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CRITICAL ILLNESS MYOPATHY: EFFECTS OF SPECIFIC INTERVENTION STRATEGIES AND MOLECULAR MECHANISMS

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Critical illness myopathy: effects of specific intervention strategies and molecular mechanisms

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Skeletal muscles are a tissue with remarkable adaptability and are essential in the body in many aspects. The homeostasis of the muscles is vital for the maintenance of the body, thus muscle damage is associated with several diseases and leads to a poor quality of life. Acquired muscle weaknesses in the intensive care unit (ICU) is a major complication that occurs in severely ill patients and has a significant impact on the immune system, energy metabolism, amino acid reserves and temperature regulation in the body. Critical illness myopathy (CIM) is a myopathy that results from critical illness and is commonly found in mechanically ventilated ICU patients. It is characterized by paralysis of the limb muscles, atrophy and reduced muscle excitability. The exact cause and underlying mechanisms of the disease remain obscure, hence, the aim of this thesis was to achieve a better understanding of the cellular and molecular mechanisms underlying the muscle wasting and weakness seen in ICU patients with CIM. In accordance with this, a rodent ICU model was used to address the mechanistic and therapeutic aspects of the disease. This thesis has investigated the intracellular pathways controlling the mechanisms underlying muscle wasting in an ICU rat model and the effects of passive mechanical loading. Passive mechanical loading induced significant positive effects on muscle function in the limb muscles and was able to attenuate myosin protein loss, associated with mechanical silencing and CIM. It was also demonstrated that both fission and fusion events as well as mitophagy are significantly affected by mechanosensing. Mitochondria dynamic alterations induced by mechanical silencing were completely counteracted by passive mechanical loading. Additionally it is demonstrated that the temporal pattern and the lack of preferential myosin loss observed in the diaphragm in response to CMV and immobilization differs dramatically to what is occurring in the limb muscles. Further, the response of cranial nerve innervated masseter is also different from that of limb and diaphragm muscles. Early activation of heat shock proteins suggest that an enriched antioxidative profile in the masseter may play a role in the mechanism of preserved masticatory function in CIM.

LIST OF SCIENTIFIC PAPERS

I. Renaud G., Llano-Diez M., Ravara B., Gorza L., Feng H-Z., Jin J-P., Cacciani N., Gustafson A-M., Ochala J., **Corpeno R**., Li M., Hedström Y., Ford G. C., Nair K. S. and Larsson L. (2013), Sparing of muscle mass and function by passive loading in an experimental intensive care unit model. The Journal of Physiology, 591: 1385–1402. doi: 10.1113/jphysiol.2012.248724

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III. **Corpeno R**., Dworkin B., Cacciani N., Salah H., Bergman H.-M., Ravara B., Vitadello M., Gorza L., Gustafson A.-M., Hedström Y., Petersson J., Feng H-Z., Jin J-P., Iwamoto H., Yagi N., Artemenko K., Bergquist J. and Larsson L. (2014), Time course analysis of mechanical ventilation-induced diaphragm contractile muscle dysfunction in the rat. The Journal of Physiology, 592: 3859–3880. doi: 10.1113/jphysiol.2014.277962

IV. Akkad H., **Corpeno R**. and Larsson L. (2014) Masseter Muscle Myofibrillar Protein Synthesis and Degradation in an Experimental Critical Illness Myopathy Model. PLoS ONE 9(4): e92622. doi:10.1371/journal.pone.009262

CONTENTS

LIST OF ABBREVIATIONS

4EBP-1	4E binding protein-1
ALK	Activin receptor-like kinase
ActR	Activin receptors
Akt	Protein kinase B (PKB)
BMPs	Bone morphogenic proteins
Cav3	Caveolin-3
CMA	Chaperone mediated autophagy
CIM	Critical illness myopathy
CIP	Critical illness polyneuropathy
CIPNM	Critical illness polyneuromyopathy
CMV	Controlled mechanical ventilation
CS	Systemic corticosteroids
CSA	Cross-sectional area
Diaphorase NADPH	NADPH-d
DEPTOR	The Dep domain-containing mTOR-interacting protein
DNPH	2.4-dinitrophenylhydrazine
ER	Endoplasmic reticulum
eIF	Eukaryotic initiation factor
F-actin	Filamentous actin

FbxO	F-box protein
FoxO	Forkhead Box O
Fst	Follistatin
G-actin	Globular actin
GC/MS/MS	Gas chromatography tandem mass spectrometry
GDF	Growth differentiation factors
HSPs	Heat shock proteins
IKKβ	IκB kinase
ICU	Intensive care unit
LAMP-2A	Lysosomal-associated membrane protein 2A
mLST8	Mammalian lethal with SEC13 protein 8
mSIN1	Stress-activated protein kinase-interacting protein 1
Mstn	Myostatin
mTOR	The mammalian (or mechanistic) target of rapamycin
MuRF1	Muscle RING finger-1
MV	Mechanical ventilation
MyHC	Myosin heavy chains
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NMB	Neuromuscular blockers
nNOS	Neuronal nitric oxide synthase

PGC-1α	PPARγ coactivator-1α
PI3K	Phosphoinositide 3 kinase
PPARγ	Peroxisome proliferator-activated receptors γ
PRAS40	The proline-rich Akt substrate of 40 kDa
PROTOR	The protein observed with RICTOR
RAPTOR	The regulatory associated protein of mTOR
ROS	Reactive oxygen species
S6	Ribosomal protein S6
S6K	p70 ribosomal protein S6 kinase
SF	Specific force
SIRS	Systemic inflammatory response syndrome
SMART	Specific of muscle atrophy and regulated by transcription
TGFβ	Transforming growth factor-β
Tn	Troponin
TNFα	Tumor necrosis factor -α
TSC2	Tuberous sclerosis complex 2
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
VIDD	Ventilator-induced diaphragmatic dysfunction

1 INTRODUCTION

1.1 SKELETAL MUSCLES

Skeletal muscles are a tissue with remarkable adaptability essential in the body in many aspects. Skeletal muscles are not only important for generating force and movements but they also functions as an amino acid pool, helps to regulate body temperature, work as passive protectors of the bones and are important producers of cytokines and growth factors. All muscles have a common structure that gives them shape and organization. The major cytoskeletal structure for muscle contraction is the myofibril, which docks the actin (thin) and myosin (thick) filaments. A myofibril consists of numerous sarcomeres, which are the main contractile components. The sarcomeres are connected in tandem with each segment lined by the Z-disc, a macromolecular complex that consists of several scaffolding proteins. Each fiber has a complex arrangement that enables its specific functions such as contraction, metabolic properties and protein synthesis regulation. Within the muscles, there is a vascular network that allows the muscle fibers to obtain oxygen, energy substrates and dispose heat. This network helps the muscles to maintain a constant environment in the close surroundings of each muscle fiber during rest and also helps to minimize disturbance of the homeostasis in response to different stimuli e.g. environmental factors, nutrition, loading conditions, and contractile activity during exercise. The homeostasis of the muscle is vital for the maintenance of several essential functions, like breathing, body movements and speaking, which are crucial for personal independence. On the contrary, when the homeostasis is perturbed, it leads to muscle damage associated with several diseases and leads to a poor quality of life.

1.1.1 Contractile proteins

1.1.1.1 Myosin

Myosin is a superfamily of molecular motor proteins that power muscle contraction, movement and load-dependent anchoring on actin filament in eukaryotic cells. Skeletal muscle myosin belongs to myosin class II and is the motor protein of the sarcomere. Myosin generates muscle contraction by transducing chemical energy into mechanical energy through ATPase activity in order to move along the actin filaments (Baldwin and Haddad, 2002; Sweeney and Houdusse, 2010).

The myosin molecule consists of two globular domains (heads) and a long tail or rod. The head domain contains both ATP- and actin-binding sites and is able to hydrolyze ATP to generate force and movement. The C-terminal region of the two myosin heavy chains (MyHC) forms the α-helical coiled coil that constitutes the rod region of the myosin molecule. The myosin rods create the "thick" or myosin filament decorated with hundreds of myosin heads responsible for force generation and movement.

8

Different fiber types differ in their structural and metabolic properties as different muscles have varied requirements. The fiber types are divergent in their contractile speed and are usually classified with regard to the MyHC isoform into one slow and three fast types of muscle fibers. The mechanical and energetic aspects of slow fibers make them more suitable for low-intensity and long-lasting activity. Fast fibers, on the other hand are preeminent for short and strong contractile performances. In both cases, stability is required between the characteristics of the molecular motors and the metabolic processes aimed to ATP restoration. There are a number of MyHC isoforms. Type I fibers (slow) contain the MyHC1 isoform and the fast types IIA, IIX and IIB (fastest) contain MyHC-IIa, MyHC-type IIx and MyHC-IIb respectively (Pette and Staron, 2001; Schiaffino and Reggiani, 2011).

1.1.1.2 Actin

Myosin would not be a motor without its interaction with actin filaments (Sweeney and Houdusse, 2010). Actin is an abundant protein and highly evolutionarily conserved. The human genome has six actin genes that encode six functional actin isoforms that are expressed in serval muscle and non-muscle tissues. The most important are the *ACTA1*- and *ACTC1*- genes encoding skeletal muscle α-actin and its cardiac counterpart, cardiac muscle α-actin (Perrin and Ervasti, 2010; Tondeleir et al., 2009). The actin protein is composed of a single polypeptide chain of 375 amino acids, containing 4 subdomains in a double helical structure. In eukaryotic cells actin exists in two forms, globular actin (G-actin) and filamentous actin (F-actin).

1.2   REGULATION OF MUSCLE MASS

Skeletal muscle mass is ultimately determined by the balance between the rate of protein synthesis and protein degradation (Goodman et al., 2011). How skeletal muscles respond to a certain stimuli is controlled by two conflicting events, muscle growth and muscle degradation. These two activities are mechanistically linked with a common set of molecules controlling cellular pathways that regulate how the muscle will respond; with increased protein synthesis and stimulation of cell growth (muscle hypertrophy) or with increased protein breakdown and reduced cell growth (muscle atrophy) (Sartorelli and Fulco, 2004). Regulation of muscle mass is hence a coordinated balance between protein degradation and synthesis that reflects the physiological state of the muscle fiber. Hypertrophy occurs during development, in response to mechanical overload or anabolic hormonal stimulation. In contrast, muscle atrophy occurs in response to aging, starvation, cancer, diabetes, bed rest, denervation or catabolic hormonal stimulation (Schiaffino et al., 2013). Additionally, fiber cross-sectional area (CSA), is a particularly adaptive and important property of skeletal muscles that undergoes continuous modification to regulate muscle strength and metabolism (Bodine and Baehr, 2014).

1.2.1 Skeletal muscle growth (muscle protein synthesis)

Skeletal muscles have a remarkable ability to adapt and previous studies have shown that various stimuli such as nutrients, growth factors, and mechanical load, can regulate protein synthesis in skeletal muscles (Horiba et al., 2015; Kimball et al., 2002; Raskin et al., 2009; Wong and Booth, 1985). This regulation is mainly controlled at the level of translational initiation rather than the result of an increase in RNA content (Kimball and Jefferson, 2010; Laurent et al., 1978), the regulation of stimuli effect is primarily controlled by the protein kinase mammalian (or mechanistic) target of rapamycin (mTOR).

1.2.1.1 mTOR

mTOR occurs in two complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is the rapamycin sensitive complex that consist of the regulatory associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (mLST8; a.k.a. GbL), the Dep domain-containing mTOR-interacting protein (DEPTOR) and the proline-rich Akt substrate of 40 kDa (PRAS40). In contrast, mTORC2 is the rapamycin-insensitive complex, composed of the rapamycin insensitive companion of mTOR (RICTOR), mLST8, DEPTOR, stress-activated protein kinase-interacting protein 1 (mSIN1) and the protein observed with RICTOR (PROTOR) (Weigl, 2012).

Several studies have shown that signaling by mTORC1, regulates initiation of translation through the phosphorylation of substrates such as eukaryotic initiation factor (eIF), 4E binding protein-1 (4EBP-1), ribosomal protein S6 (S6) and p70 ribosomal protein S6 kinase (S6K) (Baar and Esser, 1999; Haghighat et al., 1995; Hara et al., 1997; Heitman et al., 1991). These substrates are key regulators of protein synthesis, and are expressed in all cell types (Weigl, 2012). The efficiency of an mRNA is mainly controlled by the eukaryotic initiation factor 4E (eIF4E) which is also part of the eIF4F-complex, together with eIF4G and eIF4A. eIF4A is an RNA helicase which is able to unwind hairpin structures in the 5´untranslated region (UTR) of mRNAs, hence promoting the translational process (Ray et al., 1983; Rogers et al., 2001). 4E-BP1 is an inhibitory phospho-protein, that in the hypo-phosphorylated state, is bound to eIF4E and thereby hindering the association of the eIF4F-complex (**Figure 1**). When mTOR phosphorylates 4EBP-1, 4EBP-1 detaches from eIF4E and increases the formation of the eIF4F complex and can drastically improve the protein synthesis rate (Weigl, 2012). S6K1 is a positive regulator of protein translation initiation. mTOR phosphorylates S6K which in turn phosphorylates several other proteins that also affect translation initiation like S6 and eIF4B, another member of the initiation eIF4F-complex (Marina et al., 2005). Thus, the control of translation initiation by mTOR is key for the regulation of protein synthesis in skeletal muscles (You et al., 2015).

The upstream regulators of mTOR are linked with growth factors, nutrients, energy and stress (**Figure 1**). Growth factors like insulin and IGFs stimulates phosphoinositide 3 kinase (PI3K), which leads to the activation of Akt (Aka. protein kinase B, PKB). Activated Akt phosphorylates the tuberous sclerosis complex 2 (TSC2) which is a protein that forms the

TSC1/2 complex, together with TSC1 (Inoki et al., 2002). Phosphorylation of TSC2, by Akt, prevent its inhibition of Rheb from promoting mTORC1 activity (Stocker et al., 2003; Weigl, 2012). In addition to Akt, mTOR responds to many upstream signals, including amino acids. Further, mTOR controls several cellular processes in addition to protein synthesis such as autophagy (Sandri, 2010). It has been shown that rapamycin can inhibit mechanically induced growth and mechanically induced changes in protein synthesis in skeletal muscles (Hornberger et al., 2007; Kubica et al., 2005), this together with studies with transgenic mice models of mTORC1 inactivation indicate that mechanical stimulation of skeletal muscles is sufficient to activate mTORC1, and that the activation of mTORC1 is the basis for the increased protein synthesis and muscle growth observed under mechanical stimulation (Weigl, 2012).
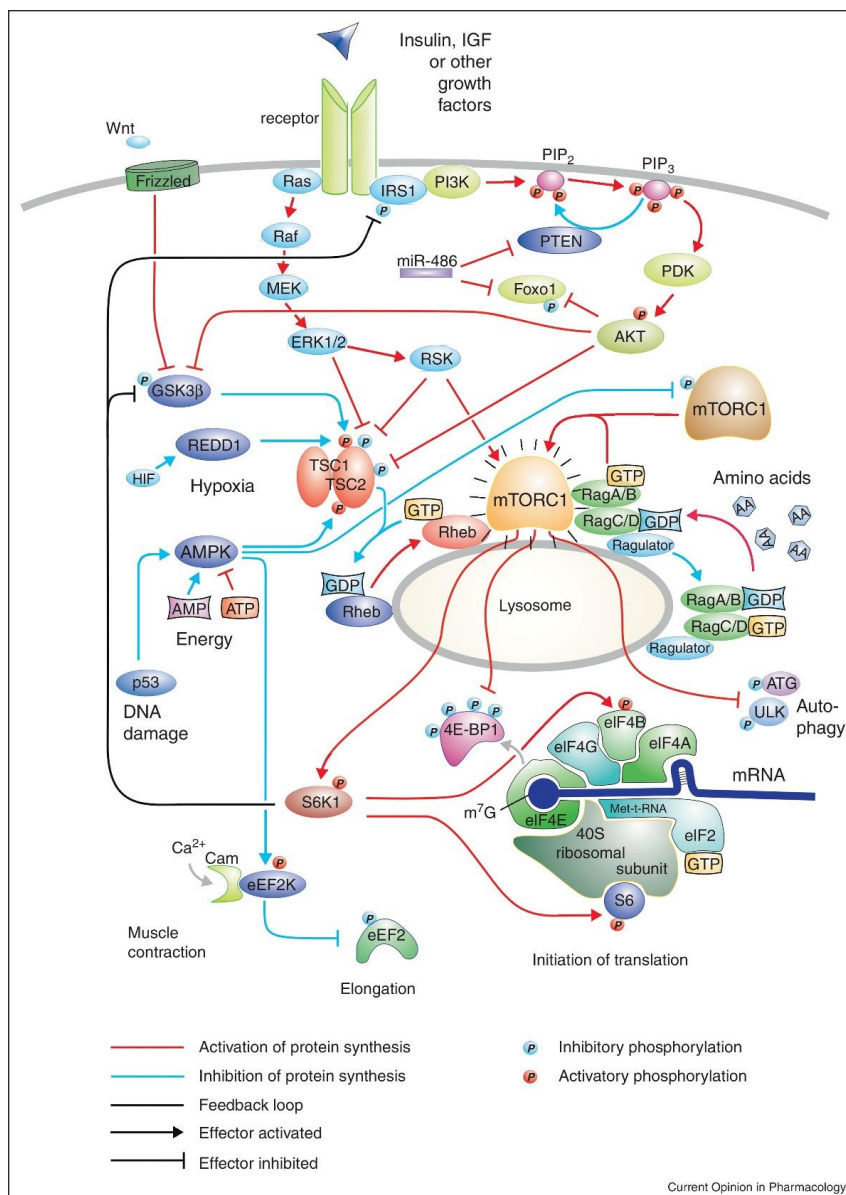
Figure 1. mTORC1 Signaling
Simplified map of the mTORC1 signaling pathway. Numerous cellular signals associated by the mTORC1 to regulate cell proliferation and growth (Weigl, 2012). With permission from Elsevier.

1.2.1.2   Myostatin-Smad2/Smad3 pathway

Smad signaling can be activated by the transforming growth factor-β (TGFβ) superfamily of receptor ligand proteins, with Myostatin (Mstn) being their most famous member. Additionally Smad signaling can also be activated by activins, some growth differentiation factors (GDF) and also bone morphogenic proteins (BMPs) (Miyazono et al., 2005; Moustakas and Heldin, 2009). Regulation of muscle mass by Smad signaling was initially discovered by the finding that inhibition of the Mstn (Aka., GDF-8) gene and of Mstn receptor binding, resulted in prominent skeletal muscle hypertrophy (Lee and McPherron, 2001; McPherron et al., 1997). Later it was shown that an increase in Mstn is associated with skeletal muscle atrophy (Gonzalez-Cadavid et al., 1998; Zimmers et al., 2002). Hence, Mstn has proven to be a negative regulator of skeletal muscle mass, in part via Smad signaling (Goodman and Hornberger, 2014; Sartori and Sandri, 2015; Sartori et al., 2013). Mstn-mediated Smad signaling is activated when the Mstn peptide binds activin type IIB and type IIA receptors (ActRIIB/IIA). This activation leads to the activation of activin receptor-like kinase-4 and -5 (ALK4 and ALK5), that in turn phosphorylates Smad 2 and 3 (Ross and Hill, 2008). These transcription factors can then form a complex with another Smad2 or 3 molecule, and additionally a Smad 4. This will result in the translocation of the complex to the nucleus where it regulates gene transcription.(Ross and Hill, 2008; Tsuchida et al., 2008; Walton et al., 2012).

Sartori et al (Sartori et al., 2009), first demonstrated that Smad signaling is essential in Mstn/activin/ TGF-β-induced muscle atrophy. It was demonstrated that overexpression of a constitutively active mutant of ALK5, up-regulated atrogin-1 and induced muscle fiber atrophy via a Smad3 (Sartori et al., 2009). It was also demonstrated that an increase in Mstn is enough to prompt a decrease in protein synthesis and a possible mechanisms for this is by atrogin-1 degradation or via the inhibition of signaling through the Akt and mTORC1, or both (Amirouche et al., 2009; Lagirand-Cantaloube et al., 2008; Lokireddy et al., 2011; Sartori and Sandri, 2015; Trendelenburg et al., 2009).

1.2.1.3   Peroxisome Proliferator-Activated Receptor-γ Coactivator-1α (PGC-1α)

Peroxisome proliferator-activated receptors γ (PPARγ) coactivator-1α (PGC-1α) is a metabolic transcription co-activator in mammals. PGC-1α is activated by signals that control energy and nutrients in the cell. Specifically, PGC-1α induces and coordinates gene expression that stimulates mitochondrial biogenesis, fiber-type switching in skeletal muscles, and metabolic pathways linked to the fasted response in the liver (Puigserver, 2005). The expression of PGC-1α in skeletal muscles is greatly influenced by levels of physical activity, with endurance exercise increasing PGC-1α expression and physical inactivity leading to decreased expression (Aoi et al., 2010; Correia et al., 2015; Pilegaard et al., 2003; Scarpulla, 2008). Several studies have shown that PGC-1α expression decreases in muscle wasting (Adhihetty et al., 1985; Baker et al., 2006; Ling et al., 2004; Roberts-Wilson et al., 2010; Sainz et al., 2009), but there are also models that have shown the contrary, an increase in PGC-1α expression (Abruzzo et al., 2010; Sainz et al., 2009; Wagatsuma et al., 2011). So far

no one has shown that the loss of PGC-1α expression *per se* is sufficient to induce muscle atrophy. However, it has been confirmed that an increased expression of PGC-1α is sufficient to mitigate protein degradation and protect skeletal muscle mass from different atrophic stimuli (Cannavino et al., 2014; Goodman et al., 2011)

1.2.2 Skeletal muscle atrophy (muscle protein degradation)

The loss of skeletal muscle mass is a complex process that occurs as a consequence of a variety of stressors. Atrophy includes the reduction of muscle fibers CSA due to a net loss of proteins, organelles and cytoplasm. This occurs as a result of alterations in the balance between anabolic and catabolic processes, with the net result being a loss of muscle mass when protein breakdown exceeds protein synthesis (Bodine and Baehr, 2014). Acute muscle atrophy occurs in many pathological conditions, including neural inactivity, mechanical unloading, inflammation, metabolic stress, and elevated glucocorticoids levels. Acute muscle atrophy is then due to hyper-activation of the main cellular degradation pathways like ubiquitin-proteasome system (UPS) and the autophagy lysosome pathways (Sandri, 2013; Schiaffino et al., 2013). The activation of proteolytic systems in the cells is regulated at the gene level and the transcripts associated with muscle atrophy have been studied by gene expression profiling (Gomes et al., 2001; Bodine et al., 2001a). A subset of genes that are commonly up- or down- regulated in muscle atrophy, irrespective of the underlying cause, have been identified and are called atrophy-related genes or "atrogenes" (Bodine et al., 2001; Gomes et al., 2001; Lecker et al., 2004; Sacheck et al., 2007). Among these atrogenes there are several transcripts associated with the UPS and autophagy (Sandri, 2008).

Atrogenes as well as protein breakdown in general, are blocked by the IGF1/Akt pathway. Akt phosphorylates Forkhead Box (Fox) O, thus inhibiting the UPS. In times of food deprivation when insulin levels are low, muscle mass is reduced by protein degradation, whereas high nutritional supply leads to a blockade of protein degradation and increased protein synthesis by activation of mTORC1. Members of the FoxO family (FoxO1, 3 and 4), downstream Akt, have been identified as key transcription factors controlling the expression of MuRF1 and atrogin-1. Additionally it has been shown that FoxO3, specifically, also regulates autophagy (Mammucari et al., 2007; Sandri et al., 2004; Zhao et al., 2007). Recent work by Milan et al. (Milan et al., 2015) demonstrates that muscle-specific deletion of FoxO members protects the muscle from atrophy, further demonstrating the role of FoxOs in the induction of the UPS and the autophagy lysosome pathway. Furthermore, it was demonstrated that FoxOs control several stress-response pathways such as the unfolded protein response, ROS detoxification, DNA repair and translation (Milan et al., 2015).

1.2.2.1 Ubiquitin proteasome system

The UPS is an ATP-dependent proteolytic system that targets proteins with ubiquitin (Ub) molecule substrates, through a cascade of conjugating enzymes (ligases), for identification of degradation (Murton et al., 2008). The ubiquitin ligase enzymes (E3), binds the protein substrate and once a protein is ubiquitinated, it is unfolded and fed into the proteasome in an ATP-dependent process. (Sandri, 2013). Two important atrogenes that have been found in several animal models of muscle atrophy including burn injury, diabetes, denervation, unloading, dexamethasone administration, and sepsis, are muscle-specific E3s; atrogin-1 and muscle RING finger-1 (MuRF1) (Kisselev and Goldberg, 2001). These two atrogenes have been extensively studied in muscle protein degradation and their involvement in muscle atrophy is well known. Mice lacking atrogin-1 and MuRF1 are resistant to muscle atrophy in response to denervation and knockdown of atrogin-1 prevents muscle wasting induced by fasting (Bodine et al., 2001; Cong et al., 2011).

It has been shown that MuRF1 interacts with and controls the half-life of several essential muscle structural proteins like troponin I (Kedar et al., 2004), MyHC (Clarke et al., 2007; Fielitz et al., 2007) and actin (Polge et al., 2011). So far only a few muscle proteins have been identified as substrates for atrogin-1. The substrates found are however all related to growth-associated processes (Csibi et al., 2010; Tintignac et al., 2005). Even though MuRF1 and atrogin-1 are significant Ub-ligases involved in muscle atrophy, there are likely to be other E3s related to muscle wasting. Additionally, particular Ub ligases can be involved in different muscle atrophy models and in different stages of the process. Recently a new set of Ub ligases have been discovered as significant atrogenes that are under the control of the FoxO-family. This set of Ub-ligases includes MUSA1, an E3, that has been found to be critical in muscle atrophy during denervation and fasting (Sartori et al., 2013), Fbxo31, an additional E3, Itch, a Ub-ligase that regulates the half-life of several transcription factors, and Fbxo21, a gene of unknown function but that contains an F-box motif and that has been given the name SMART (Specific of muscle atrophy and regulated by transcription). This novel set of E3s were all up-regulated in response to denervation induced atrophy (Milan et al., 2015).

1.2.2.2 Autophagy lysosome pathway

Autophagy is a physiological process that is vital for the cells to eliminate damaged cell-components and to remodel the cellular architecture (Neel et al., 2013). There are three types of autophagy that all promote proteolytic degradation of cytosolic components; macro-autophagy, micro-autophagy and chaperone mediated autophagy (CMA). Both macro-and micro-autophagy can ingest large structures through selective and non-selective mechanisms. Macro-autophagy transports cytoplasmic cargo to the lysosome through the intermediate of an autophagosome, a double membrane-bound vesicle. The autophagosome will then fuse with the lysosome forming an autolysosome. In contrast, in micro-autophagy, the cytosolic components are taken up by the lysosome directly through invagination of the lysosomal membrane (Glick et al.; Sandri, 2010). In the third type of autophagy CMA, proteins that have been targeted are translocated in a complex with chaperone proteins (such as heat shock

proteins), and can cross the lysosomal membrane. This way the targeted proteins can be recognized by the lysosomal membrane receptor, lysosomal-associated membrane protein 2A (LAMP-2A) and are subsequently unfolded and degraded (Saftig et al., 2008).

Autophagy is primarily considered to be a non-selective degradation pathway, but autophagy can trigger the selective elimination of specific organelles, such as mitochondria (via mitophagy). *Parkin, PINK1, Bnip3 and Bnip3L* are genes that have been identified in mammals to control mitophagy, inactivation of the genes encoding these proteins leads to abnormal mitochondria (Hara et al., 2006). During muscle wasting the mitochondrial network is drastically remodeled in response to fasting or denervation, and autophagy plays a great part in this process (Romanello et al., 2010a; Romanello and Sandri, 2010)**.** Alteration in mitochondrial dynamics is sufficient to cause muscle wasting in mice, indicating that disturbance of the mitochondrial network is essential for the muscle homeostasis (Romanello et al., 2010a; Romanello and Sandri, 2010). The critical role of the autophagy-lysosome system in skeletal muscles has repeatedly been confirmed by showing that alterations to the system contribute to the pathogenesis of several genetic muscle diseases (Chang et al., 2012; Masiero et al., 2009; Raben et al., 2008). Autophagy has two important roles in muscle homeostasis; it can be harmful and trigger muscle degeneration, but it can also work as a compensatory mechanism for cell survival (Bonaldo and Sandri, 2013).

1.2.2.3   Inflammatory cytokines

NF-κB is a known regulator of genes that encode cytokines, cytokine receptors, and cell-adhesion molecules. They all play major roles as mediators of immunity and inflammation and are also expressed in skeletal muscles. Tumor necrosis factor-α (TNFα) is an important cytokine involved in muscle wasting and cachexia (Peterson et al., 2011). When NF-κB is inactive, it is retained in the cytoplasm by IκB, a family of inhibitory proteins. However in response to TNFα, the IκB kinase (IKKβ) complex phosphorylates IκB, which results in the ubiquitination and degradation of the inhibitory protein, hence leading to the nuclear translocation of NF-κB and activation of NF-κB-mediated gene transcription (Bonaldo and Sandri, 2013; Peterson et al., 2011). NF-κB regulates the transcription of more than 400 genes that are involved in immune- and growth-regulation, inflammation, carcinogenesis, and apoptosis (Carmeli et al., 2015). Additionally the role of NF-κB in muscle atrophy has been demonstrated by its capability of controlling the expression of MuRF1. Cai et al. (Cai et al., 2004) demonstrated that the activation of NF-κB in muscle-specific transgenic mice activated IKK (MIKK) and induces significant atrophy due to an accelerated protein breakdown, which is mediated by the expression of MuRF1.

1.2.2.4   Apoptosis

Apoptosis is an extremely organized process of programed cell death that can happen both in physiological and pathological conditions (Marzetti et al., 2012; Otrocka-Domagala, 2011). The main role of apoptosis is to reduce the number of cells in excessive proliferation and to eliminate damaged or metabolically broken cells. Apoptosis can be activated by both external

and internal signals in the cell, triggering a number of molecular pathways of cell death (Marzetti et al., 2012). The apoptotic machinery includes regulatory proteins, endonucleases, protease inhibitors, and proteolytic enzymes, known as caspases. Upon stimulus for cell death, initiator caspases (caspases 8, 9, and 12) are activated which in turn leads to activation of effector caspases (caspases 3, 6, and 7) that regulates the cellular degradation and DNA fragmentation (Carmeli et al., 2015). In addition, apoptotic signaling in skeletal muscles can also activate degradation of muscle proteins through the activation of the UPS (Carmeli et al., 2015).
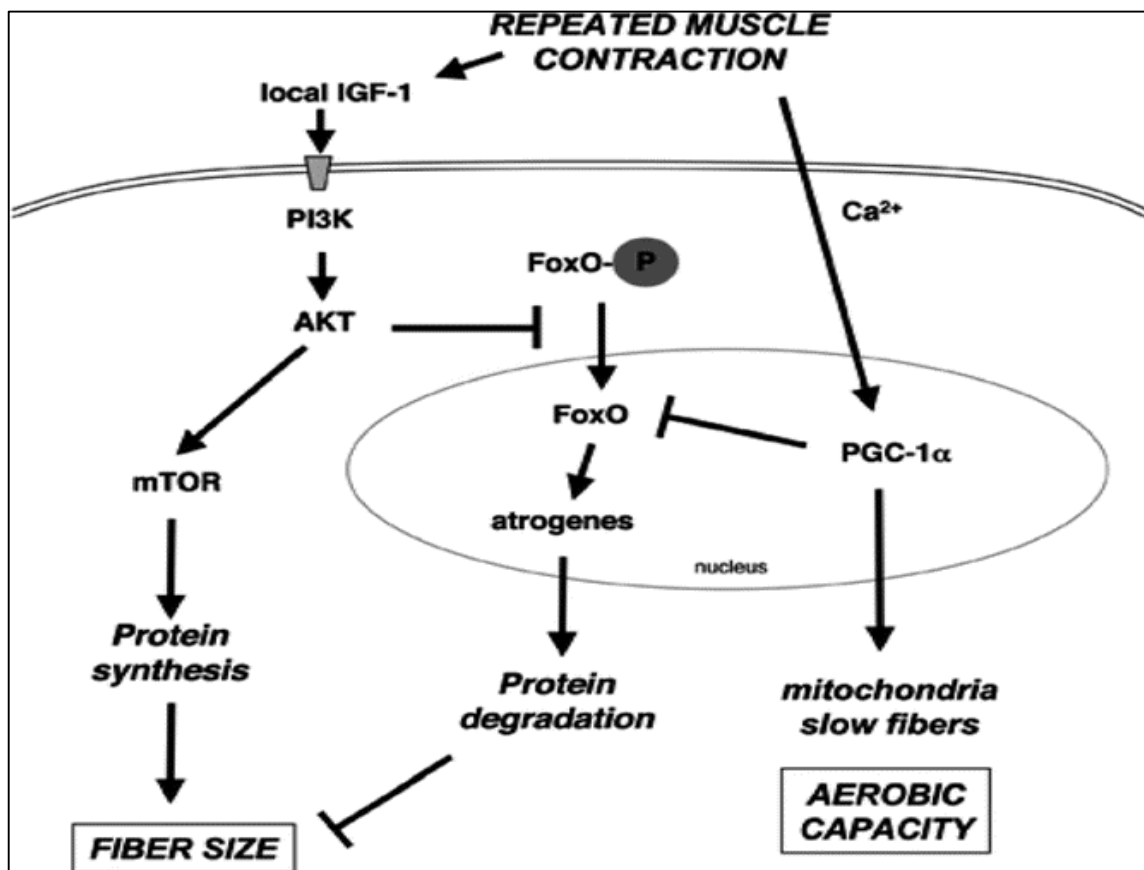
Figure 2. Muscle mass regulating signaling pathways.
IGF-1, PI3K, Akt, mTOR, FoxO, PGC-1α (Puthucheary et al., 2010a). With permission from Wolters Kluwer Health, Inc.

1.3   MUSCLE SPECIFIC DIFFERENCES

Skeletal muscles are heterogeneous both at the level of whole muscles, motor units and individual muscle fibers. Different muscle properties will decide the specific movements that each muscle can perform. An important aspect of muscle diversity lies in their embryological origin (Merrell and Kardon, 2013; Sambasivan et al., 2009). Craniofacial muscles, are evolutionarily and developmentally distinct from trunk and limb muscles (Noden and Francis-West, 2006; Sambasivan et al., 2011). Trunk and limbs muscle are derived from somites that originate from the paraxial mesoderm of the trunk, whereas the cranial mesoderm gives rise to head muscles like extraocular muscles and cheek muscles like the masseter and the buccinators (Grifone and Kelly, 2007; Harel et al., 2009; Nathan et al., 2008). MyoD, Myf5 and Mrf4 are myogenic regulatory factors that work together to control the entry to the myogenic differentiation program and that applies to all skeletal muscles. However, the regulatory hierarchies that act upstream of the myogenic factors are diverse in somatic and cranial mesoderm (Czajkowski et al., 2014). Significant differences between muscles also lies in further development into mature skeletal muscles (Merrell and Kardon, 2013).

In adult skeletal muscles and muscle fibers, the same stimulus can cause drastic divergent responses. The same fiber type may undergo opposite changes in different muscles. Even within the same muscle different fiber types react differently (Blaauw et al., 2013; Schiaffino et al., 2013). The nervous system utilizes the capacity of the muscles to generate force and movement for a variety of motor tasks. These tasks can roughly be divided into three main types; postural joint stabilization, long lasting repetitive activities (like respiration) and fast and powerful actions (such as jumping or kicking). In mammals the motor units are functionally organized into separate components, the motor units each consist of a motoneuron and the muscle fibers that it exclusively innervates. Muscle fibers attain a degree of specialized molecular structure and physiological parameters to perfectly suit the needs of the motor units (Blaauw et al., 2013). The heterogeneity of skeletal muscle fibers, therefore, mainly reflects an adaptation to the different patterns of activity; in addition it also indicates specialization in membrane properties, calcium shuttling mechanisms, contractile machinery and the structure of the cytoskeleton (Polla et al., 2004; Schiaffino and Reggiani, 2011).

The diaphragm is a muscle engaged in a nonstop rhythmic activity which does not permit any break to rest. Therefore the diaphragm muscle fibers must be very resistant to fatigue (Merrell and Kardon, 2013; Polla et al., 2004). In addition to ventilation, the diaphragm muscle is also required for coughing, talking and singing, activities which are phasic and occasional. Diaphragm fibers generally have a smaller CSA than limb muscles. However the number of capillary vessels surrounding each fiber is the same, the diffusion distance is reduced which makes the oxygen supply more efficient in the diaphragm than in other muscles. This might improve oxygen diffusion and contribute to the increased resistance of the diaphragm to fatigue. Respiratory muscle fibers do not undergo the same changes in response to training and inactivity as limb muscle fibers (Merrell and Kardon, 2013; Polla et al., 2004).

1.4   MUSCLE WASTING IN THE INTESIVE CARE UNIT

Acquired muscle weaknesses in the intensive care unit (ICU) is a major complication that occurs in severely ill patients and has a significant impact on the immune system, energy metabolism, amino acid reserves, and temperature regulation (Puthucheary et al., 2010b). It affects the limb and respiratory muscles, and, as a consequence it usually complicates weaning from the ventilator, increases the length of stay in the intensive care unit, and prolongs physical rehabilitation. This condition can either affect the peripheral nerves (critical illness polyneuropathy, CIP), skeletal muscles (critical illness myopathy, CIM) or both (critical illness polyneuromyopathy, CIPNM) (Latronico and Bolton, 2011; Latronico and Guarneri, 2008; Visser, 2006). CIP and CIM are the primary cause of muscle weakness and paralysis in the ICU (Latronico and Bolton, 2011). The prevalence of CIP and CIM in the general ICU population is currently unknown (Tennila et al., 2000; Zink et al., 2009), however, in subpopulations in which sepsis is complicated by multiple organ failure, the incidence of CIP and/or CIM could even reach 80-100% (Tennila et al., 2000). The weakness seems to be caused by critical illness and the ICU course regardless of the underlying primary condition (Lacomis et al., 1996; Maramattom et al., 2004). Muscle wasting and weakness is increasingly being recognized and the increasing prevalence of diagnosed ICU acquired weakness is both due to a growing awareness and improved survival of patients with prolonged organ failure (Puthucheary et al., 2010b). Muscle atrophy occurs within 10 to 21 days of muscle disuse when healthy older adults are bound to bedrest. Similar adverse skeletal muscle changes happen as early as days 5 to 7 after ICU admission in critical ill patients (Puthucheary et al., 2010b).

1.4.1   Critical illness myopathy

CIM is a myopathy that results from critical illness and is commonly found in deeply sedated immobilized and mechanically ventilated ICU patients. MacFarlane and Rosenthal were the ones to describe CIM, almost 40 years ago (MacFarlane and Rosenthal, 1977). The illness is characterized by paralysis of the limb muscles, apparent atrophy of the skeletal muscles and reduced muscle excitability but with intact nerve conduction velocities (Larsson et al., 2000; MacFarlane and Rosenthal, 1977). A unique feature of CIM is a specific myosin filament loss, which leads to muscle mass wasting and loss of function (Larsson et al., 2000; Sander et al., 2002). Other key findings are decreased generation of muscle force and unbalanced muscle metabolism, disorganization of sarcomeres, altered protein turnover and impaired autophagy (Friedrich et al., 2015). Among critically ill adults, CIM is linked to prolonged mechanical ventilation (MV), persistent functional disability, loss of independence, and decreased life expectancy.

Systemic factors cause muscle damage and intracellular signals alter bioenergetics and increases muscle protein break-down. Even though the development of muscle impairment associated with CIM is being described with increasing accuracy as a result of advances in genetic and proteomic science, the exact cause and underlying mechanisms of the disease remain unknown.

Many triggering factor have been suggested for CIM and muscle wasting in the ICU including sepsis, systemic inflammatory response syndrome (SIRS) and multiple organ failure. Animal models have, however, allowed the separation of sepsis and other confounding risk factors, e.g., mechanical ventilation and muscle unloading, which are usually present in ICU patients. Sepsis on its own has then not been able to replicate the CIM phenotype (Friedrich et al., 2015). Other potential triggering factors evolve from the interventions used in modern anesthesiology and the ICU, that is; prolonged mechanical ventilation, neuromuscular blockers (NMB), systemic corticosteroid (CS), hormone treatment and muscle unloading (Larsson, 2007).

1.4.2 Ventilator-induced diaphragm dysfunction

MV is often a life-saving procedure for patients with respiratory failure, however, there is increasing suggestion that prolonged disuse of the diaphragm results in rapid development of diaphragmatic weakness due to both atrophy and contractile dysfunction. This harmful effect of prolonged MV has been named ventilator-induced diaphragmatic dysfunction (VIDD) (Powers et al., 2013; Vassilakopoulos and Petrof, 2004). Most patients are without difficulty removed from MV, but about 25% of patients experience difficult weaning (Daniel Martin et al., 2013). Furthermore, for these patients, weaning procedures account for 40% - 60% of the total time on the ventilator (Esteban et al., 1994; Esteban et al., 1995). There is still little information available regarding the underlying mechanism in humans. By using both microscopic and molecular analyses of diaphragm tissue, it has been shown that VIDD induces significant decreases in fiber cross-section and a substantial up-regulation of oxidative stress enzymes and activation of several intracellular proteolytic pathways including the UPS (Levine et al., 2008; Petrof et al., 2010; Shanely et al., 2002).

1.4.3 Preventive and therapeutic approaches

Some therapeutic strategies have been proposed for the prevention of CIM and muscle wasting in the ICU, including nutritional interventions, antioxidant therapy, application of testosterone derivatives and growth hormones (Garnacho-Montero et al., 2001; Hermans et al., 2014; Hermans et al., 2009). However, none of these interventions has conclusively shown to have beneficial effects on muscle function (Zink et al., 2009). Strategies used today to inhibit muscle weaknesses and wasting in the ICU, mainly focus on avoiding and reducing risk factors, like sepsis, hyperglycemia, prolonged immobilization, prolonged duration of mechanical ventilation as well as the use of corticosteroids and neuromuscular blocking agents (Hermans et al., 2014; Zink et al., 2009). Additionally, strategies that prevent the catabolic state associated with critical illness are now being used and could be beneficial. Thus, therapeutic strategies are evidently limited and with uncertain beneficial effects, further research is clearly needed to achieve a better understanding of the cellular and molecular mechanisms underlying the muscle wasting and weakness seen in ICU patients.

2 AIM

The aim of this thesis is to achieve a better understanding of the cellular and molecular mechanisms underlying the muscle wasting and weakness seen in ICU patients with CIM. The objective is to evaluate specific therapeutic strategies and efficient rehabilitation programs. In accordance with the stated objectives, a rodent ICU model was used to address the mechanistic and therapeutic aspects of the disease. The long term goal of this project is to unravel underlying mechanisms, identify new therapeutic strategies and efficient rehabilitation programs.

2.1 SPECIFIC AIMS

- Determine the effects of passive mechanical loading on mucle wasting in response to mechanical silencing in an experimental rat ICU model (Paper I).
- Gain insight into the molecular pathways regulating the process of mechanical activation of skeletal muscles that are affected by the ICU condition (Paper II).
- Follow the time course changes of the diaphragm muscle structure and function, as well as gene and protein levels affected by the ICU condition.
- Gain understanding of mechanisms of the relative sparing of a cranial nerve innervated muscle in response to mechanical silencing, in comparison to spinal nerve innervated muscles.

3 METHODS

3.1 ANIMALS

Female Sprague-Dawley rats were anaesthetized, treated with α-cobra toxin and mechanically ventilated for durations varying from 6 hours to 14 days. The sham-operated control animals underwent the same interventions as the controls, but were not pharmacologically paralyzed with α-cobra toxin, that is, sham operated controls were anaesthetized (isoflurane), spontaneously breathing, given intra-arterial and intravenous solutions, and euthanized within 2 hours after the initial anesthesia and surgery. Animals were euthanized by removing the heart during deep isoflurane anesthesia and controlled mechanical ventilation.

3.2 MECHANICAL LOADING

The mechanisms underlying the effects on skeletal muscle structure and function by passive mechanical loading in animals exposed to immobilization and mechanical ventilation ("ICU intervention") was investigated. The left leg of the animal was activated for 6 hours at the shortest duration and 12 hours day $^{-1}$ at durations 12 hours and longer throughout the experiment, using a mechanical lever arm that produced continuous passive maximal ankle joint flexions–extensions at a speed of 13.3 cycles min $^{-1}$ (**Paper I and II**). Experiments were terminated at durations varying between 6 hours and 14 days

3.3 MUSCLE BIOPSIES AND PERMEABILIZATION OF MUSCLE FIBER MEMBRANE

The EDL, soleus, plantaris and the gastrocnemius muscles from the loaded left leg and the unloaded right leg (**Paper I and II**), the masseter (**Paper III**) and the diaphragm (**Paper IV**), were dissected immediately after the animals were killed. The masseter, plantaris and the gastrocnemius muscle and one half of the diaphragm, EDL and soleus muscle was quickly frozen in liquid propane cooled by liquid nitrogen and stored at -160°C for further analyses. The other half of the diaphragm, EDL and soleus muscle were placed in relaxing solution at 4°C and bundles of ~50 fibers were dissected free and tied with surgical silk to glass capillary tubes at slightly stretched lengths. The bundles were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 hours at 4°C, after which they were transferred to -20°C. The muscle bundles were treated with sucrose, a cryo-protectant, within 1-2 weeks for long-term storage (Frontera and Larsson, 1997). Subsequently, the muscle bundles were detached from the capillary tubes and snap frozen in liquid nitrogen-chilled propane and stored at -160°C. One day before the experiments, a bundle was transferred to a 2.0 M sucrose solution for 30 min, then incubated in solutions of decreasing sucrose concentration (1.5-0.5 M) and finally kept in a skinning solution at -20°C.

3.4 PROTEIN EXPRESSION ANALYSIS

3.4.1 Total protein content

Total protein content was determined from 10μm EDL, soleus **(Paper I and II)**, gastrocnemius, plantaris **(Paper II)**, diaphragm **(Paper III)** and masseter **(Paper IV)** muscle cross-sections dissolved in 100μl 8M urea buffer after centrifugation and heating (90°C for 2 min) using the NanoOrange® protein quantification kit **(Paper I)** and the Pierce® 660 protein assay **(Paper II and IV)** according to manufactures instructions.

3.4.2 Myosin and actin content

Myosin and actin contents were quantified on 12% SDS-PAGE **(Paper I)**. The total acrylamide concentration was 4% (w/v) in the stacking gel, 12% in the running gel and the gel matrix included 10% glycerol. Samples were loaded together with the standard dilutions. The standard was prepared by pooling sections from control rat EDL and soleus muscles. The myofibrillar protein standards were prepared, assuming that actin and myosin contents were 12.5 and 25% of the total protein content, respectively. Linear actin and myosin curves were observed within the 5-200 μg ml $^{-1}$ range, but the calibration curves were not parallel. Gel electrophoresis was performed at a constant current of 16 mA for 5 hours at 15°C. The gels were stained with coomassie blue and subsequently scanned in a soft laser densitometer. The volume integration function was used to quantify the amount of protein.

3.4.3 Myosin and actin ratio

Myosin and actin ratios were measured on 12% SDS-PAGE **(paper I and IV)**. The total acrylamide concentration was 4% (w/v) in the stacking gel, 12% in the running gel and the gel matrix included 10% glycerol. Gel electrophoresis was performed at a constant current of 16 mA for 5 hours at 15°C. The gels were stained with coomassie blue and subsequently scanned in a soft laser densitometer. The volume integration function was used to quantify myosin and actin.

3.4.4 Myosin heavy chain isoform expression

MyHC isoform expression was determined by 6% SDS-PAGE **(Paper I and IV)**. The total acrylamide concentration was 4% (w/v) in the stacking gel, 6% in the running gel, the gel matrix included 30% glycerol. The gel electrophoresis was performed at a constant voltage of 120 V for 20-22 hours at 10°C. The gels were silver-stained and subsequently scanned in a soft laser densitometer.

3.4.5 Troponin isoform expression

Troponin (Tn) isoform expression was measured in TA muscle samples homogenized in SDS-gel sample buffer **(Paper I)**. After heating (5 min at 80°C) and centrifuging muscle samples were stored at -80°C. For SDS-PAGE the protein extracts were resolved on 14% Laemmli gel with an acrylamide/bis ratio of 180:1. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue R250.

3.4.6 Western blots

To study the expression of specific proteins, immunoblot analysis were done. In **paper I** troponin isoform expression was measured in TA muscle. Atrogin-1 and MuRF1 was measured in the soleus and Grp94 protein levels were studied in the plantaris muscle. In **paper II** S6, P-S6 (Ser240/244), 4EBP1, P-4EBP1 (Thr37/46), Akt, P-Akt (Ser473) was measured in soleus and in plantaris muscles. Caspase 3 was measured in the gastrocnemius muscle. Additionally AIF and cytochrome C was quantified in the cytosolic and mitochondrial fraction of the gastrocnemius muscle. In **Paper III** MuRF1, Grp94, Caspase 3, Calpain and LC3B protein levels were studied in the diaphragm muscle. In **paper IV** atrogin-1, MuRF1, Akt, P-Akt (Ser473), LC3B, HSP70, HSP90, β-crystallin, MMP-2 and TIMP-2 were quantified in the masseter muscle. The immunoblots were subsequently scanned in a soft laser densitometer, the signal intensities were quantified using the volume integration function (arbitrary units) and normalized to actin, actinin or complex IV.

3.4.6.1 *Protein Carbonylation Levels*

To assess the formation of protein carbonyl groups, the OxyBlot protein oxidation detection kit (Chemicon, Chandlers Ford, UK) **(Paper I and III)** was used. About 12μg of total protein was used for derivatization with 2.4-dinitrophenylhydrazine (DNPH) according to the manufacturer's detailed protocol and processed for Western blot analysis. A positive control included derivatization of 3μg of oxidized bovine serum albumin (BSA), whereas the negative control was performed with an equal amount of total protein reacted in the absence of DNPH. Levels of oxidated protein were quantified using the NIH Image J analysis software.

3.5 GENE EXPRESSION ANALYSIS

3.5.1 Total RNA isolation and quantification

Total RNA was extracted from frozen gastrocnemius muscle (proximal part) **(Paper I and II)**, plantaris muscle **(Paper II)**, diaphragm **(Paper III)** and masseter **(Paper IV)** tissue (10-30 mg) using a Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA). RNA concentrations were then quantified using the fluorescent nucleic acid stain Ribogreen (Molecular Probes, Eugene, OR, USA), on a fluorescence spectrophotometer.

3.5.2 Real time Polymerase chain reaction (qPCR)

qPCR was used to analyze mRNA levels for MyHC isoforms **(Paper I, III and IV)**, actin **(Paper III and IV)**, MuRF1, atrogin-1 **(Paper II and IV)**, IGF-1, Fbxo 31, SMART, Mgn, HDAC4, HDAC5, PGC-1α, PGC-1α4, Fis1, OPA1, Mfn1, Mnf2, Bnip3, Pink1, Parkin **(Paper II).** cDNA synthesis and qPCR was performed as described in the papers I, II, III and IV.

3.5.3 Gene expression profiling (Paper I)

Three micrograms of total RNA from the proximal gastrocnemius muscle samples was extracted and processed to generate biotin-labelled cDNA. Each sample was then hybridized to an Affymetrix Rat Gene 1.0 ST Array. Microarray data were background-adjusted, normalized and log transformed summarized values. In order to search for the differentially expressed genes between the samples from the different days an empirical Bayes moderated t-test was applied. A linear model was fitted to the data, controls *vs* unloaded and unloaded *vs* loaded at the following time durations: 0.25-4 days, 5-8 days and 9-14 days. To address the problem with multiple testing, the *p*-values were adjusted according to Benjamini and Hochberg. Probe sets with a minimum fold change of ±1.5 and adjusted p-value < 0.5 at least in one of the time points were included for further analysis.

3.6 IMMUNOHISTOCHEMISTRY (PAPER III)

3.6.1 Immunohistochemistry nNOS

After a 10 min fixation at room temperature with 4% buffered paraformaldehyde, cryosections were incubated overnight at 4°C with anti-neuronal nitric oxide synthase (anti-nNOS) antibody, extensively rinsed with PBS and incubated for 2 h at room temperature with secondary antibody conjugated with Alexa 568. Slides were observed with a Leica SP5 confocal microscope equipped with a helium 543 nm laser (Leica Microsystems).

3.6.2 MyHC immunohistochemistry

Serial consecutive transverse cryosections of, diaphragm muscle, were assayed for immunohistochemistry using anti-MyHC antibodies. The following antibodies were used: clone BA-D5 for type I-β MyHC, clone SC-71 for type IIa MyHC, and clone BF-35, which labels all MyHCs except for type IIx, and therefore allows the identification of the unreacting myofiber as type IIx.

3.6.3 DNPH immunohistochemistry

Serial cryosections labelled with anti-myosin antibodies were reacted with DNPH for 1 hour at room temperature (Vitadello et al., 2003). After a 10 min rinse, sections were saturated with 3% BSA for 20 min, and incubated with the anti-DNPH antibody. Bound antibody was revealed by peroxidase immunohistochemistry.

3.6.4 NADPH-d histochemistry

Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) activity was demonstrated by histochemistry. Cryosections were fixed for 10 min, after rinsing with PBS, sections were incubated in medium containing Tris-HCl, Triton X-100, nitroblue tetrazolium chloride and NADPH for 45 min at 37°C. Reaction was stopped by rinsing briefly with distilled water. Consistency of the labelling was validated by independent analysis of adjacent

cryosections. Histochemical and immunohistochemical analysis was performed using the Zeiss Axioplan microscope and ×10 and ×40 Plan-Neofluar objectives. Images were acquired using a Leica digital DFC 300FX camera and the IM50 software. Myofiber cross-sectional circumference (CSC) was evaluated using ImageJ software.

3.7 CONTRACTILE MEASUREMENTS OF SINGLE MUSCLE FIBERS

On the day of an experiment, a fiber segment 1-2 mm long was left exposed to the experimental solution between connectors leading to a force transducer and a lever arm system **(Paper I and III)**. The apparatus was mounted on the stage of an inverted microscope. While the fiber segments were in relaxing solution, the sarcomere length was set to 2.65-2.75 μm by adjusting the overall segment length. The diameter of the fiber segment between the connectors was measured through the microscope at a magnification of ×320 with an image analysis system prior to the mechanical experiments. Fiber depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the fiber. The focusing control of the microscope was used as a micrometer. Fiber CSA was calculated from the diameter and depth, assuming an elliptical circumference, and was corrected for the 20% swelling that is known to occur during skinning. Maximum active tension (P_0) was calculated as the difference between the total tension in the activating solution (pCa 4.5) and the resting tension measured in the same segment while in the relaxing solution. All contractile measurements were carried out at 15°C. Specific force (SF) was calculated as maximum tension (P_0) normalized to CSA. After the mechanical measurements, each fiber was placed in urea buffer and stored at -80°C for subsequent electrophoretic analyses.

3.8 FRACTIONAL PROTEIN SYNTHESIS RATE (PAPER I)

Fractional protein synthesis rate was used to measure the rate of muscle protein synthesis in the superficial and deep part of the gastrocnemius muscle using [ring-^{13}C^6] phenylalanine ($15\mu g \ g^{-1}$ body weight) as a tracer. The level of enrichment of [ring-^{13}C^6] phenylalanine in the tissue was analyzed using chromatography tandem mass spectrometry (GC/MS/MS).

3.9 MASS SPECTROMETRY (PAPER III)

LC/MS analysis of myosin heavy chain isoforms was performed to investigate post translational modifications of myosin

3.9.1 In-gel digestion

The type I, IIa, IIx and, in some instances, IIb, MyHC isoforms were separated on a 6% SDS-PAGE gel. The bands were cut out and washed with 100 mM NH_4HCO_3 and then reduced. The samples were alkylated and then dried at 50°C before treating with trypsin and then kept at 37°C overnight. Samples were sonicated, on the following morning and the liquid was collected and then dried under vacuum. The peptides were stored at -20°C until further analysis.

3.9.2 Liquid chromatography (LC)/mass spectrometry (MS) analysis

The LC/MS analysis was performed using the linear ion trap. The precursor ions were isolated within a 1Da window and fragmented by collision-induced dissociation with 35% normalized collision energy. The activation time was set to 10 ms and $q = 0.25$. Dynamic exclusion was set to 30 s and the exclusion mass width was set to 5 p.p.m. relative to the reference mass.

3.10 X-RAY DIFFRACTION (PAPER III)

Structural analysis of diaphragm bundles was assessed by x-ray diffraction. On the day of x-ray recordings, fiber bundles were placed in a plastic dish containing a relaxing solution and washed thoroughly to remove the glycerol. They were then transferred to a specimen chamber filled with the same relaxing solution. Each fiber bundle was then fixed (clamped ends) and slightly stretched. Subsequently, X-ray diffraction patterns were recorded at 15°C. For each fiber bundle, approximately 20–40 diffraction patterns were recorded at the BL45XU beamline of Spring-8.

3.11 STATISTICS

Sigmaplot software (systat Software, Inc., CA, USA) was used to generate descriptive statistics. Means, standard error of the means (SEM) and linear regression analysis were calculated according to standard procedures. Paired t-test in pairwise comparison, between the loaded and unloaded One and Two-way analysis of variance (ANOVA) and the Tukey *post-hoc* test were used when comparing multiple groups. When normality test failed, a one way ANOVA on ranks, i.e., Kruskal-wallis and the Dunn's *post-hoc* were performed. Differences were considered significant at $p < 0.05$.

4 RESULT AND DISCUSSION

4.1 BENEFICIAL EFFECTS OF PASSIVE MECHANICAL LOADING ON SKELETAL MUSCLES (I)

Mechanical silencing (absence of external and internal strain) has been shown to be a key factor underlying muscle wasting associated with the ICU condition (Ochala et al., 2011a). Hence it was hypothesized that passive loading would alleviate the muscle wasting and decreased force-generation capacity associated with CIM and mechanical silencing. A physical therapy intervention was consequently tried using a rat ICU model. Animals were subjected to unilateral passive mechanical loading for 12 hours per day for up to 14 days. Results showed a significant decrease in muscle atrophy associated with the ICU condition, independent of muscle type. At the single fiber level, a 44% higher CSA and a doubling of the force generating capacity was observed. This was accompanied by an up-regulation of transcriptional levels of contractile proteins from 5-8 days of the ICU intervention, thus leading to a reduced myosin loss in the soleus. This is in accordance with other studies that have shown that static passive stretching suppresses muscle wasting associated with denervation, hind-limb suspension and cast immobilization (Agata et al., 2009; Coutinho et al., 2004; Sasa et al., 2004; Williams, 1990).

The beneficial effects of passive mechanical loading on alleviating the muscle atrophy in sedated pharmacologically paralyzed and mechanically ventilated rats support the importance of early physical mobility therapy in deeply sedated and mechanically ventilated ICU patients, resulting in shortening of ventilator days and ICU stay. This also reduces the financial costs and enhances patients prognosis and the quality of life of survivors (Brahmbhatt et al., 2010; Morris, 2007; Morris et al., 2008; Needham et al., 2010; Schweickert et al., 2009).

4.2 MYOSTATIN-SMAD2/SMAD3 PATHWAY MAY BE INVOLVED IN THE BENEFICIAL EFFECTS OF PASSIVE MECHANICAL LOADING

Caveolin-3 (Cav3) is a muscle specific, membrane protein that directly binds to and regulates specific lipid and lipid-modified proteins (Ohsawa et al., 2008). Several mutations in the Cav3 gene have been detected in autosomal dominant limb-girdle muscular dystrophy and autosomal dominant rippling muscle disease (Betz et al., 2001; Galbiati et al., 1999; Minetti et al., 1998). Further, it has also been shown that Cav3 is an inhibitor of Mstn that works by suppressing Mstn's intracellular signaling to phosphorylate Smad2, inter alia (Ohsawa et al., 2008).

Follistatin (Fstn), is a protein which has been found to inhibit TGF-β family members (Lee, 2010). Specifically Fstn has been shown to function as a potent inhibitor of Mstn both *in vitro* and *in vivo* (Lee and McPherron, 2001; Zimmers et al., 2002).

Both Cav3 and Fstn were consequently up-regulated after 5 days of ICU intervention in response to passive mechanical loading. This suggests a stimulation of muscle growth through the mysotatin/Smad2/Smad3 pathway, and possible by prompting protein synthesis by Akt and mTORC1 (Amirouche et al., 2009; Lagirand-Cantaloube et al., 2008; Lokireddy et al., 2011; Sartori and Sandri, 2015; Trendelenburg et al., 2009).

However, further investigation is needed to resolve how passive mechanical loading causes the up-regulation of Cav3 and Fstn to stimulate muscle growth through the mysotatin/Smad2/Smad3 pathway.

4.3 MITOCHONDRIAL DYNAMICS PLAY A CRITICAL ROLE IN THE BENEFICIAL EFFECTS OF PASSIVE MECHANICAL LOADING (II)

Mitochondria are constantly changing in number, shape and localization. This feature is a result of the continuous alteration between fusion and fission events. Mitochondrial dynamics are important to secure proper mitochondria maintenance and repair. Alteration of the mitochondrial morphology allows exchange of mitochondrial content and discrimination of terminally damaged mitochondria to enable degradation by a controlled autophagy called mitophagy (Chen and Chan, 2010; Tondera et al., 2009). Alterations in mitochondria functions have long been characterized in different conditions of muscle wasting such as aging, diabetes and muscular dystrophies (Bernardi and Bonaldo, 2008; Larsson, 2010; Mootha et al., 2004; Patti et al., 2003). Furthermore, it was similarly shown that an imbalance in mitochondrial dynamics plays a role in the development of muscle atrophy induced by denervation, fasting and FoxO3 overexpression (Romanello et al., 2010b). In accordance with these results we found alterations in mitochondrial dynamics in response to mechanical silencing associated with CIM in the experimental ICU rat model. Transcription levels of Fis1, Opa1, Mfn1 and Mfn2, all mediators of mitochondrial fission and fusion, were significantly up-regulated after 5 days of mechanical silencing and remained up-regulated after 9-14 days. This up-regulation was however restricted to the unloaded side, i.e. on the loaded side these transcripts remained at control levels (**Figure 3**).

Additionally, Bnip3, Pink1 and Parkin were analyzed to study the effect of the ICU treatment and passive mechanical loading on mitophagy. Significant changes at the transcriptional levels of Pink1 were observed between the loaded and the unloaded side both after 5-8 days and 9-14 days. Similarly, Parkin was significantly induced after 5 days and passive mechanical loading counteracted this induction **(Figure 3)**.

Consequently, this indicates a key role of mitochondrial dynamic events as well as mitophagy in muscle wasting induced by mechanical silencing associated with ICU treatment and CIM, furthermore, these effects were completely counteracted by passive mechanical loading.
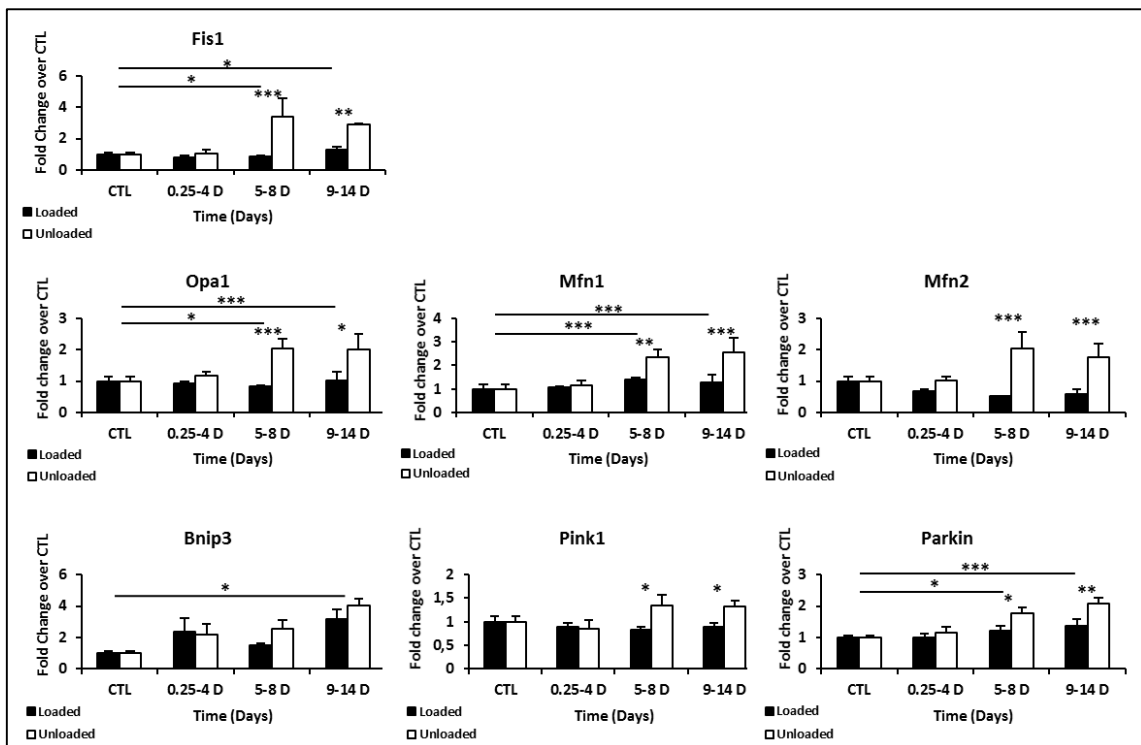
Figure 3. Mitochondria dynamics and Mitophagy

Transcription levels of Fis1, OPA1, Mfn1 mfn2, BNIP3, Parkin and Pink1 in plantaris muscle from control animals and from animals exposed to sedation, NMB, mechanical ventilation and unilateral passive mechanical loading for 0.25-4, 5-8, 9-14 days. Values are fold changes over controls and are means +SEM and Significant differences are indicated by * p< 0.05, ** p<0.01, *** p< 0.001

4.4 A NOVEL SET OF UBIQUITIN LIGASES ARE INDUCED BY MECHANICAL SILENCING (II)

Milan et al. (Milan et al., 2015) recently demonstrated that specific deletion of FoxOs in skeletal muscles prevents muscle loss and force decline in response to fasting and denervation. It was demonstrated that the FoxO family is required for the induction of several atrogenes including a set of novel E3s. A novel gene that encodes an F-box protein (FbxO21) was identified and given the name SMART, however its substrates are still unknown. In accordance with this, SMART and FbxO30 was up-regulated in response to mechanical silencing after 5-8 days of ICU intervention in the rat model. Transcriptional levels of both these E3 ligases also remained elevated after 9-14 days. Additionally, the up-regulation of these E3s was only observed on the unloaded side, on the loaded side, the transcriptional levels did not differ from controls. This suggests that mechanosensing plays a more important role than motoneuron discharge pattern in regulating the expression of these E3 ligases.

Gain- and loss-of-function experiments also showed that FoxOs are required for SMART regulation and that FoxO1 and FoxO3 are recruited on the promoter. Deletion of FoxO3 and not of FoxO1, however, completely blunted SMART induction after denervation (Milan et al., 2015) suggesting that SMART is mainly under FoxO3 regulation and that FoxO3 may play a significant role in disuse muscle wasting.

4.5 CONTROLLED MECHANICAL VENTILATION CAUSES EARLY CONTRACTILE DYSFUNCTIONS IN THE DIAPHRAGM (III)

Controlled mechanical ventilation (CMV) plays a key role in triggering the impaired diaphragm muscle function and the concomitant delayed weaning from the respirator in critically ill ICU patients (named VIDD). Experimental and clinical studies done in order to investigate the effect on diaphragm by CMV have mainly focused on early effects (Gayan-Ramirez et al., 2003; Racz et al., 2003) or at specific time points (De Jonghe et al., 2007; Radell et al., 2002; Yang et al., 2002).

A time-resolved analyses was performed, ranging from 6 hours to14 days using an experimental rat ICU model allowing detailed studies of the diaphragm in response to long-term CMV to improve our understanding of the mechanisms underlying VIDD. In accordance with former studies a rapid and early decline in maximum muscle fiber force was seen. However, on the contrary to previous studies that have shown a severe atrophy in response to short durations of CMV (De Jonghe et al., 2007; Gayan-Ramirez et al., 2003; Racz et al., 2003), muscle fiber size was maintained for the initial 4 days of mechanical ventilation in our model. Further these results are in accordance with previous observation seen in an experimental pig model, where no decrease in CSA was seen in response to CMV for 5 days (Ochala et al., 2011b). The combined effects of mechanical ventilation on fiber size and SF indicated a 25, 64 and 85% reduction of diaphragm muscle function in response to 0.25-4, 5-8 and 9-14 days of CMV compared with control fibers, respectively. The disagreement between the results may be due to other confounding factor, besides MV when commercially available ventilators are used even at duration's shorter than 24 hours (Larsson et al., 2015).

4.6 DIAPHRAGM ATROPHY, IN RESPONSE TO UNLOADING, DIFFERS FROM UNLOADED INDUCED ATROPHY IN THE LIMB MUSCLES (III)

The atrophy and loss of SF observed in the diaphragm, in response to CMV in this study, was more severe than what previously has been observed in limb muscles of animals exposed to the same type of intervention (Ochala et al., 2011a). In this study myosin:actin ratios remained unchanged until 9-14 days of CMV which is a significant difference from what has been observed in the limb muscles where myosin:actin ratios are reduced after 5 days of the ICU intervention. A decrease of myosin content was observed over time, hence indicating that protein degradation is not specific to myosin, but is also affecting actin, in the diaphragm muscle. This is in accordance with another study that demonstrated that myosin and actin degradation occurs in the diaphragm following 18-72 hours of CMV in brain dead, organ donors (Levine et al., 2011). On the other hand transcriptional levels of both myosin and actin remained unchanged until 9-14 days of exposure to CMV also in sharp contrast to what has been observed in the limb muscles, where the transcriptional levels of myosin and actin were dramatically decreased (Ochala et al., 2011a). Collectively these results demonstrate a

significant difference in the transcriptional regulation of contractile proteins between the diaphragm and limb muscles.

However, the contractile protein proteolysis does not correlate with the dramatic decrease in force generating capacity suggesting that protein degradation alone is not responsible for the impaired force production.

Mechanical ventilation has repeatedly been shown to increase ROS production and induce oxidative stress targeting proteins for degradation (Leijten et al., 1995; Powers et al., 2002; Shanely et al., 2002; Zergeroglu et al., 2003). In accordance with these studies, an increased amount of protein carbonylation was observed in the experimental ICU model. An increasing trend was detected early and was significant after 5 days of CMV. Altered ROS levels result in mitochondrial dysfunction (Blakemore et al., 1996; Suliman et al., 1999) that can lead to apoptosis and activation of the proteasome pathway targeting PTMs induced by oxidative stress (Boudriau et al., 1996; Zergeroglu et al., 2003). It has furthermore been shown that PTMs can effect muscle fiber function (Prochniewicz et al., 2008; Ramamurthy et al., 2001). Using mass spectrometry analyses it was demonstrated that CMV induced myosin PTMs, all of which were located in the rod region which is essential for the packing of myosin molecules into myosin filaments and for the binding of myosin-associated proteins. Unfortunately, less is known about modifications in this region of the myosin molecule than in the globular head. Nevertheless, mutation in the rod region of myosin results in specific myopathies (Ochala and Larsson, 2012).

4.7 MUSCLE MASS REGULATION MECHANISMS RESPOND DIFFERENT IN MASSETER COMPARED TO LIMB AND DIAPHRAGM MUSCLES IN RESPONSE TO MECHANICAL SILENCING (IV)

CIM primarily affects the limb and trunk muscles while cranial nerve innervated muscles are spared or less affected. The mechanisms underlying these muscle-specific differences, however, remain unknown. Some of the hallmarks of muscle atrophy and CIM, were therefore studied, to try to enhance our understanding of the mechanisms of the relative sparing of the masseter in response to mechanical silencing.

It was demonstrated that the 2:1 relationship between myosin and actin content was well preserved until the longest duration (9–14 days) in the masseter in contrast to what we previously have seen in the limb muscles where myosin:actin ratios and fiber size show a progressive decline after 5 days of the intervention (Ochala et al., 2011a). Furthermore transcriptional regulation of myofibrillar proteins in the masseter was not affected by the intervention and transcriptional levels of myosin and actin were unaffected during the two week observation period. In limb muscles, on the other hand, a dramatic transcriptional down-regulation of both myosin and actin was observed at durations longer than five days in the same experimental ICU model (Ochala et al., 2011a) as well as in ICU patients with CIM (Llano-Diez et al., 2012; Norman et al., 2009). Thus, the cranial nerve innervated masseter

muscle seems to resist the atrophy-inducing condition for longer periods than distal hind limb muscles.

In this study, the brief down-regulation of Akt phosphorylation does not seem to affect myosin and actin synthesis. Akt also inhibits the activation of FoxO and consequently the up-regulation of atrogenes, thus protects from protein degradation. The role of Akt was confirmed in the masseter as Akt phosphorylation levels were inversely proportional to the transcriptional levels of MuRF1 and atrogin-1, as well as to LC3b, which is also regulated by FoxO. The significant increase in Akt reactivation in the 9–14 day group may be explained as a muscle-specific adaptation to stress.

4.8 MASSETER MUSCLES IMPROVED ANTIOXIDATIVE PROFILE MAY BE THE MECHANISM OF PRESERVED MASTICATORY FUNCTION IN CIM (IV)

Heat shock proteins (HSPs) represent a cellular defense mechanism involved in muscle tissue remodeling and adaptation to e.g. oxidation, inflammation or energy changes (Liu and Steinacker, 2001). HSPs in the masseter were activated more rapidly than in limb muscles. HSP70 increased significantly after 5 days of ICU treatment, while αB-crystallin showed a slight but early increase. An up-regulation of HSPs has also been seen in limb muscles in response to the ICU intervention (Ochala et al., 2011a), these increases were more delayed compared with the masseter. The up-regulation of HSP70 and αB-crystallin and the stability of HSP90 protein levels in the masseter accords with our previous cranial-versus-spinal response to critical illness in a porcine ICU model, where these three proteins were induced to a larger extent in the masseter compared with limb muscles (Aare et al., 2011).

HSP70 and αB-crystallin also regulate redox signaling of the mitochondria and have an anti-apoptotic function (Arrigo et al., 2007; Ferrer-Martinez et al., 2006; Parcellier et al., 2005). Additionally in one of our porcine critical illness models studies, HSP70 and αB-crystallin changes correlated with oxidative stress, in both cranial- and spinal-nerve innervated muscles (Aare et al., 2011). Further HSP90 has been shown to be involved in the inflammatory signaling via corticosteroid receptors (Dalman et al., 1991; Stephanou et al., 1998). Based on previous reports and present observations, we may speculate that mitochondrial oxidation and the subsequent inflammatory and apoptotic responses are highly involved in the pathology of CIM.

5 CONCLUSIONS

This thesis has investigated the intracellular pathways controlling the mechanism underlying muscle wasting in ICU patients with CIM and the effects of passive mechanical loading in an experimental ICU rat model.

Passive mechanical loading of limb muscles, induces significant positive effects on muscle function and is able to attenuate myosin protein loss associated with mechanical silencing and CIM. Further, both fission and fusion events as well as mitophagy are significantly affected by mechanical signaling. However, mitochondria dynamic alterations induced by mechanical silencing are completely counteracted by passive mechanical loading indicating that mitochondria may be a key player in the role of mechanosensing. Additionally, the novel discovered ubiquitin ligases Fbxo31 and SMART are induced by mechanical silencing, an induction that similarly is prevented by passive mechanical loading.

This demonstrates that the mechanism underlying the effects of passive mechanical loading are part of an intricate biological system involving activation and inhibition of different protein synthesis- and degradation- pathways, as well as mitochondria function. The beneficial effects of passive mechanical loading on muscle structure and function, are however, strongly supportive to the use of early physical therapy among immobilized ICU patients.

CMV plays a key role in triggering the impaired diaphragm muscle function in critically ill ICU patients. CMV induces early structural changes of the sarcomere that results in impaired SF in muscle fibers of the diaphragm. In this study it is demonstrated that CMV induces oxidative stress that may further be a key factor triggering post-translational protein modifications and the myofibrillar protein disorganization and atrophy. The temporal pattern and the lack of preferential myosin loss observed in the diaphragm in response to CMV and immobilization differs dramatically to what is occurring in the limb muscles.

Craniofacial muscles are usually spared or less affected in CIM. Here we demonstrate that the masseter muscle sustains a high myosin:actin ratio and muscle fiber size for longer periods of exposure to the ICU condition in the experimental rat ICU model. Studying different cell signaling pathways it is suggested that an enriched antioxidative profile in the masseter may play a role in the mechanisms of preserved masticatory function in CIM.

Accordingly, these findings demonstrates muscle type differences in the mechanisms behind the loss of muscle function associated with the ICU condition between the limb, diaphragm and masseter muscles. This important knowledge needs to be taken into account when designing strategies for reducing muscle wasting and weakness found in immobilized mechanically ventilated intensive care patients.

6 ACKNOWLEDGEMENTS

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