published “Die Elementarorganismen und ihre Beziehungen zu den Zellen” (Altmann 1894; Mazzarello 1999). This book is considered as an early histological description of what Altmann denominated as “bioblasts” (life germs), and what is now known as mitochondria (O’Rourke 2010). Altmann believed that these bioblasts were autonomous elementary organisms responsible for metabolic and genetic functions.

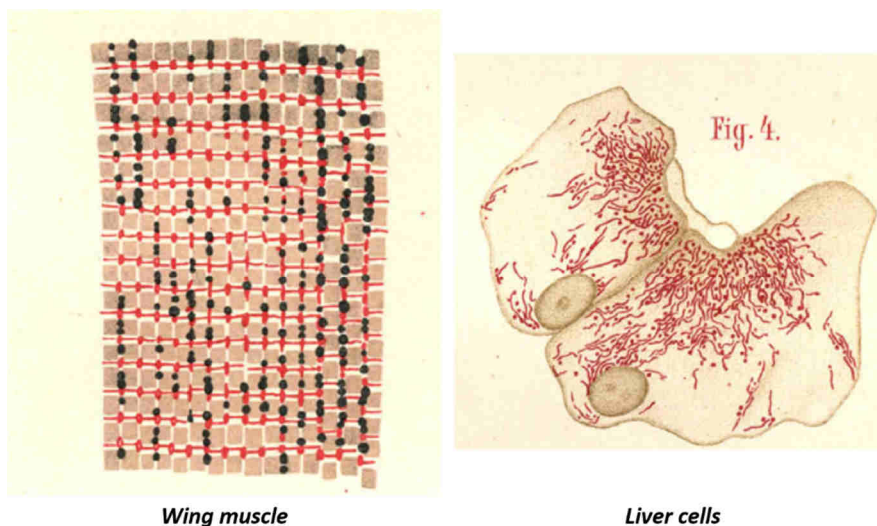


Figure 1: Histological drawings of “Bioblasts” from Richard Altmann (19th century).

Adapted from (O’Rourke 2010).

Few years later, in 1898, Carl Benda, a German microbiologist, observed these organelles during spermatogenesis (van der Giezen 2011; Tao et al. 2014). Because of their aspect, and tendency to form long chains, Benda designated them as “mitochondria”. The word *Mitochondrion* being a combination of the Greek words *mitos* (thread), and *chondros* (granule). In 1900, Leonor Michaelis, a German biochemist, and physical chemist, discovered that Janus green B is a supravital stain for mitochondria. This staining will remain the sole glimpse of the mitochondria until 1952, with the first publication of electron micrograph pictures of mitochondria were published by George Palade, a Romanian-American cell biologist (Palade 1952). In 1913, Otto Heinrich Warburg, a German physiologist, discovered that cellular respiration is associated with insoluble subcellular structures, which he called “grana” (Warburg 1913). In 1918, Paul Portier, a French zoologist and marine biologist, emitted the hypothesis that mitochondria were bacterial symbionts that were living inside eukaryotic cells (van der Giezen 2011). Although we know today that this hypothesis was correct, at this time, Portier encountered strong opposition from the scientific community. In 1937, Hans Adolf Krebs, a German-British biochemist and physician, formulated the citric acid cycle, and localized its enzymes in the mitochondria (Ernster & Schatz 1981; van der Giezen 2011). In 1952, Mary Mitchell and Herschel Mitchell, two American Biochemists, reported that mitochondrial inheritance does not follow Mendelian rules (Mitchell & Mitchell 1952). Six years later, McLean discovered that mitochondria synthesize their own proteins (McLEAN et al. 1958). In 1963, Margit Nass, and Sylvan Nass discovered that mitochondria contain their own genome (Nass & Nass 1963). Nine years later, it was shown that this mitochondrial DNA (mtDNA) was transmitted in an uniparental fashion (maternal inheritance) in animals (Dawid & Blackler 1972). Nine years later, the first mitochondrial genome to be sequenced was published: the human mitochondrial genome, with its 16,589 base pairs (Anderson et al. 1981).

In 1970, the book *Origin of Eukaryotic cells*, by Lynn Margulis revived the theory first proposed by Paul Portier in 1918: the serial endosymbiosis theory. Symbiosis can be described as life forms living together for mutual advantage (Jinn & Lucas 1999). This theory proposes

that a bacterial endosymbiont established itself inside a proto-eukaryote organism, thus becoming the mitochondrion (van der Giezen 2011). Although this theory was strongly rejected at the beginning of the century, new evidences supporting this idea appeared. In 1978, Robert Schwartz and Margaret Dayhoff realized a phylogenetic analysis, and showed that the mitochondrial-encoded cytochromes originated from an alphaproteobacteria (Schwartz & Dayhoff 1978). Increasing data in support of this alphaproteobacterial origin has been published since then, and this endosymbiotic origin has now been accepted by the scientific community. However, the exact place of the mitochondrion in the alphaproteobacteria class is still debated (Tao et al. 2014). Interestingly, although the presence of mitochondria was thought to be mandatory in eukaryote organisms, a eukaryote without a mitochondrial organelle has recently been discovered (Karnkowska et al. 2016).

2) Structure

Mitochondria are highly specialised organelles, and are often depicted as ellipsoid isolated single entities floating in the cytosol. However, this view is certainly too simplistic. Mitochondria are organized in a dynamic complex network, that can adapt to energy demand, and interact with the cell's cytoskeleton. These organelles can be dissected in four sub-compartments: mitochondria are delimited by two highly specialised, and different membranes, which delimitate two mitochondrial compartments: the intermembrane space and the matrix.

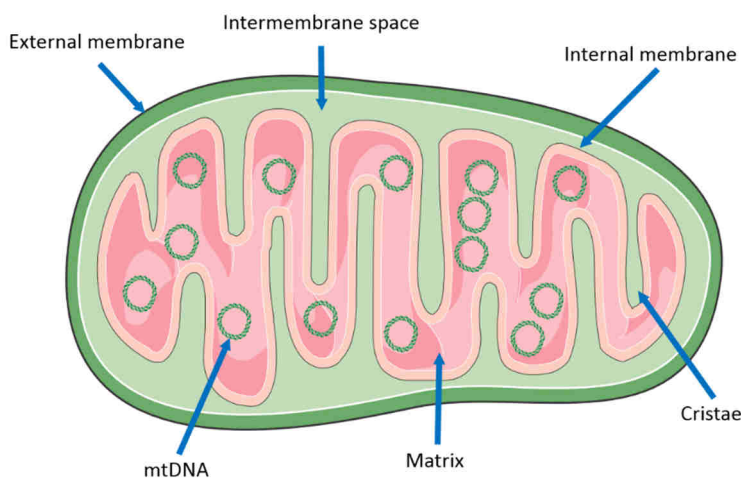


Figure 2: Schematic representation of a mitochondrion.

a) External membrane

The external membrane separates the mitochondria from the cytosol. It is composed by a lipidic bilayer, which contains numerous protein complexes. These proteins form pores, that act like a molecular sieve, enabling the transfer of small molecules of less than 5kDa, and small proteins. This transporter is called voltage dependant anion channel (VDAC), which is composed by a β -Barrel forming the porin ion channel. VDAC is the most abundant protein in the mitochondrial outer membrane, and small ions, such as Ca^{2+} , Na^+ , K^+ , Cl^- , or OH^- , and water soluble mitochondrial metabolites, such as ATP, ADP, pyruvate, succinate, and inorganic phosphate, all cross the outer membrane via this porin. There are three VDAC isoforms in mammals, which are encoded by three different genes, VDAC1, VDAC2, and VDAC3 with more than 70% of sequence homology, VDAC1 being the most abundant isoform (Noskov et al. 2016). The transport of larger proteins is realised via the transporter of the outer membrane complex (TOM complex, also called TOM40 complex). The TOM complex is composed by the core complex made up by Tom40, Tom22, Tom5, Tom6, and Tom7, and peripherally associated receptors, Tom20 and Tom70 (and a minor component Tom71) (Endo & Yamano 2010). Tom20 acts as an initial recognition site for proteins and transfers them to the central receptor Tom22. From here, they are inserted into the Tom40 channel (Chacinska et al. 2009), which will transfer them to the intermembrane space. The outer mitochondrial membrane also contains a transporter for long-chain fatty acids. Indeed, fatty acids are catabolised within the mitochondria through β -oxidation. However, although short-chain and medium-chain fatty acids can enter the mitochondria by simple diffusion, long-chain fatty acids require the intervention of Carnitine palmitoyltransferase 1 (CPT1). There are three identified tissue-specific isoforms of CPT1: CPT1-A is the liver isoform, CPT1-B is the muscle isoform, and CPT1-C is the brain isoform (Bonfont et al. 2004). Long-chain fatty acids are first activated by a long-chain fatty Acyl-CoA synthetase on the outer mitochondrial membrane. Long-chain fatty acyl-CoAs are then associated to carnitine by CPT1 to form acyl-carnitine (and CoA-SH), which can pass the outer mitochondrial membrane through a porin, and reach the intermembrane space.

b) Intermembrane space

The intermembrane space is delimited on one side by the outer mitochondrial membrane, and on the other side by the inner mitochondrial membrane. As most small molecules can cross the outer membrane, the intermembrane space can be considered chemically equivalent to the cytosol.

c) Internal membrane

The mitochondrial internal membrane is impermeable to ions and small molecules, contrary to the outer mitochondrial membrane. This membrane is constituted by a highly specialised lipid bilayer, containing cardiolipin in high quantities (Alberts et al. 2002). As for the outer membrane, transporters are necessary for the import of proteins inside the mitochondrial matrix, the transporters of the inner membrane (TIM) complexes. The TIM23 complex transports precursor proteins across the inner membrane and into the matrix (Chacinska et al. 2009). On the other hand, the TIM22 complex is responsible for the integration of proteins in the inner membrane. The inner mitochondrial membrane also contains the components of the mitochondrial respiratory chain, which is composed by five complexes which lead to the production of ATP via oxidative phosphorylation. This respiratory chain will be described in details later. The respiratory chain expulses protons from the matrix to the intermembrane space in order to create a proton gradient that will be used by complex V to generate ATP. However, the inner mitochondrial membrane contains proteins that enable the leakage of protons from the intermembrane space to the matrix, the uncoupling proteins (UCP). UCPS have been thought to have a role in inducible proton leak, and in control of reactive oxygen species (ROS) production (Mailloux & Harper 2011). During basal respiration, it has been shown that uncoupling represents 20–50% of the energy consumption by the mitochondria of a normally functioning cell (Rolfe & Brand 1996; Rolfe et al. 1999; Harper et al. 2001). As ADP and ATP cannot cross the inner membrane, an ADP/ATP translocator is necessary to import ADP in the mitochondria and export the newly formed ATP in the intermembrane space, and so in the cytosol. This translocator is called the adenine nucleotide translocator (ANT), and

exists in three paralogous isoforms. The first isoform, ANT1, is predominantly expressed in heart and skeletal muscle. ANT2 and ANT3 being predominantly expressed ubiquitously and in liver respectively. Within the internal mitochondrial membrane, a translocase enables the transfer of acyl-carnitine from the intermembrane space to the mitochondrial matrix, where CPT2 will catalyse the transformation of acyl-carnitine and CoA-SH to Acyl-CoA and carnitine. Acyl-CoA will then be subjected to β -oxidation, and carnitine will be recycled to the cytosol. Unlike CPT1, only one isoform of CPT2 exists (Bonnetfont et al. 2004). In order to increase its surface (up to 5 times compared to the inner mitochondrial membrane), the mitochondrial internal membrane is folded, forming cristae protruding into the matrix.

d) Matrix

The matrix constitutes the inner part of the mitochondria, and is delimited by the inner mitochondrial membrane. This compartment contains the enzymes necessary for two major metabolic pathways: the citric acid cycle, and the β -oxidation of fatty acids. The mitochondrial matrix also contains its own protein synthesis machinery, with mitochondrial specific ribosomes, tRNAs, rRNAs, and its own DNA.

e) mtDNA

Within the cell, mitochondria are the only location of extra-chromosomal DNA (Taylor & Turnbull 2007). Mitochondrial content varies according to the metabolic activity of the cell and corresponds to the energetic demand of a given tissue. Moreover, mitochondria grow and divide independently of nuclear division. This particularity is due to the presence of a mitochondrial DNA (mtDNA), which is circular, double-stranded, and approximately 16 kb long in animals (Tao et al. 2014).

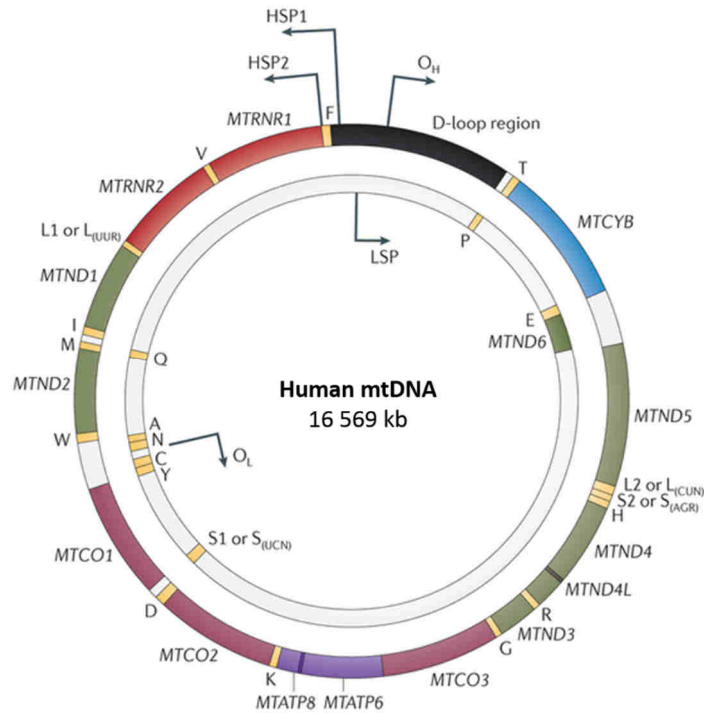


Figure 3: Circular map of the Human mtDNA. Adapted from (Stewart & Chinnery 2015).

In humans, this mtDNA is 16 569 base pairs long, and approximately 100-10 000 separate copies are present per cell. The two strands of the mtDNA are referred as the heavy strand (H-strand) which has two promoters (HSP1 and HSP2), and the light strand (L-strand) which has one promoter (LSP). These two strands are differentiated by their nucleotide content: the H-strand being guanine-rich, and the L-strand being cytosine-rich. mtDNA encodes for 37 genes, most information is encoded by the H-strand, with genes for 2 rRNAs, 14 tRNAs, and 12 polypeptides, and the L-strand codes for 8 tRNAs and one polypeptide (Taanman 1999). All these 13 polypeptides are essential proteins of the OXPHOS system.

Mitochondrial genes encoding for			
Proteins	tRNAs		rRNAs
ND1	TRNF (t-RNA-Phe)	TRNK (t-RNA-Lys)	RNR1 (12SrRNA)
ND2	TRNV (t-RNA-Val)	TRNS1 (t-RNA-Ser)	RNR2 (16SrRNA)
ND3	TRNP (t-RNA-Pro)	TRNS2 (t-RNA-Ser)	
ND4L	TRNI (t-RNA-Ile)	TRNL1 (t-RNA-Leu)	
ND4	TRNQ (t-RNA-Gln)	TRNL2 (t-RNA-Leu)	
ND5	TRNM (t-RNA-Met)	TRNE (t-RNA-Glu)	
ND6	TRNW (t-RNA-Trp)	TRNT (t-RNA-Thr)	
COX1	TRNA (t-RNA-Ala)	TRNR (t-RNA-Arg)	
COX2	TRNN (t-RNA-Asn)	TRNG (t-RNA-Gly)	
COX3	TRNY (t-RNA-Tyr)		
ATP6	TRNH (t-RNA-His)		
ATP8	TRNC (t-RNA-Cys)		
CYTB	TRND (t-RNA-Asp)		

Table 1: Mitochondrial-encoded genes. Adapted from (Tao et al. 2014).

Due to low efficiency of DNA repair pathways and a more mutagenic intracellular environment due to local reactive oxygen species production, the mutation rate of mtDNA is believed to be 5-10 times higher than in nuclear DNA (Bonné-Tamir et al. 2003; Tao et al. 2014). The polyploid nature of the mitochondrial genome, combined with this high mutation rate leads to an important feature of mtDNA: homoplasmy and heteroplasmy (Taylor & Turnbull 2007). Homoplasmy could be defined as when all copies of mtDNA are identical, and heteroplasmy as when two or more different mitochondrial genotypes are present within the cell.

Although mitochondria possess their own genome, most of mitochondrial genes have been incorporated to nuclear DNA (approximately 850). Mitochondria are hence under the dual genetic control of both nuclear DNA and mtDNA (Taylor & Turnbull 2007).

3) Transcriptional regulation of mitochondrial biogenesis

Mitochondrial biogenesis can be described as the growth of the pre-existing mitochondrial network, and requires coordinated synthesis and import of 1000-1500 nuclear-encoded proteins, that will be synthesised by cytosolic ribosomes (Jornayvaz & Shulman 2010). Mitochondrial biogenesis can be induced by environmental stress, like oxidative stress, caloric restriction (without malnutrition), cold exposure, cell division, and exercise. These stimuli will trigger the transcription cascade of mitochondrial biogenesis. At the top of this cascade stand the peroxisome proliferator-activated receptor- γ -coactivator-1 (PGC-1) family members.

a) The PGC-1 family

This family is composed by three founding members: PGC-1 α , PGC-1 β , and PRC. Although these proteins are encoded by different genes (Martínez-Redondo et al. 2015), they have partly overlapping functions and have similar modular structures. The members of the PGC-1 family possess no intrinsic enzymatic activity. Their biological activity only relies on interactions with DNA-bound transcription factors, and coactivator complexes (Correia et al. 2015). PGC-1s play a role not only in the regulation of gene expression, but also collaborate with co-transcriptional processing of nascent mRNAs (Martínez-Redondo et al. 2015; Monsalve et al. 2000).

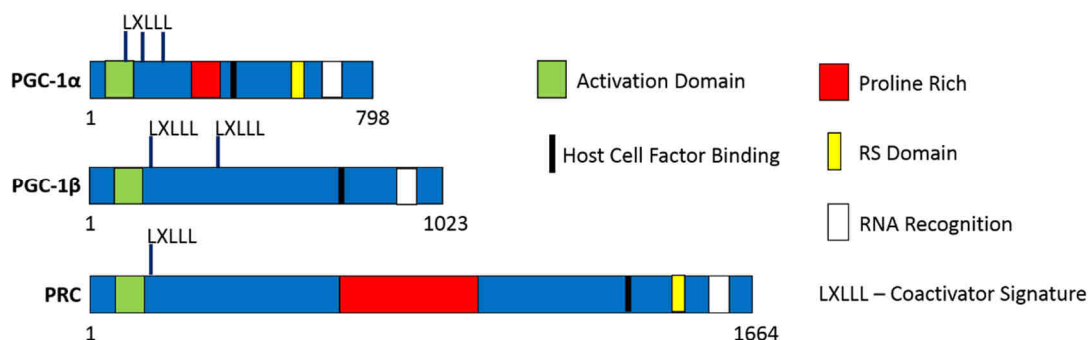


Figure 4: Arrangement of conserved domains among the PGC-1 family coactivators. Adapted from (Scarpulla 2011).

It has been shown that individual inhibition of PGC-1 α or PGC-1 β has only a mild effect on mitochondrial biogenesis. However, simultaneous inhibition of both PGC-1 α and PGC-1 β leads to a massive reduction in mitochondrial gene expression (Rowe et al. 2013). Interestingly, PGC-1 α has been shown to induce a fibre switch to oxidative type skeletal muscle fibres (Lin, Wu, et al. 2002)

i. PGC-1 α

The first member of the PGC-1 family that has been identified is PGC-1 α , which has been described as a regulator of genes involved in cold-induced thermogenesis in brown adipose tissue and skeletal muscles (Puigserver et al. 1998). This member is the most studied of the family and is currently considered to be the master regulator of mitochondrial biogenesis and function (Puigserver & Spiegelman 2003; Palikaras & Tavernarakis 2014). The importance of PGC-1 α in mitochondrial biogenesis control is supported by gain of function experiments in both cells (Wu et al. 1999), and transgenic mice (Lehman et al. 2000). Wu et al. showed that overexpression of PGC-1 α in C₂C₁₂ muscle cells lead to an increased expression of mRNA of both COX IV (nuclear encoded), and COX II (mitochondrial encoded) subunits of the mitochondrial respiratory chain complex IV, and in an increase in mtDNA copy number. Moreover, Lehman and al. showed that muscle-specific overexpression of PGC-1 α in transgenic mice lead to mitochondrial proliferation, and in an increase of the expression of mitochondrial genes involved in OXPHOS. Alternative splicing variants of PGC-1 α have been shown to not only enhance mitochondrial biogenesis, but also increase the expression of carnitine palmitoyltransferase-1b (CPT-1b), and uncoupling protein 1 (UCP1) (Zhang et al. 2009). PGC-1 α also possesses anti-atrophic effects in skeletal muscles, by suppressing Forkhead box O3 (FoxO3) transcriptional activity (Correia et al. 2015). This has for consequences a reduced expression of the E3 ubiquitin ligase muscle RING-finger protein-1 (MuRF-1), and of Atrogin-1, which leads to a decreased protein degradation (Brault et al. 2010; Cannavino et al. 2015; Hindi et al. 2014; Wenz et al. 2009). Interestingly, Wenz et al., and Cannavino et al. both showed in atrophic skeletal muscle that PGC-1 α expression limits

autophagy activation. In the skeletal muscle of transgenic mice, overexpression of PGC-1 α promotes the switch to slow, oxidative type I and IIA muscle fibres (Lin, Wu, et al. 2002). Recently, PGC-1 α has been shown to modulate systemic ketone body homeostasis, and to ameliorate diabetic hypercholesterolemia in mice (Svensson et al. 2016).

PGC-1 α is a transcriptional co-activator, which means that it does not directly interact with DNA, but increases the activity of nuclear receptors through the interaction of its amino-terminal activation domain with docking transcription factors and coactivators (Scarpulla 2011; Jornayvaz & Shulman 2010). It has been shown to be involved in an auto-regulatory loop controlling its own expression (Ljubicic et al. 2010), by co-activating MEF2. MEF2 then binds to the PGC-1 α promoter, hence regulating its expression (Handschin et al. 2003). PGC-1 α activates the expression of the Nuclear respiratory factors (NRF-1 and NRF-2), and interacts with them to transactivate transcription factors of mtDNA, such as TFAM, TFB1M, and TFB2M (Ljubicic et al. 2010; Gleyzer et al. 2005; Scarpulla 2006)

ii. PGC-1 β

Formerly PGC-1 β has also been designated as PERC (PGC-1 related Estrogen Receptor Coactivator), and is a homologue of PGC-1 α (Lin, Puigserver, et al. 2002). This protein has first been identified four years after PGC-1 α , by a team in Basel (Kressler et al. 2002). This member has been much less described than PGC-1 α , and to this day, the specific role of PGC-1 β is still debated in the literature. It shares similar function and molecular structure with PGC-1 α , including nuclear-receptor binding and transcriptional activation, leading to mitochondrial biogenesis (Jornayvaz & Shulman 2010). Just as PGC-1 α , PGC-1 β also suppresses FoxO3 transcriptional activity, and hence protein degradation (Brault et al. 2010). Recently, in an inducible muscle specific knock out, PGC-1 β has been shown to control anti-oxidant defence in skeletal muscles via SOD2 (Gali Ramamoorthy et al. 2015). However, compared to PGC-1 α , PGC-1 β lacks the arginine/serine domain necessary for RNA processing (Lin, Puigserver, et al. 2002). In the skeletal muscle, in contrast to overexpression of PGC-1 α , which promotes the switch to type I and IIA fibres, overexpression of PGC-1 β

induces the switch to highly oxidative, but fast twitch type IIX muscle fibres (Arany et al. 2007). Interestingly, contrary to PGC-1 α , PGC-1 β expression has been shown to be unaffected, or even reduced in exercised skeletal muscle (Koves et al. 2005; Mortensen et al. 2007), and is not up-regulated after cold exposure (Meirhaeghe et al. 2003). In addition, in reason of differences in proton leak, PGC-1 β promotes coupled respiration on a much higher level than PGC-1 α , suggesting a difference in metabolic efficiency between these two proteins (St-Pierre et al. 2003). PGC-1 β appears to be similar to PGC-1 α in its functional interaction with NRF-1 (Scarpulla 2011). Altogether, these observations suggest that although PGC-1 α and PGC-1 β appear to be stimulated independently, both clearly control mitochondrial biogenesis through NRF-1 (Jornayvaz & Shulman 2010).

iii. PRC

PRC (PGC-1 Related Coactivator) is the least known member of this family. It resembles its two other members in binding to nuclear transcription factors associated with mitochondrial function, such as NRF-1, NRF-2, TFAM, TFB1M, TFB2M, CREB, and ERR α (Vercauteren et al. 2006; Vercauteren et al. 2008; Andersson & Scarpulla 2001; Gleyzer et al. 2005). Knocking out PRC in mice leads to an embryonic lethal phenotype (He et al. 2012), and its silencing in proliferating cells to aberrant mitochondrial biogenesis, and cell cycle arrest (Vercauteren et al. 2009). As PGC-1 α , expression of PRC is induced after acute exercise (Egan et al. 2013). PRC has been shown to induce myogenesis in C₂C₁₂ myotubes (Philp et al. 2011). Like PGC-1 β , PRC is not induced by cold exposure, and is not implied in adaptive thermogenesis (Andersson & Scarpulla 2001). In mice, although PGC-1 α and PGC-1 β mRNAs are abundantly expressed in tissues with high mitochondrial content, it is not the case for PRC (Andersson & Scarpulla 2001).

b) The Nuclear Respiratory Factors (NRFs)

The nuclear respiratory factors were the first regulatory factors implicated in mitochondrial biogenesis and function that have been identified. NRF-1 has been discovered via its binding to the cytochrome c promoter, and has been associated with the expression of genes involved

in the expression and function of the mitochondrial respiratory chain (Evans & Scarpulla 1989; Scarpulla 2011). On the other hand, NRF-2 has been identified as a multisubunit activator of cytochrome oxidase expression (complex IV of the mitochondrial respiratory chain) (Scarpulla 2006). The NRFs have both been reported to be implicated in the *in vivo* control of all 10 subunits of the cytochrome oxidase subunits which are encoded by the nucleus (Ongwijitwat et al. 2006; Dhar et al. 2008). Most notably, NRF-1 and NRF2 act on human genes encoding constituents of the mtDNA transcription machinery including mitochondrial transcription factor A (TFAM), mitochondrial transcription factors B 1 (TFB1M) and 2 (TFB2M) (Palikaras & Tavernarakis 2014). Additionally, NRF-1 acts on the expression of the mitochondrial DNA-directed RNA polymerase (POLRMT) (Scarpulla 2006). Recently, it has been demonstrated that NRF2 protects mitochondria from oxidative injury, directly by interacting with the outer mitochondrial membrane (Strom et al. 2016).

c) Mitochondrial Transcription Factor A (TFAM)

Mitochondrial biogenesis is dependent on the simultaneous expression of both mitochondrial and nuclear genomes. Thus, a coordinating system is mandatory for the proper functioning of this organelle. This key component is the mitochondrial transcription factor A (TFAM, or also denominated mtTFA in the literature). As written previously, TFAM expression can be triggered via the PGC-1s, and via the NRFs. Not only TFAM is considered as the most important transcription factor involved in mitochondrial biogenesis, this protein is essential for mtDNA transcription and replication (Ljubicic et al. 2010). After transcription of this nuclear encoded gene, TFAM mRNA will be translated in the cytoplasm into a precursor protein. This precursor protein will then be imported in the mitochondrial matrix via the TOM/TIM complexes, where it will be processed into its mature form. Both L-strand and H-strand of the mtDNA contain enhancer regions, upstream of their promoter, that bind TFAM simultaneously (Parisi & Clayton 1991; Larsson et al. 1997). The binding of TFAM on the mitochondrial DNA will induce the introduction of a contortion that will allow the binding of the mitochondrial RNA polymerase (mtRNAP), with the help of TFB1M or TFB2M (Ljubicic et al. 2010). Transcription

of the mtDNA will lead to the production of a single RNA molecule, which will then be spliced into mRNAs, tRNAs, and rRNAs. Replication of the H-strand occurs simultaneously with the transcription of the L-strand. Indeed, the docking of TFAM on DNA enables the docking of the mitochondrial DNA polymerase (DNA pol- γ), hence the replication of mtDNA. Loss of TFAM protein in mitochondrial myopathy patients has been shown to be correlated with mtDNA depletion (Larsson et al. 1994; Poulton et al. 1994). Mitochondrial biogenesis being directly correlated to mtDNA content, TFAM appears as a major regulator, enabling the coordinated expression of both nuclear-encoded and mitochondrial-encoded mitochondrial proteins, and the control of mtDNA copy number.

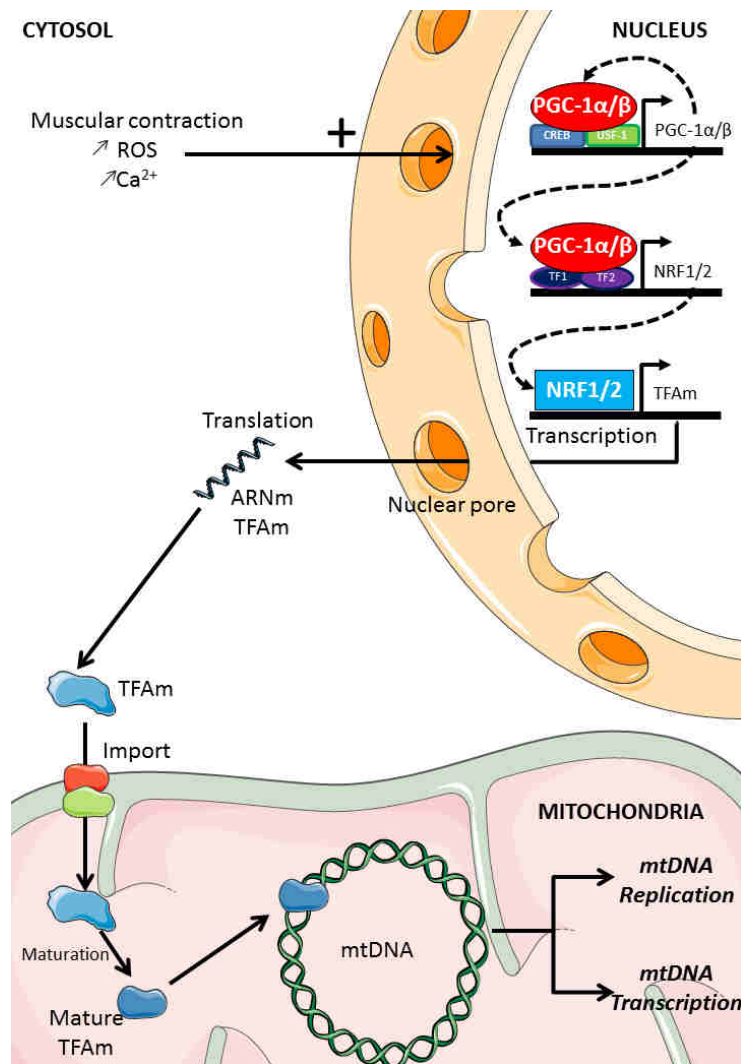


Figure 5: Schematic representation of the transcriptional control of mitochondrial Biogenesis. Adapted from (Ljubicic et al. 2010).

4) Functions

Mitochondria are organelles present in every cell type, with the exception of mammalian erythrocytes. However some other animal types, such as birds, possess functional mitochondria in erythrocytes (Stier et al. 2013). Mitochondria are often depicted as the “powerhouse of the cell”, without them, eukaryotes would be dependent on glycolysis. However, these organelles display numerous other vital functions. Indeed, they are the main actors in charge of not only the cell energy production, but are also involved in calcium homeostasis regulation, intracellular pH regulation, heme biosynthesis, lipid biosynthesis and metabolism, ubiquinol and Fe-S centres biosynthesis, steroid hormones production, and in controlling the programmed cell death. In this part, we will focus on its role in apoptosis, and on oxidative phosphorylation.

a) Programmed cell death

Apoptosis is a genetically controlled pathway and mechanism that can eliminate unwanted cells. Diverse stimuli can induce this pathway: the stimulus can be external, transmitted within the cell by a cell surface receptor, or internal, resulting from the action of a drug, a toxin, or radiation (Scheffler 2007). Both pathways lead to the elimination of the unwanted cell. Apoptosis can be triggered under three major circumstances: during development and homeostasis, during natural senescence and aging, and as a defence mechanism against genetically damaged cells. At the heart of the intrinsic pathway of apoptosis stand the mitochondria. Indeed, mitochondria can regulate caspase cleavage and activation by a phenomenon denominated mitochondrial outer membrane permeabilisation (MOMP) (Tait & Green 2013). This selective permeabilisation will have for consequences a loss in the mitochondrial membrane potential ($\Delta\Psi_m$), and the release of intermembrane space proteins in the cell cytosol. The integrity of the outer mitochondrial membrane is controlled by the members of the Bcl-2 family of proteins (Parsons & Green 2010). In one hand, Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, Boo/Diva have been identified as a potent anti-apoptotic factors that blocks MOMP by sequestering pro-apoptotic members of the Bcl-2 family. They are composed by

four BH homology domains (BH1 to BH4) and a C-terminal hydrophobic domain (TM). On the other hand, the pro-apoptotic family members are Bax and Bak, which are composed by three BH homology domains (BH1 to BH3) and a TM domain. Under normal conditions, Bax is located in the cytosol, and Bak at the outer mitochondrial membrane. After an apoptotic stimulus, Bax will be translocated to the outer mitochondrial membrane, where it will form homo-oligomers with Bak, which will form a pore: the Mitochondrial Apoptosis-Induced Channel (MAC) (Parsons & Green 2010). The opening of the MAC leads to the release of intermembrane space proteins in the cell cytosol. Moreover, this family also contains other pro-apoptotic factors, that are the BH3-only proteins (Bid, Bim, Noxa, Puma, Bmf, Bad, Bik, Blk, Hrk/DP5), which are composed by a BH3 domain and a TM domain. Contrary to Bak and Bax, they do not permeabilise the outer mitochondrial membrane, but rather activate Bak and Bax, and/or neutralise the anti-apoptotic Bcl-2 family members (Gross et al. 1999).

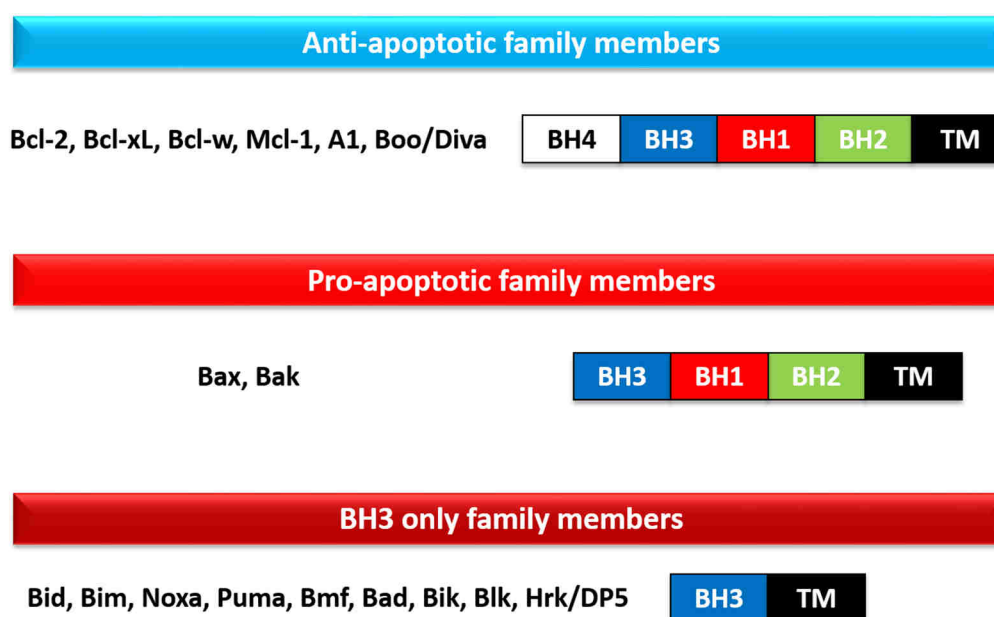


Figure 6: The Bcl-2 family members. Adapted from (Parsons & Green 2010).

The MOMP will lead to the release of cytochrome c in the cytosol, where it will bind to Apaf-1 (Apoptotic Protease Activating factor-1) monomers. These monomers will oligomerise

to form the ‘apoptosome’ (in presence of dATP). The apoptosome will then recruit the caspase-9 zymogen via its N-terminal caspase-activation recruitment domain (CARD) (Boatright & Salvesen 2003). This inactive procaspase-9 will be cleaved by the apoptosome to form the active caspase-9. Caspase-9 is an initiator caspase, that will need to recruit executioner caspases to induce apoptosis. Indeed, caspase-9, will cleave the procaspase-3 zymogen to form the active caspase-3. The active caspase-3 will then induce chromatin condensation, DNA fragmentation, nuclear fragmentation, selective proteolysis, cell shrinkage, and hence effective induction of the programmed cell death.

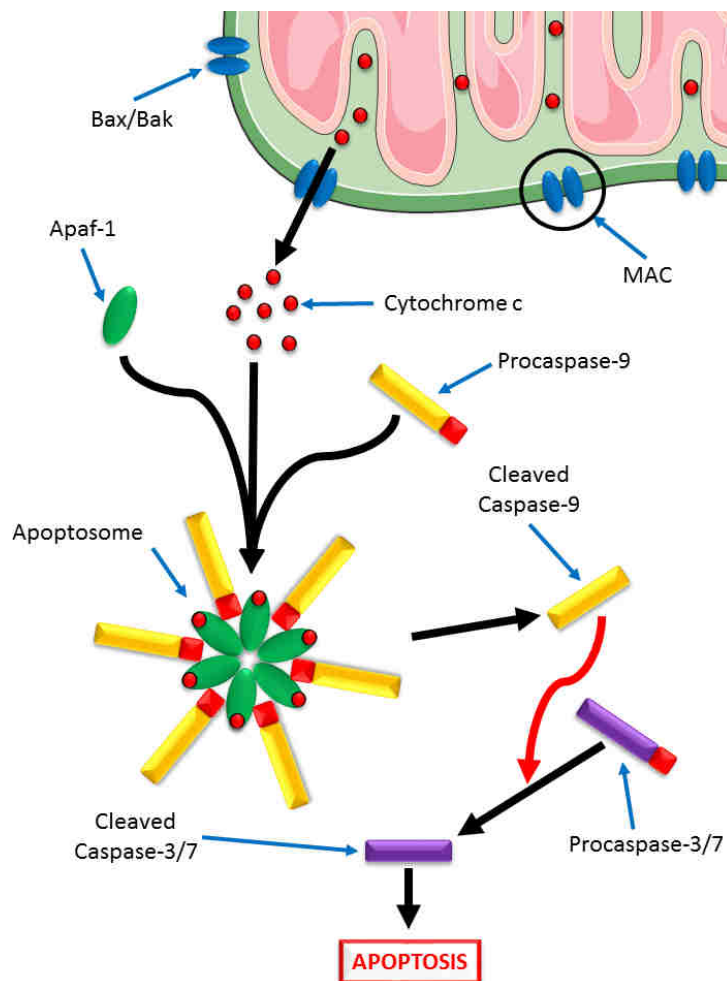


Figure 7: Schematic representation of the induction of the intrinsic pathway of apoptosis. Adapted from (Tait & Green 2013; Vucic et al. 2011)

b) The mitochondrial electron transfer chain and oxidative phosphorylation

At the end of the 18th century, Lavoisier and Laplace demonstrated that the amount of heat released per volume of CO₂ produced was equal in the combustion of charcoal, and in the respiration of a guinea pig (Scheffler 2007). Although at this time, combustion was believed to take place in the lungs, nowadays we know that the siege of respiration lies in mitochondria, at the level of the mitochondrial electron transfer chain (ETC). The electron transfer chain is composed by five complexes, which are each composed by multiple protein subunits. This ETC uses redox equivalents produced during the substrate oxidation by the citric acid cycle and the β -oxidation within the mitochondria. The ETC is an enzymatic series of electron donors and acceptors, which are located in the inner mitochondrial membrane. Each electron donor transfers electrons to an acceptor which is more electronegative. Briefly, electrons are transferred from NADH to complex I, and from FADH₂ to complex II. These two complexes will transfer their electrons to ubiquinone, which will transfer them to complex III. This third complex will transfer its electrons to cytochrome c, which will then transfer the electrons to complex IV. At the level of complex IV, electrons will be transferred to the most electronegative, and terminal electron acceptor of the chain: molecular oxygen, which will be then reduced to water. Passage of electrons from donor to acceptor releases energy. This energy will be used at the level of complexes I, III, and IV to transfer a proton from the mitochondrial matrix, to the intermembrane space. This will lead to an accumulation of protons in the intermembrane space, creating an electrochemical proton gradient, which will be used by complex V in order to phosphorylate ADP to ATP. This entire process is called oxidative phosphorylation (OXPHOS), and is responsible of the production of most of the ATP within the cell.

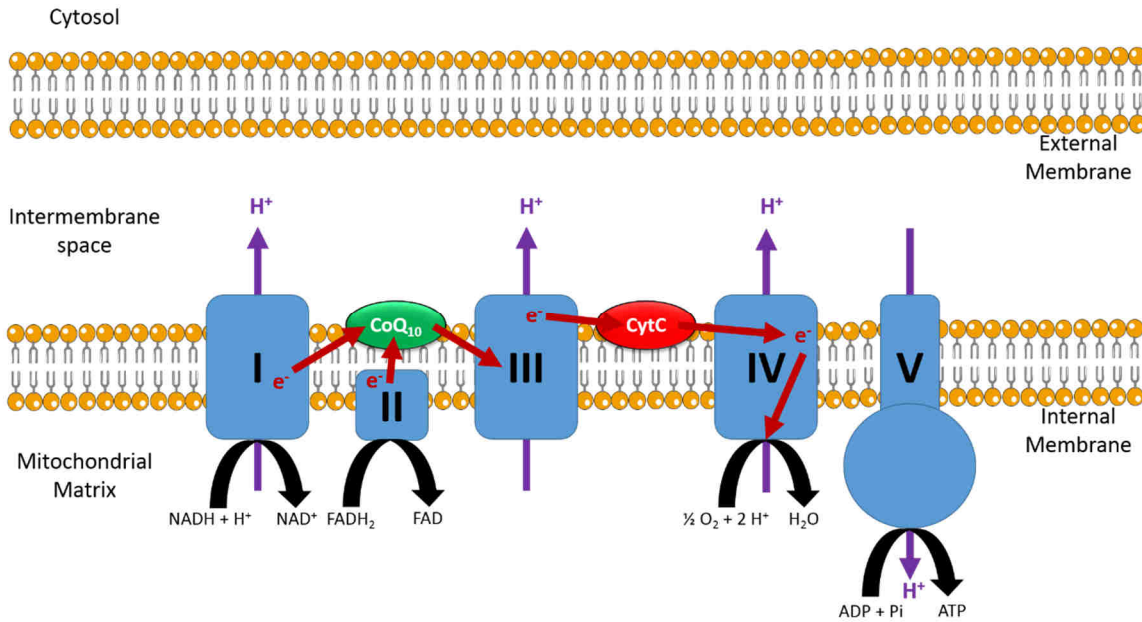
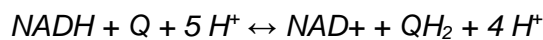


Figure 8: Schematic representation of the Mitochondrial Electron Transport Chain.

i. Complex I

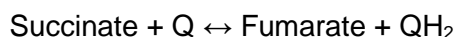
Mitochondrial Complex I of the ETC is properly referred as NADH:ubiquinone oxidoreductase (or NADH dehydrogenase). It catalyses the transfer of two electrons from NADH to ubiquinone, along with the transfer of four protons from the matrix to the intermembrane space. The reaction can be described as follow:



With Q being the oxidised and QH_2 the reduced form of ubiquinone (Scheffler 2007). This reaction requires a complex of approximately 980 kDa composed of 44 subunits. Most of these subunits are encoded by the nucleus (37), whereas only 7 are encoded by the mitochondrial genome (Wirth et al. 2016; Scheffler 2007).

ii. Complex II

Complex II of the ETC is known as succinate:ubiquinone oxidoreductase (or succinate dehydrogenase). It is the simplest complex in the ETC, as it is composed by 4 peptides only (SDHA-D). Subunits SDHA and SDHB are the largest peptides and constitute the hydrophilic domain in the matrix, and constitute the enzymatic part (succinate deshydrogenase), linking directly the citric acid cycle to the ETC (Hanstein et al. 1971; Davis & Hatefi 1971). The two other subunits (SDHC and SDHD) are integral membrane proteins of the inner mitochondrial membrane, and constitute the “anchor”. It is the only complex of the ETC which is coded by nucleus (although this statement is not true for some red algae and in a zooflagellate (Scheffler 2007)). It catalyses the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. Two protons and two electrons are necessary; however, no proton is transferred to the intermembrane space. The reaction can be described as follow:



In some tissues, complex II content is higher than other matrix enzymes. As a consequence, succinate:ubiquinone oxidoreductase delivers to ubiquinone a higher quantity of electrons than what complex III can transport, inducing an over-oxidation of ubiquinone. This phenomenon has for consequences the appearance of a reverse electron flux towards complex I (Favier et al. 2005). This induces a reduction of complex I, which promotes the production of reactive oxygen species (ROS).

iii. Ubiquinone

Ubiquinone is also known as Coenzyme Q₁₀ (CoQ₁₀). It is a fat-soluble mobile substance that is located in the inner mitochondrial membrane, and transfers the electrons from complex I or II to complex III. CoQ₁₀ is the only endogenously synthesized lipid-soluble radical-scavenging antioxidant (Crane 2007), and exists under three different redox states. Ubiquinone corresponds to the fully reduced form. Reduction by two electrons of ubiquinone converts it to ubiquinol, which has the capacity to act as a two electron carrier. Reduction by

one electron of ubiquinone, or oxidation by loss of an electron of ubiquinol, converts it to ubisemiquinone, which has the capacity to act as a one electron carrier. It has to be noted that maximal ADP-stimulated mitochondrial respiration depends on convergent electron flow through complexes I & II to the Q-junction of the electron transport chain (Gnaiger 2009).

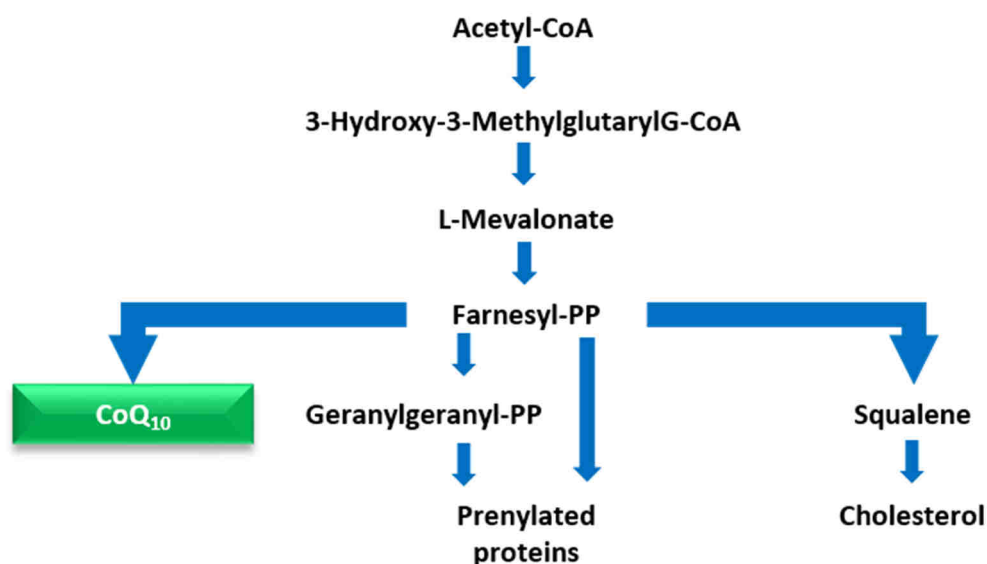


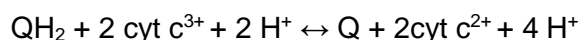
Figure 9: Schematic representation of the CoQ₁₀ biosynthesis pathway. Adapted from (Mugoni et al. 2013)

Biosynthesis of CoQ₁₀ is dependent on mevalonate production within the cell. Mevalonate-derived farnesyl-pyrophosphates (FPP) is the key intermediate for protein isoprenylation, cholesterol and CoQ₁₀ biosynthesis (Mugoni et al. 2013).

iv. Complex III

Complex III of the ETC is known in the literature as ubiquinone-cytochrome c oxidoreductase (or cytochrome c reductase, or bc₁ complex). It contains four active redox prosthetic groups: 2 cytochrome b, cytochrome c₁, and a Fe/S centre. Complex III is composed by 11 subunits, which are mostly encoded by the nucleus (10). Only one subunit containing the two cytochrome b is encoded by the mtDNA (Borisov 2002). This enzyme catalyses the

transfer of two electrons from ubiquinol to cytochrome c. The reaction can be described as follow:



Complex III has two reaction centers for ubiquinone: Q_i and Q_o . The Q_i center (also known as Q_N) is located at the matrix side of the inner mitochondrial membrane and can be inhibited by Antimycine A. The Q_i center is associated with the uptake of protons from the matrix and recycling half of the electrons back to the quinone pool. The Q_o center (also known as Q_P) is located at the intermembrane space side of the inner mitochondrial membrane and can be inhibited with myxothiazol. The Q_o center is associated with the release of protons in the intermembrane space and electrons are divided in two pathways: half are recycled and half are transferred to cytochrome c. This step is associated with the release of 4 protons at the Positive side (P, intermembrane space), whereas only 2 protons are uptaken at the Negative side (N, matrix) during the Q cycle (Scheffler 2007).

v. Cytochrome c

Cytochrome c is a small (12.3 kDa), hydrophilic haemoprotein located at the inner mitochondrial membrane, and is encoded by the nucleus (Cai et al. 1998). Cytochrome c transfers electrons from complex III to complex IV of the mitochondrial ETC and also plays a role in the triggering of the intrinsic pathway of apoptosis when released from the mitochondrion.

vi. Complex IV

Complex IV of the mitochondrial ETC is referred as cytochrome c oxidase. In humans, it is composed by 13 subunits, which are mostly encoded by the nucleus (10). The three largest subunits are encoded by the mitochondria: COXI, COXII, and COXIII (Scheffler 2007; Borisov 2002). Complex IV transfers electrons from cytochrome c, which will be reoxidised, to the terminal electron acceptor: molecular oxygen. The reaction can be described as follow:



During this step, four protons are consumed, and four others are translocated to the intermembrane space of the mitochondria. The overall process can be perturbed by the premature departure of partially reduced oxygen. This will have for consequence the formation of the reactive oxygen species superoxide (O_2^*) (Scheffler 2007).

vii. Complex V

Complex V is denominated as F_1F_0 -ATPase in the literature. This complex will use the electrochemical proton gradient created by the four previous complexes in order to produce ATP. Complex V is a multiple subunit complex of over 500 kDa, composed by two sub-complexes. The first subcomplex is an insoluble membrane complex referred as F_0 . This F_0 complex possesses the ability to translocate protons across the inner mitochondrial membrane from the intermembrane space (high potential), to the matrix. The F_0 complex contains nine different subunits: a, b, c, d, e, f, g, F6, and A6L (Walker et al. 1995). The second subcomplex allows to synthetise ATP driven by the proton motive force, and is referred as F_1 -ATPase. It is composed by 6 different subunits: α , β , γ , δ , ϵ , and OSCP (Oligomycin Sensitivity Conferral Protein) (Scheffler 2007). As the F_0 subcomplex provides a channel for the protons, the proton flow will be converted to a torque on the rotor of the F_1 -ATPase. This rotor possesses three binding sites for ADP. Three protons must be translocated through the membrane to induce a 120° turn of the rotor, and the conversion of one ADP to one ATP. Under physiological condition, the rotor is estimated to rotate at approximately 100 Hz (Scheffler 2007), hence inducing a flow of 900 protons per second, and converting 300 ADP to ATP per second.

B. OXIDATIVE AND REDUCTIVE STRESS

1) The oxygen paradox

The paradox of aerobic life consists in the fact that although higher eukaryotes require oxygen to exist (in order to keep the mitochondrial respiratory chain in function), oxygen is inherently dangerous for them (Davies 1995). Indeed, oxygen exists under its molecular form (O_2), and as a free radical. A free radical can be defined as any species (atom or molecule)

capable of independent existence that contains one or more unpaired electrons in an outer valence shell (Halliwell & Gutteridge 2007). The outer valence shell of O contains one unpaired electron, then when two atoms of oxygen combine to form O₂, the outer valence shell electrons do not spin-pair but remain as two unpaired electrons (Davies 1995).

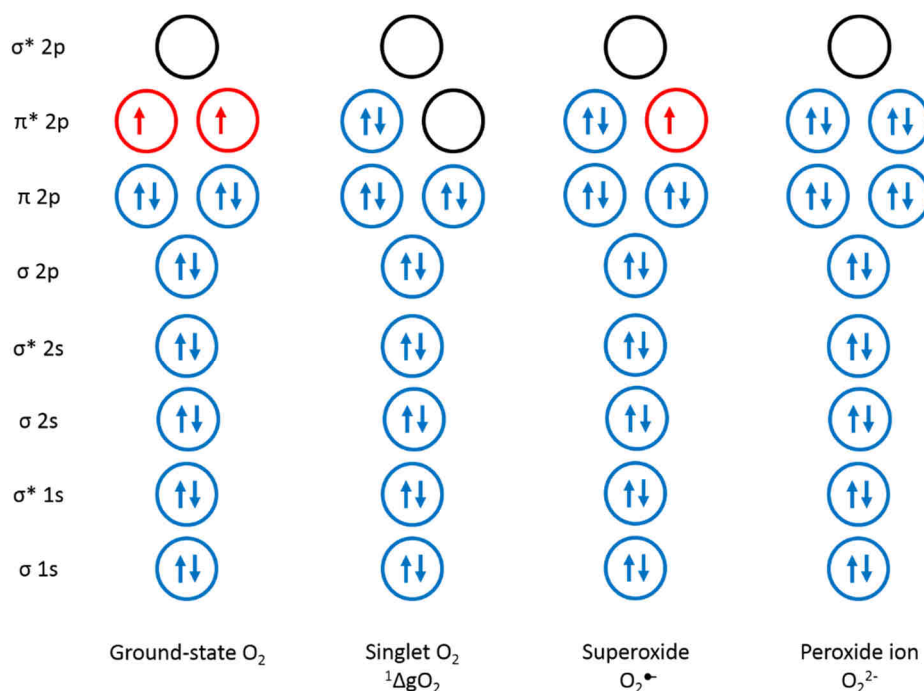
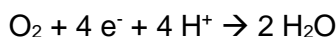
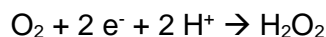


Figure 10: Simplified version of bonding in the diatomic oxygen and its derivatives. Adapted from (Halliwell & Gutteridge 2007).

Although singlet oxygen (¹ΔgO₂) is not considered as a free radical (no unpaired electrons), its oxidising ability is greatly enhanced compared to O₂. Singlet oxygen can be formed by simple input of energy. One-electron reduction of oxygen generates the superoxide anion radical O₂^{•-}. Addition of one electron to superoxide will form the peroxide ion (O₂²⁻), which is non radical, and has a weaker bond between the two oxygen atoms than O₂. Adding two more electrons to the peroxide ion totally removes the bond, leading to the formation of two oxide ions (O²⁻). However, in biology only the protonated form of O₂²⁻ and O²⁻ exist: H₂O₂ and water (Halliwell & Gutteridge 2007). The reactions can be described as follow:



2) Free Radicals in Biology

The term reactive oxygen species (ROS) includes the oxygen radicals, but also H₂O₂, HOCl (hypochlorous acid), and O₃ (ozone). This implies that all oxygen radicals are ROS, but not all ROS are oxygen radicals.

Radicals		Non-radicals	
Name	Symbol	Name	Symbol
Superoxide	O ₂ ^{•-}	Hydrogen peroxide	H ₂ O ₂
Hydroxyperoxyl	HO ₂ [•]	Peroxynitrite	ONOO ⁻
Hydroxyl	OH [•]	Peroxynitrous Acid	ONOOH
Peroxyl	RO ₂ [•]	Nitrosoperoxycarbonate	ONOCO ₂ ⁻
Alkoxy	RO [•]	Hypochlorous Acid	HOCl
Carbonate	CO ₃ ^{•-}	Hydrobromous Acid	HOBr
Carbon dioxide	CO ₂ ^{•-}	Ozone	O ₃
Singlet dioxygen	¹ Σg ⁺	Singlet oxygen	¹ Δg

Table 2: Reactive Oxygen Species. Adapted from (Halliwell & Gutteridge 2007).

In cells, there are several sites of ROS production, such as enzymes, auto-oxidation reactions, haem proteins, endoplasmic reticulum, peroxisomes, and mitochondria.

a) Enzymatic sources of ROS

i. Xanthine oxidase

In healthy cells, xanthine dehydrogenase (XDH) oxidises xanthine or hypoxanthine into uric acid, and transfers electrons not to O₂, but to NADH. When cells are damaged, XDH can be converted to xanthine oxidase (XO) by reduction of thiol groups. Under hypoxic conditions, depletion of ATP causes accumulation of hypoxanthine. When the tissue is reoxygenated, this accumulated hypoxanthine will be oxidised by XO, having for consequences, generation of superoxide and hydrogen peroxide. XO and XDH also catalyse O₂-dependant oxidation of NADH, hence generating superoxide (Halliwell & Gutteridge 2007).

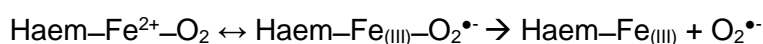
ii. NADPH oxidase

Superoxide can also be produced deliberately by the cells, for example it is used in the respiratory burst. Activation of the neutrophil NADPH oxidase leads to the conversion of NADPH to NADP^+ , and releases two electrons that will be used to reduce molecular oxygen to superoxide (Halliwell & Gutteridge 2007). The reaction can be described as follow:



b) Haem proteins

In haemoglobin and myoglobin, iron is in the Fe^{2+} state. Electron delocalisation occurs when molecular oxygen binds. As a consequence, the bonding is intermediate between Fe^{2+} to O_2 , and $\text{Fe}_{(\text{III})}$ to superoxide. Occasionally, superoxide is released, and the $\text{Fe}_{(\text{III})}$ formed cannot bind molecular oxygen again. Approximately 3% of haemoglobin in humans is oxidised per day in humans (Klotz et al. 2002). The reactions can be described as follow:



c) Endoplasmic Reticulum

Isolated microsomal fractions containing endoplasmic reticulum (ER) produce superoxide and hydrogen peroxide in presence of NADPH (Halliwell & Gutteridge 2007). ER contains enzymes capable of detoxifying lipophilic molecules and metabolites: the cytochromes P450 (Turrens 2003). They oxidise unsaturated fatty acids, and xenobiotics, hence producing superoxide (Morel & Barouki 1999). In addition, the mechanism used by cells to fold proteins in the ER might also participate to ROS generation (Halliwell & Gutteridge 2007).

d) Peroxisomes

Peroxisomes are probably the major sites of intracellular H_2O_2 production, as a result of their essentially oxidative type of metabolism (Del Río & López-Huertas 2016). For example, in rat liver, peroxisomes may be responsible for up to 20% of the oxygen consumption and 35% of the H_2O_2 production (Fransen et al. 2012).

e) Mitochondria

In cells, the most important source of ROS is the mitochondria. Indeed, it has been estimated that approximately 90% of cellular ROS are produced by mitochondria (Balaban et al. 2005). An intramitochondrial superoxide concentration of 1 to 10 picomolars during normal respiration has been estimated (Zhao et al. 2005). Within the mitochondria, complex I and complex III of the mitochondrial respiratory chain are potential sites of ROS generation. Indeed, complex II can deliver to ubiquinone a higher quantity of electrons than what complex III can transport, inducing an over-oxidation of ubiquinone. This will lead to the appearance of a reverse electron flux towards complex I (Favier et al. 2005). This induces a reduction of complex I, which promotes the production of reactive oxygen species (ROS). This reverse electron flux also has for consequences the reduction of NAD^+ to NADH. At the level of complex III, ROS generation takes place at the level of the Q_o ubiquinone site (Liu et al. 2002). ROS production at this level is thought to be the result of the partial reduction of Q_o ubiquinone to semiquinone, which is unstable. A second electron, hence the ubiquinone form, is indeed necessary to stabilize the proton transfer. However, there is a probability that ubisemiquinone meets a molecular oxygen molecule before stabilisation, hence forming superoxide (Turrens et al. 1985). Such model implies that ubisemiquinone is formed only transiently at the Q_o site, and never accumulates significantly. Hence the occupancy of ubisemiquinone is expected to be extremely low and formation of a Q_o site-associated ubisemiquinone has been proven very difficult to show experimentally (Bleier & Dröse 2013). Superoxide production at the Q_o site and not at the Q_i site has been shown by the use of specific inhibitors that bind to the two distinct ubiquinone binding sites. Indeed, superoxide production is strongly enhanced after the use of the Q_i site inhibitor antimycin A (Bleier & Dröse 2013). On the other hand, 'distal' Q_o site inhibitor stigmatellin completely abolishes the superoxide production. Moreover, binding of the 'proximal' Q_o site inhibitor myxothiazol causes only a partial reduction (Muller et al. 2002; Muller et al. 2003; Starkov & Fiskum 2001). It has been proposed that the UCPs allow proton leak in order to avoid electrons to "back up" to O_2 by decreasing the membrane potential (Halliwell & Gutteridge 2007).

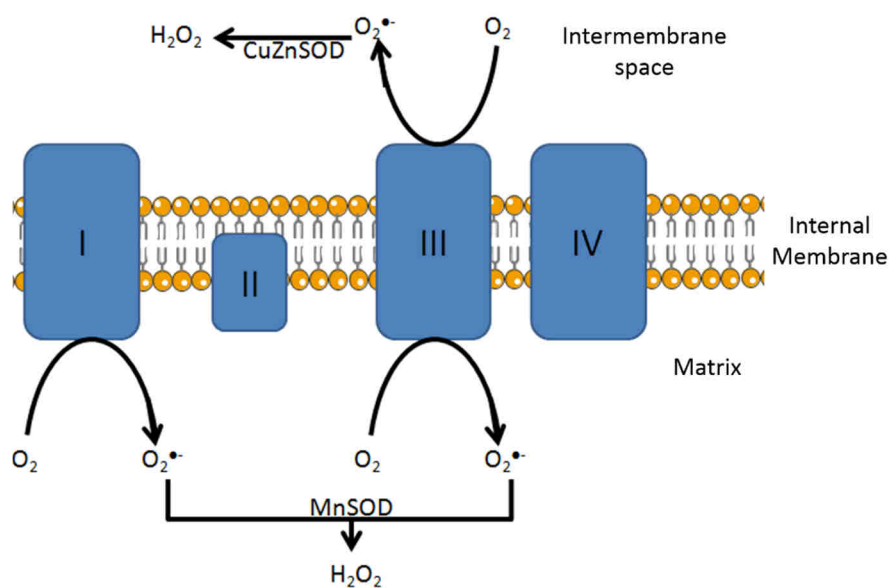


Figure 11: ROS production sites within the mitochondrial respiratory chain.

3) Oxidative Stress

An overproduction of ROS within the cell will lead to the appearance of oxidative stress. Oxidative stress can be defined as an imbalance between ROS production and their detoxification. Indeed, an excess in ROS production will lead to the appearance of damages within the cell. However, oxidative stress could also be the result of diminished antioxidant defences. Cells tolerate mild to moderate oxidative stress, which leads to an increased antioxidant defences synthesis (Halliwell & Gutteridge 2007). On the other hand, high oxidative stress will lead to cell injury. Oxidative stress will damage many different types of molecules, and could lead to cell senescence. Lipid peroxidation can be defined as the oxidative deterioration of polyunsaturated fatty acids. Polyunsaturated fatty acids contain two or more carbon-carbon double bonds that will get oxidised by ROS. This will have for consequences a decrease in the membrane fluidity, which is essential for the proper functioning of biological membranes (Halliwell & Gutteridge 2007). A single event of lipid peroxidation has the potential to induce a chain reaction leading to the generation of multiple lipid peroxides. Oxidative stress can provoke damage to proteins by direct attack of ROS, or by secondary damage. These

oxidations will either induce a break in the peptide chain, hence modifying the protein length, or modify their structure by adding products of lipid peroxidation. These alterations will lead to a modification of the protein structure, hence inducing a loss in the catalytic function (Stadtman & Levine 2002). This damaged protein will either be repaired when possible, or removed by lysosomes and proteasomes. Finally, oxidative stress can lead to the deterioration of DNA. Indeed, addition of H_2O_2 to cells leads to an increase of DNA strand breakage within minutes. Damage to DNA by ROS will have for consequences the apparition of mutations within the DNA, but could also lead to misincorporations by incorporation of ROS-modified nucleotides, and could lead to a change in gene expression if the mutation induced is located in an intron (in a promoter region for example). As 90% of ROS production is from mitochondrial origin, mtDNA presents a ROS susceptibility 10 folds higher than nuclear DNA (Richter et al. 1988). This could explain the higher mutation rate of mtDNA, and heteroplasmy. Moreover, these ROS-induced mutations in mtDNA could impair mitochondrial proteins expression, and lead to defects in the mitochondrial respiratory chain, hence inducing more ROS production, and the appearance of a “vicious circle”. This overproduction of ROS will then lead to the triggering of the intrinsic pathway of apoptosis, and to cell death. Hence, antioxidant defences are required to defend cells against these oxidative stress injuries. Often, the only hint of a past oxidative stress within the cell may be the increase in antioxidant defences, indicating a counteract of the cell against oxidative stress (Halliwell & Gutteridge 2007).

4) Antioxidant defences

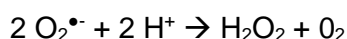
Antioxidants can be defined as any substance that delays, prevents, or removes oxidative damage to a target molecule (Halliwell & Gutteridge 2007). Antioxidant defences comprise agents that catalytically remove ROS, agents that decrease ROS formation, such as uncoupling proteins, chaperones molecules that protect biomolecules against oxidative injuries, physical quenchers of ROS (such as carotenoids quench singlet oxygen ($^1\Delta gO_2$)), and agents that will be preferentially oxidised by ROS in order to protect other molecules (Halliwell & Gutteridge 2007). In this part, I will focus on the agents that remove catalytically ROS. These

can be divided into two categories: enzymatic and non-enzymatic defence systems (Powers & Lennon 1999).

a) Antioxidant defence enzymes

i. Superoxide dismutases

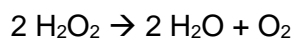
Superoxide dismutases (SODs) catalyse the dismutation of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2). The simplified equation can be described as follow:



In humans, there are three types of SODs (SOD1 to SOD3) that catalyse this reaction. These SODs are differentiated by their metallic cofactor, and their localisation. SOD1 possesses a copper-zinc cofactor, and is hence also named CuZnSOD. Most of this SOD1 is located in the cytosol of cells, but is also present in the nucleus, in lysosomes, in peroxisomes, and in the mitochondrial intermembrane space (Halliwell & Gutteridge 2007). SOD2 possesses a manganese cofactor (MnSOD), and is located within the mitochondrial matrix. The third type is SOD3, which is a CuZnSOD like SOD1. However, SOD3 is extracellular, and possesses the ability to bind membranes.

ii. Catalase

As shown previously, dismutation of superoxide generates hydrogen peroxide. This H_2O_2 will directly be decomposed to molecular oxygen by catalase. The equation can be described as follow:



Most of catalase activity is located in the peroxisomes. Mitochondria of skeletal muscle and liver, and endoplasmic reticulum contain no catalase. However, in the heart some catalase is present in the mitochondrial matrix (Halliwell & Gutteridge 2007).

iii. Peroxiredoxines

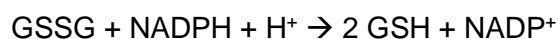
Peroxiredoxines (Prx) are cysteine-dependent peroxidases that catalyse the reduction of different peroxides. Within the mitochondria, Prx3 has been estimated to reduce 90% of the hydrogen peroxide produced there (Quijano et al. 2016).

iv. Glutathione peroxidases

As catalase, glutathione peroxidases (GPx) decompose hydrogen peroxide to water. However, peroxidases remove hydrogen peroxide by using it to oxidise another molecule. GPx couples the reduction of H_2O_2 to H_2O with the oxidation of two molecules of reduced glutathione (GSH) to oxidised glutathione (GSSG) (Matés et al. 1999). The equation can be described as follow:



GPx exists in four different types: GPx1 is located in the cell cytosol. Gpx2 is located in cells lining the gastrointestinal tract, and could serve to metabolise peroxides from the ingested food (Halliwell & Gutteridge 2007). GPx3 is located in mammalian plasma, and extracellular fluids. GPx4 is also known as phospholipid hydroperoxide glutathione peroxidase (PHGPx), and is associated to the mitochondrial membrane (Matés et al. 1999). Contrary to the other GPx, GPx4 possesses the ability to reduce fatty acids and cholesterol hydroperoxides. As GPx consumes GSH, it is necessary to maintain the GSH pool within the cell. The enzyme responsible for this reaction is glutathione reductase, which converts GSSG back to GSH. The equation can be described as follow:



b) *Non-enzymatic defence systems*

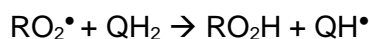
i. Reduced glutathione

GSH is synthesized in the cytosol of all animal cells. It is estimated that mitochondria contain 10 to 15% of the total cellular glutathione pool (Ribas et al. 2014). Apart from its role

with GPx, GSH also reacts directly as an antioxidant with OH^\bullet , HOCl , ONOO^- , RO^\bullet , RO_2^\bullet , $\text{CO}_3^{\bullet-}$, NO_2^\bullet , carbon-centered radicals, and singlet oxygen, but not superoxide (Halliwell & Gutteridge 2007). The GSH/GSSG ratio is often used as a marker of oxidative stress, as GSH pool decreases, and GSSG pool increases in presence of ROS (Ji et al. 1991).

ii. Coenzyme Q₁₀ and cytochrome c

Within the mitochondrial respiratory chain, CoQ₁₀ and cytochrome c participate to the antioxidant defence. Indeed, ubiquinol can scavenge RO_2^\bullet radicals and inhibit lipid peroxidation (Halliwell & Gutteridge 2007). The reaction can be described as follow:



Moreover, CoQ₁₀ is implied in the regeneration of vitamin E, which amplifies its role against oxidative stress (Podda et al. 1996). Cytochrome c, which is present in the mitochondrial intermembrane space, can reduce the superoxide and hydrogen peroxide produced at the level of the electron transport chain (Bowman & Bren 2008).

iii. Compounds derived from the diet

Ascorbic acid (vitamin C) is an antioxidant that can be found in citrus fruits, berries, mango, guava, broccoli, and peppers. Ascorbate is hydrophilic, and can be found in cells in the cytosol and in extracellular fluids. Vitamin E is lipophilic, and can be found in wheat-germ, vegetable oils, nuts, grains, and green leafy vegetables. Technically, vitamin E does not correspond to a specific chemical, but a nutritional term. Indeed, eight substances have been shown to possess vitamin E activity: d- α -, d- β -, d- γ -, and d- δ -tocopherols, and d- α -, d- β -, d- γ -, and d- δ -tocotrienols (Halliwell & Gutteridge 2007).

5) Reductive stress

Although antioxidants display numerous beneficial effects against oxidative stress, just as an excess of oxidants is deleterious for the cell, an excess of reducing agents could also be harmful. Even though oxidative stress is a well-known phenomenon, the impact of reductive stress remains poorly understood. Reductive stress can be defined as an excess of reducing

equivalents (NAD(P)H and/or GSH) in the presence of intact oxido-reductive systems (Brewer et al. 2013). Numerous studies trying to evaluate the benefits of antioxidant therapies in tens of thousands of patients have been realised. However, the use of antioxidants in preventing the complications of atherosclerotic disease has been disappointingly ineffective (Steinhubl 2008). Although appropriate agents have been evaluated, they were tested at inappropriate dose or inadequate durations. Higher levels of vitamin E supplementation has not only been ineffective, but has also been associated with an increased mortality (Miller et al. 2005; Bjelakovic et al. 2007). Removal of NADPH oxidase (Nox) in heart, using transgenic mice, and the subsequent reductive stress has been shown to lead to exacerbation of ischemia/reperfusion injury (Yu et al. 2014). Surprisingly, antioxidant vitamins, and N-acetylcysteine have been shown to induce an increased reactive oxygen species production during reductive stress (Herbert 1996; Brewer et al. 2013; Zhang et al. 2012; Pimentel et al. 2012). Interestingly, antioxidant supplementation has been shown to attenuate skeletal muscle mitochondrial biogenesis following exercise in both rodents (with Vitamin E) (Strobel et al. 2011), and in human (with N-acetylcysteine) (Petersen et al. 2012). In addition, supplementation with the antioxidants vitamins C and E in humans has been shown to reduce the mRNA expression levels of PGC-1 α and PGC-1 β (Ristow et al. 2009). Hence, antioxidants can block the benefic adaptive response to exercise-induced ROS.

6) Mitochondrial Hormesis

ROS have been shown to have many deleterious effects. However, they can also induce beneficial effects by activating ROS-dependant pathways. The phenomenon involved in this dual effect of ROS is called mitochondrial hormesis. Hormesis relies on the principle of mithridatism. This term derives from Mithridates VI, who was the son of Mithridates V, king of Pontus, a region on the southern coast of the Black Sea. After his father assassination by poisoning in 120 BC, he escaped by fear of being poisoned by his own mother. He spent seven years in the wild, where he tried to find a way to protect himself against an assassination attempt with poison. His solution was to ingest a potion containing 54 known poisonous

compounds mixed together in small doses, believing that he would hence develop a resistance, protecting him against higher doses (Yun & Finkel 2014). After his exile he took back the throne and imprisoned his mother. Several years later, he was defeated by Pompey, and attempted suicide by poison without success, because of his immunity to poison. To achieve his goal, he then asked his bodyguard to kill him by the sword. Several centuries later, Paracelsus, who held the chair of medicine at the University of Basel and was the city physician for less than a year, wrote in the 16th century: “*sola dosis facit venenum*”, which can be translated as “the dose makes the poison”. After this small historical interlude, the reader can now better discern what is the principle of hormesis. Hormesis can be described as the activation of cellular protective and reparative properties induced by mild physiologic stress (Schwartz & Sack 2008). In other terms, a low level of stress leads to subsequent protection against bigger insults (Halliwell & Gutteridge 2007). The term “hormesis” has been proposed in 1943 by Southam and Ehrlich in their work on an antibiotic substance extracted from the heartwood of Western Red Cedar (*Thuja plicata*). They observed an apparent stimulation in growth of otherwise sensitive organisms at certain concentrations (Randall et al. 1947). Mitochondrial hormesis is based on the assumption that a moderate increase in ROS production during mitochondrial activity leads to the triggering of cellular defence systems (Gems & Partridge 2008; Ristow & Zarse 2010; Sano & Fukuda 2008). The easiest example of mitochondrial hormesis is the muscular adaptations to exercise. Indeed, exercise has been known for a long time to induce the production of ROS in skeletal muscles. However, if the intensity of the exercise is sufficient, but not too high, this sub-lethal dose of ROS will trigger adaptive pathways, resulting in the induction of mitochondrial biogenesis and aerobic capacity, augmented antioxidant capacity, improved vascularization, and insulin sensitivity (Alleman et al. 2014). Reduction of glucose uptake and metabolism has been shown to extend *Caenorhabditis elegans* life span, by inducing mitochondrial respiration and increasing the production of ROS (Schulz et al. 2007). Moreover, impaired insulin and IGF-1 signalling has also been shown to extend life span by increasing mitochondrial metabolism in *C. elegans* (Zarse et al. 2012). Significant effort has been made in the past to decrease ROS formation, with the assumption that it could at least

ameliorate aging process in humans (Ristow & Zarse 2010). However, in the past two decades, studies have shown that sub-lethal levels of ROS are acting as essential signalling molecules, that transduce signals from the mitochondria. These signals induce the triggering of mitochondrial biogenesis pathways via the PGC-1 family members, and lead to an increased mitochondrial content and an improvement of antioxidant defence systems. Among the different tissue types within the body, the one that shows the greatest potential of adaptations to physiological demand and reactive oxygen species, is the skeletal muscle.

C. Skeletal muscles

In mammals, four different types of cells specialised in contraction exist: skeletal muscle cells, cardiac muscle cells, smooth muscle cells, and myoepithelial cells (Alberts et al. 2002), each type having a particular function, structure, and development. In this thesis, we will focus on skeletal muscle cells, which are responsible for almost all voluntary movements. There are approximately 640 skeletal muscles in humans, which constitute the most abundant tissue type in the organism. Indeed, skeletal muscles account for approximately 38.4% of the body weight in men, and approximately 30.6% in women (Janssen et al. 2000).

1) Structure

Skeletal muscles are composed by muscle fibres, that are maintained together by connective tissue. Muscles are generally connected to bones by tendons, which are mainly composed by unidirectional fibres of collagen. Each muscle is enclosed in a fascia composed of connective tissue, and known as epimysium. Within the epimysium, muscle fibres are organised in fascicles that are surrounded by another layer of connective tissue: the perimysium. Each fascicle is composed by many muscle fibres or myocytes, which are enclosed in a third layer of connective tissue: the endomysium. Myocytes are multi-nucleated cells, that result from the fusion of myoblasts during embryogenesis. Once the fusion is over, the nuclei will never replicate their DNA again (Alberts et al. 2002). However, some myoblasts persist as quiescent stem cells in adults: satellite cells. If a myocyte is destroyed after birth,

satellite cells, which are adjacent to muscular fibres, will form new fibres by fusion. This ability to form new myocytes is limited and cannot restore complete muscular strength in case of severe damage. In that case, muscle loss will be compensated by increasing the size of the remaining myocytes by hypertrophy. Muscle fibres have a diameter of 10 to 100 μM and a length that can reach 20 cm (Widmaier et al. 2009). Nuclei are located at the periphery of the myocytes, and the major part (85-90%) of a myocyte is composed by myofibrils.

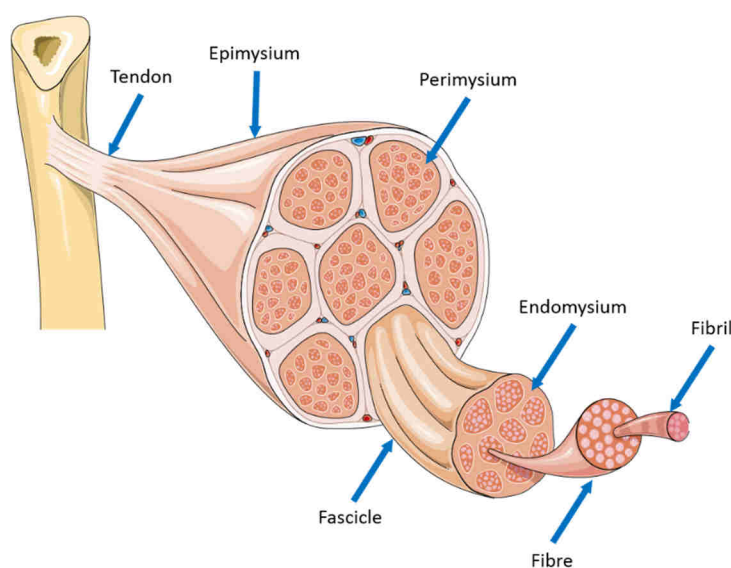


Figure 12: Skeletal muscle organisation.

In brief, myofibrils are composed by sarcomeres, which consist of the basic contractile subunit of the muscle (Shadrin et al. 2016). Each sarcomere contains a central myosin-rich dark anisotropic (A) band, and two actin light isotropic (I) bands. The A band is composed by thick filaments of myosin, and the I band is composed by two halves thin filaments. One of the extremities of the thin filament is anchored to a protein network named Z line. The H zone corresponds to a narrow band located at the middle of band A. The M line constitutes a dark thin line in the middle of the H zone, and is constituted by proteins that link adjacent thick filaments. Myosin heads will hydrolyse ATP and lead to the sliding of filaments, sarcomere shortening, and hence muscular contraction.

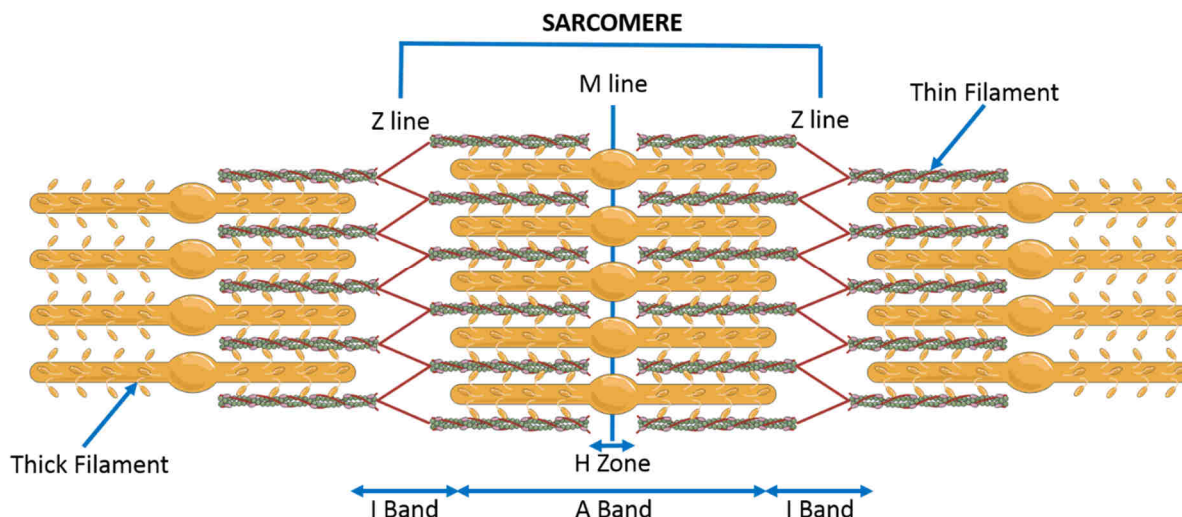


Figure 13: Schematic organisation of the sarcomere.

2) Fibre types

All skeletal muscle fibres do not have the same metabolic and mechanic characteristics. Indeed, these fibres can be assigned to different types, depending on the maximal movement rate (slow or fast), their response to neural input, and their metabolic style (oxidative or glycolytic) (Talbot & Maves 2016). Skeletal muscles can first be classified by their maximal rate to degrade ATP as “slow twitch” (type I), or “fast twitch” (type II). Type I fibres have a low contraction rate, and develop few strength, however these fibres are resistant to fatigue. Typically, these fibres can for example be found on marathon runners. Type II fibres have a high contraction rate, and allow to develop a lot of strength, however these fibres are easily fatigable. Typically, these fibres can be found on sprinters. In a second time, these fibres can be further classified by their ATP synthesis machinery. Indeed, certain fibres possess a large amount of mitochondria, and rely mainly on oxidative phosphorylation. These fibres can be defined as “oxidative”. These fibres require more oxygen, and depend hence on the vascularisation to synthetize ATP. They also possess large amounts of myoglobin, a protein that can bind oxygen and increase its diffusion within the fibre, and create a reserve (Widmaier et al. 2009). On the other hand, other fibres possess few mitochondria, and mainly rely on

glycolysis to produce ATP. These fibres can be identified as “glycolytic”, and contain low amounts of myoglobin. Moreover, the analysis of the expression myosin heavy chains enables to differentiate three subtypes of fast twitch fibres: IIA, IIX, and IIB. However, humans do not appear to possess MYH4-expressing type IIB fibres (Talbot & Maves 2016). Type IIA fibres use both oxidative and glycolytic metabolisms to produce ATP, and have a relatively high mitochondrial content (however lower than type I fibres). These fibres are relatively resistant to fatigue (Schiaffino 2010). Type IIB fibres possess a low amount of mitochondria and rely mainly on glycolysis. These fibres are easily fatigable, but develop tremendous amounts of strength. Type IIX fibres possess an intermediate phenotype between type IIA and type IIB fibres.



Overall fibre type	Slow-twitch	Fast-twitch		
Fibre type	Type I	Type IIA	Type IIX	Type IIB
Speed of fatigue	Slow	Fast	Fast	Fast
Speed of contraction	Slow	Fast	Fast	Fast
Metabolic type	Oxidative	Oxidative & Glycolytic	Glycolytic	Glycolytic
Glycogen content	Low	Intermediate	High	High
Representative myosin	MYH7	MYH2	MYH1	MYH4
Mitochondrial content	Very high	High	Low	Low
Myoglobin content	Very high	High	Low	Low
Fibre diameter	Small	Intermediate	Big	Big
Example				

Table 3: Skeletal muscle fibre types characteristics. Adapted from (Talbot & Maves 2016; Widmaier et al. 2009)

Interestingly, the proportion of fibre type in a muscle is plastic, and muscles can adapt to the different needs. For example, endurance exercise training can modestly increase the proportion of slow-twitch fibres (Talbot & Maves 2016). On the other hand, muscle inactivity leads to the conversion of oxidative fibres to type IIX fibres.

3) Mitochondria in skeletal muscles

Skeletal muscle metabolic plasticity in response to physiological and pathophysiological stimuli is a remarkable feature, enabling adaptation of energy production to energy demands (Ljubicic et al. 2010; Vainshtein et al. 2015). At the heart of this unique property, stand the mitochondria. Within skeletal muscle, there are two subfractions of mitochondria that can be isolated in fiber. Subsarcolemmal (SS) mitochondria can be found immediately underneath the sarcolemmal membrane, and intermyofibrillar (IMF) mitochondria are located between the myofibrils (Adhihetty et al. 2005). SS mitochondria are estimated to account for approximately 20% of the muscle cell mitochondrial content. On the other hand, IMF mitochondria constitute the remaining 80% (Hoppeler 1986). These two population possess specific functional, biochemical, and compositional properties (Cogswell et al. 1993). Indeed, the subsarcolemmal fraction displays higher adaptive capacities to diverse stimuli than the IMF fraction. Interestingly, IMF mitochondria have a higher rate of activation of apoptotic pathways when subjected to oxidative stress, revealing a potentially dominant role for the IMF subfraction in the triggering of apoptotic pathways within skeletal muscles (Adhihetty et al. 2005). In addition, fibre type (and thus mitochondrial content) has been shown to have an impact on apoptotic susceptibility (McMillan & Quadriatero 2011). Indeed, McMillan & Quadriatero observed significantly higher levels of ROS generation, and apoptosis-related mitochondrial events in the red gastrocnemius (mostly glycolytic) compared with the white gastrocnemius (mostly oxidative) muscle, possibly indicating a greater basal level of apoptotic signalling. Hence fibre type and mitochondrial content constitute crucial considerations when considering the triggering of apoptotic pathways in skeletal muscles. Several mitochondrial toxicants have

been shown to trigger apoptosis within skeletal muscles, leading to muscular impairments. Among these mitochondrial toxicants, we will focus on the mechanisms involved in statin myotoxicity.

D. Statins

1) Definition

Statins are a class of molecules also known as 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors. These molecules have revolutionised the treatment of hypercholesterolemia and the management of patients with increased cardiovascular disease (CVD) risk. CVD is one of the major cause of premature death, and a cause of morbidity in western countries. For example, in the UK, CVD is estimated to account for 35% of premature death in men, and 27% in women (Ward et al. 2007). Indeed, statins have been shown to reduce morbidity and mortality from the three manifestations of CVD: coronary heart disease (Allen Maycock et al. 2002), cerebrovascular disease (Amarenco et al. 2006), and peripheral arterial disease (Mohler et al. 2003). These molecules indirectly decrease the de novo synthesis of cholesterol by inhibiting the HMG-CoA reductase enzyme. This enzyme is responsible for the conversion of HMG-CoA to mevalonate, which is a rate-limiting step in cholesterol biosynthesis. An adult on a low-cholesterol diet typically synthesizes about 800 mg of cholesterol per day. The synthesis of cholesterol mainly takes place in the liver, but the intestine also participates significantly (Berg et al. 2002).

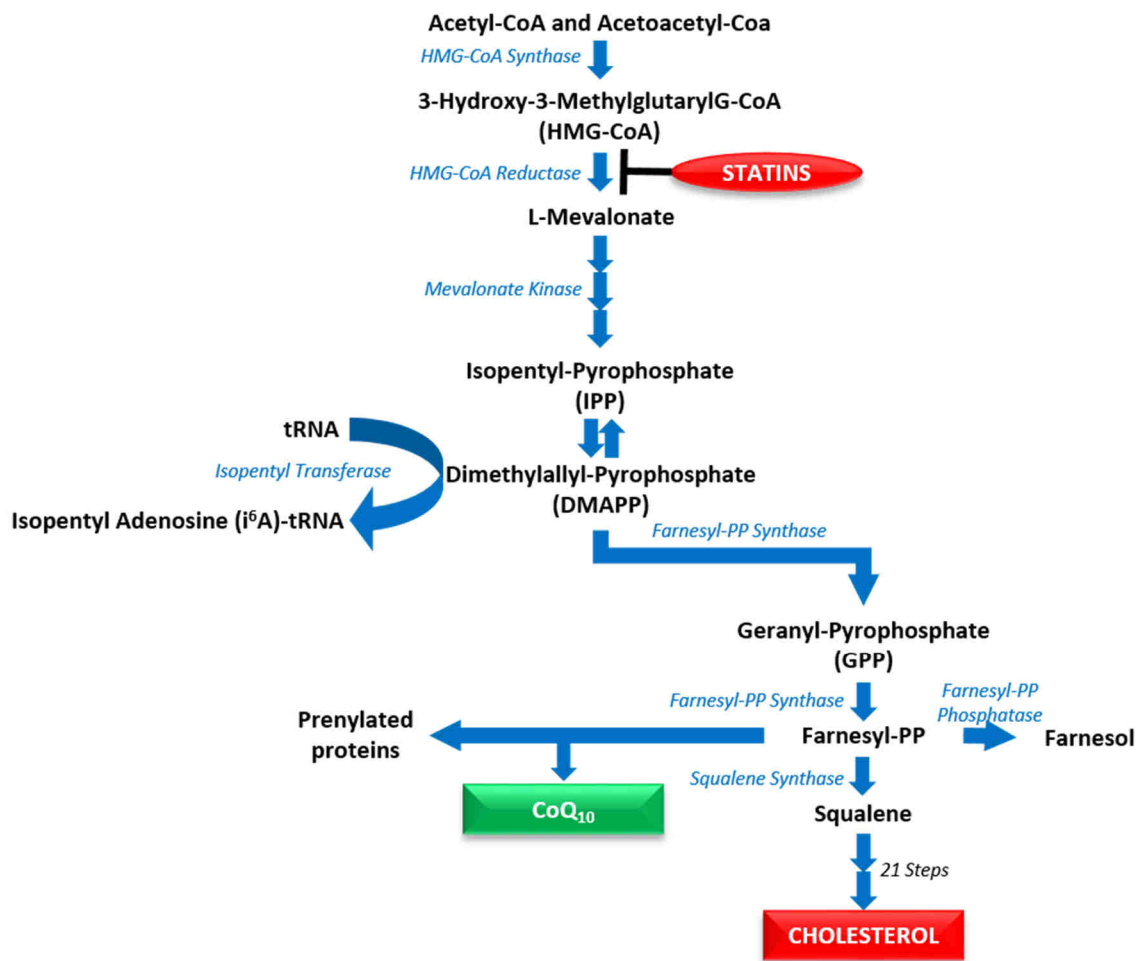


Figure 14: Cholesterol biosynthesis pathway.

2) Usage

Statins are among the most commonly prescribed drugs worldwide, and are used in both primary and secondary prevention. In Europe in 2000, the widest use of statins was in Norway, with 59.28 daily doses per 1000 inhabitants, and the lowest use was Italy with 14.74 doses per 1000 inhabitants (Walley et al. 2004). In the United States of America, during 2011-2012, 27.2% of adults aged 40 and over used a prescription cholesterol-lowering agent in the last month. During the same period, 83% of adults who used a cholesterol-lowering agent used statins alone, and 10% statins in combination with another non-statin agent (Gu et al. 2014).

SKELETAL MUSCLE TOXICITY AND STATINS: ROLE OF MITOCHONDRIAL ADAPTATIONS

Country	Simvastatin		Lovastatin		Pravastatin		Fluvastatin		Atorvastatin		Cerivastatin		All statins		Average annual increase in statin use 1997-2002 (%)‡
	Total use*	Rate use†	Total use*	Rate use†	Total use*	Rate use†	Total use*	Rate use†	Total use*	Rate use†	Total use*	Rate use†	Total use*	Rate use†	
Austria	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	64.96	21.94	37
Belgium	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	146.9	39.32	NK
Denmark	14.83	7.60	1.37	0.70	3.32	1.70	0.98	0.50	8.97	4.60	0.78	0.40	30.25	15.50	38
Finland	23.05	12.12	6.45	3.39	3.97	2.09	6.55	3.44	18.12	9.53	0.52	0.27	58.65	30.85	37
France	206.81	13.57	0.00	0.00	145.17	9.58	29.79	1.97	357.52	23.56	107.60	7.09	846.88	55.82	NK
Germany	144.10	5.54	31.20	1.20	55.90	2.15	41.30	1.59	299.70	11.52	116.20	4.47	688.40	26.47	26
Ireland	1.27	3.02	0.00	0.00	4.63	11.05	0.50	1.19	4.24	10.12	4.16	1.00	14.80	26.38	NK
Italy	132.51	6.29	0.00	0.00	41.18	1.96	5.15	0.24	93.84	4.46	37.04	1.79	309.72	14.74	52
Netherlands	115.30	22.13	0.00	0.00	32.58	6.25	7.94	1.53	96.87	16.72	3.60	0.69	256.29	47.28	27
Norway	48.70	29.79	1.80	1.10	9.40	5.75	0.70	0.43	34.41	21.05	1.91	1.17	96.91	59.28	28
Portugal	14.13	5.29	7.38	2.76	8.69	3.25	8.68	3.25	9.21	3.44	2.85	1.07	50.93	19.06	NK
Spain	101.83	6.89	37.88	2.56	57.36	3.88	9.00	0.61	111.81	7.56	42.59	2.88	360.30	24.13	31
Sweden	59.46	18.60	0.00	0.00	11.49	3.59	2.13	0.66	34.46	10.78	2.11	0.66	109.65	34.29	34
UK	178.03	9.72	0.00	0.00	48.52	2.65	12.02	0.66	172.01	9.39	26.47	1.44	437.03	23.86	48

NK=Not known. *Total use in million defined daily doses. †Rate use in defined daily doses/1000 of population covered/day.

‡Data available only for the following periods: Austria, Norway, Spain 1997-2001; Finland, Sweden 1998-2002; Italy 2002; Germany, Netherlands, UK 1997-2002.

Table 4: Use of statins in European countries in 2000 (Walley et al. 2004).

The first statin to be discovered was Mevastatin in 1971, by a Japanese researcher. Akira Endo isolated the first HMG-CoA reductase inhibitor from *Penicillium citrinum*. However, Mevastatin was never marketed, because of its adverse effects on muscle deterioration, and sometimes death in laboratory dogs. Mevastatin was then derivatised to produce Pravastatin. The first statin available on the market was Lovastatin, which has been approved by the US FDA in 1987, and is produced by Merck. Nowadays, there are seven statins approved on the market: Atorvastatin (Tahor®), Fluvastatin (Lescol®), Lovastatin (Mevacor®), Pitavastatin (Livalo®), Pravastatin (Pravacol®), Rosuvastatin (Crestor®), and Simvastatin (Zocor®). However, another statin (Cerivastatin) has been on the market until its withdrawal in 2001 because of its unacceptable incidence of rhabdomyolysis and death (Baker & Tarnopolsky 2001).

	Drug	Cerivastatin	Simvastatin	Fluvastatin	Lovastatin	Atorvastatin	Rosuvastatin	Pravastatin
	Lipophilicity	+++	++	+	+	+	-	-
LDL Reduction	<25%		5	20	10-20	10	5	10-20
	25-35%	0,2	10-20	40-80	20-30	10	5	20-30
	35-45%	0,4	20-40		40-80	10-20	5-10	40-80
	45-55%		80			20-40	10-20	
	55-60%					80	20-40	
	60-65%						40-80	

Table 5: Statin lipophilicity and dose (mg) necessary to decrease LDL cholesterol to a set percentage. “+” indicates lipophilicity, and “-” indicates hydrophilicity. Adapted from (White 2002; Hunninghake 1998; Yasunobu et al. 1997; Joshi et al. 1999)

3) Toxicity

Although statins display beneficial effects on the risk of CVD, display pleiotropic effects (that will not be described in this thesis), and are considered as clinically relatively safe, these molecules are associated with skeletal muscle, metabolic, neurological, and other possible side effects (Thompson et al. 2016). Indeed, statins have been associated with cases of cognitive problems, memory loss, or even dementia (Wagstaff et al. 2003). Moreover, statins have been shown to increase the risk of diabetes mellitus significantly (Thakker et al. 2016; Suneja et al. 2014). Statin can also lead to an elevated liver function, a decreased renal function, tendon rupture, interstitial lung disease, depression, low testosterone, and sleep disturbance (Thompson et al. 2016). However, the most frequent adverse event encountered with statins is the apparition of statin-associated muscle symptoms (SAMS).

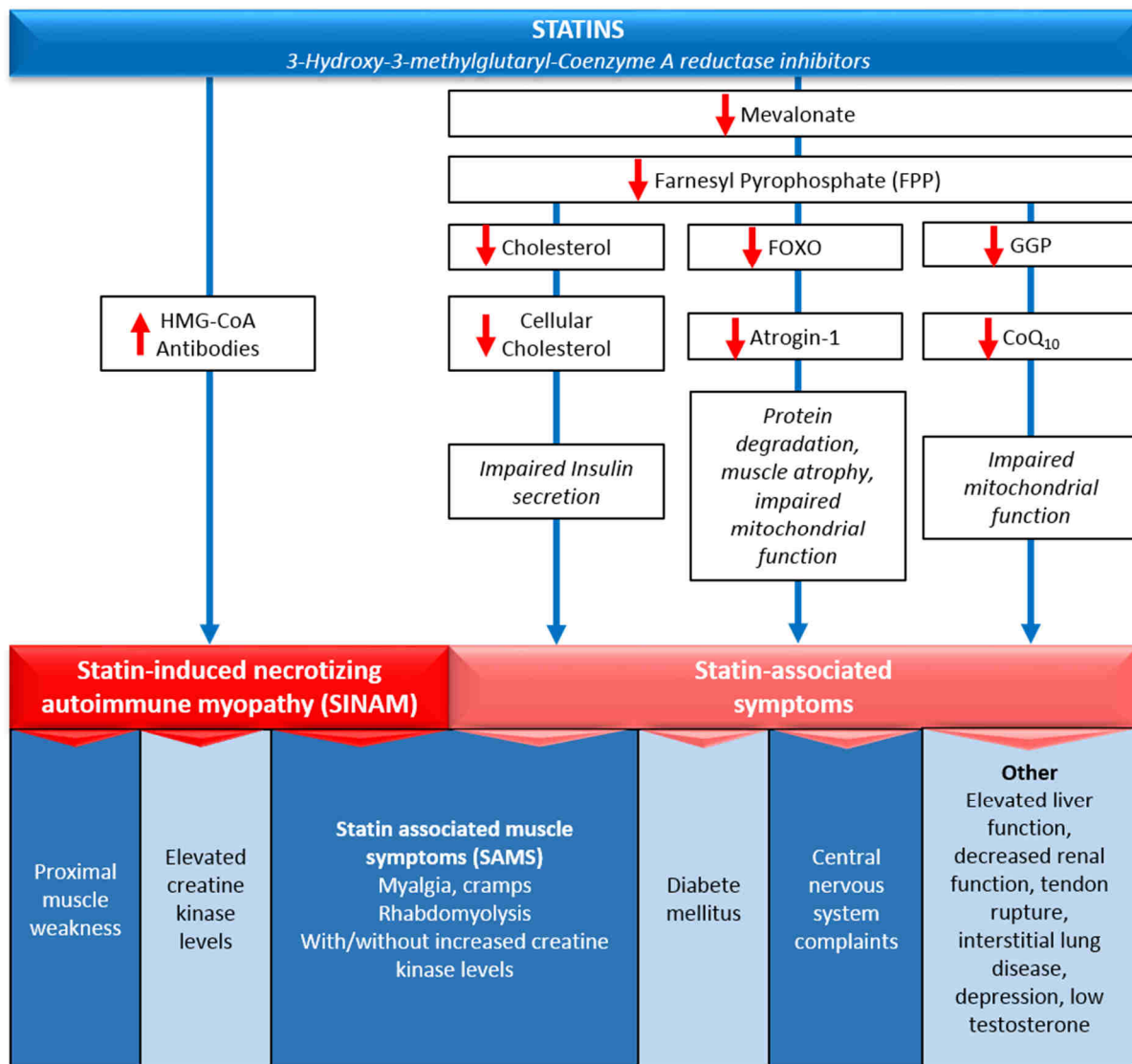


Figure 15: Statin-associated side effects. Adapted from (Thompson et al. 2016)

a) *Statin Associated Muscle Symptoms*

SAMS include fatigue, muscle pain, muscle weakness, muscle tenderness, and cramps. These symptoms range from clinically asymptomatic muscle disease recognised by plasmatic creatine kinase (CK) elevation, to a life-threatening condition of rhabdomyolysis (Maghsoodi & Wierzbicki 2016). The occurrence of these SAMS is debated in the literature, due to the absence of a consensus definition. For example, the PRIMO study studied questionnaires on 7924 French patients treated for at least 3 months with high-dosage statins, and reported

muscular symptoms in 10.5% of participants (Bruckert et al. 2005) but other studies have reported rates of musculoskeletal pain as high as 23% in statin users (Buettner et al. 2012). The reported incidence of myalgia during therapy with the more powerful statins has varied from 1% in pharmaceutical company reports (Anon 2002) to 25% of patients (Phillips et al. 2002). Moreover, the incidence of less serious side effects of muscle pain and weakness, as opposed to rhabdomyolysis, are estimated to be underreported (Tomlinson & Mangione 2005). However, recently, this statin-related myotoxicity (SRM) has been classified into seven categories (SRM 0-SRM 6) (Alfirevic et al. 2014). Asymptomatic rise in plasmatic creatine kinase (SRM 0) has been reported with statins, and exercise has been associated as a risk factor in 25% of cases. However, the American College of Cardiology, the American Heart Association, a Canadian Working Group, the National Lipid Association, and the European Phenotype Standardization Project have defined that SAMS can occur without CK elevation, and that it is the most frequent presentation of SAMS (Thompson et al. 2016).

i. Myalgia

Statins can provoke two types of myalgia. Tolerable symptomatic myalgia (SRM 1) can be defined as the presence of muscle ache without any elevation in the creatine kinase levels (Joy & Hegele 2009), and can occur in up to 33% of patients (Alfirevic et al. 2014). On the other hand, intolerable myalgia (SRM 2) can be defined as the presence of muscle ache with an elevation of CK inferior to 4 times the upper limit of normal.

ii. Myopathy

Myopathy (SRM 3) can be defined as muscle pain and weakness unrelated to trauma with CK elevation superior to 4 times the upper limit of normal, but inferior to 10 times, with a complete resolution on dechallenge (Alfirevic et al. 2014). Myopathy is also characterised by the presence of inflammation within the muscles (myositis). The incidence of myopathy in sedentary patients has been estimated to range between 1% to 10% of patients (Meador & Huey 2010). This occurrence can be aggravated by polymedication, or vigorous exercise. Indeed, in patient exercising, symptoms can occur in 25% of statin users (Thompson et al.

1997). In comparison, the incidence of these symptoms occur in 1% to 5% of the population who exercise but do not take statins (Alfirevic et al. 2014). In athletes who suffer from familial hypercholesterolemia, especially in sports with high risk of dehydration, or exercise-induced rhabdomyolysis, like marathon running, statin myopathy has been reported in up to 75% of patients (Sinzinger & O'Grady 2004). Severe myopathy (SRM 4) can be defined as muscle pain and weakness unrelated to trauma with CK elevation superior to 10 times the upper limit of normal, but inferior to 50 times, with a complete resolution on dechallenge. SRM 4 is estimated to happen in 0.11% of patients (Alfirevic et al. 2014).

iii. Rhabdomyolysis

Rhabdomyolysis (SRM 5) is the most severe and potentially life-threatening adverse effect of statins (Maghsoodi & Wierzbicki 2016). SRM 5 can be described as an elevation of CK elevation superior to 10 times the upper limit of normal with evidence of renal impairment, and muscle pain and weakness unrelated to trauma or an elevation of CK elevation superior to 50 times the upper limit of normal (Alfirevic et al. 2014). Rhabdomyolysis is characterised by muscle necrosis, with the release of myoglobin in the bloodstream, which can lead to renal failure and can proceed to disseminated intravascular coagulation, multiorgan failure, and even death (Maghsoodi & Wierzbicki 2016; Alfirevic et al. 2014). The incidence of this adverse event is low, and is estimated to take place in 2.0 cases / 10 000 person-years of treatment (Thompson et al. 2016).

iv. Statin-induced necrotising autoimmune myopathy

Statin-induced necrotising autoimmune myopathy (SINAM, or SRM 6) is characterised by the presence of autoantibodies directed against HMG-CoA reductase, the pharmacological target of statins. Statins block the activity, but also increase the production, of HMG-CoA reductase. Moreover, HMG-CoA reductase is also expressed on the cell surface of regenerating muscle fibres, leading to the degradation of muscles. SRM 6 is also characterised by an incomplete resolution on dechallenge. It is estimated that SINAM affects 1 on 100 000 patients (Thompson et al. 2016).

Statin Related Myotoxicity Classification	Phenotype	Definition	Incidence
SRM 0	Asymptomatic rise in plasmatic creatine kinase	No muscle symptoms CK elevation <4x ULN	1-25%
SRM 1	Tolerable myalgia	Muscle symptoms without CK elevation	0,3-33%
SRM 2	Intolerable myalgia	Muscle symptoms, CK <4x ULN, Complete resolution on dechallenge	
SRM 3	Myopathy	CK elevation >4x ULN <10x ULN ± muscle symptoms, complete resolution on dechallenge	Sedentary: 5-10% Exercising: 25% Athletes: 75%
SRM 4	Severe myopathy	CK elevation >10x ULN <50x ULN, Muscle symptoms, complete resolution on dechallenge	0,11%
SRM 5	Rhabdomyolysis	CK elevation >10x ULN with evidence of renal impairment + muscle symptoms or CK >50x ULN	2/10 000 person-years
SRM 6	Statin-induced necrotising autoimmune myopathy	HMGCR antibodies, HMGCR expression in muscle biopsy, incomplete resolution on dechallenge	1/100 000 patients

Table 6: Statin-related myotoxicity phenotype classification. Adapted from (Alfirevic et al. 2014; Meador & Huey 2010; Thompson et al. 1997; Sinzinger & O’Grady 2004; Thompson et al. 2016)

b) Statins and mitochondria

i. Ubiquinone synthesis

As stated previously, biosynthesis of CoQ₁₀ is dependent on mevalonate production within the cell. Hence, statins by inhibiting mevalonate synthesis also decrease the biosynthesis of ubiquinone. As a lipid-soluble component of virtually all cell membranes, it is an essential electron carrier in the mitochondrial respiratory chain and an important antioxidant in the mitochondrial inner membrane (Ernster & Dallner 1995). Indeed, ubiquinone constitutes the Q junction where the electrons converge from complex I and complex II. A decrease in CoQ₁₀ within muscles would hence induce an impaired mitochondrial function, and a decreased ATP production. In patients taking statins, a decrease of about 40% in the CoQ₁₀ plasmatic pool has been reported (Ghirlanda et al. 1993). There are publications in which the authors tried to rescue mitochondrial ubiquinone deficiency induced by statins with a supplementation of CoQ₁₀ in animals. For instance, Muraki et al. showed that the CoQ₁₀ supplementation at 10 mg/L via drinking water in mice is able to reverse the mitochondrial dysfunction induced by

atorvastatin (Muraki et al. 2012). However, in humans, it is established that statins decrease the serum CoQ₁₀ concentration (Banach, Serban, Ursoniu, et al. 2015), but the data regarding the effect of CoQ₁₀ in patients with statin-associated myopathy are currently inconsistent (Banach, Serban, Sahebkar, et al. 2015). The skeletal muscle CoQ₁₀ content appears to stay high enough in patients treated with statins to maintain the function of the electron transport chain. In addition, CoQ₁₀ and CoQ₉ concentrations in C₂C₁₂ myotubes exposed to 10 µM simvastatin were measured in a previous investigation (Mullen et al. 2010). In this investigation, cholesterol synthesis was blocked by simvastatin immediately, whereas the CoQ₁₀ and CoQ₉ contents were maintained over the entire observation period of 18h (indicating a long half-life of these compounds). Moreover, the authors observed a rapid decrease in the prenylation of Rap1 and Ras, two small GTPases involved in cell survival which are prenylated by geranylgeranylate. Thus, the cellular geranylgeranylate stores appear therefore to be much more volatile than the ones of CoQ₁₀. Finally, CoQ₁₀ is also an antioxidant, rendering it difficult to differentiate between an effect of CoQ₁₀ by its function in the respiratory chain or as an antioxidant.

ii. Mitochondrial respiratory chain inhibition

Statins have been shown to have a direct inhibitory effect on the mitochondrial respiratory chain *in vitro* and *in vivo*. On isolated mitochondria, lipophilic statins have been shown to decrease glutamate-driven state 3 mitochondrial respiration (Kaufmann et al. 2006). On permeabilised glycolytic human and rat skeletal muscles, simvastatin has been shown to acutely inhibit Complex I of the mitochondrial respiratory chain (Sirvent et al. 2005). Interestingly, these authors also observed that contrary to skeletal muscles, the heart was protected. In line with these results, treatment with statins has been shown to increase mitochondrial oxidative capacities in heart biopsies of patients, whereas the deltoid skeletal muscle, which was impaired (Boutbir, Charles, et al. 2012). These results were also observed in the heart and the plantaris of rats treated during two weeks with atorvastatin 10 mg.kg⁻¹.day⁻¹. Hence statins seem to have opposite effects in heart, and in skeletal muscles.

At the heart of these antagonistic effects, stand the mitochondrion and reactive oxygen species.

iii. Induction of Apoptosis

Statins are known to be toxic *in vitro* for many cell lines, and the induction of apoptotic pathways has been shown to be induced in human skeletal muscle cell lines (Sacher et al. 2005). Indeed, these authors observed an activation of caspases 3 and 9, and only a partial prevention while adding mevalonate. This induction of pro-apoptotic pathways has also been shown in primary human myotubes with simvastatin (Kwak et al. 2012). Kwak et al. showed that this apoptosis activation was linked to an increase of mitochondrial superoxide production. In addition, the induction of apoptosis by simvastatin has been shown in C₂C₁₂ myotubes, and in the gastrocnemius muscle of mice (Bonifacio et al. 2015). However, the induction of apoptosis by mitochondrial ROS remained to be proven. In addition, it has been shown that mitochondrial content is decreased in skeletal muscles of patients treated with statins (Stringer et al. 2013).

iv. Hormesis

As stated previously, statins can have opposite effects in heart and skeletal muscles and this effect is seemingly due to reactive oxygen species (Bouitbir, Charles, et al. 2012). In rat skeletal muscles, with a low mitochondrial content and antioxidant defences, statins induce a massive increase of oxidative stress via a direct inhibition of the mitochondrial respiratory chain, which leads to a decreased mitochondrial content. Interestingly, the treatment of rats with the antioxidant quercetin prevented partially the deleterious effects of statins. On the other hand, in the heart which has a high mitochondrial content and high antioxidant defences, statins increase mildly oxidative stress, which leads to the triggering of mitochondrial biogenesis pathways. This hormetic activation of mitochondrial biogenesis leads to an increased mitochondrial content, and increased antioxidant defences to counteract more efficiently the stress induced by statins.

E. Problematic

According to these observations, we clearly have two types of effects from HMG-CoA reductase inhibitors in muscles. Interestingly, the highly oxidative heart muscle is preserved from the deleterious effects of statins, thanks to mitochondrial hormesis. On the other hand, glycolytic skeletal muscles are impaired by statins via excessive mitochondrial ROS production. Statins have been shown to induce apoptosis *in vitro*, on various cell lines and on primary human myotubes. However, the link between excessive ROS production and oxidative stress, and the induction of apoptosis pathways is not clear, and is yet to be proven.

Surprisingly, contrary to glycolytic skeletal muscles, oxidative skeletal muscles seem to be protected from the deleterious effects of statins (Seachrist et al. 2005). In addition, fibre type (and thus mitochondrial content) has been shown to have an impact on apoptotic susceptibility (McMillan & Quadrilatero 2011). Exercise training, which is known to increase mitochondrial content in skeletal muscles, and hence change muscle phenotype, induces *in vitro* a higher tolerance to statins (Bouitbir, Daussin, et al. 2012). Indeed, when acutely challenged by atorvastatin, skeletal muscles from trained rats display a higher half-maximal inhibitory concentration (IC₅₀). Hence muscle phenotype appears to have a crucial importance in statin tolerance and the possible triggering of apoptotic pathways. However, a better characterisation of the effect of statins in glycolytic and oxidative skeletal muscles on oxidative stress and on the potential subsequent activation of apoptotic pathways, would enable us to get a better understanding of statin myotoxicity.

Interestingly, an increase of mitochondrial content in L₆ myoblasts has been shown to protect against both caspase-dependant and caspase-independent apoptosis (Dam et al. 2013). Hence we could hypothesise that an increase of the mitochondrial content *in vitro* could at least provide a partial protection against the deleterious effects of statins. In addition, a better understanding of mitochondrial adaptations to oxidoreductive stress would help us to get a better insight on the mode of action of HMG-CoA reductase inhibitors in skeletal muscles.

Indeed, although oxidative stress is a well-known phenomenon, the effects of reductive stress remains poorly understood. Diverse antioxidants have been shown to induce an increased reactive oxygen species production during reductive stress (Herbert 1996; Brewer et al. 2013; Zhang et al. 2012; Pimentel et al. 2012). However, the mechanism involved in this paradoxical effect remains unknown. These observations raise the following questions: what is the effect of reductive stress on mitochondria? And how could mitochondria defend themselves against it?

In the highly oxidative cardiac muscle, statin protect mitochondria by triggering mitochondrial hormesis, via PGC-1 β overexpression in both humans and rats (Bouitbir, Charles, et al. 2012). On the other hand, in glycolytic skeletal muscles, a repression of mitochondrial proliferation through both PGC-1 α and PGC-1 β can be observed. Although conventional wisdom attributes oxidative stress response to PGC-1 α in skeletal muscles, the function and regulation of PGC-1 β is still debated in the literature (Zechner et al. 2010; Rowe et al. 2013; Handschin & Spiegelman 2011). The role of PGC-1 β remains hence poorly understood, and potentially underestimated. Thus, not only mitochondrial content and antioxidant power, but also PGC-1 β driven adaptations appear to be key pillars in mitochondrial oxidative stress adaptations, and statin tolerance.

Altogether, these observations bring to light what will be the common thread running through this thesis: *mitochondrial content appears to be a determining factor in statin tolerance*.

This raises several questions:

- Is there a link in oxidative and glycolytic muscles between mitochondrial ROS production and the triggering of apoptotic pathways *in vivo* in response to statins?
- What is the effect of reductive stress on mitochondria, and how could mitochondria defend themselves against it? Could reductive stress trigger mitochondrial hormesis?
- Could an increase in mitochondrial content provide a protection against statin-induced apoptosis *in vitro*?

- If an increase in mitochondrial content is supposed to protect cells, what would be the consequences of an impairment in the mitochondrial biogenesis pathways? Would mice be more prone to develop statin myopathy?

Along this thesis, we will try to bring light on these unanswered interrogations.

— PART II —

RESULTS

*I searched everywhere for a proof of reality, when all the while I understood quite well that
the standard of reality had changed*

— Algernon Blackwood —

Article I

Statins Trigger Mitochondrial ROS-Induced Apoptosis in Glycolytic Skeletal Muscle

Also known as “The 8 reviewers paper”

Mitochondrial content appears to be a determining factor in statin tolerance.

Within this article, we will try to find an answer to the following question: Is there a link in oxidative and glycolytic muscles between mitochondrial ROS production and the triggering of apoptotic pathways *in vivo* in response to statins?

ORIGINAL RESEARCH COMMUNICATION

Statins Trigger Mitochondrial Reactive Oxygen Species-Induced Apoptosis in Glycolytic Skeletal Muscle

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Abstract

Aims: Although statins are the most widely used cholesterol-lowering agents, they are associated with a variety of muscle complaints. The goal of this study was to characterize the effects of statins on the mitochondrial apoptosis pathway induced by mitochondrial oxidative stress in skeletal muscle using human muscle biopsies as well as *in vivo* and *in vitro* models. **Results:** Statins increased mitochondrial H₂O₂ production, the Bax/Bcl-2 ratio, and TUNEL staining in deltoid biopsies of patients with statin-associated myopathy. Furthermore, atorvastatin treatment for 2 weeks at 10 mg/kg/day in rats increased H₂O₂ accumulation and mRNA levels and immunostaining of the Bax/Bcl-2 ratio, as well as TUNEL staining and caspase 3 cleavage in glycolytic (plantaris) skeletal muscle, but not in oxidative (soleus) skeletal muscle, which has a high antioxidative capacity. Atorvastatin also decreased the GSH/GSSG ratio, but only in glycolytic skeletal muscle. Cotreatment with the antioxidant, quercetin, at 25 mg/kg/day abolished these effects in plantaris. An *in vitro* study with L₆ myoblasts directly demonstrated the link between mitochondrial oxidative stress following atorvastatin exposure and activation of the mitochondrial apoptosis signaling pathway. **Innovation:** Treatment with atorvastatin is associated with mitochondrial oxidative stress, which activates apoptosis and contributes to myopathy. Glycolytic muscles are more sensitive to atorvastatin than oxidative muscles, which may be due to the higher antioxidative capacity in oxidative muscles. **Conclusion:** There is a link between statin-induced mitochondrial oxidative stress and activation of the mitochondrial apoptosis signaling pathway in glycolytic skeletal muscle, which may be associated with statin-associated myopathy. *Antioxid. Redox Signal.* 24, 84–98.

Introduction

3-HYDROXY-3-METHYLGLUTARYL COENZYME A (HMG-CoA) reductase inhibitors (statins) are the most widely used cholesterol-lowering agents for the prevention of obstructive cardiovascular events (13). In addition, statins have been shown to exhibit pleiotropic cellular effects and may have potential use in the treatment of many other conditions, including ventricular arrhythmias, peripheral arterial disease, idiopathic dilated cardiomyopathy, and/or cancer. The number of individuals using statins will continue to increase

due to the rising incidence of cardiovascular diseases and due to the ongoing research efforts on clinical use of statins.

Treatment with statins is relatively safe. However, myopathy is the most common adverse reaction, with symptoms mainly including fatigue, weakness, and pain. In some cases, the symptoms are associated with rhabdomyolysis, which is a life-threatening condition. These symptoms can reduce the quality of life, limit physical activity, and reduce medication compliance. Although the adverse effects of statin treatment are not well understood, mitochondrial dysfunction could play a central mechanistic role in statin-associated myopathy (6, 20).

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Innovation

The present study demonstrates that mitochondrial oxidative stress is a key element in the pathogenesis of statin-related myopathy. Statins induced excessive mitochondrial oxidative stress mainly in glycolytic skeletal muscles, which have a low antioxidative capacity. Cotreatment with mitochondria-specific antioxidants directly demonstrated the link between mitochondrial reactive oxygen species accumulation and activation of apoptosis in glycolytic skeletal muscle fibers. Since such findings were not observed in oxidative soleus muscle exposed to atorvastatin, the current study shows that activation of apoptosis by atorvastatin depends on the metabolic phenotype of muscle fibers.

The mitochondrion plays a central role in initiating the intrinsic pathway of apoptosis, which is a unique physiological process of cell death, and is essential for morphogenesis, tissue homeostasis, and host defense (12). When cytochrome c is released from mitochondria into the cytosol, it initiates the formation of an apoptosome in combination with other molecules, such as Apaf-1, adenosine triphosphate (ATP), and procaspases.

Statins have been reported to cause apoptosis in various cell lines (14). Furthermore, *in vitro* exposure of isolated rat skeletal muscle mitochondria to statins triggered a Ca^{2+} -induced opening of the mitochondrial permeability transition pore (mPTP) and the loss of the mitochondrial membrane potential ($\Delta\Psi_m$) (43). Elevated cytoplasmic calcium concentrations are associated with Bax translocation to the mitochondria, leading to the formation and opening of the mPTP, which eventually results in cytochrome c release and apoptosis (36, 42).

Statins block *de novo* synthesis of cholesterol by inhibiting the enzyme, HMG-CoA reductase. The product of this reaction, mevalonic acid, is also a precursor of isoprenoids, molecules that are required for prenylation and activation of small GTPases such as Ras. Signal transduction pathways involving Ras are important for cell survival and inhibition of their prenylation could be linked to the onset of apoptosis during statin exposure in several cell types (14).

The role of mitochondria concerning the mechanisms of statin-induced apoptosis is largely unknown. Previously, we showed that statin treatment induced mitochondrial dysfunction *via* an oxidative stress mechanism in skeletal muscle and that antioxidant treatment could prevent these deleterious effects (6). Because oxidative stress has been reported to trigger apoptosis by several mechanisms (46), we hypothesized that the increase in oxidative stress following statin-induced mitochondrial impairment could participate in activating the mitochondrial apoptosis signaling pathway.

Our present study provided *in vivo* and *in vitro* evidences that statins can trigger apoptosis in fast glycolytic skeletal muscle and that this mechanism is linked to an increase in mitochondrial reactive oxygen species (ROS). On the other hand, the slow oxidative soleus muscle, which has a high antioxidative capacity, was protected from this deleterious effect. Cotreatment with the antioxidant, quercetin, abolished statin-induced apoptosis in rats and a mitochondria-specific

antioxidant was protective against atorvastatin-associated toxicity in L_6 cells, indicating a relationship between mitochondrial ROS production and apoptosis.

Results*Oxidative stress and apoptosis were increased in the deltoids of patients with statin-induced myopathy*

We analyzed deltoid muscle biopsies of patients ($n=5$, M-STAT group) suffering from statin-induced myopathy, using age-matched control subjects ($n=5$, M-CONT group). Morphological observation after hematoxylin and eosin staining revealed the presence of myofibers with central nuclei in the deltoid muscle of patients under statin treatment, indicating that some fibers were in the process of de-/regeneration (Fig. 1A). Plasma creatine kinase activity, which is a sign of muscle damage, was 15-fold higher in patients treated with statins compared with controls ($p<0.01$; Fig. 1B). Mitochondrial H_2O_2 production was 10-fold higher in the M-STAT group compared with the M-CONT group with glutamate/malate as substrates for complex I and succinate as substrate for complex II ($p<0.01$ and $p<0.01$, respectively; Fig. 1C).

TUNEL staining data revealed that statin treatment enhanced significantly the percentage of myonuclei that were positive for DNA strand breaks (4.4% vs. 1.6% for M-STAT vs. M-CONT, respectively, $p<0.05$; Fig. 1D, E). To show the potential implication of the mitochondrial pathway of apoptosis in humans, we studied the gene expression of *Bax* and *Bcl-2* at the mRNA level in these patients. The sequences of the primers are listed in Table 2. The ratio between the proapoptotic *Bax* and the antiapoptotic *Bcl-2*, which is considered to be a trigger for apoptosis, increased three-fold in the M-STAT group compared with the M-CONT group ($p<0.05$; Fig. 1F). These data suggested that in patients with statin-associated myopathy, statins increased mitochondrial ROS production, which triggered the mitochondrial apoptosis signaling pathway.

Atorvastatin treatment impaired muscle structure and increased oxidative stress only in glycolytic skeletal muscle and cotreatment with quercetin prevented these effects in rats

We treated rats with atorvastatin for 2 weeks at 10 mg/kg/day. Cotreatment with the antioxidant, quercetin, was used to demonstrate a possible link between statin-associated cellular oxidative stress and triggering of the mitochondrial apoptotic pathway in two distinct skeletal muscle phenotypes (the glycolytic plantaris and the oxidative soleus muscle). Morphological observation of muscles after hematoxylin and eosin staining revealed the presence of myofibers with central nuclei, fibrosis, small rounded, and very big fibers, indicating phases of muscle de-/regeneration after atorvastatin treatment in the plantaris muscle (Fig. 2A). In comparison, the oxidative soleus muscle, which has a high antioxidative capacity, was protected from the deleterious effects of statins (Fig. 2B).

Cotreatment with quercetin prevented the plantaris muscle from the deleterious effects of atorvastatin, underscoring the importance of ROS. This cotreatment allowed this muscle to maintain the polygonal shape of fibers and the homogenous fiber size distribution. H_2O_2 production was increased in the

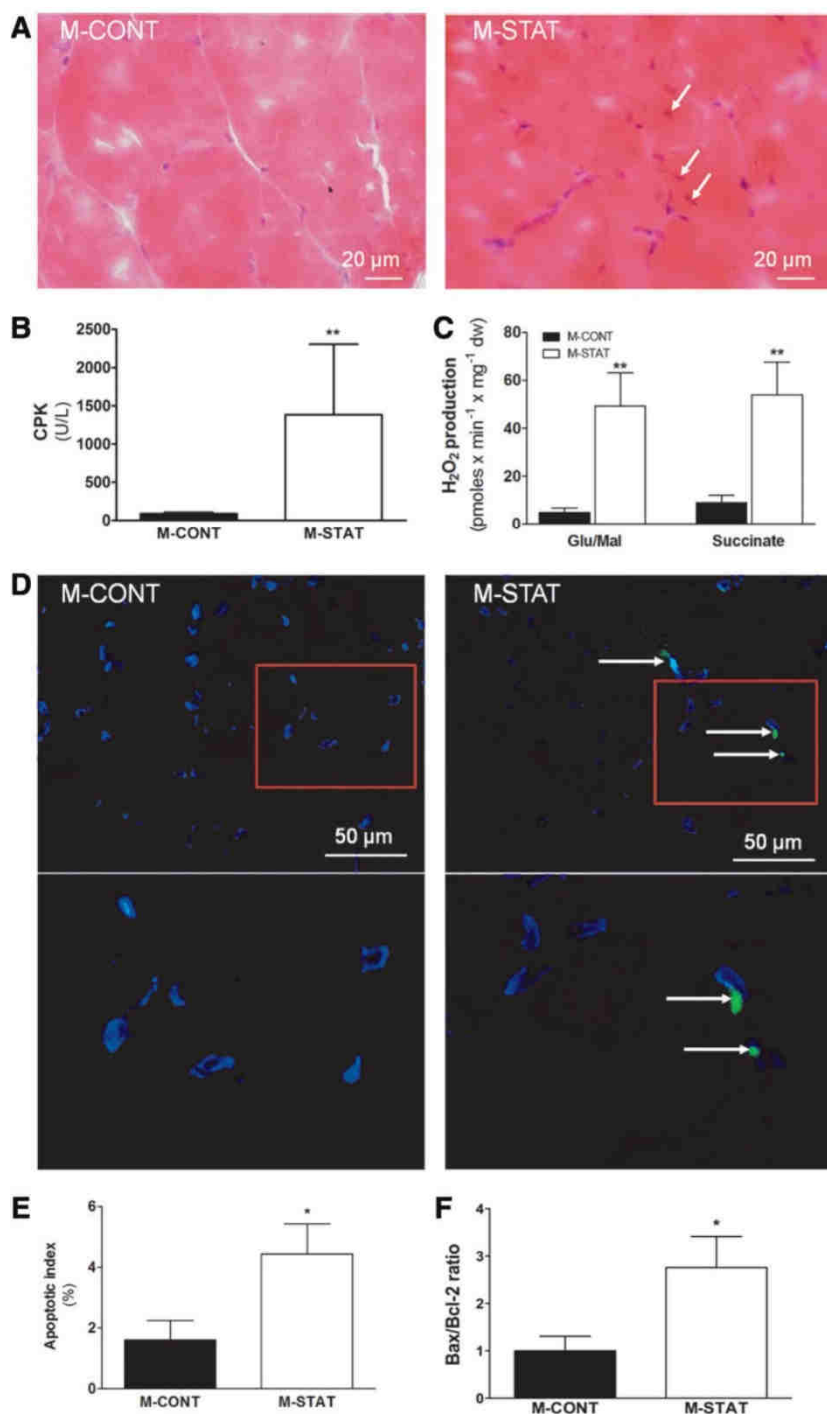


FIG. 1. Statin treatment enhanced apoptosis in deltoid muscles from patients treated with statins. (A) Hematoxylin and eosin staining of deltoid sections, *white arrows* indicate myofibers with central nuclei. (B) CPK measured in plasma from patients treated (M-STAT) or not (M-CONT) with statins. (C) Mitochondrial H₂O₂ measured in deltoid permeabilized fibers. (D) Nuclei were stained with DAPI shown in *blue*, and TUNEL nuclei were visualized with *green* fluorescein in both groups. Merged DAPI and TUNEL nuclei from different groups are shown, and *white arrows* indicate apoptotic nuclei. Lower picture corresponds to a magnification of the *red rectangle* area. (E) Apoptotic index calculated from TUNEL staining. (F) *Bax/Bcl-2* ratio, which triggers cell apoptosis. The results are expressed as the mean \pm SEM; $n = 5$; * $p < 0.05$; ** $p < 0.01$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

plantaris muscle of rats treated with atorvastatin compared with the control group ($p < 0.01$; Fig. 2C), whereas cotreatment with quercetin prevented this increase when compared with the control group. No significant differences were found in any group in the soleus. Total antioxidant capacity (TAC), which reflects the nonenzymatic antioxidant capacity, was lower in the plantaris compared with the

soleus (Fig. 2D). Atorvastatin treatment did not significantly decrease TAC compared with control in the plantaris, but cotreatment with quercetin increased TAC compared with atorvastatin alone ($p < 0.05$). In the soleus, there were no differences between the four groups. The total glutathione content was three-fold higher in control soleus compared with control plantaris muscle (Fig. 2E).

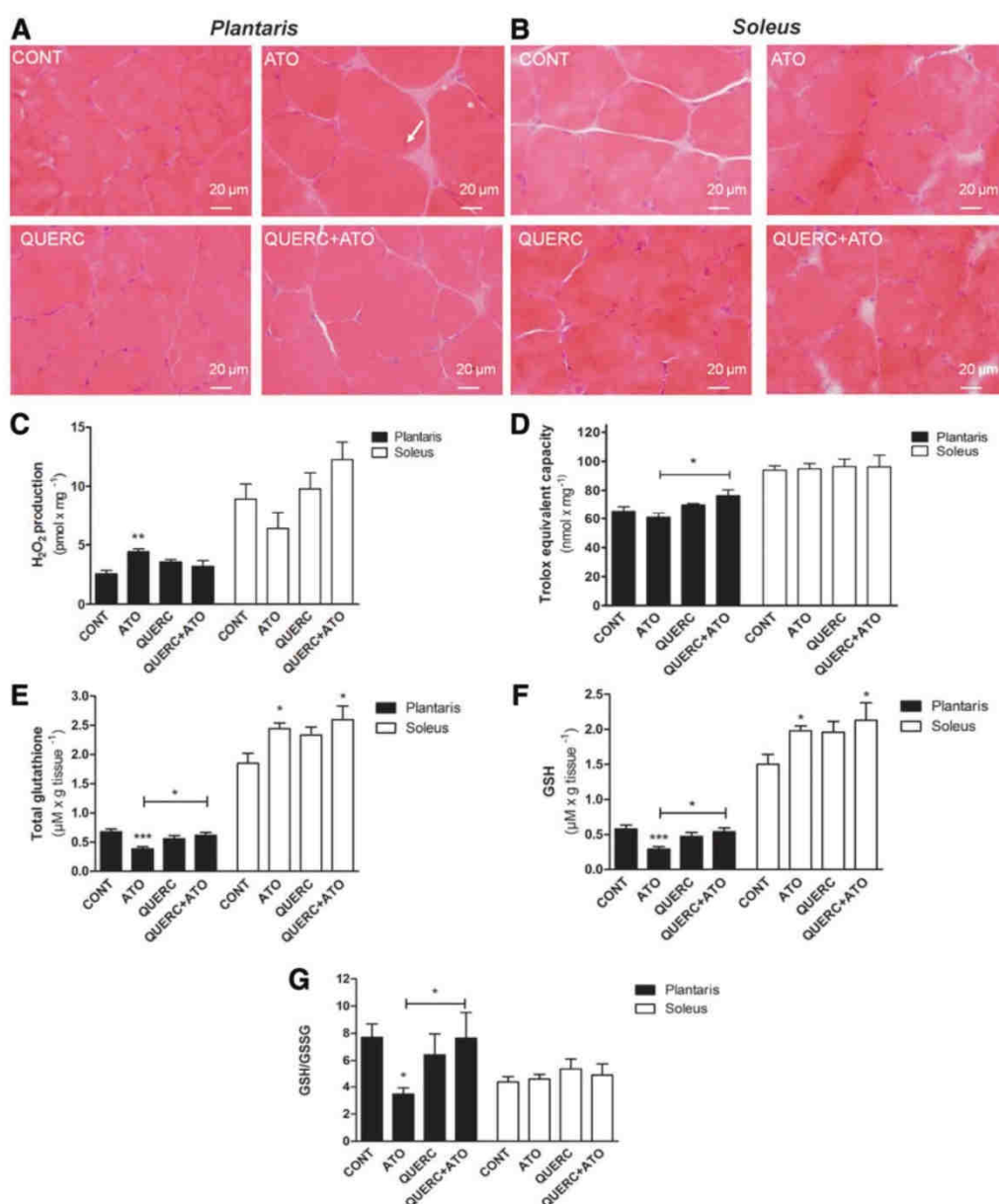


FIG. 2. Atorvastatin treatment in rats enhanced ROS and decreased the antioxidant capacities in the plantaris, whereas the cotreatment with quercetin abolished these effects. (A) Hematoxylin and eosin staining of plantaris muscle sections, *white arrows* indicate myofibers with central nuclei. (B) Hematoxylin and eosin staining of soleus muscle sections. (C) H₂O₂ production in plantaris and soleus muscles. (D) Total antioxidant capacity of plantaris and soleus muscles expressed as Trolox equivalent. (E) Total glutathione, (F) GSH measurements, and (G) the GSH/GSSG ratio in plantaris and soleus muscles. The results are expressed as the mean ± SEM; n=6–8; *p<0.05; ** p<0.01, ***p<0.001. ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Atorvastatin treatment decreased the reduced (GSH) and the total glutathione content in the plantaris muscle (p<0.001 for GSH and total glutathione; Fig. 2E, F). Cotreatment with quercetin prevented this drop in the GSH and total glutathione content (p<0.05 for total and reduced glutathione). In comparison, in the soleus muscle, ator-

vastatin treatment increased total glutathione and GSH (p<0.05 for total glutathione and GSH; Fig. 2E, F). The GSH/GSSG ratio, which is an indicator of oxidative stress, only decreased in the plantaris muscle of animals treated with atorvastatin (p<0.05; Fig. 2G). Again, cotreatment with quercetin prevented the drop in this ratio (Fig. 2G).

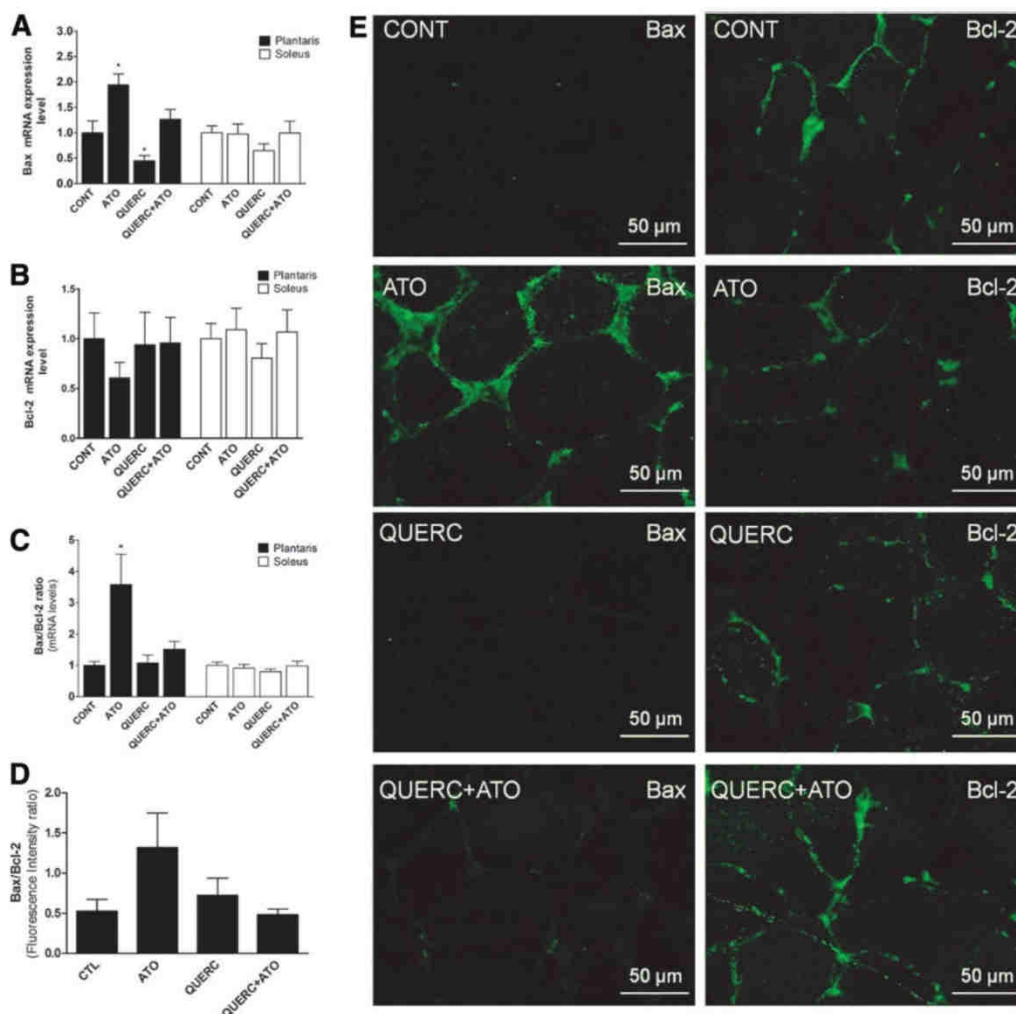


FIG. 3. Atorvastatin treatment increased the Bax/Bcl-2 ratio in the plantaris, but not in the soleus muscle, whereas cotreatment with quercetin abolished these effects. (A) *Bax* mRNA expression level. (B) *Bcl-2* mRNA expression level. (C) *Bax/Bcl-2* mRNA ratio in plantaris and soleus muscles from different groups. (D) Bax/Bcl-2 fluorescence intensity ratio of plantaris muscle immunostainings. (E) Bax and Bcl-2 immunostaining pictures in the plantaris muscle sections. The results are expressed as the mean \pm SEM; $n = 6-8$; $*p < 0.05$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Atorvastatin treatment increased apoptosis in glycolytic skeletal muscle of rats, whereas oxidative muscle was protected, and quercetin prevented atorvastatin-associated apoptosis in glycolytic muscle

The *Bax* mRNA expression was higher in the atorvastatin group compared with the control group in the plantaris ($p < 0.05$), but not in the soleus muscle (Fig. 3A). Treatment with quercetin alone decreased *Bax* mRNA expression compared with control in the plantaris ($p < 0.05$), whereas in rats treated with atorvastatin, cotreatment with quercetin partially prevented the atorvastatin-associated increase. *Bcl-2* mRNA expression was similar in all groups in plantaris and soleus muscles (Fig. 3B). Consequently, the *Bax/Bcl-2* ratio was higher in the plantaris of the atorvastatin group ($p < 0.05$; Fig. 3C), but not in soleus muscle. Cotreatment with quer-

etin partially prevented the atorvastatin-associated increase of the *Bax/Bcl-2* ratio in plantaris.

To confirm these results, we performed immunostainings of Bax and Bcl-2 in both muscles. Both Bax and Bcl-2 immunofluorescence levels were detectable in the plantaris muscle after atorvastatin treatment (Fig. 3D, E). Treatment with atorvastatin was associated with a 2.4-fold increase in the Bax/Bcl-2 ratio without reaching statistical significance due to a high variability ($p = 0.15$; Fig. 3D). Cotreatment with quercetin partially prevented this increase in the Bax/Bcl-2 ratio. Concerning soleus, no visible differences in the Bax/Bcl-2 expression ratio between atorvastatin-treated and control rats were observed (data not shown).

Western blot of caspase 3 (Fig. 4A-C) revealed a 4.5-fold increase in cleaved caspase 3 protein content under atorvastatin treatment in the plantaris, but not in the soleus ($p < 0.01$; Fig. 4B). Cotreatment with quercetin prevented caspase 3

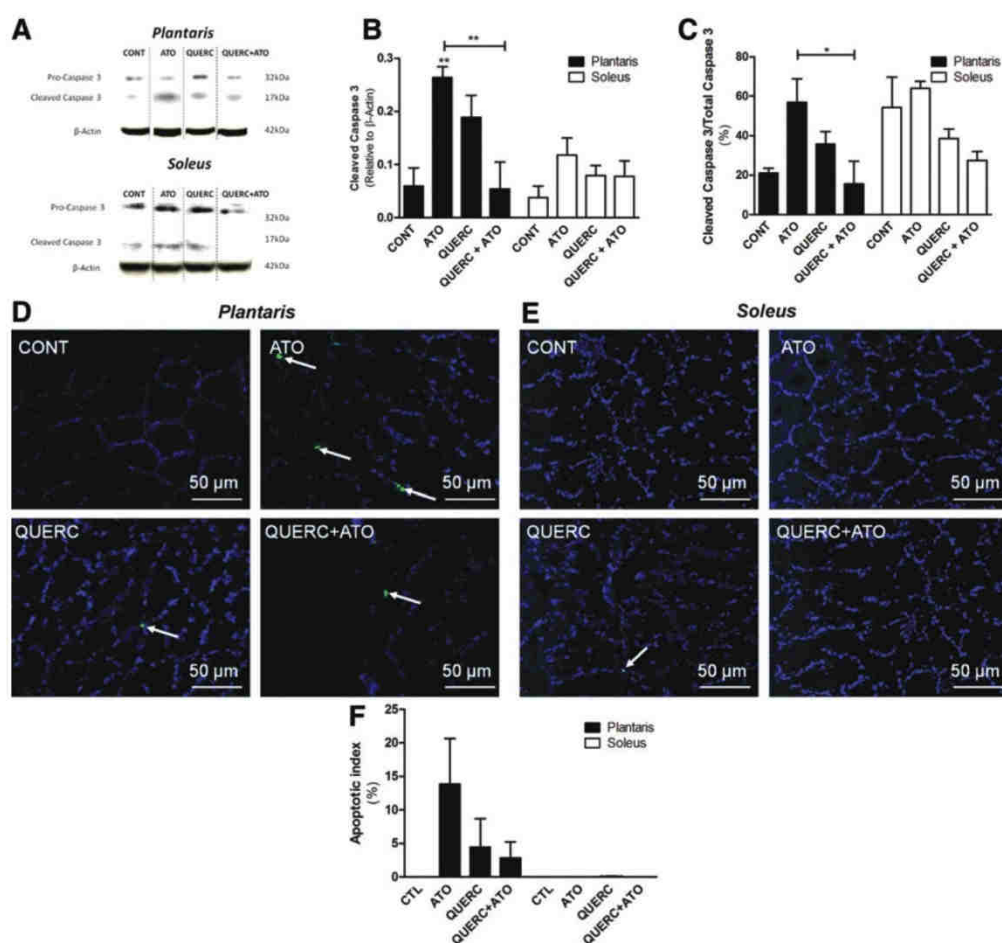


FIG. 4. Atorvastatin treatment induced apoptotic pathways only in the plantaris, but not in the soleus muscle, whereas the cotreatment with quercetin abolished these effects. (A) Immunoblots of procaspase 3 and cleaved caspase 3. **(B)** Cleaved caspase 3 quantification. **(C)** Cleaved caspase 3 expression relative to total caspase 3 expression. **(D–F)** Nuclei were stained with DAPI shown in blue, and TUNEL nuclei were visualized with green fluorescence in the plantaris muscle and in the soleus. Merged DAPI and TUNEL nuclei from different groups are shown, and white arrows indicate apoptotic nuclei. **(D)** Merged TUNEL images of plantaris muscle sections. **(E)** Merged TUNEL images of soleus muscle sections. **(F)** Apoptotic index calculated from TUNEL staining of the plantaris muscle. Values are expressed as mean \pm SEM, * $p < 0.05$; ** $p < 0.01$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

cleavage compared with atorvastatin alone ($p < 0.01$). Similar results were obtained for the ratio, cleaved caspase 3/total caspase 3 ($p < 0.05$; Fig. 4C). TUNEL staining data showed that the proportion of myonuclei positive for DNA strand breaks increased in the plantaris of the atorvastatin-treated group, but was similar in atorvastatin-treated and control rats concerning the soleus muscle (Fig. 4D–F). In the plantaris muscle, cotreatment with quercetin reduced the presence of myonuclei positive for DNA strand breaks compared with atorvastatin alone.

Acute atorvastatin exposure impaired mitochondrial respiration, increased mitochondrial ROS production, and triggered the mitochondrial apoptosis pathway in L₆ myoblasts

Maximal OXPHOS respiratory rates (C_{I+II}-linked state) and H₂O₂ production in permeabilized cells were studied

simultaneously after acute exposure to atorvastatin. Atorvastatin decreased C_{I+II}-linked state oxygen consumption in a concentration-dependent manner (Fig. 5A). The decrease in the C_{I+II}-linked state oxygen consumption became significant at 50 μ M ($p < 0.05$) and reached -42% at 100 μ M ($p < 0.001$). Figure 5B shows H₂O₂ production from permeabilized L₆ cells. Atorvastatin increased C_{I+II}-linked state H₂O₂ production in a concentration-dependent manner. The increase in C_{I+II}-linked state H₂O₂ production started to be significant at 10 μ M ($p < 0.01$) and reached +40% at 100 μ M ($p < 0.001$).

The free radical leak (FRL) also increased in a concentration-dependent manner regarding exposure to atorvastatin (Fig. 5C), reaching statistical significance at 100 μ M ($p < 0.01$). After 1 h of incubation with atorvastatin, the percentage of cells displaying caspase 3 and 7 activity was increased in a concentration-dependent manner, reaching statistical significance at 100 μ M ($p < 0.01$; Fig. 5D).

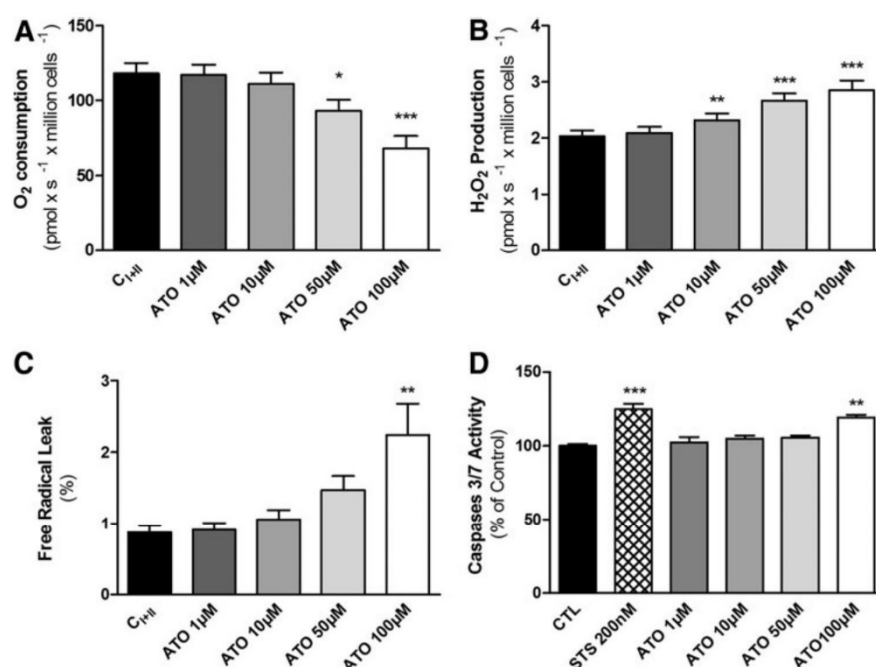


FIG. 5. Atorvastatin acutely impaired mitochondrial respiratory chain function and triggered apoptotic pathways in *L*₆ cells. Concentration-dependent effect of atorvastatin (A) on mitochondrial maximal OXPHOS respiratory rates (C_{I+II}) (*n*=4); (B) mitochondrial H₂O₂ production (*n*=4); and (C) on free radical leak (*n*=4). (D) Caspase 3/7 activity assay after 1 h of incubation with 1, 10, 50, and 100 μM atorvastatin or 200 nM staurosporine (*n*=4). Values are expressed as mean ± SEM, **p*<0.05; ***p*<0.01; ****p*<0.001. (A–C) Repeated measure ANOVA, followed by a Tukey post-test. (D) One-way ANOVA, followed by a Tukey post-test.

Atorvastatin treatment for 24 h triggered apoptotic pathways, whereas cotreatment with different antioxidants abolished these effects

Cytotoxicity (*i.e.*, release of adenylate kinase) increased in a concentration-dependent manner (Fig. 6A), reaching statistical significance at 50 μM atorvastatin (*p*<0.01). Coincubation with mevalonate completely prevented cytotoxicity in the presence of 50 μM, but only partially in the presence of 100 μM atorvastatin. The intracellular concentration of ATP was lowered by exposure to 100 μM atorvastatin for 24 h (*p*<0.001; Fig. 6B). Coincubation of cells with the antioxidant, quercetin 10 μM or MitoTEMPO 1 nM, partially prevented the reduction of the cellular ATP content following atorvastatin exposure (both *p*<0.05).

Mitochondrial superoxide production in myoblasts was increased by exposure to 100 μM atorvastatin for 24 h (*p*<0.01; Fig. 6C). Coincubation of cells with quercetin 10 μM or MitoTEMPO 1 nM prevented the atorvastatin-associated increase in superoxide anion production (*p*<0.05 or *p*<0.01, respectively). Similarly, caspase 3 and 7 activity was increased by 100 μM atorvastatin compared with the control (*p*<0.01; Fig. 6D) and coincubation with either 10 μM quercetin or 1 nM MitoTEMPO prevented this increase (*p*<0.05 for both antioxidants).

Discussion

In the current study, we showed that (i) ROS production and number of nuclei with DNA fragmentation were increased in deltoid muscle of patients with statin-induced myopathy compared with control subjects; (ii) in our experimental rat model, atorvastatin treatment increased ROS production, reduced antioxidative capacities, and triggered the mitochondrial apoptosis pathway in a fast glycolytic skeletal muscle, while a slow oxidative skeletal muscle was

resistant; (iii) cotreatment with the antioxidant, quercetin, prevented mitochondrial apoptosis in a glycolytic skeletal muscle of rats; and (iv) in an *L*₆ cell culture model, atorvastatin increased mitochondrial ROS production, which triggered the mitochondrial apoptosis pathway and led to cell death. These findings provide a new mechanism of how statins affect the integrity of skeletal muscle.

Statins induced oxidative stress and apoptosis in human deltoid muscle

Statins are associated with a number of adverse muscular effects, including a decline in skeletal muscle function and strength, as well as an increase in muscle fatigue, weakness, soreness, and cramps (19). The cellular mechanisms explaining these effects are numerous, and dysfunction at the level of mitochondria seems to be largely implicated as shown in our previous and other studies (6–8, 17, 40). In the present study, we showed that deltoid muscles from patients treated with statins exhibited an increase in mitochondrial ROS production, which is probably related to the inhibition of the mitochondrial electron transport chain. Indeed, we have already shown previously that statins inhibit mitochondrial respiration in human skeletal muscle (6) and isolated rat skeletal muscle mitochondria (17).

Moreover, deltoid muscle from patients treated with statins showed an increase in the Bax/Bcl-2 ratio and in fibers with DNA fragmentation. These findings suggested that statins increased mitochondrial ROS formation, which triggered apoptosis pathways in skeletal muscle. However, the number of patients studied was small and patients differed in terms of statin treatment (Table 1), precluding to establish a clear link between oxidative stress and apoptosis. Therefore, *in vivo* and *in vitro* experimental models were used to confirm these findings and to explore their mechanisms.

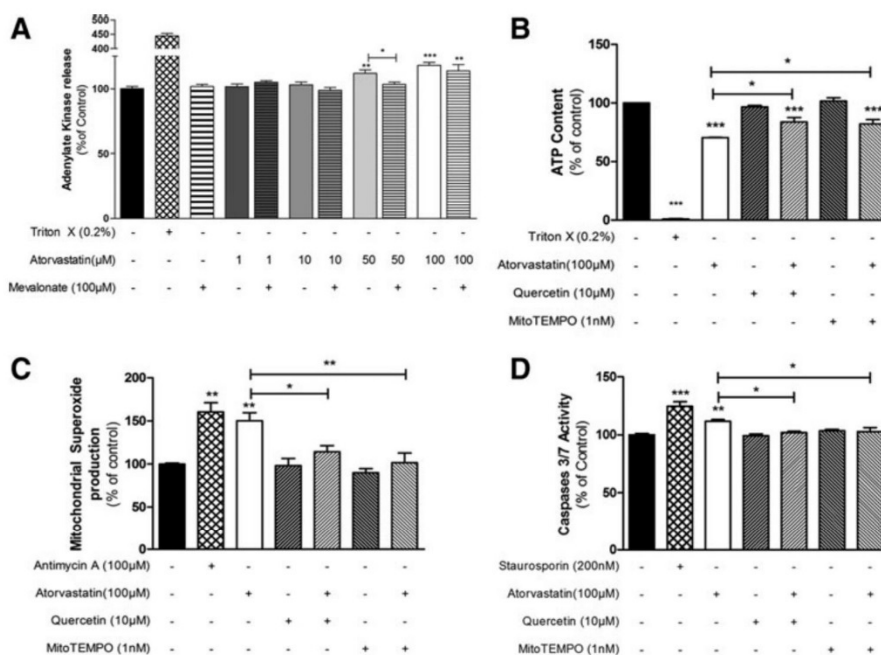


FIG. 6. After 24 h of incubation, atorvastatin induced cell death *via* a mitochondrial ROS-mediated apoptosis, and the antioxidants, quercetin and MitoTEMPO, prevented these effects in L₆ cells. (A) Cytotoxicity of statins in L₆ myoblasts determined by adenylate kinase release. L₆ cells were exposed to 1, 10, 50, or 100 μM of atorvastatin for 24 h, with or without mevalonate (100 μM). (B) Adenosine triphosphate content following exposure to 100 μM atorvastatin for 24 h with or without 10 μM quercetin or 1 nM MitoTEMPO. (C) Mitochondrial superoxide production following exposure to 100 μM atorvastatin for 24 h with or without 10 μM quercetin or 1 nM MitoTEMPO. (D) Caspase 3/7 activation following exposure to 100 μM atorvastatin for 24 h with or without 10 μM quercetin or 1 nM MitoTEMPO. Values are expressed as mean ± SEM, **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

In rats, atorvastatin induced oxidative stress only in the vulnerable glycolytic skeletal muscle, whereas the oxidative muscle was resistant

To understand better the effects of statins on oxidative stress and on the stimulation of apoptosis pathways, we treated rats with 10 mg/kg/day atorvastatin for 2 weeks. This dose was calculated as described by Reagan-Shaw *et al.* (35) and corresponds to ~1.6 mg/kg in humans, which is close to the maximally used dose in humans (80 mg/day).

We evaluated the effects of atorvastatin in two distinct muscle types: the oxidative soleus muscle, with a high mitochondrial content and high antioxidative capacity, and the glycolytic plantaris muscle, with a low mitochondrial content and low antioxidative capacity. We could show that atorvastatin treatment increased H₂O₂ production and decreased total and reduced glutathione content in the glycolytic muscle, leading to cellular oxidative stress. However, soleus muscle, with its high antioxidative capacity, was resistant to statin-induced oxidative stress.

Taking into account these findings, we cotreated rats with atorvastatin and the antioxidant, quercetin, to investigate the role of oxidative stress specifically in atorvastatin-associated myotoxicity. We could show that cotreatment with quercetin prevented ROS accumulation and glutathione depletion in the vulnerable glycolytic muscle. These data demonstrated that oxidative stress is a key feature in statin-associated myotoxicity.

In previous studies, we have shown that statin-associated ROS production in glycolytic skeletal muscle (7) or isolated muscle mitochondria (17) is due to inhibition of the mitochondrial electron transport chain. Similar findings have been reported in other *in vitro* models (37), and both complex I and complex III are potential sites of O₂⁻ production (6, 38). The function of the electron transport chain could also be impaired due to a lack of coenzyme Q (CoQ) 9 and 10, which transport electrons between complex I or II to complex III and have antioxidative properties (15). Statins inhibit the synthesis of farnesyl pyrophosphate, a substrate for the production of CoQ.

Due to the important role of CoQ in mitochondrial energy production and the importance of mitochondria in muscle energy metabolism, the hypothesis has been formulated that CoQ deficiency may be associated with statin-associated myopathy. In support of this hypothesis, Muraki *et al.* reported that CoQ10 supplementation *via* drinking water is able to prevent atorvastatin-associated mitochondrial dysfunction in mouse quadriceps (26). In humans, it is established that statins decrease the serum CoQ concentration (4), but the data regarding the effect of CoQ in patients with statin-associated myopathy are currently inconsistent (3). The skeletal muscle CoQ content appears to stay high enough in patients treated with statins to maintain the function of the electron transport chain.

Interestingly, in the current study, the oxidative skeletal muscle was protected from the adverse effects of statins, possibly due to its high antioxidative capacity. Similar to our

TABLE 1. HUMAN DELTOID MUSCLE BIOPSIES FROM PATIENTS

Group	Sex	Age (years)	Symptoms	EMG	Muscle pathology	Statin molecule, dosing, and treatment duration	Comedications	Diabetes	Cardiovascular disease
Patients with statin-induced myopathy (M-STAT)	F	58	Muscle pain and weakness	Myogenic features	N	Pravastatin 20 mg/d 6 months	Bisoprolol	No	Arterial hypertension
	M	82	Muscle pain and weakness	Myogenic features	Myopathic changes	Simvastatin 20 mg/d 24 months	Bisoprolol	Type II	Arterial hypertension
	F	66	Muscle pain and weakness	N	Myopathic changes	Rosuvastatin 10 mg/d 60 months	Propranolol	No	Arterial hypertension
	F	41	Muscle pain and weakness	N	N	Atorvastatin 20 mg/d 48 months	Furosemide Irbesartan Ramipril Insulin Infliximab	No	Arterial hypertension
M	53	Muscle pain and weakness	Myogenic features	Myopathic changes	Simvastatin 20 mg/d 48 months		Type I	No	
Mean		55.2±4.4							
Normal subjects (M-CONT)	M	69	NA	N	N	NA	Nicardipine	No	Arterial hypertension
	F	60	NA	N	N	NA	Ramipril Hydrochlorothiazide Urapidil	No	Arterial hypertension
	F	49	NA	N	N	NA	No	No	No
Mean	M	47	NA	N	N	NA	No	No	No
	M	46	NA	N	N	NA	No	No	No
P		60.0±6.8							
		ns							

EMG, electromyography; F, female; M, male; N, normal; NA, not applicable; Normal CK levels, <200 U/l; ns, not significant.

findings, soleus was also not affected in a study by Seachrist *et al.* (40). The total glutathione content was three-fold higher in the oxidative soleus compared with the glycolytic plantaris muscle of control rats, demonstrating the higher antioxidative capacity in the soleus muscle. Recently, we showed in cardiac muscle that atorvastatin acted through a mitohormesis mechanism by stimulating antioxidant capacity and mitochondrial biogenesis through ROS-induced PGC-1 expression (6). A similar mechanism may contribute to the observed resistance of the oxidative soleus muscle to statin-associated oxidative stress.

Rats treated with atorvastatin showed apoptosis only in the vulnerable glycolytic skeletal muscle, whereas the oxidative muscle was protected

Statins have been shown to induce apoptosis in skeletal myoblasts, myotubes, and differentiated primary human skeletal muscle cells, most often in a concentration-dependent manner (9, 16, 27, 36, 49). Nevertheless, it is currently not well explored whether activation of apoptosis is causally related to statin-associated myopathy. Seachrist *et al.* showed that cerivastatin induced muscle damage after 14 days of oral administration in female rats (40). In this study, protein levels of cleaved caspase 3 were determined 24 h after the last dose and did not differ from control rats. In comparison, in our study, we showed that atorvastatin induced apoptosis in glycolytic skeletal muscle fibers of rats treated for 2 weeks, as evidenced by an increase in the Bax/Bcl-2 mRNA ratio, increased expression of the proapoptotic Bax, TUNEL-positive nuclei, and an increased caspase 3 cleavage.

In support of our results, Bonifacio *et al.* also showed recently that in the glycolytic gastrocnemius muscle of mice treated with simvastatin for 3 weeks, cleavage of caspases 3 and 9 was increased (5). Moreover, our cell experiments showed that exposure to atorvastatin for 24 h increased the percentage of L₆ myoblasts with caspase 3 and/or 7 activity. The discrepancy between the findings of Seachrist *et al.* (40) and the current study regarding apoptosis in glycolytic skeletal muscle may be explained by the statin used (cerivastatin by Seachrist *et al.* and atorvastatin in the current study). Cerivastatin not only impairs the function of the electron transport chain but also uncouples oxidative phosphorylation (17), possibly depleting ATP in glycolytic skeletal muscle fibers to such an extent that necrosis, and not apoptosis, is favored.

In contrast to the plantaris muscle, apoptosis was not induced in the soleus muscle, which has a high antioxidative capacity. Recently, McMillian and Quadrilatero proposed that a muscle with more mitochondria may show different responses to a mitochondria-mediated apoptotic stimulus compared with a muscle with less mitochondria (24). Our results support this hypothesis. To our knowledge, this is the first study to show that the activation of the statin-induced apoptotic pathway can depend on the metabolic phenotype of skeletal muscles. In support of our findings, an increase in mitochondrial content provided protection against apoptosis induced by different stresses in cell cultures (10, 41).

Moreover, regular exercise training, which increases the mitochondrial content in skeletal muscle, has been associated with lower levels of DNA fragmentation and apoptosis (45, 47). In contrast, muscle inactivity, which typically leads to a

decreased mitochondrial content (48), is associated with increased apoptosis (21, 44). Thus, skeletal muscle with a high oxidative metabolic activity and a high mitochondrial content appears to be more resistant to apoptosis than inactive or nonoxidative muscle.

In L₆ cells, statins induced apoptosis through an increase of mitochondrial oxidative stress and these effects were partially mevalonate independent

Due to inhibition of the conversion of HMG-CoA to mevalonate, the synthesis of not only cholesterol but also of intermediates between HMG-CoA and cholesterol, such as farnesyl pyrophosphate, dolichol, and CoQ, is decreased (17). We demonstrated that 50 μ M atorvastatin was cytotoxic for L₆ myoblasts (*i.e.*, release of adenylate kinase) and that coincubation with 100 μ M mevalonate prevented cytotoxicity completely.

In previous investigations, this has also been shown for caspase 3 activation and apoptosis (36). These findings show that cytotoxicity of atorvastatin is related to the inhibition of the mevalonate biosynthesis pathway. However, in the presence of 100 μ M atorvastatin, 100 μ M mevalonate could not prevent atorvastatin-associated cytotoxicity completely, suggesting that both impaired prenylation of critical proteins and direct mitochondrial toxicity are responsible for atorvastatin-associated cytotoxicity. Acute direct mitochondrial toxicity of statins has been shown in the current (Fig. 5A) and in previous studies using isolated rat muscle mitochondria (17).

The activation of the apoptotic pathway can involve several factors, including oxidative stress (32). Enhanced production of ROS is associated with a proapoptotic shift of the expression pattern of Bcl-2 proteins, as evidenced by an increased Bax-to-Bcl-2 ratio (25, 31). Increased ROS production stimulates the proapoptotic factor Bax, resulting in the activation of the mitochondrial apoptotic pathway (2).

Sacher *et al.* reported that Bax is translocated to the mitochondria in response to statin treatment, which may trigger mitochondrial membrane permeability transition with release of cytochrome c and activation of the apoptosis signaling pathway (36). Kwak *et al.* demonstrated that human skeletal myotubes treated with simvastatin showed mitochondrial ROS accumulation and increased susceptibility of mitochondria for Ca²⁺-induced permeability transition pore opening (19). Permeability transition pore opening results in the collapse of $\Delta\Psi_m$ and in the release of cytochrome c.

To show the source and importance of ROS, we performed cell experiments. We incubated L₆ cells with atorvastatin (from 1 to 100 μ M) and showed that atorvastatin inhibited mitochondrial respiration and increased mitochondrial ROS accumulation. ROS such as H₂O₂ are reactive compounds, which can, for instance, cause DNA single-strand breaks with consecutive p53 activation (39) and activation of cell death pathways (23, 28).

To show directly the relationship between mitochondrial ROS accumulation and statin-associated toxicity, we performed coincubations with antioxidants. Coincubation with quercetin or the mitochondria-specific MitoTEMPO prevented cellular ATP depletion, ROS accumulation, and caspase activation in L₆ cells exposed to 100 μ M atorvastatin almost completely, demonstrating the link between ROS accumulation and cell damage. Quercetin is a flavonoid,

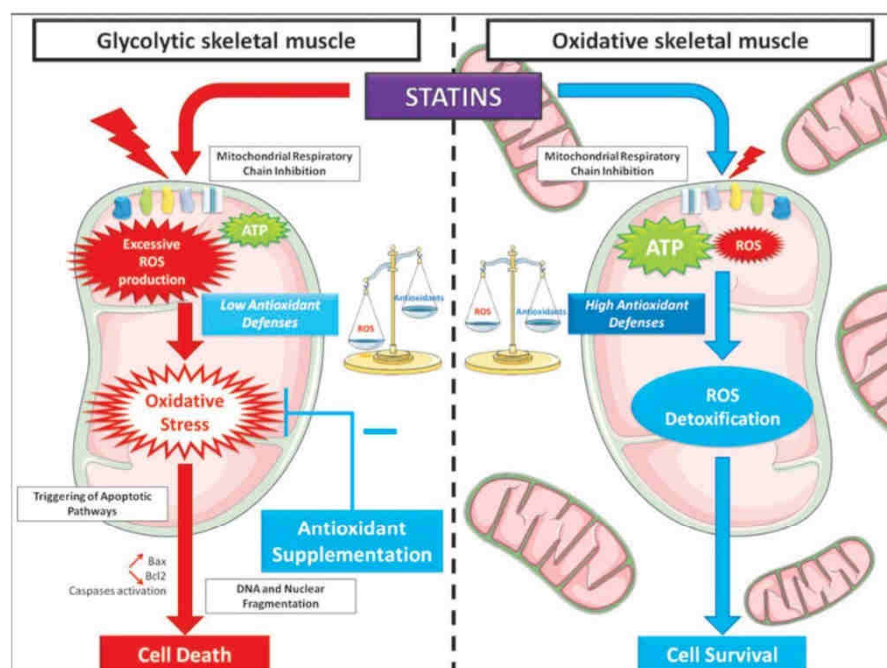


FIG. 7. Hypothesized scheme of statin-induced apoptosis in skeletal muscle. Statins affect ROS production and apoptosis differently in the glycolytic plantaris and in the oxidative soleus muscle. Oxidative muscles (e.g., soleus) have a high antioxidative capacity and can degrade ROS produced by statin-associated impairment of the function of the mitochondrial electron transport chain. In contrast, glycolytic muscles (e.g., plantaris) have a low antioxidative capacity, limiting their potential for metabolizing ROS. Mitochondrial ROS accumulation is associated with activation of apoptosis and cell death. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

which is not specific for mitochondria and has demonstrated antioxidative effects in several cell and animal models in a concentration-dependent manner (33). However, at concentrations higher than 10 μM , the concentration used in the current study, quercetin itself can be cytotoxic by inducing apoptosis (29).

In comparison, MitoTEMPO is specifically targeted to mitochondria (11), directly demonstrating the link between mitochondrial ROS production and cytotoxicity of atorvastatin. Although the atorvastatin concentrations used in our cell experiments were higher than the concentrations normally reached *in vivo* [which are below 5 μM (19)], our data are in agreement with the clinical findings in humans, showing that exposure is a strong risk factor for statin-associated myopathy (22, 34).

In conclusion, as illustrated in Figure 7, statins increased ROS production and induced apoptosis in the human deltoid

and glycolytic rat plantaris, but not in the oxidative rat soleus muscle, which has a higher antioxidative capacity than glycolytic muscles. Mitochondrial accumulation of ROS caused by statins is a consequence of direct mitochondrial damage as well as a lack of intermediates of cholesterol synthesis and is linked directly to apoptosis of glycolytic muscle fibers. Future research directions could include the development and clinical assessment of mitochondria-specific ROS scavengers to prevent and/or treat myopathy in patients treated with statins.

Materials and Methods

Human deltoid biopsies

We studied biopsies from the deltoid muscles of five patients with statin-induced myopathy; that is, patients who

TABLE 2. PRIMER SEQUENCES USED FOR QUANTITATIVE REAL-TIME PCR AMPLIFICATION

Target gene	Organism	Forward primer 5'→3'	Accession number
		Reverse primer 5'→3'	
<i>Bax</i>	Rat	GCTGGACACTGGACTTCCTC GAGGACTCCAGCCACAAAGA	NC 005100
<i>Bcl-2</i>	Rat	CGACTTTGCAGAGATGTCCA CCTGAAGAGTTCTCTCCACCA	NC 005112
<i>Beta actin</i>	Rat	TTGCTGACAGGATGCAGAAG CAGTGAGGCCAGGATAGAGC	BC 166732
<i>Bax</i>	Human	GGGGACGAAGTGGACAGTAA CAGTTGAAGTTGCCGTCAGA	NM 138761
<i>Bcl-2</i>	Human	TTGTTCAAACGGGATTCACA GGCTGGGCACATTTACTGTT	NM 000633
<i>GCB</i>	Human	TGGGCATTAAGGGACAGAG GGGAAAGTGAGTCACCCAAA	M 19285

developed muscle weakness and/or rhabdomyolysis while being treated with a statin, in whom muscle weakness and rhabdomyolysis regressed after statin treatment discontinuation and in whom other causes of myopathy were excluded. Muscle biopsy parameters were compared with those of five healthy volunteer subjects. All subjects signed a consent form approved by the institutional review board of the New Civil Hospital of Strasbourg.

Animal experiments

Experiments were performed on adult male Wistar rats (Dépre) weighing ≈ 250 – 300 g (6 weeks old). The rats were housed in a neutral temperature environment ($22^\circ\text{C} \pm 2^\circ\text{C}$) on a 12:12-h photoperiod and were provided food and water *ad libitum*. All experiments were performed in agreement with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by our local ethics committee (AL/02/11/05/08). Atorvastatin (Tahor[®]) was generously provided by Pfizer, and quercetin was kindly provided by C. Damgé and D. Betbeder (Lille, France).

After 1 week of acclimation, 32 rats were randomly divided into four groups as follows: (i) animals treated with water (CONT, $n=8$); (ii) animals treated with atorvastatin (ATO, $n=8$, 10 mg/kg/day); (iii) animals treated with quercetin (QUERC, $n=8$, 25 mg/kg/day); and (iv) animals treated with atorvastatin and quercetin (QUERC + ATO; $n=8$; 10 mg/kg/day and 25 mg/kg/day, respectively). Atorvastatin and quercetin were both administered by oral gavage; atorvastatin was administered for 2 weeks and treatment with quercetin was started 5 days before and continued during treatment with atorvastatin.

Tissue processing

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). The oxidative soleus muscle and the glycolytic superficial part of the plantaris muscle were excised and cleaned of adipose and connective tissues. A part of each muscle tissue was used freshly for assessing H_2O_2 production. Another part was immediately frozen in isopentane, cooled by liquid nitrogen, and stored for later analysis.

Mitochondrial H_2O_2 production in permeabilized fibers

After dissection, fiber bundles were permeabilized by gentle agitation for 30 min in ice-cold buffer solution supplemented with 50 $\mu\text{g}/\text{ml}$ saponin. Then, fibers were placed in ice-cold buffer Z containing 110 mM K-methane sulfonate, 35 mM KCl, 1 mM EGTA, 5 mM K_2HPO_4 , 3 mM MgCl_2 , 6 mM H_2O , 0.05 mM glutamate, and 0.02 mM malate with 0.5 mg/ml BSA (pH 7.1, 295 mOsmol/kg H_2O). Permeabilized fibers remained in buffer Z on a rotator at 4°C until analysis without any deterioration in mitochondrial function.

H_2O_2 production was measured with Amplex Red (Invitrogen), which reacts with H_2O_2 in a 1:1 stoichiometry catalyzed by HRP (horseradish peroxidase; Invitrogen) to yield the fluorescent compound, resorufin, and a molar equivalent of O_2 (1, 30). Resorufin has excitation and emission wavelengths of 563 and 587 nm, respectively, and is extremely stable once formed. Fluorescence was measured

continuously with a Fluoromax 3 (Jobin Yvon) spectrofluorometer with temperature control and magnetic stirring. After a baseline (reactants only) was established, the reaction was initiated by adding a permeabilized fiber bundle to 600 μL of buffer Z. Buffer Z contained 5 mM Amplex Red and 0.5 U/ml HRP. After 5 min, we added 5 mM glutamate and 2 mM malate as substrates for complex I and 10 mM succinate for complex II at 37°C . At the end of each experiment, the fibers were harvested and dried for 15 min at 150°C . The results are reported in $\text{pmol} \times \text{min}^{-1} \times \text{mg}^{-1}$ dry weight.

Western blot of caspase 3

Approximately 20 mg of rat skeletal muscle (plantaris and soleus) was homogenized with a microdismembrator for 1 min at 2000 rpm (Sartorius Stedim Biotech). Muscle homogenates were then lysed on ice for 15 min with 100 μl (per mg tissue) of RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, and Complete Mini protease inhibitor [Roche]). After lysis, the mixture was vortexed and centrifuged for 10 min at 4°C at 10,000 rpm. The supernatant was collected and the protein concentration determined using the Pierce BCA protein assay kit. For each sample, 60 μg of protein was separated on a NuPAGE 4–12% Bis-Tris gel (Life technologies).

The antibody against caspase 3 was used at a dilution of 1:1000 (Rabbit polyclonal anti-active + pro caspase 3 antibody [ab13847] from Abcam PLC). Peroxidase-labeled goat anti-rabbit IgG (1:2000 dilution; sc-2004, Santa Cruz Biotechnology) in combination with a chemiluminescent substrate (Clarity Western ECL substrate; Bio-Rad Laboratories) was used for the analysis. Quantification was performed using the ImageJ software (National Institutes of Health). Images were modified with ImageJ to remove background.

L_6 cells

L_6 rat myoblasts were grown in Dulbecco's modified Eagle's medium (Low glucose, Milerium; VWR International) supplemented with 20% fetal calf serum and 1% antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin; Gibco) at 37°C under a humidified 5% CO_2 atmosphere. L_6 cells were obtained from the American Type Culture Collection (ATCC).

Simultaneous high-resolution measurement of mitochondrial respiration and hydrogen peroxide production in L_6 cells

Mitochondrial respiration was studied simultaneously with H_2O_2 production in digitonin-skinned cells to keep mitochondria in their architectural environment. The method was adapted from Krumschnabel *et al.* (18). The analysis took place in a thermostated oxygraph chamber at 37°C with continuous stirring (Oxygraph-2k; Oroboros instruments). Mitochondrial H_2O_2 production was measured with the Amplex Red reagent (Invitrogen), which reacted with H_2O_2 in a 1:1 stoichiometry catalyzed by HRP (Fluka Biochemika) to yield the fluorescent compound, resorufin, and molar equivalent O_2 .

Briefly, $\sim 1,500,000$ cells were placed in Mir05Cr medium (110 mM sucrose, 60 mM K^+ -lactobionate, 0.5 mM EGTA, 3 mM MgCl_2 , 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES adjusted to pH 7.1 with KOH at 30°C , 20 mM creatine, and 1g/l BSA essentially fatty acid free) containing

HRP (1 U/ml) and Amplex Red (10 μ M) in the oxygraph chamber and were permeabilized with digitonin (2 μ M). Maximal OXPHOS respiration rate (C_{I+II} -linked state) was measured in the presence of saturating amounts of glutamate (10 mM), malate (2 mM), ADP (2.5 mM), and succinate (10 mM) as substrates. The C_{I+II} -linked state characterizes the electron flow through complexes I, II, III, and IV. For the acute effects experiments, atorvastatin (1, 10, 50, and 100 μ M) was directly injected into the oxygraph chamber after the C_{I+II} -linked state. Results are expressed in $\text{pmol} \times \text{s}^{-1} \times 10^{-6}$ cells.

Mitochondrial FRL

H_2O_2 production and O_2 consumption were measured simultaneously in the same sample under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence, which reduce O_2 to ROS in the respiratory chain (the percentage of FRL) instead of reaching cytochrome oxidase to reduce O_2 to water (1). Because two electrons are needed to reduce 1 mol of O_2 to H_2O_2 , whereas four electrons are transferred in the reduction of 1 mol of O_2 to water, the percent of FRL was calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption, and then the result was multiplied by 100.

Mitochondrial superoxide production

Mitochondrial superoxide production was measured with MitoSOXTM Red mitochondrial superoxide indicator for live cell imaging (Molecular Probes; Life technologies). L₆ myoblasts were incubated for 24 h with 100 μ M atorvastatin, with or without antioxidants (quercetin 10 μ M, or MitoTEMPO 1 nM [Sigma]). Incubation with antimycin A 100 μ M for 30 min was used as a positive control. Cells were incubated with 2.5 μ M MitoSOX Red and different atorvastatin concentrations at 37°C for 10 min, and fluorescence was measured with a Tecan M200 Pro Infinity plate reader. Excitation was set at 510 nm and emission at 580 nm. Each bar represents the mean of four independent experiments carried out in triplicate.

Statistical analyses

Data are presented as the mean \pm SEM. Statistical analyses were performed using Student's *t*-test, one-way ANOVA, or two-way ANOVA, followed by a Tukey post-test (GraphPad Prism 5; Graph Pad Software, Inc.). Statistical significance is displayed as **p* < 0.05; ***p* < 0.01, and ****p* < 0.001.

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Abbreviations Used

$\Delta\Psi_m$ = mitochondrial membrane potential
 AK = adenylate kinase
 Apaf-1 = apoptotic peptidase activating factor 1
 ATP = adenosine triphosphate
 CoQ = coenzyme Q
 CPK = creatine phosphokinase
 CTF = corrected total fluorescence (CTF)
 DTNB = 5,5'-dithio-bis-(2-nitrobenzoic acid)
 EDTA = ethylenediaminetetraacetic acid
 FRL = free radical leak
 GSH = reduced glutathione
 GSSG = oxidized glutathione
 H₂O₂ = hydrogen peroxide
 HDL = high-density lipoprotein
 HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A
 HRP = horseradish peroxidase
 mPTP = mitochondrial permeability transition pore
 PCR = polymerase chain reaction
 PBS = phosphate-buffered saline
 ROS = reactive oxygen species
 TAC = total antioxidant capacity

Supplementary Data

Supplementary Methods

Blood parameters

Blood was collected by cardiac puncture immediately before death in ethylenediaminetetraacetic acid (EDTA)-rinsed tubes, and then centrifuged at 3400 rpm for 15 min at 4°C. The plasma was separated and stored at -20°C until analysis. The analysis of plasma biochemical parameters was performed on randomly selected plasma samples. Total cholesterol and high-density lipoprotein (HDL) cholesterol were analyzed by standard spectrophotometric analysis using a diagnostic kit (Biomerieux), and creatine kinase was analyzed using a diagnostic kit (Bioassay System).

Hematoxylin and eosin (H&E) staining

H&E staining was performed on frozen sections of human deltoid muscle and rat plantaris and soleus muscles. H&E staining photographs were captured on an Olympus IX83 microscope (60× objective; Olympus). Evaluation and interpretation of the results were realized according to the MDC1A_M.1.2.004 SOP.

Measurement of GSH and GSSG

Reduced (GSH) and oxidized glutathione (GSSG) were measured in different tissues according to the Akerboom and Sies method by monitoring the reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoate by GSH at 412 nm (1). Fifty milligrams of skeletal muscle was homogenized with a microdismembrator for 1 min at 2000 rpm (Sartorius Stedim Biotech). To determine the total glutathione content, tissue samples were mixed with 400 μ l cold 5% 5-sulfosalicylic acid. After a 10-min incubation on ice, the mixture was centrifuged at 4000 rpm for 10 min at 4°C to remove proteins. The samples were then diluted (1/10) in 0.1 M cold MOPS buffer and thereafter diluted one more time (1/2 to 1/10) in MOPS at room temperature. The diluted sample was added to a mixture of 10 μ l glutathione reductase (24 units/ml) and 100 μ l MOPS (0.1 M) containing 1 mM EDTA, 0.3 mM NADPH, and 0.22 mM DTNB. The absorbance was continuously monitored for 1 min as an index of DTNB reduction by the GSH and GSSG contained in the sample. The amount of GSSG was determined by calibration curves under the same experimental conditions as the total glutathione assay, except after inhibiting GSH with 2-vinyl pyridine for 1 h at 4°C. The GSH level was calculated by subtracting the GSSG level from the total glutathione level to establish the ratio, GSH/GSSG.

Total antioxidant capacity

Muscle total antioxidant capacity was determined using the Total Antioxidant Capacity Assay kit (colorimetric) supplied by Abcam, according to the manufacturer's instructions. Briefly, 10 mg of skeletal muscle was homogenized with a microdismembrator for 1 min at 2000 rpm (Sartorius Stedim Biotech). To determine the total antioxidant capacity, tissue samples were mixed with 500 μ l ice-cold

phosphate-buffered saline (PBS) and sonicated for 5 min. The mixture was then centrifuged at 13,200 rpm for 5 min at 4°C and supernatant was collected. The samples were then diluted (1/10) in ice-cold PBS, and 100 μ l of the mixture was added to 100 μ l of Cu²⁺ working solution. Samples were then incubated for 1.5 h on an orbital shaker protected from light, and optical density was measured at 570 nm using a Tecan M200 Pro Infinity plate reader. A standard curve was realized using the antioxidant Trolox. Results were then expressed as trolox equivalent capacity (nmol) per mg of muscle.

Hydrogen peroxide assay

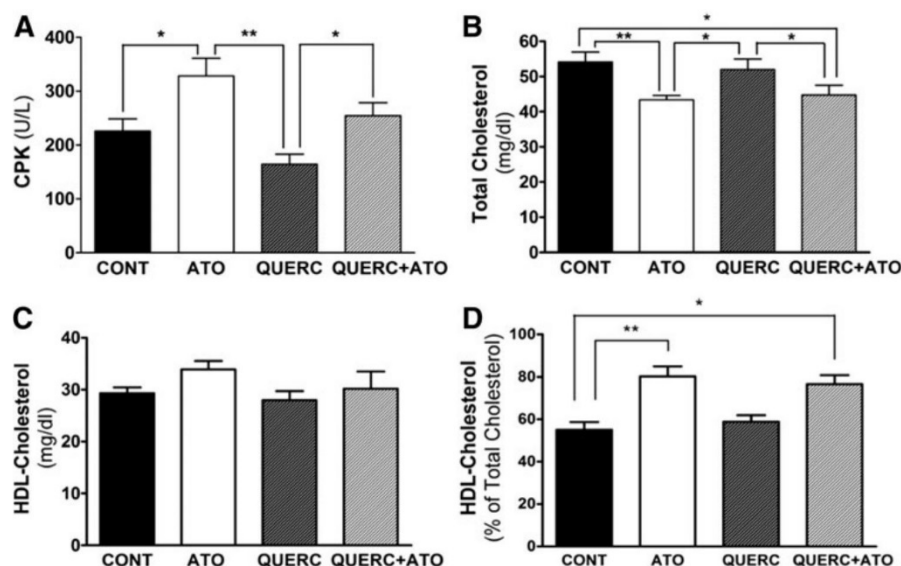
Hydrogen peroxide production was determined using the Hydrogen peroxide assay kit supplied by Abcam. Approximately 10 mg of skeletal muscle was homogenized with a microdismembrator for 1 min at 2000 rpm (Sartorius Stedim Biotech), and H₂O₂ production was determined according to the manufacturer's instructions. In the presence of horseradish peroxidase, the OxiRed Probe reacts with H₂O₂ to produce fluorescence. Fluorescence was measured (Ex/Em = 535/587 nm) using a Tecan M200 Pro Infinity plate reader. Results are expressed as pmol H₂O₂/mg of muscle.

TUNEL assessment of apoptosis

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining of myonuclei positive for DNA strand breaks was determined using a fluorescence detection kit (Calbiochem) and fluorescence microscopy. Cross sections (10 μ m) of muscles cut with a cryostat microtome were fixed with 4% paraformaldehyde for 15 min, and then the fixed sections were permeabilized with 2 mg/ml proteinase K. The TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-dUTP was added to the sections in 60 μ l drops and incubated for 60 min at 37°C in a humidified chamber in the dark. The sections were rinsed thrice in PBS for 1 min each. Following embedding, the sections were visualized with an epifluorescence microscope (Nikon Eclipse E800; 40× epifluorescence objective). After acquisition, background noise was removed using the ImageJ software (National Institutes of Health). Apoptotic index was calculated by counting the number of TUNEL-stained nuclei divided by the total number of nuclei multiplied by 100.

Immunostaining of Bax and Bcl-2

Cross sections (10 μ m) of muscles cut with a cryostat microtome were fixed with -20°C acetone for 10 min. Nonspecific binding was blocked by preincubation with 3% BSA in PBS for 30 min at room temperature. The primary antibodies were diluted 1:100 for Bax and Bcl-2 (Genetex) and were incubated with sections overnight at 4°C. The secondary antibodies coupled with Alexa Fluor were incubated with the sections at a dilution of 1:500 for 1 h at room temperature. The sections were rinsed thrice in PBS for 1 min each. Following embedding, the sections were visualized with an epifluorescence microscope (Nikon Eclipse E800;



SUPPLEMENTARY FIG. S1. Atorvastatin treatment at 10 mg/kg/day for two weeks increased CPK and decreased total cholesterol in rats. (A) CPK, (B) total cholesterol, (C) HDL cholesterol and (D) the proportion of HDL-cholesterol expressed in percentage of the total cholesterol measured in rats treated or not with atorvastatin. Values represent the mean \pm SEM; n = 8; * p < 0.05; ** p < 0.01.

40 \times epifluorescence objective), and the emission signal was recorded with a Zeiss 573–637 nm filter. Corrected total fluorescence (CTF) was determined using ImageJ as follows: CTF = integrated density – (mean fluorescence of background reading \times area). After acquisition, background noise was removed using the ImageJ software (National Institutes of Health).

Quantitative real-time polymerase chain reaction

Total RNA was obtained from muscles using Trizol reagent™ (Invitrogen Life Technologies) as previously described (3) and following the manufacturer’s instructions. RNA was stored at -70°C until the reverse transcription reaction was performed. cDNA was synthesized from total RNA with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. To perform the real-time polymerase chain reaction (RT-PCR), 2.5 μl of cDNA was used in a final volume of 8 μl , containing 10 μM of each primer (sense and antisense), SYBR green (Invitrogen Life Technologies) as the fluorescent dye, and H_2O . The real-time PCR measurement of individual cDNAs was performed in triplicate using SYBR green dye to measure duplex DNA formation with the Light-Cycler System (Roche Diagnostics). The sequences of the primers were designed using information contained in the public database GenBank of the National Center for Biotechnology Information. The sequences of the primer sets used are listed in Table 2. The quantification of gene expression was conducted by the method described by (1), using the glucocerebrosidase gene as the inner control, which is the most stable gene for RT-PCR measurements in muscles. The amplification efficiency of each sample was calculated as described by (2).

Cytotoxicity assay

In vitro cytotoxicity was assessed by the release of adenylate kinase (AK), which results from the loss of cell membrane integrity. AK was quantified using the ToxiLight

assay kit supplied from Lonza, according to the manufacturer’s instructions. AK was measured after 24 h of incubation with atorvastatin (1, 10, 50, and 100 μM) with or without mevalonate 100 μM . Triton X 2% was used as a positive control.

Adenosine triphosphate measurement

The cellular adenosine triphosphate content was determined using the CellTiter Glo assay kit from Promega following the manufacturer’s instructions. L_6 myoblasts were incubated for 24 h with atorvastatin 100 μM , with or without antioxidants: quercetin 10 μM or Mito-TEMPO 1 nM. Incubation with Triton X 2% was used as a negative control. Briefly, 100 μl assay buffer was added to each 96-well containing 100 μl culture medium. After incubation in the dark for 30 min, luminescence was measured using a Tecan M200 Pro Infinity plate reader. Each bar represents the mean of five independent experiments carried out in triplicate.

Apoptosis measurement assays

Caspase activation was determined using the Caspase-Glo 3/7 assay kit from Promega following the manufacturer’s instructions. For the acute effects of statins, cells were incubated for 1 h with atorvastatin (1, 10, 50, and 100 μM). For the 24-h experiments, cells were incubated with atorvastatin 100 μM , with or without antioxidants: quercetin 10 μM or MitoTEMPO 1 nM. For both acute and 24-h experiments, 1 h of incubation with staurosporine 200 nM was used as a positive control. Luminescence was measured using a Tecan M200 Pro Infinity plate reader.

Supplementary Results

Atorvastatin treatment increased plasma creatine kinase and decreased total cholesterol in rats

After 2 weeks of treatment, plasma CK increased by 45% in the ATO group compared with the CONT group (p < 0.05;

Supplementary Fig. 1A). No significant differences in plasma CK were found in the QUERC and QUERC+ATO groups compared with CONT. Total cholesterol decreased by 20% in the ATO group ($p < 0.01$; Supplementary Fig. 1B) and by 17% in the QUERC+ATO group ($p < 0.05$) compared with the CONT group. HDL cholesterol levels did not change in any group (Supplementary Fig. 1C). However the proportion of HDL cholesterol expressed in percentage of the total cholesterol increased in both ATO and QUERC+ATO groups compared with CONT (80% vs. 55%, $p < 0.01$ and 77% vs. 55%, $p < 0.05$ respectively; Supplementary Fig. 1D).

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Article II

Reductive stress impairs myoblasts mitochondrial function and triggers mitochondrial hormesis

Mitochondrial content appears to be a determining factor in statin tolerance.

Within this article, we will try to provide answers to the following questions: What is the effect of reductive stress on mitochondria, and how could mitochondria defend themselves against it? Could reductive stress trigger mitochondrial hormesis? Could an increase in mitochondrial content provide a protection against statin-induced apoptosis *in vitro*?



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Reductive stress impairs myoblasts mitochondrial function and triggers mitochondrial hormesis



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ABSTRACT

Even though oxidative stress damage from excessive production of ROS is a well known phenomenon, the impact of reductive stress remains poorly understood. This study tested the hypothesis that cellular reductive stress could lead to mitochondrial malfunction, triggering a mitochondrial hormesis (mitohormesis) phenomenon able to protect mitochondria from the deleterious effects of statins. We performed several *in vitro* experiments on L₆ myoblasts and studied the effects of N-acetylcysteine (NAC) at different exposure times. Direct NAC exposure (1 mM) led to reductive stress, impairing mitochondrial function by decreasing maximal mitochondrial respiration and increasing H₂O₂ production. After 24 h of incubation, the reactive oxygen species (ROS) production was increased. The resulting mitochondrial oxidation activated mitochondrial biogenesis pathways at the mRNA level. After one week of exposure, mitochondria were well-adapted as shown by the decrease of cellular ROS, the increase of mitochondrial content, as well as of the antioxidant capacities. Atorvastatin (ATO) exposure (100 μM) for 24 h increased ROS levels, reduced the percentage of live cells, and increased the total percentage of apoptotic cells. NAC exposure during 3 days failed to protect cells from the deleterious effects of statins. On the other hand, NAC pretreatment during one week triggered mitochondrial hormesis and reduced the deleterious effect of statins. These results contribute to a better understanding of the redox-dependant pathways linked to mitochondria, showing that reductive stress could trigger mitochondrial hormesis phenomenon.

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1. Introduction

The mitochondrion is the most important organelle in determining continued cell survival and cell death. In most cell types, mitochondria are the major source of reactive oxygen species (ROS), which affect apoptosis both directly and indirectly via the activation of transcription factors [1]. For a long time, ROS were considered exclusively unwanted by-products of oxidative phosphorylation, as high concentrations of ROS cause lipid peroxidation and damage to cell membranes, proteins, carbohydrates, and DNA [2]. In the last decade, it has been shown that low, non-cytotoxic concentrations of ROS can serve as signals, triggering the activation of specific pathways [3–10]. The preservation of this non-cytotoxic level of ROS is ensured by antioxidant systems, which play a major role in cellular redox homeostasis. A deficiency or defect in these systems leads to an increase in tissue damage. In such cases, ROS can act either as second messengers or as a source of cellular damage, depending on the level of ROS production [4,6,7,11]. The concept of

mitochondrial hormesis, or “mitohormesis”, proposes that low doses of mitochondrial ROS can activate mitochondrial biogenesis and antioxidant capacities in order to counteract oxidative stress and to re-establish homeostasis [4,5,7,8,12].

The development of chronic oxidative stress has been implicated in the metabolic myopathy that is a secondary symptom of numerous pathologies such as diabetes mellitus, heart failure, or chronic obstructive pulmonary disease (COPD) [13,14]. This myopathy is also the most common adverse event encountered in patients treated with statins (HMG-CoA inhibitors, used to lower the plasmatic cholesterol levels), the most frequently prescribed treatment in developed countries. Recently, we found that statins protect mitochondria in the highly oxidative cardiac muscle by triggering a mitohormesis mechanism but impair mitochondrial function in glycolytic skeletal muscle [4].

Therefore, strategies involving moderate mitochondrial stress, inducing a mitohormesis mechanism may be used for the development of new ROS-targeting drugs. This would result in reducing the symptoms of metabolic myopathies by strengthening muscular mitochondrial function [11,15].

Even though oxidative stress damage from excessive production of ROS is a well known phenomenon, the impact of reductive stress

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remains poorly understood. Reductive stress can be defined as an excess of reducing equivalents (NAD(P)H and/or GSH) in the presence of intact oxido-reductive systems [2]. N-acetylcysteine (NAC) is the acetylated form (efficiently metabolized) of cysteine and is the most immediate precursor of glutathione (GSH). The GSH system is the main mechanism of detoxification of free radicals and ionized metabolites [16,17], and it is partly localized in the mitochondria. NAC is mainly used for its mucolytic action on the disulfide bonds of mucoproteins [18] and its modulating action on oxidative stress [19]. Interestingly, it has been shown that NAC treatment in H₉C₂ cells led to reductive stress [20]. This condition paradoxically increased the level of mitochondrial oxidation. However, the consequences as well as the potential beneficial effects following this reductive stress-inducing mitochondrial oxidation remained undiscovered [2].

We hypothesized that NAC treatment could change the redox environment of the cell, inducing mitochondrial oxidation (as described by [2,20]) and triggering a mitohormesis phenomenon, protecting L₆ myoblasts from the deleterious effects of statins.

Therefore, we investigated the effects of N-acetylcysteine on L₆ myoblasts. We found that NAC induced cellular reductive stress, which had for consequence an inhibitory effect on the mitochondrial respiratory chain, leading to an increase in mitochondrial ROS production and mitochondrial oxidation. This triggered mitochondrial biogenesis pathways and led to an increase in the number of mitochondria and in antioxidant capacities after 7 days of incubation. Finally, we showed that mitochondrial hormesis, triggered by NAC, protected cells against statin-induced apoptosis.

2. Materials and methods

2.1. Cell culture

L₆ rat myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM Low glucose, Milerium, VWR International) supplemented with 20% fetal calf serum (FCS) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco) at 37 °C under a humidified 5% CO₂ atmosphere. These cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

The acute effects of N-acetylcysteine (Sigma) were studied at several doses (50 µM, 500 µM, 1 mM, and 5 mM) for mitochondrial respiration, H₂O₂ production, and for the detection of superoxide production by electron spin resonance (ESR).

The chronic effects of N-acetylcysteine on L₆ myoblasts were studied by incubation with 1 mM of NAC in the culture medium for 24 h, three days, and one week. For the three days and one-week experiments, cells were also incubated with 100 µM atorvastatin for the last 24 h, in DMEM containing 10% FCS and 1% antibiotics.

2.2. Cell viability assays

Cell viability assays were performed after 24 h or one week of incubation with or without 1 mM NAC in DMEM containing 20% FCS, using a Muse™ Cell Analyzer (Merck Millipore), with the Muse Count and Viability Assay kit (Merck Millipore, Cat. Number: MCH100102), following the manufacturer's indications.

2.3. Study of mitochondrial respiration by oximetry

Mitochondrial respiration was studied in saponin-skinned cells to keep mitochondria in their architectural environment [4]. The analysis took place in a thermostated oxygraphic chamber at 37 °C with continuous stirring (Oxygraph-2 k, Oroboros instruments, Innsbruck, Austria). Cells were collected with trypsin and placed in R⁺ medium (2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 6.56 mM MgCl₂, 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 50 mM K-methane sulfonate, 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/ml of

BSA; pH = 7) in the oxygraphic chamber with saponin (0.125 mg/ml per chamber). Maximal respiration rate (V_{max}) was measured in the presence of saturating amounts of succinate (25 mM) and ADP (2 mM) as substrates. V_{max} characterizes the electron flow through complexes I, II, III, and IV. NAC was directly injected into the oxygraphic chamber after V_{max} . After the experiments, cells were collected for total protein content determination. Results are expressed as a percentage of the V_{max} or of the control group.

2.4. H₂O₂ production in permeabilized cells

H₂O₂ production was measured with the Amplex Red reagent (Invitrogen), which reacted with H₂O₂ in a 1:1 stoichiometry catalyzed by HRP (Horse Radish Peroxidase; Fluka Biochemika) to yield the fluorescent compound resorufin and molar equivalent O₂ [21]. Resorufin has excitation/emission characteristics of 563/587 nm and is extremely stable once formed. Fluorescence was measured continuously [change in fluorescence (F)/s] with a Fluoromax-4 (Jobin Yvon) spectrofluorometer with temperature control (set at 37 °C) and under continuous magnetic stirring.

Approximately 300,000 cells were added to 600 µl of buffer Z (110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K₂HPO₄, 3 mM MgCl₂, and 0.5 mg/ml BSA) with HRP (0.5 U/ml) and Amplex Red (5 µM). Saponin was directly added to allow cell permeabilization (0.125 mg/ml saponin). H₂O₂ production was then measured with glutamate (5 mmol/l), malate (2 mmol/l), succinate (25 mM), and ADP (2 mM) to stimulate electron flow through complexes I, II, III, and IV. To study the acute effects of NAC, 1 mM of NAC was then added in the spectrofluorometer's quartz cell. H₂O₂ production rate was calculated from the slope of F/s, after subtracting background, from a standard curve established with the appropriate reaction conditions. At the conclusion of each experiment, cells were collected for total protein content determination. Values are expressed as percentage of the control group.

2.5. Mitochondrial free radical leak (FRL)

H₂O₂ production and O₂ consumption were measured in parallel in the same sample under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence which reduce O₂ to ROS in the respiratory chain (the percentage of free radical leak) instead of reaching cytochrome oxidase to reduce O₂ to water [21]. Because two electrons are needed to reduce 1 mol of O₂ to H₂O₂, whereas four electrons are transferred in the reduction of 1 mol of O₂ to water, the percent of FRL was calculated as the rate of H₂O₂ production divided by twice the rate of O₂ consumption, and the result was multiplied by 100.

2.6. NAD⁺/NADH determination

NAD⁺/NADH ratio was determined after 1H incubation with NAC 1 mM, using the EnzyChrom™ NAD⁺/NADH assay kit (BioAssay Systems, Cat. Number: E2ND-100), following the manufacturer's instructions.

2.7. Electron spin resonance measurement of superoxide production

Superoxide (O₂^{•-}) production was determined after incubation with 1 mM NAC for either 24 h, three days, or one week. For the three days and one-week experiments, cells were also incubated with 100 µM atorvastatin for the last 24 h, in DMEM containing 10% FCS and 1% antibiotics. Cells were placed into a 24-well plate with Krebs-Hepes Buffer containing 25 µmol/l deferoxamine and 5 µmol/l DETC. Cells were then incubated at 37 °C with the spin probe CMH (1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine HCl, 200 µM) for 30 min under 2.7% oxygen, and 20 mm Hg partial pressure using a Gas-Controller (Noxygen Sciences Transfer, Elzach, Germany). The reaction was then stopped on ice. All ESR experiments measuring the

concentration of oxidized CM were conducted at 15 °C in a Bruker e-scan M (Bruker biospin) in disposable capillary tubes, as previously described [22]. Detection of superoxide was conducted under the following ESR settings: center field $g = 3482.579$; sweep width 60 G; microwave power 21.85 mW; modulation amplitude 2.40 G; sweep time 5.24 s; number of lag curve points 1.

2.8. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was obtained from cells using TRIzol™ (Invitrogen Life Technologies, Rockville, MD, USA), as previously described [23] and following the manufacturer's instructions. RNA was stored at -80 °C until the reverse transcription reaction was performed. cDNA was synthesized from total RNA with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. To perform the real-time PCR reaction, 1 µg of cDNA was mixed with 10 µM of each primer (sense and antisense), SYBR Green (Invitrogen Life Technologies, Rockville, MD, USA) as a fluorescent dye and H₂O. The real-time PCR measurement of individual cDNAs was performed in triplicate using SYBR Green dye to measure duplex DNA formation with the LightCycler System (Roche Diagnostics, Meylan, France). The primer sequences were designed using information contained in the public database GenBank of the National Center for Biotechnology Information (NCBI). The sequences of the primer sets used are listed in Table 1. Quantification of gene expression was performed by the method described in [24], using the β-actin gene as the internal control because it is a stable gene for real-time RT-PCR measurements in muscles. The amplification efficiency of each sample was calculated as described by Ramakers et al. [25].

2.9. ATP monitoring assay

The intracellular concentration of ATP in myoblasts was measured with the ATPLite 1-step kit from PerkinElmer Life and Analytical Sciences (Shelton, CT, USA). Luminescence was detected with a Victor3

Wallac 1420 multilabel counter (PerkinElmer). ATP concentrations were quantified according to a standard curve and expressed as percentage of the control group.

2.10. mtDNA content

DNA was isolated from L₆ myoblasts that had been treated with 1 mM NAC for one week. Briefly, cells were placed in KTT lysis buffer and vortexed. After centrifugation, phenol/chloroform premixed with isoamyl alcohol (25:24:1; pH = 8; Interchim Uptima) was added to the supernatant, followed by a centrifugation. Then chloroform was added to the supernatant before another centrifugation. The aqueous supernatant was collected; ammonium acetate (4 M, pH = 7) and absolute ethanol were added. Samples were precipitated for 1 h at -20 °C. After centrifugation, pellets of DNA were washed two times with 70% ethanol. Each pellet was dried and resuspended in 10 µl Tris-EDTA buffer (TE). DNA in samples was quantified spectrophotometrically at 260 nm. The DNA was subjected to real-time PCR in duplicate (100 ng/µl). Relative amounts of nuclear and mtDNA were determined by comparison of the amplification kinetics of pyruvate kinase and cytochrome b (Primer sequences in Table 1). Results were expressed as percentage of the control group.

2.11. Measurement of total glutathione

Total glutathione was measured in cells after one week of incubation with NAC 1 mM according to the Akerboom and Sies method [26] by monitoring the reduction of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) to 5-thio-2-nitrobenzoate (TNB) by GSH at 412 nm. Results were expressed as µM/mg protein.

2.12. Expression of PGC-1α, and SOD2

After one week incubation with NAC 1 mM, cells were lysed on ice for 15 min with 200 µl of NET lysis buffer (0.05 M Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 1% NP-40 and a protease inhibitor tablet from Roche, Basel, Switzerland). After lysis, the mixture was vortexed and centrifuged for 10 min at 4 °C at 10,000 rpm. The supernatant was collected and the protein concentration determined using the Pierce BCA protein assay kit (Darmstadt, Germany). For each sample, 20 µg of protein was separated on a denaturing SDS polyacrylamide gel (4%). The antibodies against PGC-1α, and SOD2 were used at dilutions of 1:1000, and 1:500, respectively (Goat polyclonal anti-PGC-1α antibody (ab106814) from Abcam PLC, Cambridge, UK; and Rabbit polyclonal anti-SOD2 antibody (ab13533) from Abcam PLC, Cambridge, UK). Peroxidase-labeled anti-rabbit IgG or anti-goat IgG (1:2000 dilution) in combination with a chemiluminescent substrate (ECL Western Blot detection kit, GE Healthcare, Amersham, UK) were used for the analysis. Quantification was performed using the ImageJ software (National Institutes of Health). Results were expressed as percentage of the control group.

2.13. Apoptosis measurement assays

Apoptosis measurement assays were performed after three days and one week of incubating L₆ myoblasts with or without 1 mM NAC and/or 100 µM atorvastatin for the final 24 h, in DMEM containing 10% FCS and 1% antibiotics. Atorvastatin (Tahor) was generously provided by Pfizer. Staurosporine (200 nM) was used as a positive control (data not shown). The experiments were performed using a Muse™ Cell Analyzer (Merck Millipore), with the Muse Annexin V and dead cell kit (Merck Millipore, Cat. Number: MCH100105), following the manufacturer's instructions.

Table 1
Primer sequences used for quantitative real-time PCR amplification.

Target gene	Organism	Forward primer 5' → 3' Reverse primer 5' → 3'	Accession number
PGC-1α	Rat	CACCAAAACCCACAGAGAACAG GCAGTTCACAGAGAGTCCACA	NM_031347
PGC-1β	Rat	CCCCAGTGTCTGAAGTGGAT TCTGGAACCTGAGGCTGGTCT	NM_176075
NRF1	Rat	GGCCCTTAAACAGTGAAGCTG CATCTGGGCCATTAGCATCT	NM_001100708
NRF2a	Rat	TACAATGACACAGCCTGTGC ATCCTTGGGGACCTTTGAAC	NP_001102311
NRF2b	Rat	CCCGATGGACAGCAAGTATT CCGGTTCCTCAATATTTCCA	XM_006254075
TFAm	Rat	GAAAGCACAAATCAAGAGGA CTGCCTTTCATCATGAGACAG	NM_031326
Catalase	Rat	AGATGGCACACTTTGACAGAGAG GAGAATCCGACGGCAATAGGAG	NM_012520
SOD1	Rat	AGATGACTTGGCAAAGGTG CAATCCCAATCACACCACAA	NM_017050
SOD2	Rat	CTGGACAAACCTGAGCCCTA GAACCTTGGACTCCCACAGA	NM_017051
Citrate synthase	Rat	TATGGCATGACGGAGATGAA CATGGAAGTGGCCCTTCTA	DQ403126
Cox1	Rat	CCAGAGTCATGAGTCGAAGGA AGGCGCATGAGTACTTCTCGG	YP_665631
Cox4i1	Rat	GTTGGTACCAGGGCACTTA CACATCAGGCAAGGGTAGT	NP_058898
GPx	Rat	GCCGAGTGTGGTTTACGAAT GGCTGCAAACTCTTGATTT	NM_030826
Cytb	Rat	GCAGCTTAAACATTCGCCCAATCA TACTGGTTGGCCTCCGATTCATGT	J01436
Pyruvate kinase	Rat	TGIGGGTGATCTGGTATTGTGGT AGGCATTCAGGATACGCTCAGCA	NM_012624

2.14. Statistics

Data are represented as means ± SEM. Statistical analyses were performed using Student's t test or 1-way ANOVA followed by a Tukey post test (GraphPad Prism 5, Graph Pad Software, Inc., San Diego, CA, USA). Statistical significance was displayed as *, \$p < 0.05 or **, \$\$\$p < 0.01 or ***, \$\$\$p < 0.001.

3. Results

3.1. Acute N-acetylcysteine exposure impaired mitochondrial function

The effects of NAC were first observed at the cellular level: after 1 h exposure of L₆ cells with NAC 1 mM, NAD⁺/NADH ratio was decreased compared to control (−72%; p < 0.05; Fig. 1A).

At the mitochondrial level, maximal mitochondrial respiration rates (V_{max}) and H₂O₂ production in saponin skinned cells were studied after direct exposure. NAC decreased V_{max} in a concentration-dependent manner (Fig. 1B). The diminution of V_{max} became significant for a concentration of 0.5 mM (−19%; p < 0.05) and reached −28% at a concentration of 1 mM (p < 0.01).

Fig. 1C shows mitochondrial H₂O₂ production from permeabilized L₆ cells. When cells were exposed to NAC 1 mM, there was an increase of H₂O₂ production (+40%, p < 0.05) compared to control. The concentration of 1 mM was used for further experiments because this concentration corresponded to a significant decrease in V_{max} combined with a significant increase of H₂O₂ production.

When L₆ cells were directly exposed to NAC, the free radical leak (FRL) was significantly increased (+174%; p < 0.05; Fig. 1D) compared to control.

3.2. N-acetylcysteine increased mitochondrial ROS production and triggered activation of mitochondrial biogenesis pathways after 24 h

L₆ cells were treated with 1 mM NAC for 24 h. ESR measurements showed an increase of superoxide production (+42%; p < 0.001) (Fig. 2A). The cell viability assay did not show any difference between CTL and NAC groups (Fig. 2B). The maximal mitochondrial oxidative capacity of L₆ cells was not altered (Fig. 2C) following 24 h of exposure. It is important to underscore the fact that contrary to the acute time point, we did not add NAC in the oxygraph chamber. The acceptor control ratio (ACR) was not changed between the two groups (Fig. 2D).

The relative mRNA expression levels of the main actors in mitochondrial biogenesis, mitochondrial proteins, and of the main enzymatic antioxidant systems were measured by quantitative RT-PCR (Fig. 2E) after this 24 h incubation with NAC. The relative expression level of PGC-1α was decreased by 50% (p < 0.01), whereas the other actors of mitochondrial biogenesis were highly increased: PGC-1β (+240%; p < 0.001), NRF1 (+174%; p < 0.001), and TFAM (+179%; p < 0.001) compared to CTL. We observed significant increases in the relative mRNA expression levels of COX1 (+372%; p < 0.001), COX4i1 (+281%; p < 0.01), citrate synthase (+484%; p < 0.01), and SOD1 (+260%; p < 0.01) after NAC exposure. In contrast, concerning the genes implicated in antioxidant systems, there was no modification of the relative mRNA expression levels of SOD2 or GPx.

3.3. One week of NAC incubation induced mitochondrial biogenesis pathways, increasing mitochondrial content as well as antioxidant enzymatic capacities, and reducing basal ROS production

After one week of incubation with 1 mM NAC, the mRNA expression levels of the main actors of mitochondrial biogenesis were increased

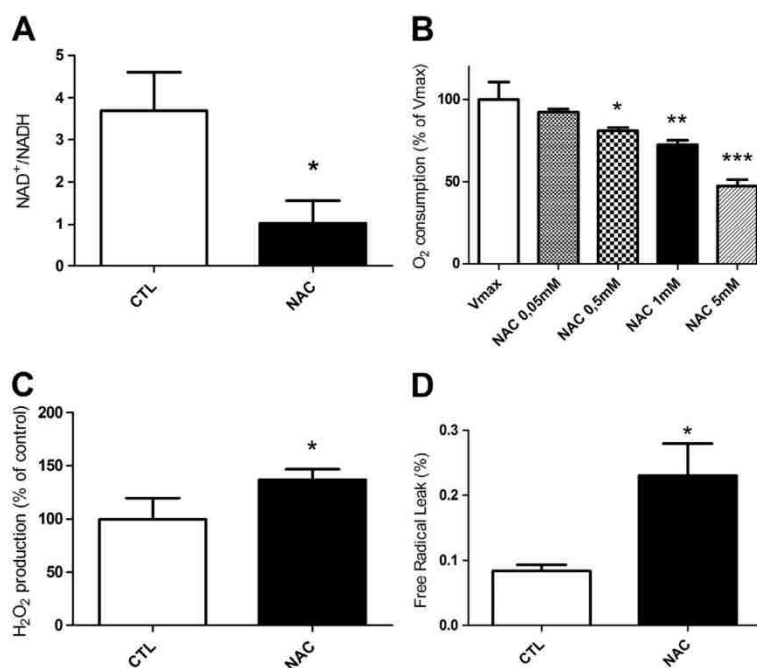


Fig. 1. Acute effects of NAC on mitochondrial function. (A) NAD⁺/NADH ratio measurement after 1 h incubation with NAC 1 mM (n = 4). (B) Dose–response of NAC effects on mitochondrial maximal respiratory rates (V_{max}) (n = 12). (C) Measurement of mitochondrial H₂O₂ production in the presence of NAC 1 mM (n = 12). (D) Free radical leak (FRL) measurement in the presence of NAC 1 mM, (n = 6). Values are expressed as means ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001; One way ANOVA with repeated measures for (B) and unpaired t-test for (A, C and D).

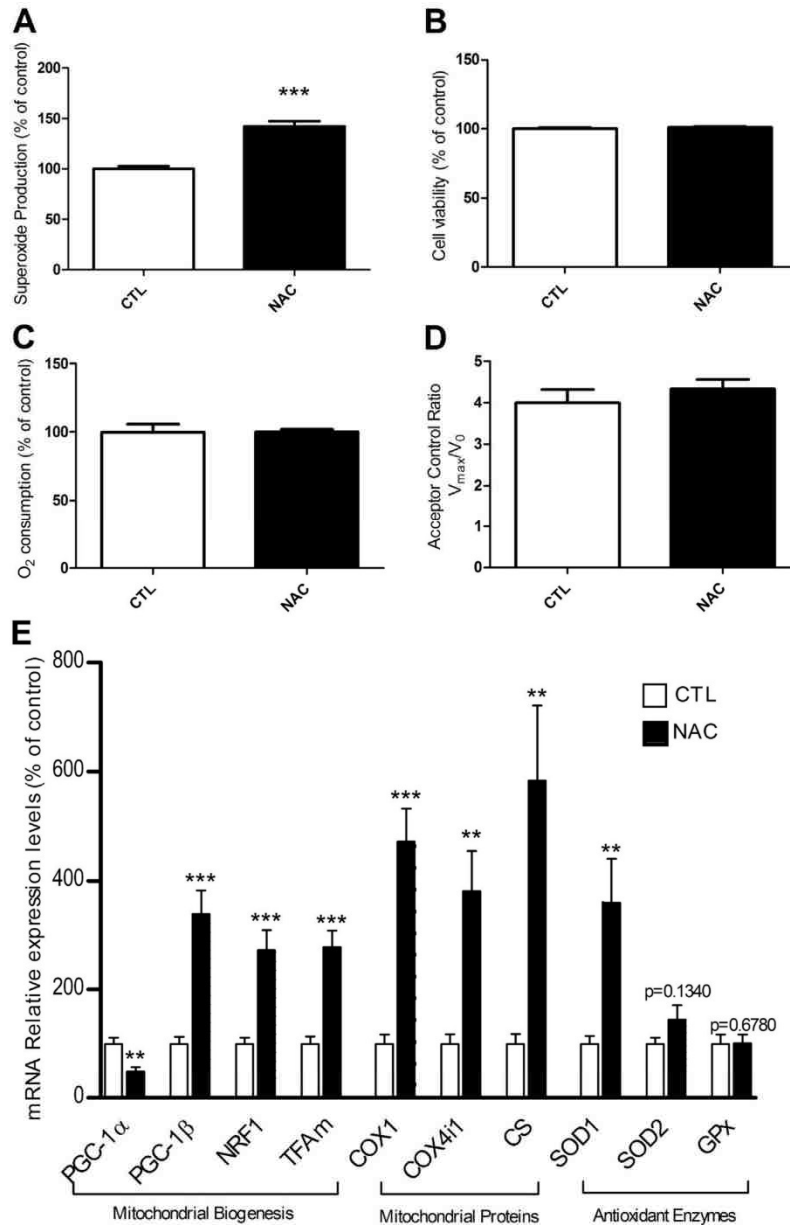


Fig. 2. Chronic effects of NAC 1 mM (24 h). (A) Superoxide production was measured by ESR (CTL: n = 16; NAC: n = 23). (B) Cell viability assay realized with the MUSE Cell Analyzer (n = 6). (C) Maximal mitochondrial oxidative capacities (V_{max}) (n = 4). (D) Acceptor control ratio. (n = 4). (E) mRNA relative expression levels of factors involved in mitochondrial biogenesis (PGC-1α, PGC-1β, NRF1, and TFAM), mitochondrial proteins (COX1, COX4i1, and citrate synthase), and antioxidant proteins (SOD1, SOD2, and GPx) were explored with or without NAC (n = 11). Values are expressed as means ± SEM, **p < 0.01; ***p < 0.001; unpaired t-test.

(Fig. 3A): PGC-1α (+64%; p = 0.06), PGC-1β (+123%; p < 0.05) as well as NRF1 (+121%; p < 0.05), NRF2a (+62%; p < 0.05), NRF2b (+106%; p < 0.01), and TFAM (+179%; p < 0.01). The relative mRNA expression levels of several mitochondrial proteins were also upregulated, including COX1 (+124%; p < 0.05), COX4i1 (+165%; p < 0.001), and citrate synthase (+87%; p < 0.05). Concerning the antioxidant systems, NAC exposure non-significantly increased the level of catalase (+41%; p = 0.08) and significantly increased the relative mRNA expression levels of SOD1 (+95%; p < 0.05), SOD2 (+107%; p < 0.01) and GPx (+145%; p < 0.001).

NAC treatment enhanced the protein expression of PGC-1α (+67%; p < 0.05) confirming the activation of the mitochondrial biogenesis pathways (Fig. 3B). The increase in SOD2 protein expression with NAC (+44%; p = 0.0683) confirmed the augmentation of antioxidant capacities obtained at the mRNA level (Fig. 3B).

The increase in mitochondrial content was shown with several techniques (Western blotting, oxygraphy, quantification of mitochondrial DNA (mtDNA), and an ATP production assay).

Interestingly, the maximal mitochondrial oxidative capacity (V_{max}, +17%; p < 0.05) (Fig. 4A) as well as the mtDNA content were increased

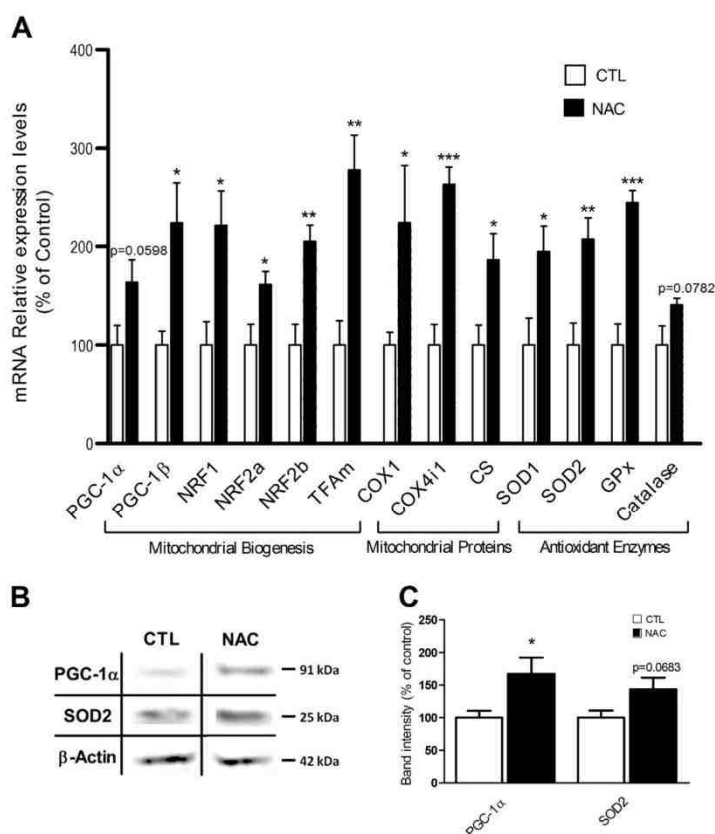


Fig. 3. Chronic effects of NAC 1 mM (1 week) on mRNA expression as well as protein expression levels. (A) mRNA relative expression levels of factors involved in mitochondrial biogenesis (PGC-1α, PGC-1β, NRF1, NRF2a, NRF2b, and TFAM), mitochondrial proteins (COX1, COX4i1, and citrate synthase), and antioxidant proteins (SOD1, SOD2, GPx, and catalase) (n = 5–6). (B–C) Band intensity of Western blots of PGC-1α and of SOD2 (n = 6–7). Values are expressed as means ± SEM, *p < 0.05; **p < 0.01; ***p < 0.001; unpaired t-test.

(+144%; p < 0.05) after one week NAC treatment (Fig. 4C). The acceptor control ratio was not changed between the two groups (Fig. 4B). The ATP monitoring assay showed an important increase in the ATP content after treatment with 1 mM NAC (+303%; p < 0.001) (Fig. 4D). These results strongly suggest an activation of the cellular metabolism after NAC exposure, leading to the increase in mitochondrial content as well as enzymatic antioxidant capacities.

We investigated the redox state of cells by studying the superoxide production by ESR and the total cellular glutathione content. In cells treated with NAC, it appeared that superoxide production was significantly diminished (−40%; p < 0.001; Fig. 4E) compared to CTL. Total cellular glutathione content was increased after treatment (+38%; p < 0.05; Fig. 4F) compared to CTL. No deleterious effects on cell viability were observed after chronic NAC exposure (Fig. 4G).

3.4. Short NAC treatment (3 days) failed to protect cells from deleterious effects of statins

Incubation of myoblasts with 100 μM atorvastatin for 24 h (ATO) impaired mitochondrial function (Fig. 5). Atorvastatin decreased the maximal mitochondrial oxidative capacity of cells (−37%; p < 0.001; Fig. 5A) and the acceptor control ratio (−31%; p < 0.05; Fig. 5B). NAC pretreatment for three days did not change either V_{max} or ACR compared to CTL. Short pretreatment with NAC did not protect maximal mitochondrial oxidative capacity of cells from the deleterious effect of ATO

(NAC + ATO, −37%; p < 0.001; Fig. 5A), and did not improve the ACR (−40%; p < 0.01; Fig. 5B) compared to CTL. Cellular ATP content (Fig. 5C) decreased after 24 h of statin treatment (−29%; p < 0.05). NAC pretreatment for three days did not change ATP content in cells. ATP content in NAC + ATO group was reduced compared to CTL (−61%; p < 0.001), as well as to ATO (p < 0.05). Incubation of myoblasts with ATO increased superoxide production (+37%; p < 0.05; Fig. 5D). Superoxide production was not altered in NAC group compared to CTL, whereas three days of pretreatment with NAC 1 mM did not protect L₆ cells from this high ROS production (NAC + ATO; +65%; p < 0.001).

Apoptosis-measurement assays showed that atorvastatin incubation for 24 h decreased cell viability (−21%; p < 0.001; Fig. 5E). Three days of NAC pretreatment (NAC + ATO) did not prevent these deleterious effects of statins (−20%; p < 0.001 compared to CTL). NAC + ATO group had a higher percentage of early apoptotic cells compared to the ATO group (13.4% vs. 7%; p < 0.001; Fig. 5F). The percentage of late apoptotic cells (Annexin V⁺/7-AAD⁺; Fig. 5G) was increased in both ATO and NAC + ATO compared to CTL (20.41% and 14.78%, respectively; p < 0.001). Moreover, the percentage of total apoptotic cells (early apoptotic and late apoptotic; Fig. 5H) was increased in the ATO condition compared to CTL (27.5% vs. 7%, respectively; p < 0.001) as well as in the NAC + ATO condition (28.2%; p < 0.001 compared to CTL). NAC pretreatment alone did not change the percentage of total apoptotic cells compared to CTL.

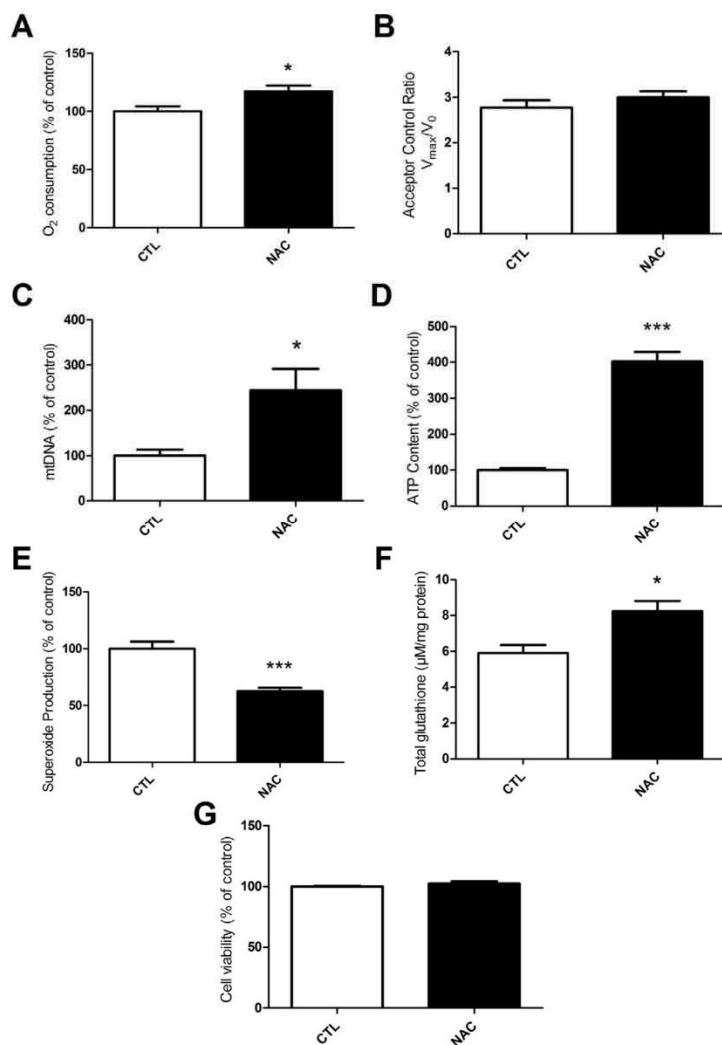


Fig. 4. Chronic effects of NAC 1 mM (1 week) on mitochondrial respiration, mitochondrial content and efficiency, oxidative stress and cell viability. (A) Maximal mitochondrial oxidative capacities (V_{max}) ($n = 14-15$). (B) Acceptor control ratio ($n = 14-15$). (C) mtDNA quantification ($n = 10-11$). (D) ATP content determination assay ($n = 12$). (E) Superoxide production measured by ESR (CTL: $n = 14$; NAC: $n = 9$). (F) Total glutathione content ($n = 4$). (G) Cell viability assay realized with the MUSE Cell Analyzer ($n = 6$). Values are expressed as means \pm SEM. * $p < 0.05$; *** $p < 0.001$; unpaired t-test.

3.5. One week of NAC treatment protected cells from the deleterious effects of statins

NAC pretreatment for one week, then 24 h with growth medium containing 10% of FCS, increased maximal mitochondrial respiratory rates (+22%; $p < 0.05$; Fig. 6A), without affecting the ACR (Fig. 6B), and increased cellular ATP content (+59%; $p < 0.001$; Fig. 6C). Seven days of pretreatment with NAC (NAC + ATO) prevented the alteration of these parameters from the deleterious effects of ATO without improving the ACR (+35% maximal respiratory rates compared to ATO, $p < 0.05$; +29% ATP content compared to ATO, $p < 0.001$). Interestingly, this NAC pretreatment protected L₆ cells from this Atorvastatin-induced ROS production (NAC + ATO, -26% compared to CTL, $p < 0.05$; -41% compared to ATO, $p < 0.001$; Fig. 6D).

Apoptosis-measurement assays showed that atorvastatin incubation for 24 h decreased cell viability (-10%; $p < 0.001$; Fig. 6E). The NAC + ATO group had a higher percentage of viable cells compared to

the ATO group (83.2% vs. 78.4%, respectively; $p < 0.001$), although this percentage remained lower than the control condition without atorvastatin (-5%; $p < 0.001$). Atorvastatin incubation tend to increase the percentage of cells in early apoptosis (Annexin V⁺/7-AAD⁻; Fig. 6F) compared to the control group (13.8% vs. 10.4%, respectively; $p < 0.05$). The NAC + ATO group had a lower percentage of early apoptotic cells compared to the ATO group (10.4% vs. 13.8%; $p < 0.05$). The percentage of late apoptotic cells (Annexin V⁺/7-AAD⁺; Fig. 6G) was increased in both ATO and NAC + ATO compared to the control (+323% and +276%, respectively; $p < 0.001$). The co-treatment did not change the percentage of late apoptotic cells compared to the ATO condition (6.4% vs. 7.2%, respectively). However, the percentage of total apoptotic cells (early apoptotic and late apoptotic; Fig. 6H) was increased in the ATO condition compared to CTL (21.5% vs. 13.5%, respectively; $p < 0.001$) and NAC + ATO had a lower percentage of total apoptotic cells compared to the ATO group (16.7% vs. 21.5%, respectively; $p < 0.05$).

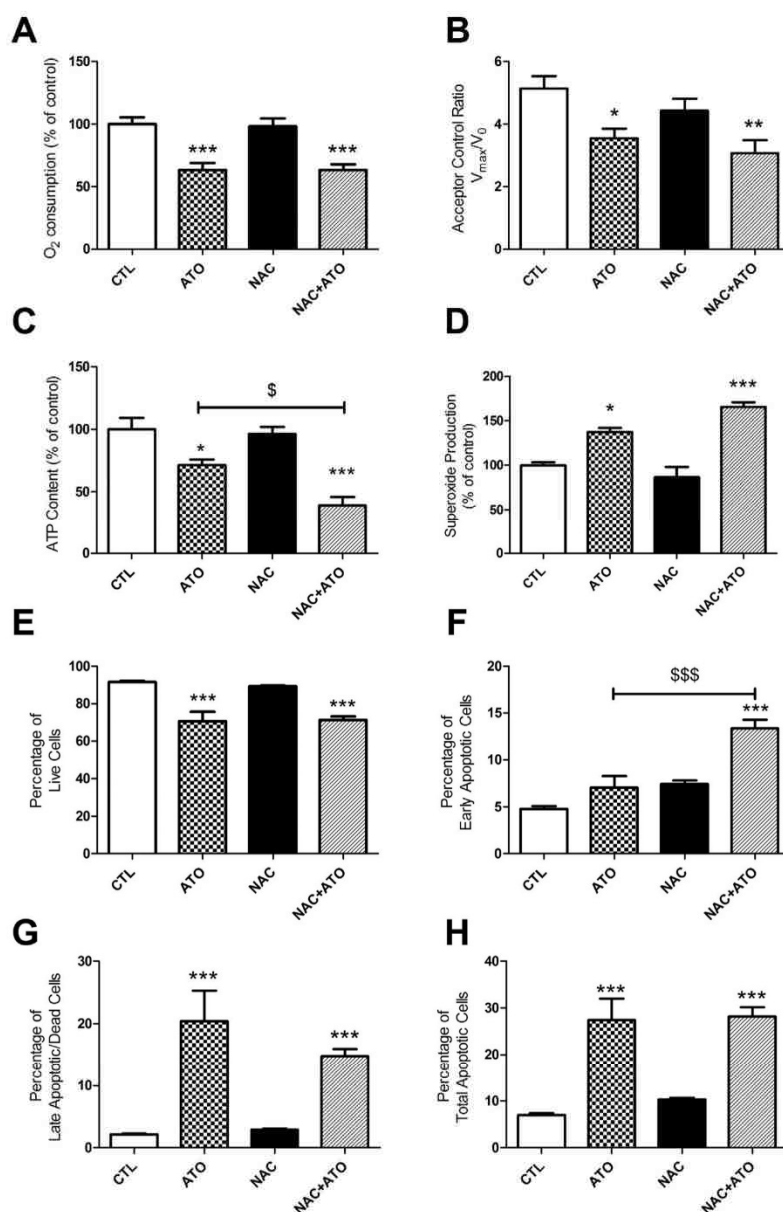


Fig. 5. NAC pretreatment for 3 days failed at protecting cells from deleterious effects of statins. (A) Maximal mitochondrial oxidative capacities (V_{max}) ($n = 7$). (B) Acceptor control ratio ($n = 7$). (C) ATP content determination assay ($n = 6$). (D) Superoxide production measured by ESR ($n = 6$). (E–H) Annexin V assay ($n = 6$): (E) Percentage of live cells (Annexin V-PE⁻/7-AAD⁻). (F) Early apoptotic cells (Annexin V-PE⁺/7-AAD⁻). (G) Late apoptotic cells (Annexin V-PE⁺/7-AAD⁺). (H) Total apoptotic cells. Values are expressed as means \pm SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to CTL; \$ $p < 0.05$; \$\$\$ $p < 0.001$ compared to ATO. One way ANOVA followed by a Tukey post test.

4. Discussion

This study showed that: (I) Acute treatment with N-acetylcysteine induced a cellular reductive stress that impaired mitochondrial function of myoblasts; (II) following this reductive stress, there was a rapid activation of mitochondrial biogenesis pathways (after 24 h of incubation); (III) three days of NAC treatment was not sufficient to induce a phenotypic adaptations allowing to protect cells from statins toxicity; (IV) after one week of NAC exposure, the mitochondrial biogenesis pathways remained activated, allowing an increase of mitochondrial content as well

as antioxidant enzymatic capacities, corresponding to a mitohormesis mechanism; (V) the activation of this mitohormesis phenomenon clearly protected cells from apoptosis triggered by short statins treatment.

4.1. The antioxidant molecule N-acetylcysteine induced cellular reductive stress, and mitochondrial oxidation by inhibiting the mitochondrial respiratory chain

Our results showed that NAC treatment induced a cellular reductive stress, as suggested by the reduction of the NAD⁺/NADH ratio. Our

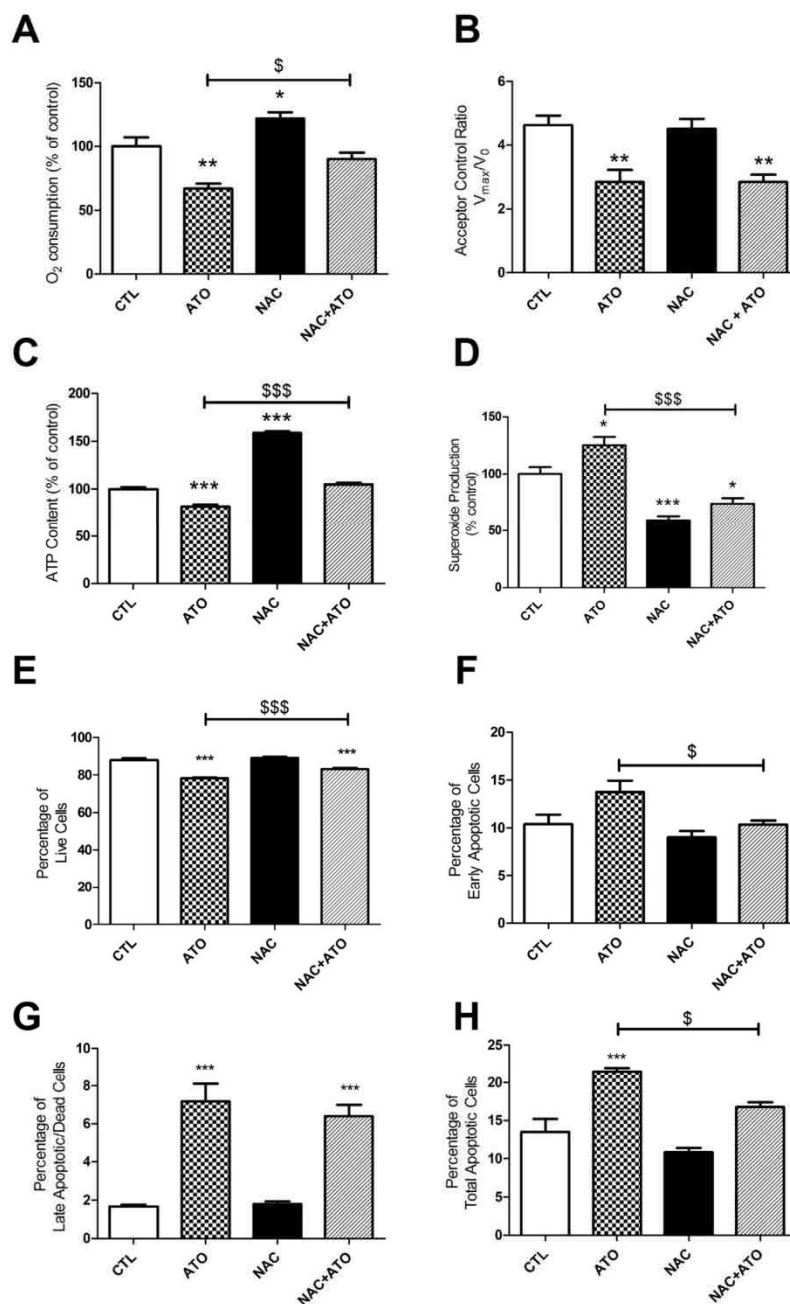


Fig. 6. Protective effects of NAC-induced mitohormesis phenomenon against deleterious effects of statins. (A) Maximal mitochondrial oxidative capacities (V_{max}) (n = 6–8). (B) Acceptor control ratio (n = 6–8). (C) ATP content determination assay (n = 6). (D) Superoxide production measured by ESR (n = 6). (E–H) Annexin V assay (n = 6): (E) Percentage of live cells (Annexin V-PE⁻/7-AAD⁻), (F) Early apoptotic cells (Annexin V-PE⁺/7-AAD⁻), (G) Late apoptotic cells (Annexin V-PE⁺/7-AAD⁺), (H) Total apoptotic cells. Values are expressed as means \pm SEM, *p < 0.05; **p < 0.01; ***p < 0.001 compared to CTL; \$p < 0.05; \$\$\$p < 0.001 compared to ATO. One way ANOVA followed by a Tukey post test.

results are in accordance with some other studies showing that NAC could provoke glutathionylation of mitochondrial proteins due to a NAC-induced reductive stress effect on H9C2 cardiomyocytes [2,20,27]. Zhang et al. observed, that causing reductive stress with NAC (4 mM) treatment for 60 min, induced an oxidation of the redox state of mito-roGFP and that Trx 2 correspondingly shifted from 56%

reduced form to 36% reduced form after NAC exposure compared with controls [20].

Then, subunits of the respiratory chain complexes could be altered by this mechanism, leading to oxidative phosphorylation (OXPHOS) dysfunction and then to an increase in ROS production as shown by the decrease of V_{max} and increase of H_2O_2 production, leading to an

increase of the free radical leak (FRL) [21,28,29]. The FRL represents the fraction of electrons out of sequence that reduce O_2 to ROS in the respiratory chain (the percent of free radical leak) instead of reaching cytochrome oxidase to reduce O_2 to water [30]. The FRL is a good index of mitochondrial oxidative stress [21] because it is independent of the mitochondrial content, which is known to influence the production of ROS [30]. Inhibition of mitochondrial respiration-induced mitochondrial ROS production has already been shown in several other *in vitro* models [4,31–33], and both complex I and complex III should be potential sites of $O_2^{\bullet -}$ production [29,34,35]. Then, in contrast to the general model in which NAC functions solely as an antioxidant, we and others observed that NAC could increase mitochondrial ROS production [2,20,36]. Our findings underscore the importance of considering the dynamic events related to oxido-reductive signals and redox-dependent pathways.

4.2. Reductive stress triggered a mitochondrial hormetic mechanism

Mitohormesis is based on the assumption that a moderate increase in ROS production during mitochondrial activity leads to the activation of cellular defense systems [4–9]. Our results suggest that NAC induced perturbations of the mitochondrial redox potential, triggering this mitohormesis phenomenon.

The transcriptional control of mitochondrial biogenesis depends on many factors, and the members of the PGC-1 family (PGC-1 α ; PGC-1 β , and PRC) are considered to be key regulators of this process [37–39]. The conventional wisdom attributes oxidative stress response to PGC-1 α , however the regulation and function of PGC-1 β is still unclear in the literature. Both are enriched in mitochondria-rich tissues, but PGC-1 β is thought to preferentially induce genes involved in the removal of reactive oxygen species and is supposed to play a role in constitutive mitochondrial biogenesis [40]. However, only PGC-1 α is thought to respond to metabolic challenges. Indeed, we found a rapid transcriptional activation leading to the increased expression of several genes implicated in mitochondrial biogenesis pathways, including PGC-1 β , the NRF family, and TFAM [12,37–39,41–43]. We observed however a significant increase in the expression of the PGC-1 α protein at 7 days despite what we observed at the mRNA level. That is why we can suggest that the adaptive response observed in our study could be driven through PGC-1 α , but we cannot exclude that this adaptive response does only affect PGC-1 α protein levels and not PGC-1 β . Then, the mRNA results suggest that the establishment of the mitohormesis phenotype at 7 days needs both PGC-1 α and β induction, with PGC-1 β expression being required earlier in the mitohormesis time course than PGC-1 α . Mitochondrial adaptations could be considered as a sequential program, requiring 6 to 10 days to obtain an effective mitochondrial biogenesis [44]. We studied the effects of one week of NAC treatment to assess the phenotypic consequences of this activation of biogenesis. In our study, we clearly showed that the activation of mitochondrial biogenesis led to an increase in cellular levels of mitochondrial content as well as cellular antioxidant capacities. These phenotypic changes were demonstrated with several techniques. First, we showed an augmentation of the relative mRNA expression of mitochondrial proteins, such as citrate synthase, as well as the simultaneous expression of nuclear and mitochondrial genomes, with an increase in the relative mRNA expression of subunits of cytochrome c oxidase (complex IV), COX1 (encoded by the mitochondrial genome) and COX4 (encoded by the nuclear genome). This simultaneous expression of both genomes could be explained by TFAM overexpression. These observations were corroborated by Western blot results showing an effective upregulation at the protein level of mitochondrial biogenesis pathways, as well as by an increase in the mtDNA copy number. At the functional level, we observed an augmentation of the mitochondrial maximal oxidative capacities (V_{max}). Interestingly, the antioxidant enzymatic systems were clearly enhanced, allowing a decrease in the cellular ROS content measured by electron spin resonance. These results are consistent with numerous findings indicating that ROS may evoke cellular signaling that promotes

metabolic health and longevity. Indeed, ROS serve as essential signaling molecules delivering messages from the mitochondria to other cellular compartments in response to physiological or pathophysiological changes [6,35,45–49]. Classically, N-acetylcysteine (NAC) is considered to be an antioxidant molecule because it is the most immediate precursor of glutathione (GSH), which is the main mechanism of detoxification of free radicals and ionized metabolites [16,17], especially in mitochondria. Our findings are quite novel in demonstrating that NAC has powerful mitochondrial and antioxidant effects, not only via its properties as a glutathione precursor (increase in GSH content) but also by enhancing the levels of mitochondrial content and antioxidant enzymes by triggering mitohormesis. According to Calabrese et al., this phenomenon could be defined as *chemical conditioning hormesis* [50]. Moreover, our results could partially explain the divergent and sometimes disappointing effects encountered with chronic NAC treatment and antioxidant supplementation [51–57]. Indeed, reductive stress could lead to an increase in mitochondrial oxidation due to these so-called antioxidants and have dramatic effects on redox pathways and on cell survival.

4.3. Mitochondrial hormesis protected cells from pro-apoptotic effects of statins

Statins are the most prescribed and most efficient medication used to lower plasma cholesterol levels [58] and are considered relatively clinically safe. However, these molecules show numerous adverse effects ranging from myalgia to rhabdomyolysis. These deleterious effects have been shown to be linked to oxidative stress in glycolytic skeletal muscles, whereas cardiac oxidative muscle, which has a higher mitochondrial content as well as higher antioxidant properties, was preserved [4]. An increase of mitochondrial content in L_6 myoblasts has been shown to protect against both caspase-dependent and caspase-independent apoptosis [59]. Therefore, statin-induced mitochondrial ROS overproduction may lead to the triggering of apoptotic pathways and lead to the appearance of statin-induced myopathy. We observed that three days of pretreatment with NAC 1 mM was not sufficient to induce mitochondrial hormesis. At this time point, NAC pretreated cells seems to be weakened by the induced mitochondrial oxidation, and so more affected by a subsequent mitochondrial stress with statins. Thus, in our conditions we observed that the switch to an effective hormetic protective phenotype occurs between an incubation time superior to 3 days, and an incubation time inferior or equal to one week. Indeed, seven days of NAC exposure seem to be necessary in order to increase mitochondrial content via mitohormesis. This phenomenon was able to protect cells against statin-induced toxic effects. Moreover, the protection provided by NAC does not occur through its known antioxidant action, but seems more likely due to the triggering of adaptive pathways. Even though NAC pretreatment does not remove totally the deleterious of statins, it enabled to maintain a mitochondrial functionality comparable to the CTL group. Statins are known to alter mitochondrial function via a direct inhibitory mechanism of the mitochondrial respiratory chain, thus decreasing ATP content and increasing ROS production [4]. The improvement of the mitochondrial function observed after NAC pretreatment protected cells against a subsequent mitochondrial stress with statins by diminishing cell death and the triggering of apoptotic pathways. Mitochondrial phenotype appears to be a key factor in the triggering of statins' myopathy, and switching muscle fiber type from glycolytic to oxidative by increasing mitochondrial content could represent a potential therapy to protect muscles from statins' myopathy.

5. Conclusions

We showed that the antioxidant N-acetylcysteine provoked a cellular reductive stress, which led to mitochondrial malfunction. The resulting mitochondrial oxidation leads to the triggering of mitochondrial biogenesis pathways via a mitohormesis mechanism, leading to

an increase of mitochondrial content combined with an improvement of antioxidant capacities in L₆ myoblasts. This process allowed a decrease in oxidative stress and provided protection against apoptosis, as previously described in another model [59]. This improved cell survival after atorvastatin treatment. To our knowledge, this is the first study showing that reductive stress could trigger the mitochondrial hormesis phenomenon.

Moreover, our findings underscore the importance of considering the dynamic events related to both oxidative and reductive stress within cells. Thus, molecules chemically considered as antioxidants might have dramatic effects on oxido-reductive signals and redox-dependant pathways. Mitochondrial content and cellular antioxidant power seem to be determining factors in the appearance of statin-induced myopathy; thus, the activation of this mitohormesis phenomenon represents a potential new therapeutic solution to reduce the toxicity of compounds affecting mitochondrial function. For example, finding a molecule able to increase mitochondrial content in skeletal muscle in vivo could provide a protection against statin-induced myopathy.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Article III

PGC-1 β is required for mitochondrial muscle adaptations in response to statins

Manuscript in preparation

Mitochondrial content appears to be a determining factor in statin tolerance.

Within this article, we will try to provide answers to the following questions: If an increase in mitochondrial content protects cells *in vitro*, what would be the consequences of an impairment in the mitochondrial biogenesis pathways *in vivo*? Would mice be more prone to develop statin myopathy?

PGC-1 β is required for mitochondrial muscle adaptations in response to statins

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Summary

Statins are generally well-tolerated, but can be associated with myopathy. Previous studies have shown that statins are mitochondrial toxicants, are associated with increased ROS production, and induce apoptosis preferentially in glycolytic and less so in oxidative muscles. The current study was performed in order to investigate the role of PGC-1 β in statin-associated myopathy. Wild type (WT) and mice with selective skeletal muscle knockout of PGC-1 β (KO mice) were received normal drinking water (WT-CTL, KO-CTL) or drinking water containing atorvastatin 5mg/kg/day (WT-ATO, KO-ATO) for 2 weeks. Plasma atorvastatin concentrations were in the nanomolar range. Mice treated with atorvastatin maintained their body weight and had reduced grip strength. In WT mice, atorvastatin reduced the fiber area, NADH-dehydrogenase staining in glycolytic gastrocnemius, but not in oxidative vastus muscle. In KO mice, atorvastatin reduced fiber area and NADH dehydrogenase staining in both muscle types. Similarly, oxidative metabolism was impaired and production of H₂O₂ increased by atorvastatin only in glycolytic WT muscle, whereas both glycolytic and oxidative muscle was affected in KO mice. Glycolytic muscle reacted to atorvastatin with an impairment of the mitochondrial proliferation pathway and oxidative defense, whereas stimulation was observed in the oxidative muscle. Similar to the previous observations, lack of PGC-1 β sensitized the oxidative vastus muscle to atorvastatin-associated toxicity. Apoptosis was detectable in WT-ATO glycolytic but not WT-ATO oxidative muscle, whereas both muscle types were affected in KO mice. In conclusion, oxidative muscle is more resistant to atorvastatin-associated toxicity than glycolytic muscle and PGC-1 β has an important role in counteracting mitochondrial toxicants such as atorvastatin.

Keywords: atorvastatin, myopathy, PGC-1 β , apoptosis, reactive oxygen species (ROS), mitochondrial proliferation

Introduction

HMG-CoA reductase inhibitors (statins) are effective drugs in cholesterol lowering with clinically important beneficial effects when used for the prevention of cardiovascular events (Downs et al. 1998). Although they are considered as relatively safe, muscle toxicity is one of the most common adverse event encountered in patients treated with statins, with rates up to 26% (Alfirevic et al. 2014). Muscle toxicity ranges from asymptomatic elevation of creatine kinase (CK) activity, myalgia with or without increased CK activity, up to rhabdomyolysis, which can be life-threatening (Roten et al. 2004). Several mechanisms leading to myopathy have been proposed, among them deregulated Ca^{2+} homeostasis (Galtier et al. 2012; Sirvent et al. 2008), proteolysis due to increased Atrogin-1 expression (Hanai et al. 2007; Bonifacio et al. 2014), decreased protein prenylation (Hanai et al. 2007), oxidative stress (Kwak et al. 2012), decreased coenzyme Q_{10} concentrations (Sirvent et al. 2008) and inhibition of Complex III of the respiratory chain (Schirris et al. 2015). Impairment of mitochondrial function triggering apoptosis appears to be a central factor in many of the proposed mechanisms (Larsen et al. 2013; Bouitbir et al. 2012; Bouitbir et al. 2016; Singh et al. 2015; Kaufmann et al. 2006). Interestingly, long-term treatment with statins has been reported to decrease mitochondrial content in skeletal muscle, underscoring mitochondrial toxicity of statins (Stringer et al. 2013; Schick et al. 2007; Vaughan et al. 2013).

The members of the peroxisome proliferator-activated receptor γ co-activator 1 family (PGC-1 α ; PGC-1 β , and PRC) are considered as key regulators of mitochondrial biogenesis and mitochondrial content (Ljubcic et al. 2010; Lin et al. 2002; Puigserver & Spiegelman 2003). Our previous data showed that statins can protect mitochondria in the highly oxidative cardiac muscle by triggering mitochondrial hormesis via stimulation of PGC-1 β expression in both humans and rats (Bouitbir et al. 2012). On the other hand, in glycolytic skeletal muscle, we observed a repression of mitochondrial proliferation by statins due to impaired expression of PGC-1 α and PGC-1 β .

Moreover, statins have been reported to induce apoptosis in skeletal muscle (Bouitbir et al. 2015; Bonifacio et al. 2015; Kwak et al. 2012). Recently, we showed in human deltoid samples and in rat plantaris that statins can trigger apoptosis in fast glycolytic skeletal muscle, and that this mechanism is ROS dependent (Bouitbir et al. 2015). In comparison, oxidative skeletal muscle such as soleus, which has a high mitochondrial content, was less or not affected by statins (Bouitbir et al. 2015). This is in agreement with experiments in L₆ myoblasts, where stimulation of mitochondrial hormesis by PGC-1 β induction was partially protective against the deleterious effects of statins (Singh et al. 2015). Glycolytic muscle appears to be more sensitive to statins than oxidative muscle and a high mitochondrial content appears to be protective.

While regulation and function of PGC-1 α in skeletal muscle is well established (Fernandez-Marcos & Auwerx 2011), the role of PGC-1 β is less clear and debated in the literature (Zechner et al. 2010; Rowe et al. 2013; Handschin & Spiegelman 2011). Recently, mice with selectively ablated PGC-1 β in skeletal muscle fibers at adulthood (PGC-1 $\beta^{(i)skm/-}$ mice) have been described (Gali Ramamoorthy et al. 2015). Interestingly, PGC-1 β appears not only to coordinate the expression of genes controlling mitochondrial structure and oxidative phosphorylation, but also genes responsible for the anti-oxidative defense system such as SOD2.

Since statins are associated with mitochondrial damage including impaired oxidative phosphorylation and mitochondrial proliferation as well as increased mitochondrial ROS production, we speculated that myotoxicity of statins would be increased in PGC-1 $\beta^{(i)skm/-}$ compared to wild-type mice. Our current study provides evidence that not only antioxidant capacity and mitochondrial content are important factors for the protection from statin-associated myotoxicity, but also the ability to trigger mitochondrial adaptive pathways such as mitochondrial proliferation.

Results and discussion

Lack of PGC-1 β impairs statin tolerance in skeletal muscle

To confirm the validity of our model, we first evaluated the plasma atorvastatin concentration by LC-MS/MS (Fig. 1A). As no differences in the plasma atorvastatin concentration were observed between WT-ATO and KO-ATO groups (data not shown), we pooled the values of these two groups. The plasma concentration reached a mean of 20 nM (range 9 nM to 33 nM) in statin treated animals, which is comparable to what is observed in patient plasma (Björkhem-Bergman et al. 2011). In the plasma of control groups (WT-CTL and KO-CTL), atorvastatin was not detectable (data not shown). Plasma creatine kinase activity is a sign of muscle damage, and can be used for the detection of drug-induced myopathies (Björkhem-Bergman et al. 2011). Atorvastatin treatment increased the plasma creatine kinase activity in both WT and KO mice, reaching statistical significance for KO-ATO compared to KO-CTL mice ($p < 0.01$) (Fig. 1B). This finding indicated muscular alterations associated with atorvastatin, in particular in PGC-1 $\beta^{(i)skm^{-/-}}$ mice. To confirm this, we performed combined forelimb and hindlimb grip strength tests, and observed that atorvastatin treatment decreased mice peak force in both groups compared to their respective control group ($p < 0.05$; Fig. 1C). No differences were found in the body weight at the end of the protocol in any group (Fig. 1D), suggesting that the decreased muscle strength was associated with muscle function and not with muscle atrophy.

To get a better insight of these muscular alterations, we performed a hematoxylin and eosin staining on sections of two metabolically different hindlimb muscles: the superficial gastrocnemius, which is mostly glycolytic, and the vastus intermedius, which is mostly oxidative. We observed a decrease of fiber area in the glycolytic muscle (Fig. 2A) of both WT-ATO ($p < 0.001$) and KO-ATO mice ($p < 0.001$) compared to their respective control group. These results in the WT groups are in line with what has been previously described by us and others (Hanai et al. 2007; Bonifacio et al. 2015), showing a statin-induced muscular atrophy in glycolytic muscle. Hanai et al. showed an increased Atrogin-1 mRNA expression in patients

with statin-induced muscle injury. This increase was also observed in C₂C₁₂ myoblasts at the mRNA and at the protein level (Bonifacio et al. 2015). These authors also showed a decrease in the phosphorylation of FoxO3, which is responsible for the increase in Atrogin-1 expression, and for the induction of an atrophic phenotype. Interestingly, fiber diameter was also decreased in the oxidative vastus intermedius (Fig. 2B) of KO-ATO mice compared to KO-CTL (p< 0.001), but not in the vastus intermedius of WT mice. These results could possibly be explained by the fact that PGC-1 β has been reported to suppress protein degradation by decreasing FoxO3 transcriptional activity (Brault et al. 2010), and that reduced PGC-1 β expression is associated with muscle wasting (Correia et al. 2015).

PGC-1 β ^{(i)skm-/-} mice are known to exhibit muscles with mitochondrial defects, as indicated by a decreased histochemical staining for the activity of mitochondrial Complex I (NADH dehydrogenase) in the glycolytic tibialis anterior muscle (Gali Ramamoorthy et al. 2015). As expected, we observed similar results for the superficial glycolytic gastrocnemius muscle (Fig. 2C; p<0.001). Moreover, treatment with atorvastatin provoked a decrease of NADH dehydrogenase staining in wild-type (p<0.001) and knockout mice (p<0.001) compared to their respective control group. The percentage of stained fibers was also decreased in the oxidative vastus intermedius (Fig.2D) of KO-ATO mice compared to KO-CTL (p< 0.01), but not in the respective WT mice. We also did not observe a significant difference NADH dehydrogenase staining between WT and KO control groups in the oxidative vastus intermedius muscle. Taken together, these results suggested that the myopathy associated with the muscular knock-out of PGC-1 β and with atorvastatin could be due to an impaired function of the mitochondrial respiratory chain.

Mitochondrial function impairments

In order to confirm this hypothesis, we then studied in depth mitochondrial function in the glycolytic superficial gastrocnemius, and in the highly oxidative soleus muscle. Atorvastatin treatment for two weeks impaired the glycolytic muscle mitochondrial function in both WT and

KO mice (Fig. 3A). Although no differences were found in the basal respiratory rates, atorvastatin treatment decreased OXPHOS C_I-linked substrate state O₂ consumption in both WT-ATO ($p < 0.05$) and KO-ATO groups ($p < 0.05$) compared to their respective control group. In addition, maximal OXPHOS respiratory rates (C_{I+II}-linked substrate state) were decreased by atorvastatin in PGC-1 $\beta^{(i)skm^{-/-}}$ ($p < 0.05$), but not in the respective control mice. In comparison, no significant differences were found in the Respiratory Control Ratio (RCR) of the gastrocnemius (Fig 3B). In the oxidative soleus muscle, mitochondrial function was impaired by atorvastatin only in PGC-1 $\beta^{(i)skm^{-/-}}$ mice (Fig. 3C). Basal mitochondrial respiration as well as C_I-linked substrate state ($p < 0.05$), C_{I+II}-linked substrate state ($p < 0.05$), and C_{II}-linked substrate state ($p < 0.05$) were decreased in soleus of KO-ATO compared to KO-CTL mice. No differences were found in the soleus RCR (Fig. 3D).

Maximal OXPHOS respiratory rate depends on convergent electron flow through Complexes I & II to the Q-junction of the electron transport chain (Gnaiger 2009). Biosynthesis of CoQ₁₀ is dependent on mevalonate production within the cell. Hence, statins decrease the biosynthesis of ubiquinone by inhibiting mevalonate production. This decrease in ubiquinone formation could potentially be a plausible explanation for the decrease of mitochondrial oxygen consumption. However, the data regarding the effect of CoQ₁₀ supplementation in patients with statin-associated myopathy are currently inconsistent (Banach et al. 2015). The skeletal muscle CoQ₁₀ content appears to remain high enough in patients treated with statins to maintain the function of the electron transport chain. Interestingly, it has recently been reported that statin-induced myopathy is associated with a direct inhibition of mitochondrial Complex III at the level of the Q_o site (Schirris et al. 2015). ROS generation by Complex III is also known to take place at the level of the Q_o ubiquinone site (Liu et al. 2002). Additionally, in that case Complex II can deliver to ubiquinone a higher quantity of electrons than Complex III can transport, inducing an over-oxidation of ubiquinone. This could lead to a reverse electron flux towards Complex I (Favier et al. 2005), putting Complex I in a reduced state, and promoting

the production of reactive oxygen species (ROS). Hence, both Complex I and III are potential ROS production sites involved in statin myotoxicity.

If the above-mentioned hypothesis is correct, the observed impairment in mitochondrial function should be associated with increased mitochondrial H₂O₂ production. This was indeed the case in the glycolytic gastrocnemius (Fig. 3E), in both WT-ATO ($p < 0.05$) and KO-ATO groups ($p < 0.05$) compared to their respective control group. In the oxidative soleus, H₂O₂ production was increased in KO-ATO mice compared to KO-CTL ($p < 0.01$), but not in WT mice. These measurements enabled us to calculate the Free Radical Leak (FRL), which was increased in the KO-ATO group compared to KO-CTL ($p < 0.05$) in the superficial gastrocnemius muscle (Fig. 3F). The cellular reduced glutathione (GSH) pool, an important constituent of the antioxidative defense system, showed a tendency to decrease in gastrocnemius of WT-ATO and KO-ATO mice as well as in vastus intermedius of KO-ATO mice compared to the respective control groups, but without reaching statistical significance (Fig. 3G). Altogether, these results are in line with data published by us and others (Bouitbir et al. 2015; Kwak et al. 2012). Indeed, Kwak et. al. observed an increase in mitochondrial ROS production in primary human myotubes treated with simvastatin due to inhibition of the mitochondrial respiratory chain. In support of these findings, in L₆ myoblasts treated with atorvastatin, we demonstrated inhibition of the mitochondrial respiratory chain and simultaneous mitochondrial ROS production in a concentration-dependent manner (Bouitbir et al. 2015). In addition, we observed an increased ROS production in both human deltoid biopsies and in the glycolytic skeletal muscle in rats.

Recently, PGC-1 β has been shown to control mitochondrial antioxidant defense in skeletal muscles via stimulation of SOD2 expression (Gali Ramamoorthy et al. 2015). The observed increase in ROS production in the presence of atorvastatin in wild type and knock-out gastrocnemius and in knock-out soleus muscle could therefore possibly be explained by the impairment of PGC-1 β (and SOD2) expression by atorvastatin.

Mitochondrial adaptive pathways are necessary for statin tolerance

Statin-induced mitochondrial oxidative stress has been reported to have opposite effects on mitochondrial biogenesis in glycolytic skeletal muscles, and in the highly oxidative heart muscle, with mitochondrial biogenesis being stimulated in heart (Bouitbir et al. 2012). The mechanism involved has been termed mitochondrial hormesis, which proposes that low doses of mitochondrial ROS can trigger mitochondrial biogenesis and thereby counteract oxidative stress and re-establish homeostasis (Singh et al. 2015; Gems & Partridge 2008; Sano & Fukuda 2008; Schulz et al. 2007; Cornelius et al. 2013). As we observed an impaired mitochondrial function in glycolytic and oxidative muscle of KO mice, but only in WT glycolytic muscle, mitochondria appear to adapt better to the toxic stress associated with statins in oxidative than in glycolytic muscle and PGC-1 β appears to be important for this adaptation. In order to confirm these assumptions, mitochondrial biogenesis activation/repression was evaluated. In the glycolytic gastrocnemius muscle (Fig. 4A), we observed an impairment of the mitochondrial biogenesis pathway in WT-ATO mice compared to WT-CTL with a significant decrease of PGC-1 α ($p < 0.05$), and PGC-1 β ($p < 0.05$) mRNA expression levels, whereas the decrease in the mRNA levels of NRF1, TFAM and NRF2 were not significant. A similar impairment was detected in KO-ATO compared to KO-CTL mice, with a decrease of PGC-1 β ($p < 0.05$), NRF2 ($p < 0.05$), and TFAM ($p < 0.05$) mRNA expression, whereas the decreases in PGC-1 α and NRF1 mRNA expression were not significant. As a consequence of impaired TFAM expression, cytosolic SOD1 ($p < 0.01$) and mitochondrial SOD2 mRNA expression were also reduced in KO-ATO mice. In contrast, in the oxidative vastus intermedius muscle (Fig. 4B), we observed an activation of mitochondrial biogenesis pathways, with an increased expression of PGC-1 α ($p < 0.05$), NRF1 ($p < 0.05$), NRF2 ($p < 0.05$), TFAM ($p < 0.05$), and SOD2 ($p < 0.05$) in WT-ATO compared to WT-CTL mice. On the contrary, in the KO-ATO group, we observed a global repression of the mitochondrial biogenesis pathway, with a decrease of the mRNA expression levels of PGC-1 α ($p < 0.05$), PGC-1 β ($p < 0.05$), TFAM ($p < 0.05$), and SOD2 ($p < 0.05$) compared to KO-CTL mice.

The study of mitochondrial adaptations by western blotting grossly confirmed what we observed at the mRNA level. In the gastrocnemius (Fig. 4C&E), we observed in WT-ATO mice a decrease in mitochondrial biogenesis-linked proteins, such as of PGC-1 β ($p<0.05$) and of TFAM ($p<0.01$) compared to WT-CTL. This repression of mitochondrial biogenesis was confirmed with a trend for a decrease in the expression of TOMM20 and Cytochrome b, and a significant decrease in the mtDNA content ($p<0.05$; Fig. 4F). In KO-ATO mice, although we did not observe a decrease in mitochondrial biogenesis-linked proteins, we observed a decrease in TOMM20 expression ($p<0.05$), and a decrease in the mtDNA content ($p<0.05$).

On the other hand, in the vastus intermedius (Fig. 4D&E), we observed in WT-ATO an activation of the mitochondrial biogenesis pathway with an increase in PGC-1 β ($p<0.05$). This was confirmed with the increase in TOMM20 expression ($p<0.05$), Cytochrome b ($p<0.01$) and an increase in the mtDNA content ($p<0.01$; Fig. 4F). In KO-ATO mice, although we did not observe a decrease in mitochondrial biogenesis-linked proteins, we observed a decrease in Cytochrome b protein expression ($p<0.05$), and in the mtDNA content ($p<0.05$).

Although it had previously been stated that physiological levels of ROS do not induce PGC-1 β expression in skeletal muscle (Gali Ramamoorthy et al. 2015), our results show that this can be so at mild ROS levels induced by atorvastatin in oxidative muscle in WT mice. Taken together, these results show that statins have opposite effects on mitochondria of glycolytic skeletal and oxidative skeletal muscle and that the reaction of mitochondria to atorvastatin is influenced by the expression of PGC-1 β . It has been shown that skeletal muscle mitochondrial DNA is decreased in patients with statin-induced myopathy (Stringer et al. 2013). Our results in glycolytic muscle (but not in oxidative muscle of WT mice) are in line with these observations. We indeed observed in glycolytic muscles a repression of the mitochondrial biogenesis, which leads to a decreased mitochondrial content in both WT and KO mice. On the other hand, we observed a triggering of the mitochondrial biogenesis pathway in oxidative skeletal muscle of wild type mice, leading to an increased mitochondrial content, which may be due to mitochondrial hormesis. Interestingly, oxidative muscle from PGC-1 β deficient mice reacted

similar to glycolytic muscle to the toxic insult of atorvastatin with an impairment of the mitochondrial biogenesis pathway and a drop in the mtDNA content. Our study therefore demonstrates that PGC-1 β has an important role in triggering mitochondrial proliferation and antioxidative defense in oxidative muscle in the presence of a mitochondrial toxicant such as atorvastatin.

A western blot of caspase 3 (Fig. 5A) revealed in the glycolytic gastrocnemius a 2-fold increase in the cleaved caspase 3/total caspase 3 ratio in WT-ATO ($p=0.05$) compared to WT-CTL, and a 2.2-fold increase for KO-ATO ($p<0.01$) compared to KO-CTL. In the oxidative vastus intermedius, no differences were observed between WT-CTL and WT-ATO, but this ratio increased 2.2-fold for KO-ATO ($p<0.05$) compared to KO-CTL. In WT mice, TUNEL staining data showed, as previously observed (Bouitbir et al. 2015), that statins increased the proportion of myonuclei positive for DNA strand breaks in the glycolytic skeletal muscle (Figure 5B&D), whereas the oxidative skeletal muscle was preserved (Figure 5C&D). In contrast to these observations, in KO mice treated with atorvastatin, both glycolytic and oxidative-type skeletal muscles displayed a higher percentage of apoptotic nuclei. These observations support our previous notion that glycolytic muscle is more sensitive to the toxic insults of statins than oxidative muscle, and that loss of PGC-1 β renders oxidative muscle as sensitive as glycolytic muscle towards statin-associated myotoxicity.

We previously showed that statins had opposite effects on mitochondria of cardiac and skeletal muscle (Bouitbir et al. 2012). Indeed, we observed that statins had deleterious effects on skeletal muscle and beneficial effects on the cardiac muscle by triggering the mitochondrial hormesis phenomenon. In a more recent publication, we showed that statins can trigger mitochondrial apoptotic pathways in glycolytic skeletal muscle, whereas oxidative skeletal muscle was resistant (Bouitbir et al. 2015). Moreover, we recently showed in vitro that increasing the mitochondrial content by triggering mitochondrial hormesis could protect L₆ myoblasts from the deleterious effects of statins (Singh et al. 2015). In the present study, we confirm that the mitochondrial content is an important factor in preventing statin associated

toxicity. In addition, we show the importance of the ability to adapt the mitochondrial biogenesis pathways to counteract stain-associated toxicity in a long-term setting. These data underscore the importance of mitochondrial biogenesis for statin tolerance, and define a hinge role of PGC-1 β in mitochondrial adaptation to oxidative stress.

Experimental procedures

Animals

Experiments were performed on adult mice (18-23 weeks at the beginning of the study; ~30-35g). PGC-1 $\beta^{(i)skm^{-/-}}$ mice, in which PGC-1 β is selectively ablated in skeletal muscle myofibers at adulthood (Gali Ramamoorthy et al. 2015), were generously provided by D. Metzger. Generation of the PGC-1 $\beta^{(i)skm^{-/-}}$ mice has been described elsewhere (Gali Ramamoorthy et al. 2015). Animals were housed in neutral temperature environment ($22^{\circ} \pm 2^{\circ}\text{C}$) on a 12:12 hour photoperiod and were provided food and water ad libitum. Breeding and maintenance of mice were performed in the accredited IGBMC/ICS animal house (A67-218-37 notification of 16/10/2013), in compliance with French and EU regulations on the use of laboratory animals for research, under the supervision of D. M. who holds animal experimentation authorizations from the French Ministry of agriculture and Fisheries (N°67-209 and A 67-227). All animal experiments were approved by the Ethical committee Com'Eth (Comité d'Ethique pour l'Expérimentation Animale, Strasbourg, France). Atorvastatin (Tahor®) was generously provided by Pfizer.

43 mice were randomly divided in 4 groups as follow: (1) control animals (WT-CTL, n=13); (2) animals treated with atorvastatin 5mg/kg/day in drinking water during two weeks (WT-ATO, n=11); (3) PGC-1 $\beta^{(i)skm^{-/-}}$ mice (KO-CTL, n=8); (4) PGC-1 $\beta^{(i)skm^{-/-}}$ treated with atorvastatin 5mg/kg/day in drinking water during two weeks (KO-ATO, n=11).

Sample collection

Animals were killed by cervical dislocation and tissues were immediately collected, weighed, and a part of each muscle tissue was immediately frozen in isopentane, cooled by liquid nitrogen and stored at -80°C for later analysis, or processed for biochemical and histological analysis. The oxidative soleus muscle and the glycolytic superficial part of the gastrocnemius muscle were excised and cleaned of adipose and connective tissues. We conducted our experiments on different muscles, separating them by muscle type. Muscles are classified by mitochondrial mass: we used the superficial part of the gastrocnemius muscle, which is mostly glycolytic, with a low mitochondrial content. For the oxidative-type muscles with a high mitochondrial content, we studied the soleus muscle, and the vastus intermedius: the mostly oxidative part of the quadriceps, which is located close to the femur bone.

Blood parameters

Blood was collected by cardiac puncture immediately after death in ethylenediaminetetraacetic acid (EDTA)-rinsed tubes and then centrifuged at 3400 rpm for 15 min at 4°C. The plasma was separated and stored at -80°C until analysis. The analysis of plasma biochemical parameters was performed on randomly selected plasma samples.

Plasmatic creatine kinase activity was determined using the Creatine Kinase Activity Assay Kit from Sigma-Aldrich (MAK116; St Louis, USA), according to the manufacturer's instructions.

Pharmacokinetic analysis of atorvastatin

Atorvastatin was analysed on an UHPLC system (Shimadzu, Kyoto, Japan) connected to an API 5500 tandem mass spectrometer (AB Sciex, Ontario, Canada). The UHPLC system consisted of four LC-30AD pumps, a SIL-30AC autosampler, a CTO-20AC column oven, a DGU-20A5 degassing unit, and a CBM-20A controller. Chromatography was performed on a kinetex 2.6 μ F5 100 Å (50x2.1 mm) analytical column (Phenomenex, Torrance, USA). Mobile phase A consisted of water plus 0.1% formic acid, while mobile phase B was methanol

supplemented with 0.1% formic acid. The following gradient program of mobile phase B was applied: 40% (0-0.25 min), 95% (0.25-1.5 min), 95% (1.5-2.0 min), 40% (2.0-2.25 min). The flow rate was set at 0.4 mL/min at 40°C. The introduced sample was pre-column diluted with water 0.1% formic acid during the first 0.25 min of each run. Atorvastatin eluted after 1.7 minutes, hence the UHPLC was only connected from minute 1-2 with the mass spectrometer to avoid superfluous contamination of the system. Atorvastatin and atorvastatin-d5 were detected by multiple reaction monitoring using electrospray ionization in the positive mode. A mass transition of 559.1→440.1 m/z and 564.1→445.1 was used for atorvastatin and atorvastatin-d5, respectively (dwell time: 25 msec, declustering potential: 126 V, entrance potential: 10 V, collision energy: 33 V, cell exit potential: 30 V). The mass spectrometer was operated at an ion source gas 1 of 45 l/min (N₂), an ion source gas 2 of 60 l/min (N₂), a curtain gas of 30 l/min, a collision gas set to medium (N₂), an ion spray voltage of 4500 V, and a source temperature of 350°C. Analyst software 1.6.2 (AB Sciex, Ontario, Canada) was used to control the LC-MS/MS system.

Calibration lines were prepared in blank mouse plasma. Thereby, blank plasma was spiked with atorvastatin at a final concentration of 2242 nM and serially diluted to 4.4 nM (≤1% DMSO). Plasma aliquots of 10 µL were precipitated with 150 µL methanol 0.1% formic acid containing the internal standard (atorvastatin-d5: 25 nM). Samples were mixed with a VX-2500 multi-tube vortexer (VWR, Dietikon, Switzerland) for about 1 min and centrifuged at 4000 rpm during 30 min (Eppendorf 5810R, Hamburg, Germany). 10 µL supernatant were injected into the LC-MS/MS system.

Grip strength Test

A Grip Strength Meter (Bioseb, Vitrolles, France) was used to measure combined forelimb and hindlimb grip strength. The test was repeated three consecutive times within the same session, and the mean value was recorded as the maximal grip strength for each mouse. All tests were performed by the same operator.

Histology

The analysis of all histological parameters was performed on randomly selected slides samples.

Hematoxylin & Eosin staining was performed on 10 µm frozen sections of 3-4 samples per group on the glycolytic muscle gastrocnemius, and the more oxidative type vastus intermedius muscle. Hematoxylin & Eosin staining photographs were captured on an Olympus IX83 microscope (Olympus, Hamburg, Germany). Evaluation and interpretation of the results were realized according to the MDC1A_M.1.2.004 SOP. Fiber area was determined using the cellSens Dimension software (Olympus, Hamburg, Germany) on at least 15 fibers per picture, and the mean of three independent pictures was calculated for each sample. Measurements were made on pictures obtained with a 20X objective. Illustrations provided in the figures have been obtained with a 40X objective. Presence of central nuclei was confirmed by color deconvolution using the ImageJ software (National Institute of Health) in order to exclude staining artefacts.

NADH diaphorase staining was performed on frozen sections of 3-4 samples per group, on the glycolytic muscle gastrocnemius, and the more oxidative type vastus intermedius muscle. NADH staining photographs were captured on an Olympus IX83 microscope (Olympus, Hamburg, Germany, 10X and 20X objectives). Determination of the percentage of oxidative-type fibers was performed by manual counting on pictures obtained with a 10X objective. Illustrations provided in the figures have been obtained with a 20X objective.

Study of muscle mitochondrial respiration

This technique ensured determination of global mitochondrial function, reflecting both the density as well as the functional properties of the muscle mitochondria (Veksler et al. 1987). The mitochondrial respiration was studied from saponin-skinned fibers that keeping mitochondria in their architectural environment, in superficial gastrocnemius, and soleus muscles. The analysis took place in a thermostated oxygraphic chamber at 37°C with

continuous stirring (Oxygraph-2k, Oroboros instruments, Innsbruck, Austria). Approximately 2 mg of fibers were placed in respiration medium (2.77 mM CaK₂EGTA, 7.23 mM K₂EGTA, 6.56 mM MgCl₂, 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 50 mM K-methane sulfonate, 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/mL of Bovine Serum Albumin [BSA]; pH=7) in the oxygraphic chamber. After the determination of the basal oxygen consumption with glutamate (5 mM) and malate (2 mM) (Basal), OXPHOS CI-linked substrate state was measured in the presence of saturating amount of adenosine diphosphate (2 mM ADP). When OXPHOS CI-linked substrate state was recorded, electron flow went through Complexes I, III, and IV. The maximal OXPHOS respiration rate CI+II-linked substrate state was then measured by adding succinate (25 mM). Complex I was blocked with rotenone (0.5 μM), allowing to measure OXPHOS CII-linked substrate state. Respiratory rates were expressed as pmol O₂ x s⁻¹ x mg⁻¹ wet weight. Respiratory Acceptor Control Ratio (RCR) was determined by calculating State 3/State 4 respiratory rates.

Mitochondrial H₂O₂ production in permeabilized fibers

H₂O₂ production was studied from saponin-skinned fibers that keep mitochondria in their architectural environment, in superficial gastrocnemius, and soleus muscles. The permeabilized bundles were placed in ice-cold buffer Z containing 110 mM K-methane sulfonate, 35 mM KCl, 1 mM EGTA, 5 mM K₂HPO₄, 3 mM MgCl₂, 6 mM H₂O, 0.05 mM glutamate, and 0.02 mM malate with 0.5 mg/ml BSA (pH 7.1, 295 mOsmol/kg H₂O). H₂O₂ production was measured with Amplex Red (Invitrogen Life Technologies, Rockville, MD, USA), which reacted with H₂O₂ in a 1:1 stoichiometry catalyzed by HRP (horseradish peroxidase; Invitrogen Life Technologies, Rockville, MD, USA) to yield the fluorescent compound resorufin and a molar equivalent of O₂ (Anderson & Neuffer 2006). Resorufin has excitation and emission wavelengths of 563 nm and 587 nm, respectively, and is extremely stable once formed. Fluorescence was measured continuously with a Fluoromax 3 (Jobin Yvon) spectrofluorometer with temperature control and magnetic stirring. After a baseline (reactants only) was established, the reaction was initiated by adding a permeabilized fiber

bundle to 600 μL of buffer Z. Buffer Z contained 5 mM Amplex Red, 0.5 U/mL HRP, 5 mM glutamate, and 2 mM malate as substrates at 37°C. Succinate (25 mM) was then added for the measurement of H_2O_2 production under reverse electron flux condition. ADP (2mM) was added in order to determine CI+II-linked substrate state H_2O_2 production for the determination of the Free Radical Leak (FRL) At the conclusion of each experiment. The results were reported in $\text{pmol H}_2\text{O}_2 \times \text{s}^{-1} \times \text{mg}^{-1}$ wet weight.

Free Radical Leak

H_2O_2 production and O_2 consumption were measured in parallel under similar experimental conditions (CI+II-linked substrate state). This allowed the calculation of the fraction of electrons out of sequence which reduce O_2 to ROS in the respiratory chain (the percentage of free radical leak) instead of reaching cytochrome oxidase to reduce O_2 to water (Anderson & Neuffer 2006). Because two electrons are needed to reduce one mole of O_2 to H_2O_2 , whereas four electrons are transferred in the reduction of one mole of O_2 to water, the percent of FRL was calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption, and the result was multiplied by 100.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was obtained from superficial gastrocnemius and the vastus intermedius muscles of six random samples per group, using the RNeasy Fibrous Tissue Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA was stored at -80°C until the reverse transcription reaction was performed. cDNA was synthesized from 1 μg total RNA with the Omniscript RT kit (QIAGEN GmbH, Hilden, Germany). To perform the real-time PCR reaction, cDNA was mixed with each primer (sense and antisense (0.3 μM final concentration), SYBR Green (Roche Diagnostics, Mannheim, Germany) as a fluorescent dye and H_2O . The real-time PCR measurement of individual cDNAs was performed in triplicate using SYBR Green dye to measure duplex DNA formation with the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The primers sequences were designed using

information contained in the public database GenBank of the National Center for Biotechnology Information (NCBI). The sequences of the primer sets used are listed in Table 1. Quantification of gene expression was performed by the method described in (Liu & Saint 2002), using the 18S gene as the internal control. The amplification efficiency of each sample was calculated as described by Ramakers et al. (Ramakers et al. 2003).

GSH content determination

GSH content was determined in superficial gastrocnemius and the vastus intermedius muscles using the GSH-Glo Glutathione Assay kit from Promega, following the manufacturer's instructions.

Western blotting

Approximately 20 mg of rat skeletal muscle (superficial gastrocnemius and vastus intermedius) was homogenized with a microdismembrator for 1 min at 2000 rpm (Sartorius Stedim Biotech, Aubagne, France). Muscle homogenates were then lysed on ice for 15 min with (100 µl per mg tissue) of RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 50mM NaF, 2mM EDTA, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, and Complete Mini protease inhibitor [Roche, Basel, Switzerland]). After lysis, the mixture was vortexed and centrifuged for 10 min at 4°C at 10,000 rpm. The supernatant was collected and the protein concentration determined using the Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). For each sample, 9 µg of protein was separated on a NuPAGE 4–12% Bis-Tris gel (Life technologies, Rockville, MD, USA). Proteins were electroblotted to PVDF membranes (Bio-Rad), and immunodetected using primary antibodies directed against PGC-1α (KP9803, calbiochem, 1/1000), PGC-1β (sc-373771, santa cruz, 1/1000), TFAM (ab131607, abcam, 1/1000), TOMM20 (ab78547, abcam, 1/1000), Cytochrome b (sc-11436, santa cruz, 1/1000), full and cleaved Caspase 3 (#9665S, cell signaling, 1/500), and Beta Actin (sc-130656, santa cruz, 1/1000). Membranes were probed with secondary antibodies conjugated to HRP directed against goat (sc-2020, santa cruz, 1/2000), rabbit (sc-2004, santa cruz, 1/2000), and mouse

(sc-2055, santa cruz, 1/2000). Membranes were revealed with a chemiluminescent substrate (Clarity Western ECL substrate; Bio-Rad Laboratories, Hercules, CA, USA), and quantification was performed using the ImageJ software (National Institute of Health). Images were modified with ImageJ to remove background.

mtDNA content

DNA was isolated from gastrocnemius and vastus intermedius muscles of six random samples per group, using the DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA in samples was quantified spectrophotometrically at 260 nm with a NanoDrop 2000 (Thermo scientific, Waltham, USA). The DNA was subjected to real-time PCR in triplicate (10ng/ μ L). Relative amounts of nuclear and mitochondrial DNA were determined by comparison of amplification kinetics of Hexokinase 2 (Hk2) and COX2 (Primer sequences in Table 1, as described by (Gariani et al. 2015)). mtDNA content was estimated by calculating the inverse of the CT_{COX2}/CT_{Hk2} ratio.

TUNEL assessment of apoptosis

TUNEL staining of myonuclei positive for DNA strand breaks was performed using the Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay kit (ThermoFisher Scientific, Waltham, MA, USA) on randomly selected slides samples. Cross-sections (10 μ m) of 3 samples per group of the glycolytic muscle gastrocnemius, and the more oxidative type vastus intermedius muscle cut with a cryostat microtome were fixed with 4% paraformaldehyde for 15 min and permeabilized with 2 mg/ml proteinase K. The TUNEL reaction mixture containing terminal deoxynucleotidyltransferase (TdT) and fluorescein-labeled dUTP was added to the sections in portions of 100 μ l and then incubated for 60 min at 37°C in a humidified chamber in the dark. Section were then washed with 3% BSA and 0.1% Triton X 100 in PBS for 5 minutes, and incubated for 30 minutes at 37°C protected from light with the Click-iT® Plus TUNEL reaction cocktail. After incubation, the sections were rinsed with PBS. Following embedding with ProLong diamond antifade mountant with DAPI (Life Technologies), the sections were

investigated with a fluorescence microscope (x40 objective; Olympus IX83). Three to five pictures were acquired per sample. After acquisition, background noise was removed using the ImageJ software (National Institutes of Health). Apoptotic index was calculated by counting the number of TUNEL-stained nuclei divided by the total number of nuclei multiplied by 100.

Statistics

Data are represented as means \pm SEM. Statistical analyses were performed using unpaired t test or 2-way ANOVA followed by a Bonferroni's post-test between atorvastatin treated mice in comparison to their respective controls (GraphPad Prism 5, Graph Pad Software, Inc., San Diego, CA, USA). Statistical significance was displayed as * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$.

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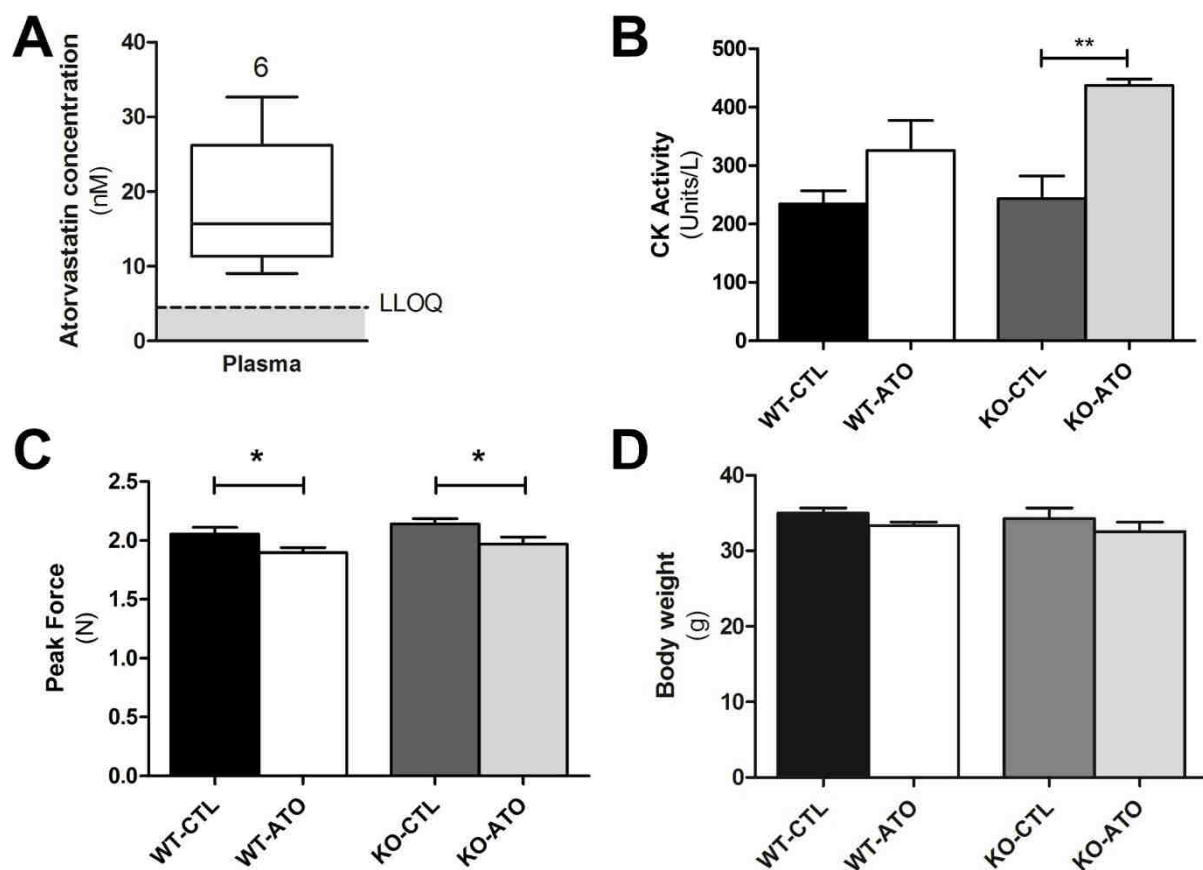


Figure 1: Atorvastatin treatment impaired skeletal muscle physiology. (A) Plasmatic atorvastatin concentration. The upper and lower limits of the boxes correspond to the interquartile range and the middle line is the median of the data set. The limits of the whiskers are the minimum and maximum values. Linear regression through the mean value of the boxes was established. (B) Plasmatic creatine kinase activity. (C) Forelimb and hindlimb peak force. (D) Mice body weight at the end of the study. (B-D) Results are expressed as mean ±SEM; *p<0.05, **p<0.01.

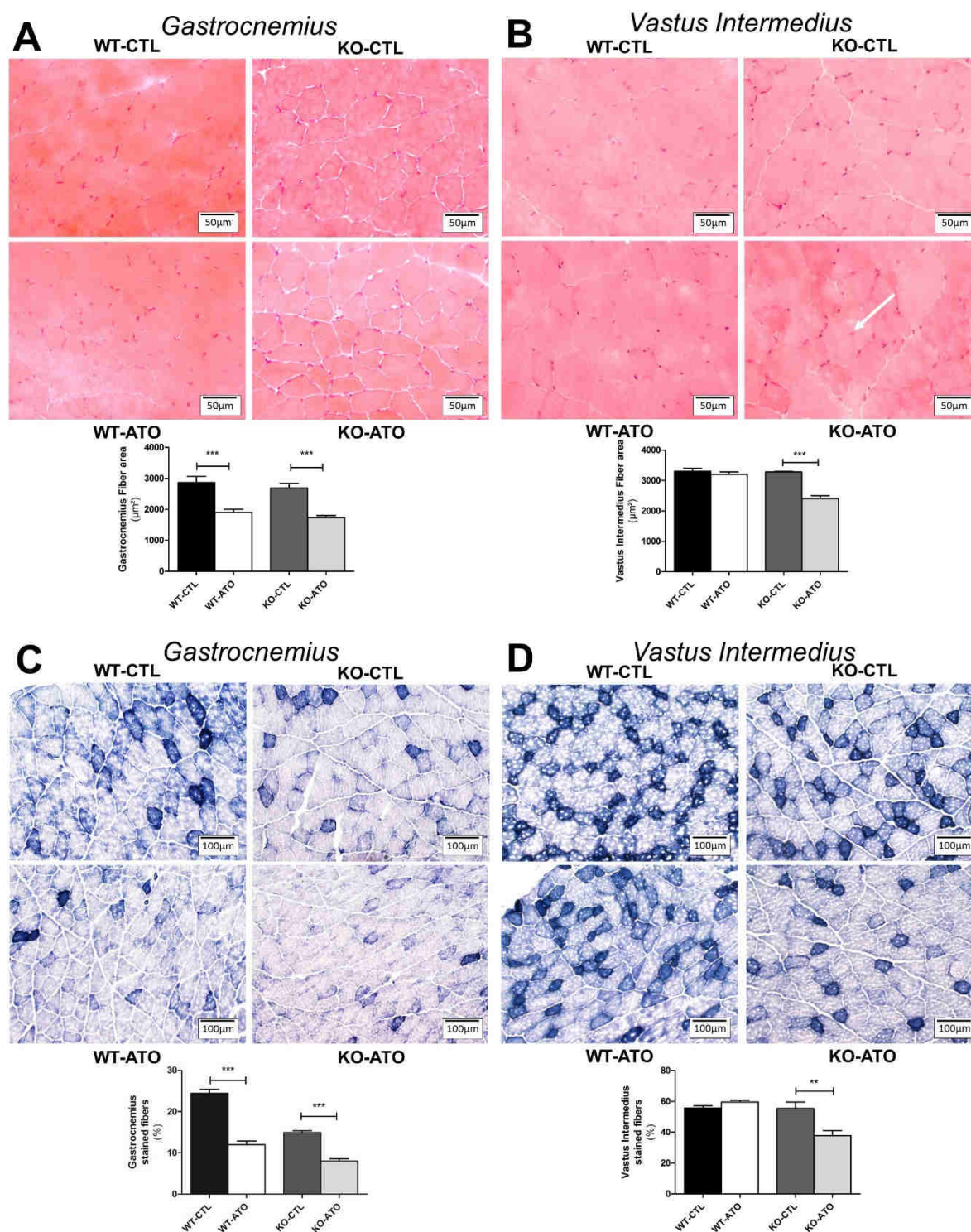


Figure 2: Atorvastatin treatment impaired muscle structure and phenotype. (A) Hematoxylin and eosin staining of superficial gastrocnemius muscle sections, and fiber area quantification. **(B)** Hematoxylin and eosin staining of vastus intermedius muscle sections, and fiber area quantification. White arrow indicates myofiber with central nuclei. **(C)** NADH diaphorase staining of superficial gastrocnemius muscle sections, and stained fibers percentage. **(D)** NADH diaphorase staining of vastus intermedius muscle sections, and stained fibers percentage. Results are expressed as mean \pm SEM; ** $p < 0.01$, *** $p < 0.001$.

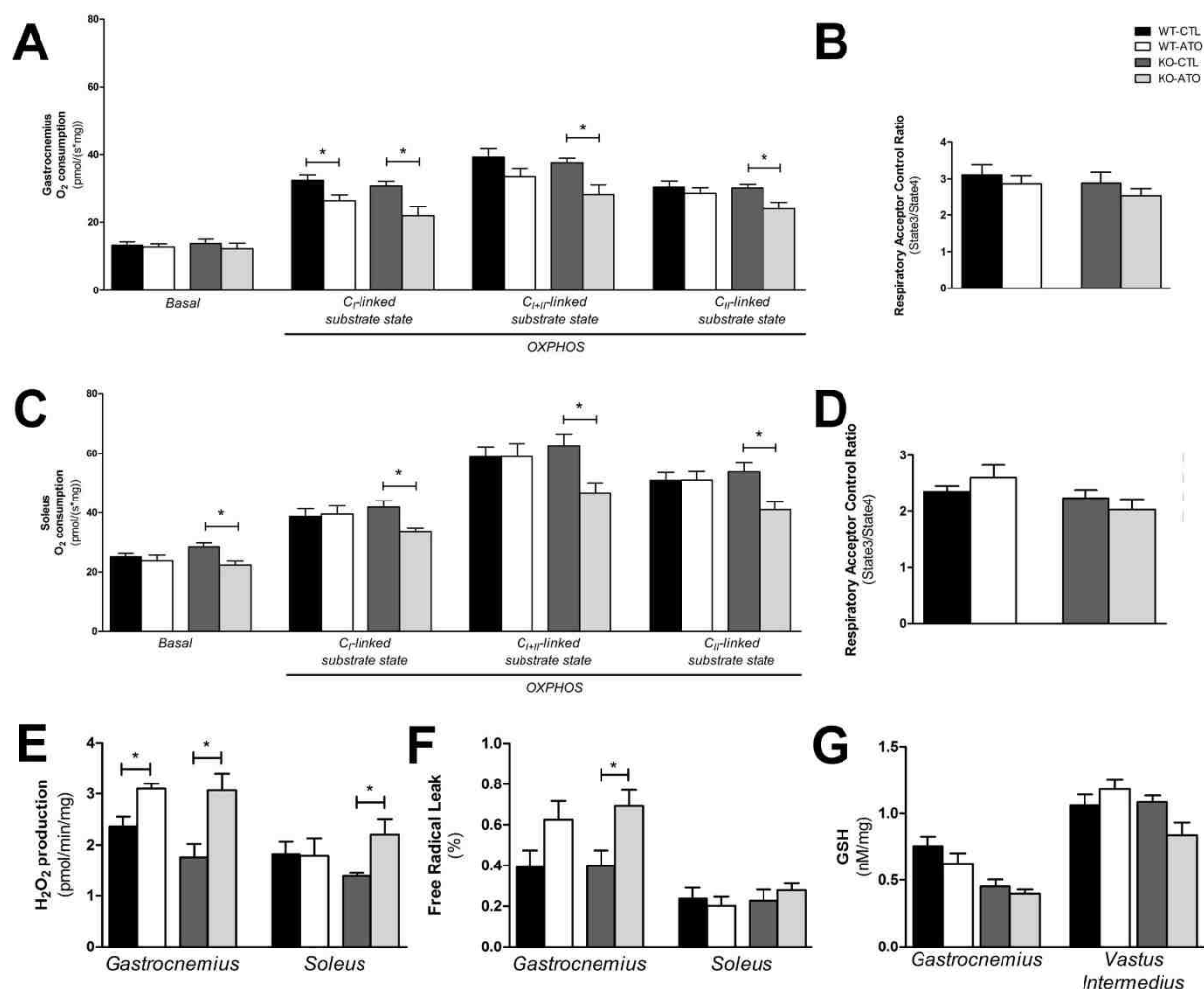


Figure 3: Atorvastatin treatment impaired mitochondrial respiratory chain and enhanced ROS production. (A) Mitochondrial oxidative capacities of superficial gastrocnemius muscle. **(B)** Superficial gastrocnemius muscle mitochondrial respiratory ratios. **(C)** Mitochondrial oxidative capacities of soleus muscle **(D)** Soleus muscle mitochondrial respiratory ratios. **(E)** Mitochondrial H₂O₂ production in superficial gastrocnemius and soleus muscles. **(F)** Mitochondrial free radical leak in superficial gastrocnemius and soleus muscles. **(G)** Reduced glutathione contents in superficial gastrocnemius and vastus intermedius muscles. Results are expressed as mean ±SEM; *p<0.05.

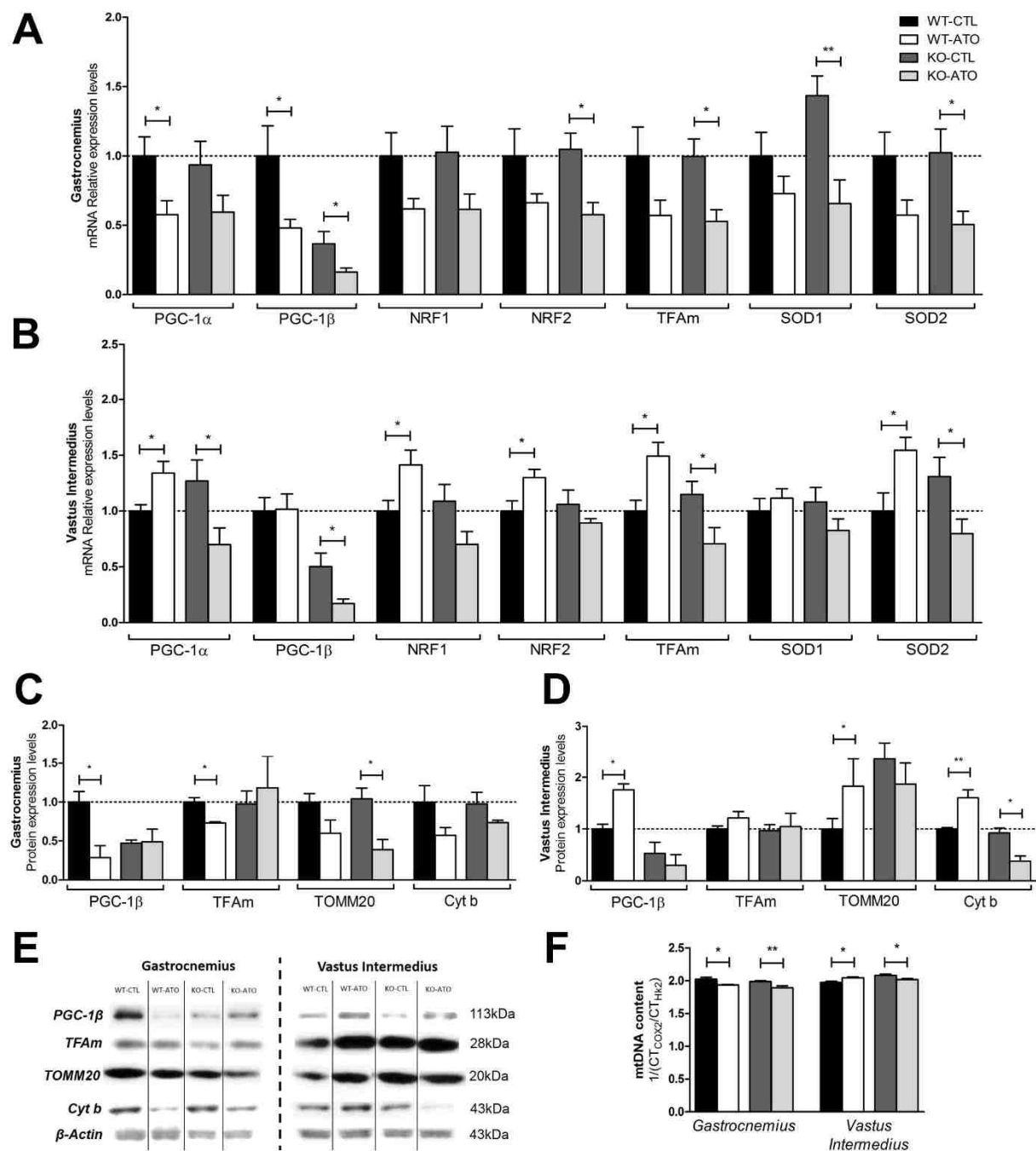


Figure 4: Opposite effects of atorvastatin treatment on mitochondrial biogenesis pathways in superficial gastrocnemius, and in vastus intermedius muscles. (A) mRNA transcript levels in superficial gastrocnemius muscle, **(B)** and in vastus intermedius muscles. **(A-B)** Values are relative to 18s expression. **(C)** muscle protein expression levels in superficial gastrocnemius, **(D)** and in vastus intermedius muscles. **(C-D)** Values are relative to β -Actin expression. **(E)** Immunoblots of PGC-1 β , TFAM, TOMM20, Cytochrome b, and β -Actin in superficial gastrocnemius and vastus intermedius muscles. **(F)** Mitochondrial DNA quantification in superficial gastrocnemius and vastus intermedius muscles. Results are expressed as mean \pm SEM; * p <0.05, ** p <0.01.

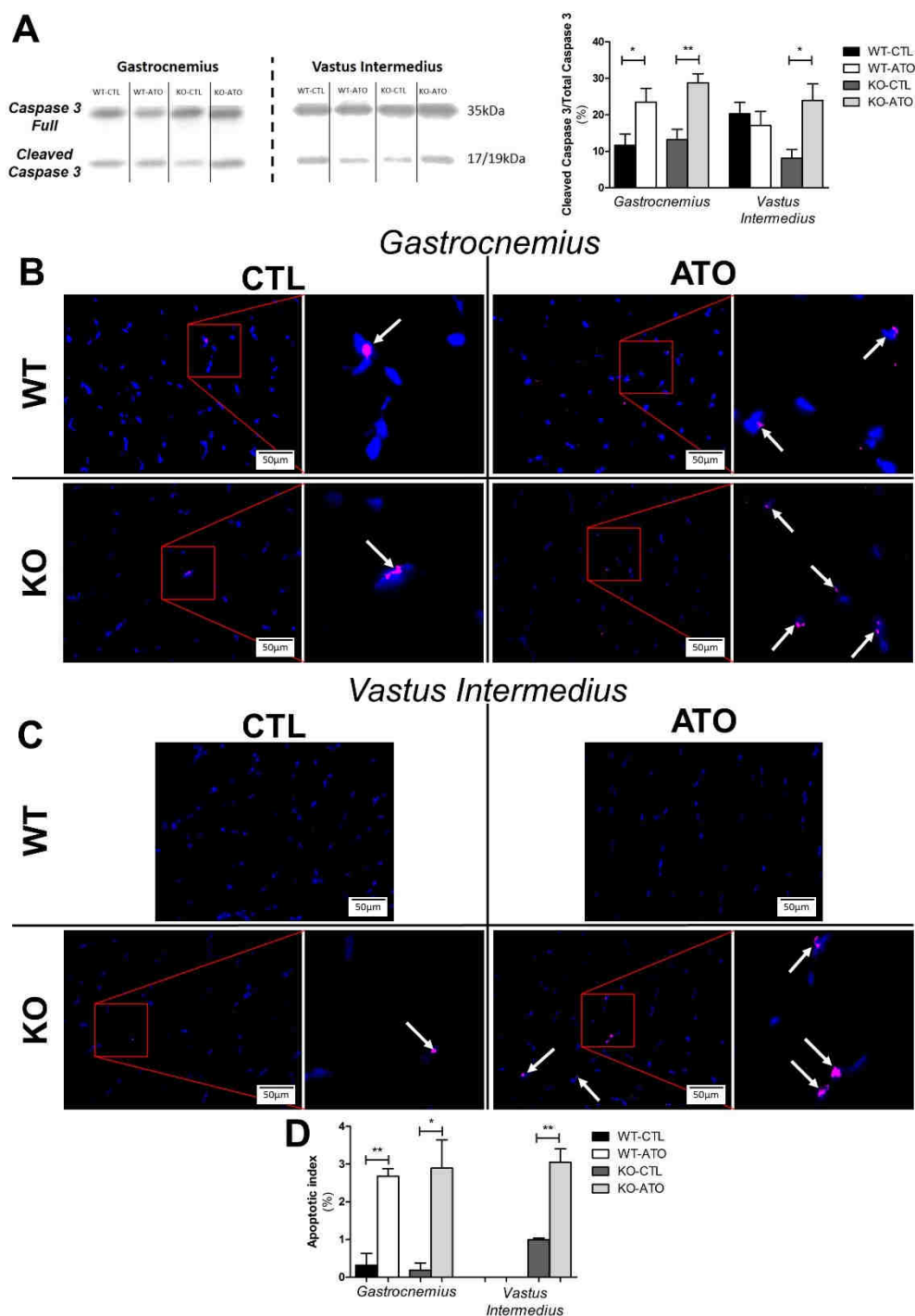


Figure 5: Atorvastatin treatment provoked the triggering of apoptotic pathways in the glycolytic skeletal muscle and in the oxidative skeletal muscle of PGC-1 β deficient mice. (A) Immunoblots of caspase 3 and cleaved caspase 3 in superficial gastrocnemius and in vastus intermedius muscles, and cleaved caspase 3 expression relative to caspase 3 expression. **(B-C)** Nuclei were stained with DAPI shown in *blue*, and TUNEL nuclei were visualized with *magenta* fluorescein in superficial gastrocnemius, and vastus intermedius muscles. Merged DAPI and TUNEL nuclei from different groups are shown and *white arrows* indicate apoptotic nuclei. Adjacent pictures correspond to a magnification of the *red square* area. **(B)** Merged TUNEL images of superficial gastrocnemius muscle sections. **(C)** Merged TUNEL images of superficial gastrocnemius muscle sections. **(D)** Apoptotic index calculated from TUNEL staining of superficial gastrocnemius and vastus intermedius muscles. Results are expressed as mean \pm SEM; * p <0.05, ** p <0.01.

Gene	Organism	Forward primer	Reverse primer
COX2	Mouse	GTT GAT AAC CGA GTC GTT CTG C	CCT GGG ATG GCA TCA GTT TT
Hk2	Mouse	GCC AGC CTC TCC TGA TTT TAG TGT	GGG AAC ACA AAA GAC CTC TTC TGG
PGC-1 α	Mouse	AAT GCA GCG GTC TTA GCA CT	ACG TCT TTG TGG CTT TTG CT
NRF1	Mouse	TTA CTC TGC TGT GGC TGA TGG	CCT CTG ATG CTT GCG TCG TCT
NRF2	Mouse	CGA GAT ATA CGC AGG AGA GGT AAG A	GCT CGA CAA TGT TCT CCA GCT T
TFAm	Mouse	GCT GAT GGG TAT GGA GAA G	GAG CCG AAT CAT CCT TTG C
SOD1	Mouse	GGC AAA GGT GGA AAT GAA GA	GTT TAC TGC GCA ATC CCA AT
SOD2	Mouse	TCA ATG GTG GGG GAC ATA TT	GCT TGA TAG CCT CCA GCA AC
18s	Mouse	TCG TCT TCG AAA CTC CGA CT	CGC GGT TCT ATT TTG TTG GT

Table 1: Primer sequences used for quantitative real-time PCR amplification.

— PART III —

DISCUSSION

Adapt or perish, now as ever, is nature's inexorable imperative.

— H.G. Wells —

A. General discussion

HMG-CoA reductase inhibitors are among the most prescribed medications in the world. Although they have undeniable beneficial effects on the cardiovascular system, this class of molecules displays undesirable side effects. The most common side effect being the appearance of statin-associated muscle symptoms, which can range from myalgia to potentially life-threatening rhabdomyolysis. SAMS can affect up to 33% of patients, and due to the increase in the obese and elderly population in Western countries, the number of statin prescriptions is on the rise. In addition, numerous studies have described pleiotropic effects of statins, opening hence the way to new potential prescriptions of these molecules. Understanding the mechanism of statin toxicity is thus of crucial importance.

All along this thesis, we tried to provide a better understanding of the mitochondrial adaptations to oxidoreductive stress, and mechanistic explanations to statin tolerance. Indeed, we first observed that mitochondrial oxidative stress plays a central role in the mitochondrial apoptotic pathway. Moreover, skeletal muscle phenotype revealed that statin only seem to affect glycolytic-type muscles, which possess a low mitochondrial content and low antioxidant defences. In order to evaluate the role of mitochondrial content in statin myotoxicity, we pharmacologically induced mitochondrial hormesis. We used that opportunity to show that cellular reductive stress leads to mitochondrial oxidation via respiratory chain inhibition and to the triggering of mitochondrial hormesis. We observed that an enhanced mitochondrial content provides a partial protection against statin toxicity, strengthening the hypothesis that mitochondrial content plays a key role in statin myotoxicity. In addition, we showed that impairing mitochondrial biogenesis pathways leads to a reduced statin tolerance in skeletal muscles. Indeed, we showed that statins induce apoptosis in glycolytic skeletal muscles, while oxidative skeletal muscles were not only preserved, but also displayed an enhanced mitochondrial function. Indeed, this is the first time that it is shown that statins trigger mitochondrial hormesis in oxidative-type skeletal muscles. In addition, we showed that not only

mitochondrial content and antioxidant power, but also mitochondrial adaptive pathways are the key pillars to statin tolerance.

The common thread of this thesis was that mitochondrial content appears to be a determining factor in statin tolerance. A difference in susceptibility in skeletal muscles, according to their phenotype, had previously been described (Seachrist et al. 2005). These authors reported no evidence of fiber degeneration in the soleus muscle (Type I fiber predominant) of rats after cerivastatin administration for two weeks, whereas glycolytic muscles were impaired. Our results are in line with these observations, and it seems that muscle phenotype, and hence mitochondrial content and antioxidant defences are indeed key factors in statin tolerance. Skeletal muscles fibres types are known to have different morphological, physiological, and biochemical properties. In an antioxidant point of view, muscle fibres with greater oxidative capacities have been shown to have a much higher total glutathione and GSH contents than the ones with lower oxidative capacities. In addition, this difference is also found in the activities of glutathione peroxidase, glutathione reductase, and catalase (Ji et al. 1992). Moreover, SOD2 expression is directly linked to mitochondrial content, and hence is more expressed in oxidative fibres. We observed deleterious effects of statins in the decompensated muscle of patients taking statins, and in the glycolytic skeletal muscles of rats and mice, but no deleterious effects in the oxidative-type skeletal muscles, which appear to be preserved via mitochondrial hormesis. In addition, it was previously reported that statins have opposite effects in skeletal muscles, and in the highly oxidative heart muscle (Bouitbir, Charles, et al. 2012). Indeed, in this study it was reported that statins provoke mitochondrial hormesis in heart, increasing mitochondrial content, and antioxidant defences. Interestingly, contrary to other observations, it has recently been shown that statins can also induce apoptosis in H₉C₂ cardiomyocytes, and can induce atrophy in mouse heart (Bonifacio et al. 2014). Based on our previous observations on the different susceptibility of oxidative and glycolytic skeletal muscles to statins, and on the report that increasing mitochondrial content in L₆ cells protects from both caspase dependent and caspase independent apoptosis (Dam

et al. 2013), we showed that an increase in mitochondrial content provides a partial protection against the deleterious effects of statins *in vitro*. It has to be noted that although mtDNA content was 2.4 times higher, and ATP content 4 times higher than the control group, mitochondrial maximal OXPHOS respiratory rate only slightly increased (+17%). Thus, mitochondrial content and antioxidant capacity appear to be important in statin tolerance. We then hypothesised that since mitochondrial content appears to be important, an impairment in the mitochondrial biogenesis pathways might lead to the exacerbation of statin myotoxicity. Although we observed no differences in mitochondrial content in the oxidative muscles of WT and KO mice, we observed opposite effects of statins. Indeed, in the oxidative skeletal muscles of WT mice, statins activated mitochondrial biogenesis pathways via a mitochondrial hormesis mechanism, and lead to an increased mitochondrial content and to statin tolerance. On the other hand, in KO mice, we observed a repression of this pathway in KO mice, leading to myotoxicity. A higher mitochondrial content seems necessary to dodge the oxidative stress induced by the first encounter with statins. However, an activation of mitochondrial biogenesis pathways appears to be necessary to establish long-term statin tolerance in the oxidative skeletal muscles.

Mechanistically, we showed that statins directly inhibit the mitochondrial respiratory chain in a dose-dependent manner, decreasing maximal OXPHOS respiratory rates, leading to simultaneous release of H₂O₂. Maximal OXPHOS respiratory rate corresponds to Complexes I+II-linked substrate state. It was recently demonstrated that statins inhibit the Q_o site of Complex III (Schirris et al. 2015). Thus, this observation could explain our findings. Indeed, the electron flow from Complexes I&II converge to CoQ₁₀, which will then transfer the electrons to Complex III. Hence, an inhibition of Complex III will decrease maximal OXPHOS respiratory rate and lead to the production of reactive oxygen species. The resulting oxidative stress is then sufficient to trigger apoptotic pathways in myocytes with a low mitochondrial content. Interestingly, the use of a “classic” antioxidant (quercetin), and a mitochondria-targeted antioxidant (MitoTEMPO) provided a partial protection against statin-induced apoptosis.

Interestingly, *in vitro* the dose of quercetin used was 10 000 times higher than the one used with the mitochondria-targeted antioxidant MitoTEMPO. However, Quercetin has been shown to activate mitochondrial biogenesis in skeletal muscles (Davis et al. 2009). Hence, with our *in vivo* results, it is difficult to distinguish whether these beneficial effects result from the antioxidant effect of quercetin, or from its effects on mitochondrial biogenesis.

Surprisingly, we observed higher caspase 3 cleavage rates in the glycolytic muscles of rats treated with the antioxidant quercetin compared to control, and we also found TUNEL positive apoptotic nuclei. In addition, antioxidant treatment gave us surprising results *in vitro* (unpublished data). Indeed, for some concentrations, we observed after 24 hours of incubation an increased mitochondrial hydrogen peroxide production for both quercetin and Mito-TEMPO. Reductive stress, just as oxidative stress impairs cell function, and could explain the divergent and disappointing results encountered with antioxidant supplementation. Indeed, the impact of reductive stress is still poorly understood, and molecules known as antioxidant could have dramatic repercussions within the cell. A better understanding of this phenomenon could provide new insights in the comprehension of the cell function. We observed that the antioxidant molecule N-acetylcysteine induces a cellular reductive stress as it could be expected. However, NAC also had acutely an inhibitory effect on the mitochondrial respiratory chain in a dose-dependent manner, which lead to mitochondrial oxidation. Recently, a role of mitochondrial glutathione and thioredoxin reductases has been proposed to explain this paradoxical effect (Korge et al. 2015). In addition, it was also recently shown that thiol-based antioxidants induce mitochondrial oxidation via Complex III (Kolossoff et al. 2015), and the role of reductive stress is gaining increasing interest. We also showed that a mild mitochondrial oxidation could lead to the triggering of mitochondrial adaptations via mitochondrial hormesis, in order to counter the stress. In tissues with a high mitochondrial content, statins provoke an increase of mitochondrial content, and higher antioxidant defences, which leads to statin tolerance. However, not only mitochondrial content seems to have an importance in statin myotoxicity, but also the induction of mitochondrial adaptive pathways. Indeed, the induction

of mitochondrial biogenesis pathways is mandatory to avoid the triggering of apoptotic pathways and muscular atrophy in oxidative skeletal muscles with similar initial mitochondrial contents.

One might also wonder about the rationale of our *in vivo* models. Indeed, statins are mainly given to obese and elderly patients, which present decompensated muscles. In these patients, we can observe the appearance of a metabolic myopathy, which is characterised by an impaired mitochondrial function. However, in our models, we used sedentary rats and mice, which are neither obese, nor old. Hence, we can suppose that using statins in an animal model of obesity or aging, might exacerbate the deleterious effects of statins in skeletal muscles. This could lead to the appearance of statin myopathy with much lower doses than the one we used in our present experiments.

In patients, statins are mostly prescribed to obese and elderly, which can present a decompensated muscle phenotype. This phenotype is associated with a lower mitochondrial content, and the predominance of glycolytic fibres. Hence, these patients are more susceptible to develop a statin myopathy. However, SAMS have been reported to affect up to 33% of patients. No accurate marker of SAMS currently exists, serum creatine kinase is often used but is not specific. Indeed, SRM1 is defined as the presence of muscle ache without any CK elevation (Thompson et al. 2016). Hence, the incidence of SAMS is divergent in the literature, and most probably underreported (Tomlinson & Mangione 2005). The initial mitochondrial content in glycolytic muscles before statin treatment initiation might clarify why some patients are affected, and why some are not. Thus, mitochondrial content measurement before the start of statin treatment could appear as a potential prediction marker of statin tolerance. However, although it is now obvious that this measurement has to be made on a glycolytic muscle, we might wonder what would be the minimal threshold of statin tolerance. Indeed, more research is necessary to determine this threshold, and hence determine a predisposition to statin tolerance or a susceptibility to their myotoxicity.

In conclusion, mitochondria are at the heart of statin myotoxicity, and modulating mitochondrial content could appear as a potential therapeutic solution to mitochondrial toxicants. The activation of mitochondrial biogenesis pathways seems to be determinant in statin tolerance. A better understanding of mitochondrial function and its adaptive pathways might provide new insights in the treatment of mitochondrial alterations.

B. Perspectives

These observations raise a number of new questions. Although we showed that an increase in mitochondrial content protects from the deleterious effects of statins *in vitro*, these benefits remain to be proven *in vivo*. Indeed, several possibilities might exist to protect skeletal muscle mitochondria from statin-associated muscular symptoms.

1) Trigger mitochondrial biogenesis pathways in glycolytic skeletal muscles.

a) Exercise

Although exercise is known to promote mitochondrial content in skeletal muscles, and could at first be perceived as an effective costless solution, this option possesses several drawbacks. Indeed, statins are mainly prescribed in obese and elderly patients with cardiovascular problems, to whom endurance training could appear as nearly impossible. In addition, the occurrence of statin-associated muscle symptoms is more frequent in exercising patients. In people who exercise (without medication), the incidence of myopathic symptoms has been reported to be comprised between 1% and 5% (Alfirevic et al. 2014). In comparison, this incidence reaches 25% in exercising patients taking statins (Thompson et al. 1997). Additionally, spontaneous physical activity levels have been reported to be reduced in individuals over age 55 treated with atorvastatin (Parker et al. 2013). Although endurance training prior to the start of statin treatment might be profitable to several patients, this solution would delay the initiation of statin therapy and might not be applicable to the majority of statin-treated patients.

b) Molecular approaches

The first plausible possibility would be to use a pharmacological inducer of mitochondrial biogenesis. In patients to which statins are prescribed, namely obese and elderly patients, this molecular approach might represent the most efficient way to increase mitochondrial content. There are several molecules which have been described as potent inducers of mitochondrial biogenesis in skeletal muscles. As an example, coffee enthusiasts might rejoice to learn that caffeine has been shown to increase mitochondrial content *in vitro* in L₆ myoblasts (McConnell et al. 2010; Ojuka et al. 2003; Ojuka et al. 2002), and primary cultured myotubes (Ding et al. 2012). Broccoli consumption has long been considered to play a role in a healthy diet. Broccoli accumulates significant amounts of glucoraphanin, which is metabolised *in vivo* to the biologically active sulforaphane (James et al. 2012). Interestingly, sulforaphane has been shown to induce NRF-2 nuclear translocation, and hence the expression of phase II enzymes and antioxidants (Negrette-Guzmán et al. 2013). Sulforaphane was shown to induce mitochondrial biogenesis in human fibroblasts (Brose et al. 2012). However, these benefits remain to be proven *in vivo*.

Theoretically, hormesis response could probably also be obtained with much lower doses of statins in the glycolytic skeletal muscles. Indeed, one of the basic principle of toxicology states that the dose makes the poison. Hence, starting statins treatment with very low doses, and progressively increase the dosage might potentially increase mitochondrial content in glycolytic-type skeletal muscles. However, this hypothesis remains to be proven, and initial very low doses might be inefficient to lower plasmatic cholesterol levels. In addition, finding a universal statin dose to start with could appear difficult due to interindividual variability.

In my humble opinion, one of the molecule which present most potential interest in skeletal muscles is AICAR. 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) is a cell-permeable AMP analogue. This molecule activates AMPK (AMP-dependent protein kinase), and has been described as an “exercise mimetic” (Narkar et al. 2008). Activation of AMPK has been shown to increase PGC-1 α expression via an increased USF-1 to the promoter of

PGC-1 α (Irrcher et al. 2008). During my PhD, I had the opportunity to conduct *in vivo* experiments with this molecule (unpublished data). Briefly, we studied the effects of this molecule in two populations of mice: sedentary mice, and mice spontaneously exercising. We observed an increase of mice strength in the treated groups compared to controls, and highly improved exercise capacities. Interestingly, we observed higher exercise capacities in untrained treated mice than in trained untreated mice. However, mitochondrial oxidative capacities only tend to increase in the glycolytic muscles of AICAR-treated mice compared to their respective control, and no difference was found in oxidative-type skeletal muscles. Based on my personal observations, it is much easier to decrease mitochondrial oxidative capacities than actually increasing them, and similar apparent oxidative capacities do not necessarily mean that there has been no change in the mitochondrial content. However, further investigations have to be conducted prior to publication. Interestingly, recently AMPK activation with AICAR has been shown to improve muscle function in a mitochondrial myopathy mouse model by promoting muscle fibre regeneration (Peralta et al. 2016). However, activation of AMPK in response to AICAR has been shown to be reduced in aging models (Ljubicic & Hood 2009; Reznick et al. 2007), and might then not be efficient in increasing mitochondrial content in this population of patients. In addition, AICAR is still relatively recent, and its potential adverse events remain unknown.

The best time point to pharmacologically induce mitochondrial biogenesis would probably be at least ten days before the start of any statin. Indeed, the *in vivo* induction of mitochondrial biogenesis pathways has been shown to require six to ten days to achieve an increase of mitochondrial content within skeletal muscles (Daussin et al. 2012). However, finding the perfect molecule able to induce safely mitochondrial biogenesis *in vivo* still requires further researches and could open new perspectives in the treatment of metabolic diseases.

c) Genetic approaches

A second possibility in the study of the role of mitochondria in statin-associated muscular symptoms would be to use genetically modified mice overexpressing a factor involved in

mitochondrial biogenesis, and treat them with statins. At the time of the writing of this thesis, we received the approval to start a new animal study with mice deficient and mice overexpressing PGC-1 α in skeletal muscles in collaboration with the team of professor Handschin in Basel. Overexpression of PGC-1 α in skeletal muscles promotes the switch to slow, oxidative type I and IIA muscle fibres (Lin, Wu, et al. 2002). We expect to observe in skeletal muscle PGC-1 α deficient mice an increased susceptibility to statin myopathy, in both glycolytic and oxidative-type skeletal muscles. In mice overexpressing PGC-1 α a potential protection against statin myotoxicity in glycolytic skeletal muscles might be revealed. The comparison of the future results observed in this study, and the one realised with PGC-1 β knock out mice, might also provide new elements in the functional differentiation of these two proteins. Since PGC-1 α alpha is a key regulator of mitochondrial biogenesis, the proposed experiments will provide much more insight in myotoxicity associated with statins. PGC-1 α is the most described mitochondrial biogenesis factor in the literature, and indubitably the use of other knock out models of genes involved in that pathway would also be appropriate in the study of statin myotoxicity. Recently, Nrf2^{-/-} mutant rats were developed using transcription activator-like effector nuclease technology in the Sprague-Dawley genetic background (Priestley et al. 2016). This model would have the advantage to use rats instead of mice. Indeed, in the study of skeletal muscles we are often facing the limitation of the number of experiments possible with one muscle imposed by the mice hind limb muscle size (for example the soleus). Hence the use of rats instead of mice would increase the amount of *ex vivo* experiments possible with a single animal. However, this model is not muscle specific and non-inducible (constitutional knock out), with potential compensation that could have taken place during development. TFAM is considered as the key regulator involved in mitochondrial biogenesis, enabling the coordinated expression of both nuclear-encoded and mitochondrial-encoded mitochondrial proteins, and the control of mtDNA copy number. Hence the use of muscle-specific TFAM knock out mice (Gineste et al. 2015) or overexpressing this factor (Ikeuchi et al. 2005) would be appropriate. A better understanding of mitochondrial function

and of the pathways involved in mitochondrial biogenesis could hence provide better insights in the tolerance of mitochondrial toxicants.

2) Targeting mitochondria

Mitochondrial network stands at the heart of statin tolerance in skeletal muscles. Hence directly targeting this compartment might enable to provide a protection against statin myotoxicity.

a) Mitochondria-targeted antioxidants

We have seen *in vitro* that the mitochondria-targeted antioxidant MitoTEMPO was similarly effective at a dose 10 000 times less important than the “classic” antioxidant quercetin. MitoTEMPO scavenges mitochondrial superoxide, and accumulates within the mitochondrial compartment. Thus, the use of antioxidants specifically targeted to the mitochondria presents tremendous advantages compared to “classic” ones, and attracts increasing interest in the scientific community. As an example, the SkQ project (Skulachev ion project) develops mitochondria-targeted antioxidants to prevent aging. They have created a potent molecule named SkQ1, which is a chimeric molecule composed by a plastoquinone (antioxidant) and of a lipophilic triphenylphosphonium cation (TPP) (Kezic et al. 2016). TPP acts as an addressing signal to the mitochondria, and enables TPP to accumulate within this compartment. SkQ1 has shown beneficial effects on many organs. In skeletal muscles, SkQ1 has been shown to delay the onset of sarcopenia in progeric OXYS rats, by retarding the development of age-related destructive pathological changes in mitochondria (Vays et al. 2014). SkQ1 has been used in several clinical trials and is approved in Russia for treatment of dry eyes (Visotimin), becoming the first mitochondria-targeted drug employed in medical practice (Skulachev 2012). In the U.S.A., SkQ1 is actually in phase III for the treatment of dry eyes, and in phase II for the treatment of uveitis. Another example is MitoQ, which is based on the same principle: an antioxidant (quinone) attached to TPP. Hence, MitoQ accumulates 500 to 600 times more in the mitochondria compared to cytosol (Sheu et al. 2006). However, Concentrations of MitoQ greater than 25 μ M resulted in the loss of mitochondrial membrane potential and showed

cytotoxicity in 143B cells. However, we have also seen that reductive stress could impair mitochondrial function, and could lead to mitochondrial oxidation. Thus, the use of mitochondria-targeted antioxidants brings new perspectives in the treatment of mitochondrial oxidative stress linked diseases, but extreme precaution has to be taken on the doses used.

b) Mitochondrial network modulation

Another potential solution could be to use molecules directly acting on proteins involved in mitochondrial dynamics. Mitochondria are dynamic organelles that can adapt to physiological demand. The mitochondrial network results from a balance between fission and fusion. For example, unopposed fusion leads to hyperfusion, whereas unopposed fission leads to fragmentation. Hyperfusion could be observed during some steps of the cell cycle, and in some stress-related conditions. Fragmentation can be observed during mitosis, in cases of increased ROS generation, and in apoptosis (Mitra 2013). Indeed, mitochondrial fission is known to be an integral step of apoptosis (Youle & Karbowski 2005). In addition, mitochondrial fusion proteins have been shown to attenuate apoptosis by inhibiting release of cytochrome c from mitochondria. Hence, inhibiting mitochondrial fission could block the triggering of apoptosis. We can then wonder if blocking mitochondrial fission could provide a partial protection against the statin associated muscle symptoms by hindering apoptotic pathways. The main molecules involved in fusion are the mitofusins (MFN1 and MFN2), which are located at the outer mitochondrial membrane, and OPA1, which is located at the inner mitochondrial membrane. Concerning mitochondrial fission, the main molecules involved are Fis1, which is located at the outer mitochondrial membrane, and is able to recruit DRP1 (Dynamin related GTPase 1). During my PhD I had the opportunity to realise in vitro experiments with the Mdivi-1 molecule. Mdivi-1 stands for Mitochondrial division inhibitor-1 and has first been described in 2008 (Cassidy-Stone et al. 2008). This molecule has been described as an inhibitor of the GTPase function of DRP1. Briefly, we observed in C₂C₁₂ myoblasts an increased tolerance to simvastatin when cells were incubated with this molecule (unpublished data). Indeed, Mdivi-1 decreased the statin's toxicity, enabled to maintain higher ATP levels, and decreased the

statin-induced mitochondrial fragmentation. However, further investigations have to be conducted prior to potential publication. One more potential field of investigation would be the study of mitophagy. Mitophagy is directly linked to mitochondrial dynamics, and corresponds to the selective elimination of mitochondria by autophagy. Indeed, we have seen that statin decrease mitochondrial biogenesis, but their effect on mitochondrial degradation has never been described in skeletal muscles. Hence a better understanding of this pathway could bring new perspectives in the comprehension of the mechanisms leading to glycolytic skeletal muscle toxicity.

Mitochondria is at the heart of statin myotoxicity. Thus there is an urge to develop mitochondrial targeted medications. This could profit not only to a better understanding of the mechanisms involved in statin myotoxicity, but also to any other disease in which mitochondria is involved.

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**PARTIES TRADUITES EN
FRANÇAIS**

PROBLÉMATIQUE

A la vue de ces observations, nous avons clairement deux types d'effets des inhibiteurs de l'HMG-CoA réductase dans les muscles. De manière intéressante, le tissu cardiaque hautement oxydatif est préservé des effets délétères des statines grâce à l'hormèse mitochondriale. D'un autre côté, le muscle squelettique de type glycolytique est affecté par les statines via une production excessive de radicaux libres dérivés de l'oxygène (RLO). Il a été montré que les statines induisent l'apoptose *in vitro* sur de nombreuses lignées cellulaires, et sur des cultures primaires de myotubes humains. Toutefois, le lien entre la production excessive de RLO et le stress oxydant, et l'induction des voies apoptotiques n'est pas clair, et reste à prouver.

Étonnamment, contrairement aux muscles squelettiques de type glycolytiques, les muscles squelettiques de type oxydatifs semblent être protégés des effets délétères des statines (Seachrist et al. 2005). De plus le type de fibres (et ainsi le contenu mitochondrial) a été démontré comme ayant un impact sur la susceptibilité au déclenchement des voies apoptotiques (McMillan & Quadrilatero 2011). L'entraînement à l'endurance, qui est connu pour augmenter le contenu mitochondrial dans le muscle squelettique, et ainsi de changer le phénotype musculaire, induit *in vitro* une plus grande tolérance aux statines (Bouitbir, Daussin, et al. 2012). En effet, lorsqu'ils étaient mis en présence de manière aiguë d'atorvastatine, les muscles squelettiques des rats entraînés ont montré une plus haute IC₅₀. Ainsi, le phénotype musculaire apparaît comme ayant une importance cruciale dans la tolérance aux statines et le possible déclenchement des voies apoptotiques. Toutefois, une meilleure caractérisation des effets des statines dans les muscles glycolytiques et oxydatifs sur le stress oxydant et la potentielle activation subséquente des voies apoptotiques, pourrait permettre de fournir une meilleure compréhension de la myotoxicité des statines.

De manière intéressante, une augmentation du contenu mitochondrial dans des myoblastes L₆ a été montrée comme fournissant une protection contre aussi bien l'apoptose

caspase-dépendante que caspase-indépendante (Dam et al. 2013). Ainsi, nous pourrions émettre l'hypothèse qu'augmenter le contenu mitochondrial in vitro pourrait fournir une protection au moins partielle contre les effets délétères des statines. De plus, une meilleure compréhension des adaptations au stress oxydoréducteur pourrait aider à amener une meilleure compréhension du mode d'action des inhibiteurs de l'HMG-CoA réductase dans le muscle squelettique. En effet, bien que le stress oxydant soit un phénomène bien connu, les effets du stress réducteur restent à ce jour méconnus. Divers antioxydants ont été décrits comme pouvant induire une augmentation de la production de RLO pendant un stress réducteur (Herbert 1996; Brewer et al. 2013; Zhang et al. 2012; Pimentel et al. 2012). Toutefois, le mécanisme impliqué dans cet effet paradoxal reste inconnu. Ces observations soulèvent les questions suivantes : quel est l'effet du stress réducteur sur la mitochondrie ? Et comment les peuvent-elles se défendre contre celui-ci ?

Dans le muscle cardiaque hautement oxydatif, les statines protègent la mitochondrie en déclenchant l'hormèse mitochondriale, via une surexpression de PGC-1 β aussi bien chez les rats que chez l'Homme (Bouitbir, Charles, et al. 2012). D'un autre côté, dans les muscles squelettiques glycolytiques, une répression de la prolifération mitochondriale via PGC-1 α et PGC-1 β est observée. Bien que la réponse au stress oxydant soit conventionnellement attribuée à PGC-1 α dans le muscle squelettique, la fonction et la régulation de PGC-1 β est toujours débattue dans la littérature (Zechner et al. 2010; Rowe et al. 2013; Handschin & Spiegelman 2011). Le rôle de PGC-1 β reste donc méconnu, et potentiellement sous-estimée. Ainsi, non seulement le contenu mitochondrial et les défenses antioxydantes, mais aussi les adaptations liées à PGC-1 β semblent être les piliers de la tolérance aux stress oxydant d'origine mitochondriale, et à la tolérance aux statines.

Ensembles, ces observations mettent en lumière ce qui sera le fil rouge tout au long de cette thèse : le contenu mitochondrial semble être un facteur déterminant de la tolérance aux statines.

Ceci soulève plusieurs questions :

- Y-a-t-il un lien, dans les muscles oxydatifs et glycolytiques, entre la production mitochondriale de RLO et le déclenchement des voies apoptotiques en réponse aux statines ?
- Quel est l'effet du stress réducteur sur la mitochondrie ? Et comment les peuvent-elles se défendre contre celui-ci ? Le stress réducteur pourrait-il déclencher le phénomène d'hormèse mitochondriale ?
- Une augmentation du contenu mitochondrial pourrait-elle fournir une protection contre l'apoptose induite par les statines *in vitro* ?
- Si une augmentation du contenu mitochondrial est supposée protéger les cellules, quelles seraient les conséquences d'une altération des voies de biogenèse mitochondriale ? Les souris seraient-elles plus susceptibles à développer une myopathie aux statines ?
- If an increase in mitochondrial content is supposed to protect cells, what would be the consequences of an impairment in the mitochondrial biogenesis pathways? Would mice be more prone to develop statin myopathy?

Tout au long de ce manuscrit, nous essaierons de faire la lumière sur ces questions
jusque-là sans réponses.

DISCUSSION

A. Discussion générale

Les inhibiteurs de l'HMG-CoA réductase font partie des médicaments les plus prescrits dans le monde. Bien qu'ils aient des effets bénéfiques indéniables sur le système cardiovasculaire, cette catégorie de molécules provoque des effets secondaires indésirables. L'effet secondaire le plus fréquent étant l'apparition de symptômes musculaires associés aux statines, pouvant aller de la myalgie à la rhabdomyolyse, condition engageant le pronostic vital des patients. Les symptômes musculaires associés aux statines (SMAS) peuvent affecter jusqu'à 33% des patients, et dû à l'augmentation des populations obèses et âgées dans les pays occidentaux, le nombre de prescriptions de statines ne cesse d'augmenter. De plus, de nombreuses études ont montré des effets pléiotropiques des statines, ouvrant ainsi la voie à de nouvelles possibilités de prescriptions de ces molécules. Comprendre le mécanisme conduisant à la toxicité des statines est ainsi d'une importance cruciale.

Tout au long de cette thèse, nous avons essayé de fournir une meilleure compréhension des adaptations mitochondriales au stress oxydo-réducteur, ainsi que des explications mécanistiques à la tolérance aux statines. En effet, nous avons tout d'abord observé que le stress oxydant mitochondrial jouait un rôle central dans la voie apoptotique mitochondriale. De plus, le phénotype musculaire squelettique a révélé que les statines semblent n'affecter que les muscles de type glycolytique, possédant un faible contenu mitochondrial, et peu de défenses antioxydantes. Afin d'évaluer le rôle du contenu mitochondrial dans la myotoxicité des statines, nous avons pharmacologiquement induit l'hormèse mitochondriale. Nous avons utilisé cette opportunité pour montrer qu'un stress réducteur cellulaire conduisait à une oxydation mitochondriale et au déclenchement de l'hormèse mitochondriale. Nous avons observé qu'une augmentation du contenu mitochondrial permet une protection partielle contre la toxicité induite par les statines, renforçant l'hypothèse que le contenu mitochondrial joue un rôle central dans la myotoxicité des statines. De plus, nous avons montré qu'une altération

des voies de biogenèse mitochondriale conduisait à une diminution de la tolérance aux statines dans les muscles squelettiques. En effet, nous avons montré que les statines induisent l'apoptose dans les muscles squelettiques glycolytiques, alors que les muscles squelettiques oxydatifs sont non seulement préservés, mais présentent aussi une amélioration de la fonction mitochondriale. En effet, c'est la première fois qu'il est montré que les statines provoquent le déclenchement de l'hormèse mitochondriale dans les muscles squelettiques de type oxydatif. De plus, nous avons montré que non seulement le contenu mitochondrial et les capacités antioxydantes, mais aussi les voies d'adaptation mitochondriales sont les piliers de la tolérance aux statines.

Le fil rouge de cette thèse était que le contenu mitochondrial semble être un facteur déterminant dans la tolérance aux statines. Une susceptibilité différente des muscles squelettiques, en fonction de leur phénotype, avait été préalablement décrite (Seachrist et al. 2005). Ces auteurs n'avaient observé aucune preuve de dégénération des fibres musculaires dans le muscle soléaire (à prédominance en fibres de Type I) de rats après administration de cérivastatine pendant deux semaines, alors que les muscles glycolytiques étaient altérés. Nos résultats corroborent ces observations, et il semble que le phénotype musculaire, et donc le contenu mitochondrial et les défenses antioxydantes soient des facteurs clés dans la tolérance aux statines. Les fibres musculaires squelettiques sont connues pour avoir des propriétés morphologiques, physiologiques, et biochimiques différentes. D'un point de vue des défenses antioxydantes, les fibres musculaires avec des capacités oxydatives plus élevées sont connues pour avoir un contenu en glutathion total et en GSH beaucoup plus élevé que celles avec des capacités oxydatives plus faibles. De plus, cette différence se retrouve dans les activités enzymatiques de la glutathion peroxydase, de la glutathion reductase, et de la catalase (Ji et al. 1992). De plus, l'expression de SOD2 est directement liée au contenu mitochondrial, et est donc plus importante dans les fibres de type oxydatives. Nous avons observé des effets délétères des statines dans le muscle décompensé de patients prenant des statines, ainsi que dans le muscle squelettique glycolytique de rats et de souris, mais aucun

effet délétère dans le muscle oxydatif de types oxydatif, qui semble préservé via un mécanisme d'hormèse mitochondriale. En outre, il avait été préalablement observé que les statines possèdent des effets opposés dans le muscle squelettique, et dans le muscle cardiaque hautement oxydatif (Bouitbir, Charles, et al. 2012). En effet, dans cette étude, il a été observé que les statines provoquent un phénomène d'hormèse mitochondriale dans le cœur, augmentant le contenu mitochondrial et les défenses antioxydantes. De manière intéressante, contrairement aux autres observations, il a été récemment montré que les statines peuvent induire l'apoptose dans des cardiomyocytes H₉C₂, et peuvent induire une atrophie cardiaque chez les souris (Bonifacio et al. 2014). En se basant sur nos observations préalables sur la différence de susceptibilité aux statines des muscles squelettiques oxydatifs et glycolytiques, ainsi que sur l'observation qu'une augmentation du contenu mitochondrial dans des cellules L₆ protège de l'apoptose aussi bien caspase-dépendante que caspase-indépendante (Dam et al. 2013), nous avons montré qu'une augmentation du contenu mitochondrial protège des effets délétères des statines *in vitro*. Il faut toutefois souligner que bien que le contenu en ADNmt fût 2,4 fois plus important, et le contenu en ATP 4 fois plus important que dans le groupe contrôle, la capacité maximale en PHOSPHOX de la chaîne respiratoire mitochondriale n'a que très peu augmenté (+17%). Ainsi le contenu mitochondrial et les capacités antioxydantes apparaissent comme important dans la tolérance aux statines. Nous avons ensuite émis l'hypothèse que si le contenu mitochondrial apparaît comme important, une altération des voies de biogenèse mitochondriale pourrait conduire à une exacerbation de la myotoxicité des statines. Bien que nous n'ayons observé aucune différence de contenu mitochondrial dans les muscles oxydatifs des souris WT et KO, nous avons observé un effet opposé des statines. En effet, dans le muscle squelettique oxydatif des souris WT, les statines ont activé les voies de biogenèse mitochondriale via un mécanisme d'hormèse mitochondriale, et ont conduit à une augmentation du contenu mitochondrial et à une tolérance des statines. A l'opposé, dans les souris KO, nous avons observé une répression de cette voie conduisant à la myotoxicité. Un contenu mitochondrial plus élevé semble nécessaire pour affronter le stress oxydant induit par la première rencontre avec les statines. Toutefois, une

activation des voies de biogenèse mitochondriale semble nécessaire à l'établissement d'une tolérance aux statines au long terme dans les muscles squelettiques oxydatifs.

Mécanistiquement, nous avons montré que les statines inhibent de façon directe la chaîne respiratoire mitochondriale de façon dose-dépendante, diminuant les capacités maximales en PHOSPHOX, conduisant à une production simultanée d' H_2O_2 . Les capacités maximales en PHOSPHOX de la chaîne respiratoire correspondent à une stimulation à l'aide de substrats des Complexes I+II. Il a récemment été montré que les statines inhibent le site Q_o du Complexe III (Schirris et al. 2015). Ainsi, cette observation pourrait expliquer nos découvertes. En effet, le flux d'électrons des Complexes I&II converge vers le CoQ_{10} , qui transfère ensuite les électrons vers le Complexe III. Ainsi, une inhibition du Complexe III diminuera les capacités maximales en PHOSPHOX, et conduira à une production de radicaux libres dérivés de l'oxygène (RLO). Le stress oxydant résultant est suffisant pour déclencher les voies apoptotiques dans les myocytes avec un faible contenu mitochondrial. De manière intéressante, l'utilisation d'un antioxydant « classique » (la quercétine), et d'un antioxydant spécifique de la mitochondrie (le MitoTEMPO) ont permis de protéger partiellement contre l'apoptose induite par les statines. Fait intéressant, la dose de quercétine utilisée *in vitro* était 10 000 fois plus importante que celle utilisée avec l'antioxydant spécifique de la mitochondrie MitoTEMPO. Toutefois, il a été montré que la quercétine pouvait activer la biogenèse mitochondriale dans les muscles squelettiques (Davis et al. 2009). Ainsi, avec nos expériences *in vivo*, il est difficile de distinguer si ces effets bénéfiques résultent d'un effet antioxydant de la quercétine, ou de ses effets sur la biogenèse mitochondriale.

Étonnamment, nous avons observé un pourcentage de caspase 3 clivée plus élevée dans le muscle glycolytique de rats traités avec l'antioxydant quercétine en comparaison avec le contrôle, ainsi que la présence de noyaux apoptotiques positifs au marquage TUNEL. De plus, le traitement avec des antioxydants nous ont donné des résultats surprenants *in vitro* (données non publiées). En effet, pour certaines concentrations, nous avons observé après 24 heures d'incubation une augmentation de la production de peroxyde d'hydrogène mitochondriale avec

aussi bien la quercétine que le MitoTEMPO. Le stress réducteur, tout comme le stress oxydant affecte les fonctions cellulaires et pourrait expliquer les résultats divergents et décevants rencontrés dans la littérature avec la supplémentation en antioxydants. En effet, l'impact du stress réducteur est toujours méconnu, et des molécules connues comme des antioxydants peuvent avoir des répercussions dramatiques au sein des cellules. Une meilleure compréhension de ce phénomène pourrait fournir une meilleure compréhension du fonctionnement cellulaire. Nous avons observé que la molécule antioxydante N-acétylcystéine (NAC) induit un stress réducteur cellulaire, comme ce à quoi on pouvait s'attendre. Toutefois, la NAC inhibe de façon aiguë la chaîne respiratoire mitochondriale de manière dose-dépendante, conduisant à une oxydation mitochondriale. Récemment, le rôle du glutathion mitochondrial et des thioredoxine réductases a été proposé pour expliquer cet effet paradoxal (Korge et al. 2015). De plus, il a également récemment été montré que les antioxydants à base thiol induisent une oxydation mitochondriale via le Complexe III (Kolossoff et al. 2015), et le rôle du stress réducteur rencontre de plus en plus d'intérêt. Nous avons aussi montré qu'une légère oxydation mitochondriale peut conduire au déclenchement d'adaptations mitochondriales via un phénomène d'hormèse mitochondriale, afin de contrer le stress. Dans des tissus avec un fort contenu mitochondrial, les statines provoquent une augmentation du contenu mitochondrial, et des défenses antioxydantes, conduisant à une tolérance des statines. Toutefois, non seulement le contenu mitochondrial semble avoir une importance dans la myotoxicité des statines, mais aussi l'induction des voies d'adaptations mitochondriales. En effet, l'induction des voies de biogenèse mitochondriales est obligatoire afin d'éviter le déclenchement des voies apoptotiques et l'atrophie musculaire dans des muscles squelettiques oxydatifs avec un contenu mitochondrial initial similaire.

On pourrait aussi se demander quel est le rationnel de nos modèles *in vivo*. En effet, les statines sont principalement prescrites à des patients obèses et âgés, qui présentent une fonction musculaire décompensée. Dans ces patients, on peut observer l'apparition d'une myopathie métabolique, qui se caractérise par une fonction mitochondriale altérée. Toutefois,

dans nos modèles, nous avons utilisé des rats et souris sédentaires, qui ne sont ni obèses, ni âgés. Ainsi, nous pouvons supposer qu'utiliser des statines dans un modèle d'obésité ou de vieillissement, pourrait exacerber les effets délétères des statines au niveau des muscles squelettiques. Cela pourrait conduire à l'apparition d'une myopathie aux statines avec des doses bien inférieures que celles que nous avons utilisé dans nos présentes expériences.

Chez les patients, les statines sont principalement prescrites à des patients obèses et âgés, qui présentent une fonction musculaire décompensée. Ce phénotype est associé à un contenu mitochondrial plus faible, et à la prédominance de fibres glycolytiques. Ainsi ces patients sont plus susceptibles de développer une myopathie aux statines. Toutefois, les SMAS ont été reportés d'affecter jusqu'à 33% des patients. Aucun marqueur de SMAS n'existe actuellement, la créatine kinase plasmatique (CPK) est souvent utilisée mais non spécifique. En effet le niveau SRM1 est défini par la présence de douleurs musculaires sans aucune élévation des CPK (Thompson et al. 2016). Ainsi, l'incidence des SMAS est divergente dans la littérature, et probablement sous-estimée (Tomlinson & Mangione 2005). Le contenu mitochondrial initial des muscles glycolytiques avant le début du traitement avec des statines pourrait clarifier pourquoi certains patients sont affectés, et pourquoi d'autres ne le sont pas. Ainsi, une mesure du contenu mitochondrial avant le début du traitement avec des statines pourrait apparaître comme un marqueur de prédiction potentiel de tolérance aux statines. Toutefois, bien qu'il soit à présent évident que cette mesure doit être réalisée sur un muscle glycolytique, on peut se demander quel serait le seuil minimal de tolérance aux statines. En effet, plus d'études sont nécessaires pour déterminer ce seuil, et ainsi déterminer une prédisposition à la tolérance aux statines, ou une prédisposition à leur myotoxicité.

En conclusion, les mitochondries sont au cœur de la myotoxicité des statines, et moduler le contenu mitochondrial pourrait apparaître comme une solution thérapeutique aux composés toxiques pour la mitochondrie. L'activation des voies de biogenèse mitochondriale semble déterminante dans la tolérance aux statines. Une meilleure compréhension de la fonction

mitochondriale et de ses voies d'adaptation pourrait fournir de nouvelles perspectives dans le traitement des altérations mitochondriales.

B. Perspectives

Ces observations soulèvent nombre de nouvelles questions. Bien que nous ayons montré qu'une augmentation du contenu mitochondrial protège des effets délétères des statines, *in vitro*, ces bénéfices restent à prouver *in vivo*. En effet, quelques possibilités pourraient exister pour protéger les mitochondries musculaires squelettiques des symptômes musculaires associés aux statines.

1) Déclencher les voies de biogenèse mitochondriale dans les muscles squelettiques glycolytiques

a) Exercice physique

Bien que l'exercice physique soit connu pour stimuler le contenu mitochondrial dans les muscles squelettiques, et pourrait apparaître comme une solution effective abordable, cette solution possède quelques désavantages. En effet, les statines sont principalement prescrites à des patients obèses ou âgés avec des problèmes cardiovasculaires, pour qui un entraînement physique pourrait apparaître presque impossible. De plus, l'apparition de SMAS est plus fréquente chez les patients faisant de l'exercice. Chez les patients qui font de l'exercice (sans médicaments), l'occurrence de symptômes myopathiques a été reportée comme étant comprise entre 1% et 5% (Alfirevic et al. 2014). En comparaison, cette incidence atteint 25% chez les patients faisant de l'exercice et prenant des statines (Thompson et al. 1997). De plus le niveau d'activité physique spontanée a été reporté comme étant réduit chez les individus de plus de 55 ans traités avec de l'atorvastatine (Parker et al. 2013). Bien qu'un entraînement d'endurance avant le début du traitement avec des statines pourrait être profitable pour quelques patients, cette solution retarderait le début du traitement avec des statines et pourrait ne pas être applicable à la majorité des patients traités avec des statines.

b) Approches moléculaires

La première possibilité plausible serait d'utiliser un activateur pharmacologique de la biogenèse mitochondriale. Chez les patients auxquels les statines sont prescrites, principalement des patients obèses et âgés, cette approche moléculaire pourrait représenter la manière la plus efficace afin d'augmenter le contenu mitochondrial. Il existe plusieurs molécules qui ont été décrites comme activateurs efficaces de la biogenèse mitochondriale dans le muscle squelettique. Par exemple, les amateurs de café peuvent se réjouir d'apprendre qu'il a été montré que la caféine augmente le contenu mitochondrial *in vitro* dans des myoblastes de type L₆ (McConnell et al. 2010; Ojuka et al. 2003; Ojuka et al. 2002), et dans des cultures primaires de myotubes (Ding et al. 2012). La consommation de brocoli a longtemps été considérée comme jouant un rôle dans un régime alimentaire équilibré. Le brocoli accumule des quantités significatives de glucoraphanine, qui est métabolisée *in vivo* en la molécule biologiquement active sulforaphane (James et al. 2012). De manière intéressante, le sulforaphane a été montré comme étant un inducteur la translocation nucléaire de NRF-2, et ainsi d'enzymes de phase II et d'antioxydants (Negrette-Guzmán et al. 2013). Il a été montré que le sulforaphane induit la biogenèse mitochondriale dans des fibroblastes humains (Brose et al. 2012). Toutefois, ces bénéfices restent à prouver *in vivo*.

En théorie, il est probable qu'une réponse hormétique puisse aussi être obtenue avec des doses bien plus faibles de statines dans le muscle squelettique glycolytique. En effet, un des principes de base de la toxicologie est que la dose fait le poison. Ainsi, commencer un traitement avec une très faible dose de statines, puis progressivement augmenter le dosage pourrait potentiellement augmenter le contenu mitochondrial dans les muscles squelettiques de type glycolytique. Toutefois, cette hypothèse reste à prouver, et des doses initiales très faibles de statines pourraient être inefficaces pour diminuer les niveaux de cholestérol plasmatique. De plus, trouver une dose universelle de statines avec laquelle débiter pourrait paraître difficile du fait de la variation interindividuelle.

En mon humble opinion, une des molécules qui présente le plus grand intérêt potentiel dans le muscle squelettique est l'AICAR. Le 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) est un analogue de l'AMP perméables aux membranes cellulaires. Cette molécule active l'AMPK (protéine kinase AMP-dépendante), et a été décrite comme étant une molécule mimant l'effet de l'exercice physique (Narkar et al. 2008). L'activation de l'AMPK a été montrée d'augmenter l'expression de PGC-1 α en augmentant la liaison d'USF-1 au promoteur de PGC-1 α (Irrcher et al. 2008). Au cours de ma thèse, j'ai eu l'opportunité de réaliser des expériences *in vivo* avec cette molécule (données non publiées). En bref, nous avons étudié les effets de cette molécule dans deux populations de souris : dans des souris sédentaires, ainsi que dans des souris faisant de l'exercice spontané. Nous avons observé une augmentation de la force des souris, ainsi que des capacités à l'exercice dans les groupes traités en comparaison avec leur groupe contrôle respectif. De manière intéressante, nous avons observé des capacités à l'exercice plus élevées chez les souris traitées non entraînées que chez les souris non traitées entraînées. Toutefois, les capacités oxydatives n'ont présenté qu'une tendance à l'augmentation dans les muscles glycolytiques des souris traitées avec de l'AICAR, en comparaison avec leur groupe contrôle respectif, et aucune différence n'a été trouvée dans les muscles squelettiques de type oxydatif. En me basant sur mes observations personnelles, il est bien plus simple de diminuer les capacités oxydatives mitochondriales que de les augmenter, et des capacités oxydatives similaires ne veulent pas nécessairement dire qu'il n'y a pas eu de changement dans le contenu mitochondrial. Toutefois, des expériences supplémentaires sont nécessaires avant une potentielle possibilité de publication. De manière intéressante, il a été montré que l'activation de l'AMPK avec l'AICAR améliore la fonction musculaire dans un modèle de myopathie mitochondriale chez la souris en favorisant la régénération des fibres musculaires (Peralta et al. 2016). Toutefois, il a été montré que l'activation de l'AMPK en réponse à l'AICAR est réduite dans les modèles âgés (Ljubcic & Hood 2009; Reznick et al. 2007), et pourrait donc être inefficace pour augmenter le contenu mitochondrial dans cette population de patients. De plus l'AICAR est une molécule relativement récente, et ses effets secondaires potentiels restent inconnus à ce jour.

Le meilleur moment pour induire pharmacologiquement la biogenèse mitochondriale serait probablement au moins dix jours avant le début du traitement avec les statines. En effet, il a été montré que l'induction *in vivo* de la biogenèse mitochondriale nécessite six à dix jours pour obtenir une augmentation effective du contenu mitochondrial dans le muscle squelettique (Daussin et al. 2012). Toutefois, trouver la molécule parfaite capable d'induire de manière sûre la biogenèse mitochondriale *in vivo* nécessite davantage de recherche et pourrait ouvrir de nouvelles perspectives dans le traitement de maladies métaboliques.

c) Approches génétiques

Une seconde possibilité dans l'étude du rôle de la mitochondrie dans les symptômes musculaires associés aux statines serait d'utiliser des souris génétiquement modifiées surexprimant un facteur impliqué dans la biogenèse mitochondriale, et de les traiter avec des statines. Au moment de l'écriture de cette thèse, nous venons de recevoir l'autorisation de débiter une nouvelle étude animale avec des souris déficientes et des souris surexprimant PGC-1 α dans le muscle squelettique, en collaboration avec l'équipe du professeur Handschin à Bâle ? La surexpression de PGC-1 α dans le muscle squelettique provoque le switch des fibres musculaires vers des fibres lentes oxydatives de Type I et IIA (Lin, Wu, et al. 2002). Nous nous attendons à observer dans les muscles squelettiques aussi bien glycolytiques qu'oxydatifs des souris déficientes pour PGC-1 α une susceptibilité accrue à la myopathie aux statines. Dans les souris surexprimant PGC-1 α , une potentielle protection contre la myotoxicité des statines pourrait être révélée dans les muscles squelettiques glycolytiques. La comparaison des futurs résultats observés dans cette étude avec ceux obtenus avec les souris déficientes pour PGC-1 β pourrait fournir de nouveaux éléments permettant de différencier fonctionnellement ces deux protéines. Etant donné que PGC-1 α est un régulateur clé de la biogenèse mitochondriale, les expériences proposées fourniront beaucoup plus de compréhension dans la myotoxicité associée aux statines. PGC-1 α est le facteur impliqué dans la biogenèse mitochondriale le plus décrit dans la littérature, et indubitablement l'utilisation d'autres modèles de knock out de gènes impliqués dans cette voie métabolique

seraient aussi appropriés dans l'étude de la myotoxicité des statines. Récemment, un modèle de rats mutants $Nrf2^{-/-}$ a été développé en utilisant la technologie TALEN (transcription activator-like effector nuclease) dans un fond génétique Sprague-Dawley (Priestley et al. 2016). Ce modèle aurait l'avantage d'utiliser des rats à la place de souris. En effet, dans l'étude des muscles squelettiques, nous sommes souvent confrontés à la limitation du nombre d'expériences possibles avec un muscle imposée par la taille des muscles des pattes arrières des souris (par exemple le muscle soléaire). Ainsi l'utilisation de rats à la place de souris augmenterait le nombre d'expériences *ex vivo* potentiellement réalisables avec un animal unique. Toutefois, ce modèle n'est pas spécifique du muscle squelettique (knock out constitutionnel), avec de potentielles compensations qui peuvent avoir eu lieu durant le développement. TFAM est considéré comme étant le régulateur clé de la biogenèse mitochondriale, permettant l'expression coordonnée des protéines encodées aussi bien par le génome nucléaire que par le génome mitochondrial, ainsi que le contrôle du nombre de copies de l'ADNmt. Ainsi, l'utilisation d'un modèle de knock out spécifique du muscle dans des souris (Gineste et al. 2015) ou de surexpression de ce facteur (Ikeuchi et al. 2005) serait approprié. Une meilleure compréhension de la fonction mitochondriale et des voies impliquées dans la biogenèse mitochondriale pourrait ainsi fournir une meilleure perception de la tolérance aux molécules toxiques pour la mitochondrie.

2) Cibler la mitochondrie

Le réseau mitochondrial est au cœur de la tolérance aux statines dans les muscles squelettiques. Ainsi, cibler directement ce compartiment pourrait permettre de fournir une protection contre la myotoxicité liée aux statines.

a) Antioxydants spécifiques de la mitochondrie

Nous avons vu *in vitro* que l'antioxydant spécifique de la mitochondrie MitoTEMPO était aussi efficace, à une dose 10 000 fois moins importante, que l'antioxydant classique quercétine. Le MitoTEMPO réagit avec l'anion superoxide, et s'accumule dans le compartiment mitochondrial. Ainsi, l'utilisation d'antioxydants spécifiques de la mitochondrie

présente d'énormes avantages en comparaison avec les antioxydants classiques, et reçoit de plus en plus d'intérêt de la part de la communauté scientifique. Par exemple, le projet SkQ (Skulachev ion project) développe des antioxydants spécifiques de la mitochondrie pour contrer les effets de l'âge. Ces chercheurs ont créé une molécule efficace nommée SkQ1, qui est une molécule chimérique composée d'une plastoquinone (antioxydant) et d'un cation triphenylphosphonium (TPP) lipophile (Kezic et al. 2016). Le TPP agit comme un signal d'adressage à la mitochondrie, et permet au TPP de s'accumuler dans ce compartiment. Le SkQ1 a montré des effets bénéfiques sur de nombreux organes. Dans le muscle squelettique, le SkQ1 retarde l'apparition de la sarcopénie dans des rats progériques OXYS, en retardant le développement des changements pathologiques mitochondriaux liés à l'âge (Vays et al. 2014). Le SkQ1 a été utilisé dans quelques essais cliniques, et a reçu l'autorisation de mise sur le marché en Russie pour le traitement de la sécheresse oculaire (Visotimin), devenant le premier médicament ciblant la mitochondrie utilisé en pratique médicale (Skulachev 2012). Aux Etats-Unis, le SkQ1 est actuellement en phase III pour le traitement de la sécheresse oculaire, et en phase II pour le traitement de l'uvéite. Un autre exemple est le MitoQ, qui est basé sur le même principe : un antioxydant (quinone) attaché au TPP. Ainsi MitoQ s'accumule de 500 à 600 fois plus dans la mitochondrie que dans le cytosol (Sheu et al. 2006). Toutefois, des concentrations de MitoQ supérieures à 25µM ont eu pour conséquences une perte du potentiel de membrane mitochondrial, et ont été montrées comme toxiques dans des cellules 143B. Toutefois, nous avons aussi vu que le stress réducteur peut altérer la fonction mitochondriale, et peut conduire à une oxydation mitochondriale. Ainsi, l'utilisation d'antioxydants spécifiques de la mitochondrie apporte de nouvelles perspectives dans le traitement de maladies liées au stress oxydant d'origine mitochondriale, mais des précautions extrêmes doivent être prises concernant les doses utilisées.

b) Modulation du réseau mitochondrial

Une autre solution potentielle pourrait être d'utiliser des molécules agissant directement sur des protéines impliquées dans la dynamique mitochondriale. Les mitochondries dont des

organites dynamiques qui peuvent s'adapter à la demande physiologique. Le réseau mitochondrial résulte d'un équilibre entre fission et fusion. Par exemple, une fusion sans opposition conduit à une hyperfusion du réseau, alors qu'une fission sans opposition conduit à une fragmentation. Une hyperfusion peut être observée lors de certaines étapes du cycle cellulaire, et dans des conditions liées au stress. La fragmentation peut être observée durant la mitose, dans le cas d'augmentation de la production de RLO, et pendant l'apoptose (Mittra 2013). En effet, la fission mitochondriale est connue pour être une étape intégrale de l'apoptose (Youle & Karbowski 2005). De plus, les protéines de fusion mitochondriale ont été montrées comme atténuant l'apoptose en inhibant le relargage de cytochrome c de la mitochondrie. Ainsi, inhiber la fission mitochondriale pourrait bloquer le déclenchement des voies apoptotiques. Nous pouvons ainsi nous demander si bloquer la fission mitochondriale pourrait permettre une protection partielle contre les SMAS en entravant les voies apoptotiques. Les principales molécules impliquées dans la fusion mitochondriale sont les mitofusines (MFN1 et MFN2), qui sont localisées à la membrane externe de la mitochondrie, et OPA1, qui est localisé à la membrane mitochondriale interne. En ce qui concerne la fission mitochondriale, les principales molécules impliquées sont Fis1, qui est localisée à la membrane mitochondriale externe, et est capable de recruter DRP1 (Dynamamin related GTPase 1). Au cours de ma thèse j'ai eu l'opportunité de réaliser des expériences *in vitro* avec la molécule Mdivi-1. Mdivi-1 signifie « Mitochondrial division inhibitor-1 » et a été pour décrit pour la première fois en 2008 (Cassidy-Stone et al. 2008). Cette molécule a été décrite comme étant un inhibiteur de la fonction GTPase de DRP1. En bref, nous avons observé dans des myoblastes de type C₂C₁₂ une tolérance accrue à la simvastatine lorsque les cellules étaient incubées avec cette molécule (données non publiées). En effet, le Mdivi-1 a diminué la toxicité des statines, et permis de maintenir des niveaux d'ATP plus élevés, et diminué la fragmentation mitochondriale induite par les statines. Toutefois, des expériences supplémentaires sont nécessaire avant une possibilité de publication. Un champ d'investigation supplémentaire serait l'étude de la mitophagie. La mitophagie est directement liée à la dynamique mitochondriale, et correspond à l'élimination sélective de mitochondries

par autophagie. En effet, nous avons vu que les statines diminuent la biogenèse mitochondriale, mais leur effet sur la dégradation mitochondriale n'a jamais été décrite dans le muscle squelettique. Ainsi une meilleure compréhension de cette voie permettrait d'amener de nouvelles perspectives dans la compréhension des mécanismes conduisant à la toxicité dans le muscle glycolytique squelettique.

La mitochondrie est au cœur de la myotoxicité des statines. Ainsi il y a une nécessité urgente de développer des traitements ciblant la mitochondrie. Ceci pourrait permettre non seulement une meilleure compréhension des mécanismes impliqués dans la myotoxicité des statines, mais aussi d'autres pathologies dans lesquelles la mitochondrie est impliquée.

François SINGH
**Skeletal muscle toxicity and
statins:**
role of mitochondrial adaptations

Résumé

Bien que les statines forment la classe d'hypolipémiants la plus utilisée, une toxicité musculaire a été reportée, pouvant ainsi provoquer l'apparition d'une myopathie. Dans la première partie, nous avons montré chez l'Homme et l'animal que les statines inhibent directement la chaîne respiratoire mitochondriale, et induisent la production de radicaux libres dérivés de l'oxygène (RLO), qui active les voies apoptotiques dans les muscles glycolytiques, alors que les muscles oxydatifs ne sont pas atteints. Nous avons ensuite montré *in vitro* que le stress réducteur peut engendrer une oxydation mitochondriale, pouvant conduire à une activation de la voie de biogenèse mitochondriale. De plus l'augmentation du contenu mitochondrial induite a permis de protéger les cellules contre l'apoptose induite par les statines. Enfin, nous avons montré *in vivo* que l'induction des voies de biogenèse mitochondriale est nécessaire à la tolérance des statines dans les muscles oxydatifs. En conclusion, le phénotype mitochondrial, tant au niveau quantitatif que qualitatif, semble être un facteur clé dans l'apparition de la myopathie aux statines.

Mots clés : Statines • Mitochondrie • Muscle squelettique • Stress oxydant • Stress réducteur • Apoptose
• Biogenèse mitochondriale

Abstract

Although statins are the most prescribed class of lipid-lowering agents, adverse muscular toxicity has been reported, which can lead to the appearance of a myopathy. In the first part, we showed in Humans and animals that statins inhibit directly the mitochondrial respiratory chain, and induce the production of reactive oxygen species (ROS), that trigger apoptotic pathways in glycolytic skeletal muscles, whereas oxidative muscles are not impaired. We then showed *in vitro* that reductive stress can provoke mitochondrial oxidation, that could lead to an activation of mitochondrial biogenesis pathways. Moreover, the consequent increase in mitochondrial content enabled to protect cells against statin-induced apoptosis. Finally, we showed *in vivo* that the induction of mitochondrial biogenesis is necessary for statin tolerance in oxidative skeletal muscles. In conclusion, mitochondrial phenotype, both quantitatively and qualitatively, seems to be a key factor in the appearance of statin myopathy.

Keywords: Statins • Mitochondria • Skeletal muscle • Oxidative stress • Reductive stress • Apoptosis •
Mitochondrial biogenesis