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Karolinska Institutet, Stockholm, Sweden

CRITICAL ILLNESS MYOPATHY: MECHANISMS AND PHARMACOLOGICAL INTERVENTIONS

Hazem Akkad

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Critical Illness Myopathy:
Mechanisms and pharmacological interventions

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Hazem Akkad

Principal Supervisor:
Prof. Dr. Lars Larsson
Karolinska Institutet
Department of Physiology and Pharmacology
Department of Clinical Neuroscience
Division of Basic and Clinical Muscle Biology

Co-supervisors:
Dr. Nicola Cacciani
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Basic and clinical muscle biology

Dr. Meishan Li
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Basic and clinical muscle biology

Dr. Stefano Gastaldello
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Basic and clinical muscle biology

Opponent:
Prof. Dr. med. Werner Z'Graggen
University Hospital Bern
Department of Neurology and Neurosurgery

Examination Board:
Prof. Dr. Göran Hedenstierna
Uppsala University
Department of Medical Sciences, Clinical Physiology

Prof. Dr. Olav Rooijackers
Karolinska Institutet
Department of Clinical Science, Intervention and Technology

Prof. Dr. Eva Blomstrand
The Swedish School of Sport and Health Sciences
The Åstrand Laboratory

ABSTRACT

The human body typically contains over 600 skeletal muscles that make up about 40% of total body weight. These muscles work together to perform functions such as locomotion, breathing, mastication, heat regulation and speech. Skeletal muscles can change their size and protein content in response to endogenous or exogenous signals, including physical, neural or chemical ones. Critically ill patients, usually treated intensively, are prone to developing a condition of muscle wasting and paralysis, called critical illness myopathy (CIM), where limb and truck muscles suffer severe atrophy and loss of force production capacity coupled with a preferential myosin loss, but craniofacial muscles remain less affected. Triggers of CIM are thought to be the exposure to the intensive care unit (ICU) interventions *per se*, such as unloading, mechanical ventilation (MV) and high doses of certain drugs such as muscle relaxants and glucocorticoids (GCs). The rapidly compromised diaphragm function due to the impact of the ventilator has been given a specific name, ventilator induced diaphragm dysfunction (VIDD). CIM and VIDD have dire consequences and research into their underlying mechanisms is urgently needed. This research is inherently difficult in patients and thus suitable animal models mimicking the ICU condition must be implemented. In this thesis, we used a pig and a rat ICU models with extended periods of immobilization, deep sedation and MV, in which we used different analyses to understand the muscle specific mechanistic differences of the masticatory, limb and the diaphragm muscles. In addition, we explored the effects of two new drugs on skeletal muscles: BGP-15, a chaperone co-inducer and vamorolone, a first-in-class dissociative GC. In paper I, we report that a 5-day GC treatment in the pig ICU model induces numerous transcriptional changes that affect myofiber function in a limb muscle. In paper II, we conclude that the masseter, the main masticatory muscle, is partially protected from CIM effects by several mechanisms that reduce proteolysis, including early heat shock protein (HSPs) activation. In paper III, we report that treatment with BGP-15 activates HSP70 and improves the diaphragm muscle fiber function in young but not old rats. In the last paper, we report differences between the new GC, vamorolone and the traditional GC, prednisolone, in the rat ICU model where the former drug shows less negative effects on fast twitch EDL muscle and both show positive effects on the slow twitch soleus muscle. These results emphasize the uniqueness of each muscle response to ICU interventions and also shed some light on a couple of promising pharmacological interventions that may counteract CIM deleterious effects.

LIST OF SCIENTIFIC PAPERS

I. Aare, S., Radell, P., Eriksson, L. I., **Akkad, H.,** Chen, Y. W., Hoffman, E. P., and Larsson, L. (2013) Effects of corticosteroids in the development of limb muscle weakness in a porcine intensive care unit model. Physiological Genomics 45, 312-320

II. **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

III. Ogilvie, H., Cacciani, N., **Akkad, H.,** and Larsson, L. (2016) Targeting Heat Shock Proteins Mitigates Ventilator Induced Diaphragm Muscle Dysfunction in an Age-Dependent Manner. Frontiers in Physiology 7, 10

IV. **Akkad, H.,** Cacciani, N., Llano-Diez, M., Corpeno Kalamgi, R., Tchkonia, T., Kirkland, J.L., Larsson, L. Vamorolone Treatment Improves Skeletal Muscle Outcome in a Critical Illness Myopathy Rat Model. *Submitted to Acta Physiologica*.

CONTENTS

LIST OF ABBREVIATIONS

4E-BP-1	Translation initiation factor binding protein
BMP4	Bone morphogenetic protein 4
CIM	Critical illness myopathy
CSA	Cross sectional area
EDL	Extensor digitorum longus
eIF-4E	Eukaryotic translation initiation factor 4E
Fbxo33	F-Box Protein 33
FoxO	Forkhead box O
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H&E	Hematoxylin and eosin
HDAC9	Histone Deacetylase 9
HSF-1	Heat shock factor 1
HSP	Heat shock protein
ICU	Intensive care unit
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IL1RAP	Interleukin-1 receptor accessory protein
JNK	c-Jun N-terminal kinases
LC3B	Microtubule-associated protein light chain 3
MAFbx/atrogin-1	Muscle atrophy F-box
MEF2	Myocyte enhancer factor-2
MnSOD	Manganese-dependent superoxide dismutase
mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING finger 1
MV	Mechanical ventilation
MyHC	Myosin heavy chain
MyoD	Myogenic differentiation
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMBA	Neuromuscular blocking agent
NR2F2	Nuclear Receptor Subfamily 2 Group F Member 2
P_0	Maximal force

p70S6K	Ribosomal protein S6 kinase beta-1
PARP-1	Poly [ADP-ribose] polymerase 1
PTM	Post-translational modification
Rac1	Ras-related C3 botulinum toxin substrate 1
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	Sarco/endoplasmic reticulum calcium-ATPase
SF	Specific force
TGF-beta	Transforming growth factor beta
TNF-α	Tumor necrosis factor *alpha*
VIDD	Ventilator induced diaphragm dysfunction

1 INTRODUCTION

1.1 SKELETAL MUSCLE

Humans are averagely made up of 36-42% skeletal muscle of total body mass in females and males, respectively (1). Skeletal muscles are vital for survival; they move the body around and perform essential functions such as breathing, mastication, swallowing, speech production and energy balance. Skeletal muscle consists of several bundles of muscle cells wrapped by a layer of connective tissue called the fascia. Tendons connect the muscles to the skeleton in order to produce skeletal movements such as locomotion and posture maintenance[i].

Muscle is a soft tissue with motor properties that allow changing the length and shape of the muscle and thus generate force, i.e., muscle contraction. The muscle tissue has three main subtypes that differ in forms and functions: skeletal (striated) muscle, cardiac (sometimes called striated or semi-striated) muscle that forms the heart chambers, and smooth (non-striated) muscle that makes up the walls of hollow organs such as the stomach and the blood vessels. Skeletal muscle contracts voluntarily while cardiac and smooth muscle contract involuntarily. Embryonically, muscle tissue develops through the process of myogenesis, where embryonic myoblasts fuse together to form multinucleated myotubes.

The muscle cells, also known as myofibers or myocytes, have a long cylindrical shape and contain multiple peripheral nuclei, many mitochondria and many chains of myofibrils. The latter, also called muscle fibrils, are rod-like units containing a repetition of the basic functional unit in striated muscle; sarcomeres[ii]. This repeated pattern between two Z lines[iii] gives rise to the striated appearance of dark and light bands visible in microscopy. Sarcomeres, in turn, are made up of long fibrous proteins such as actin, myosin and titin, etc. arranged into thick and thin myofilaments (Figure 1). In contrast, smooth muscle, does not feature sarcomeres and hence looks “smooth”. Either muscle contraction or relaxation happens when the thick and thin filaments “walk” along each other in one direction or the other.

Myosin forms the thick filament and actin the thin one. Myosin is a ubiquitous highly conserved motor protein with ATP-hydrolyzing and actin-binding properties, important for contraction in smooth and striated muscle alike. Myosin has a long tail and globular head where the ATPase enzyme activity is located. Myosin isoforms are coded by a vast family of genes and are involved in many cellular functions but class II myosin, the conventional myosin, is the one responsible for muscle contraction. The thick filament is formed by joining

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i - Posture maintenance is done subconsciously through proprioception.
ii - The word sarcomere derives from two Greek words: “sarx” and “meros” meaning “flesh” and “part”, respectively.
iii - Z is from German "Zwischenscheiben" or “discs in between”.

the long tails together, while the head region has two myosin heavy chains sticking out of the filament. Actin, on the other hand, is a family of globular proteins involved in many basic cellular functions. The alpha actin isoform is the polymerized form that constitutes the thin filaments upon which myosin filament can "ratchet" to generate motion.

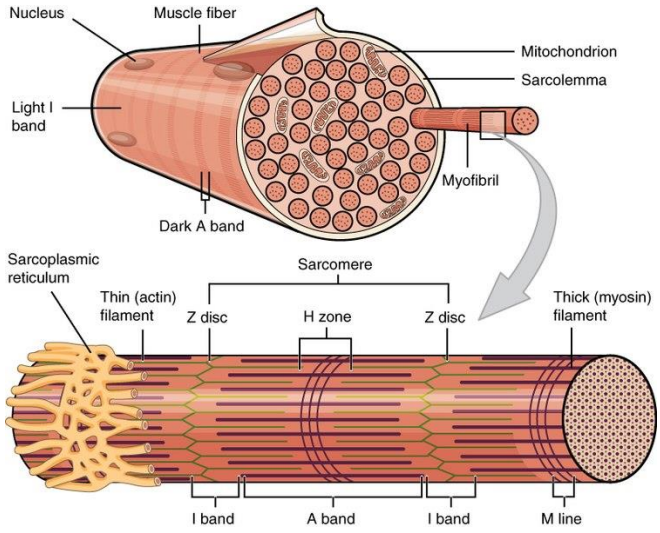

Figure 1: Muscle structure. *A single muscle fiber, wrapped by the sarcolemma (cell membrane) and contains many myofibrils that feature a repeated functional unit, the sarcomere. The latter has a thin and a thick filament that can slide past each other producing muscle contraction. Image source: OpenStax, May 2016 (https://cnx.org/contents/FPtK1zmh@8.25:fEI3C8Ot@10/Preface)*

Mature muscle tissue also contains small multipotent cells called myosatellite cells[i], in the space between the basement membrane (extracellular matrix) and the sarcolemma (cell membrane). These cells are normally quiescent but can be activated after injuries or mechanical stress to proliferate and differentiate into muscle cells or otherwise fuse with and supplement their parent cell (2).

1.2 MUSCLE CONTRACTION

Contrary to the connotation of the word "contraction", in physiology, muscle contraction does not always translate into a shorter length, rather, muscle contraction can be isometric (of constant length), concentric (shortening) or eccentric (lengthening). Natural motions are combinations of all these types. The initiation of activated state of muscle fibers where "tension" is generated is what constitutes muscle contraction. When muscle contraction ends, muscle fibers return to their state of low tension (3) and muscle relaxation ensues.

Muscle contraction originates in motor neurons[ii]. One motor neuron may innervate several muscle fibers that contract simultaneously upon the neuron signal. The cyclical binding of myosin head on the actin filament which forms actomyosin complex is called cross bridge cycling or the "sliding filament mechanism" of muscle contraction (4). Briefly, when an action potential arrives at the meeting point of a motor neuron and a muscle fiber, the neuromuscular junction, acetylcholine is released into the synaptic cleft and binds to the

i - Satellite cells are considered a type of stem cells.
ii - In contrast, cardiac and smooth muscle cells contraction is myogenic; originates in the muscle cell itself without nerve stimulation.

nicotinic acetylcholine receptors on the neuromuscular junction. This binding gives rise to the end-plate potential that briefly reverses the sarcolemma polarity, opening sarcoplasmic voltage-sensitive sodium channels and spreading the action potential across the myofiber surface (5) (Figure 2). Depolarization of the myofiber activates the dihydropyridine receptors on the sarcolemma, that in turn physically activates the ryanodine receptors on the calcium storing sarcoplasmic reticulum. The sarcoplasmic reticulum releases Ca^{+2} into the cytosol, which, in turn, bind to troponin C that initiates the cross-bridge cycling.

One cross-bridge cycle consists of the following steps: First, an ATP molecule binds to a myosin head, detaching myosin from actin[i]. Myosin hydrolyzes ATP into inorganic phosphate and ADP, a reaction that produces energy used to move myosin along actin to bind weakly onto another actin molecule. This binding is weak because actin binding site is partially covered by tropomyosin at this stage. When Ca^{+2} binds to troponin C on the actin filament, tropomyosin moves away to completely unblock the actin binding site, thus allowing the myosin heads to strengthen their binding to actin. At this stage myosin releases the inorganic phosphate, starts a power stroke that brings actin closer, and shorten the sarcomere. Finally, myosin releases the ADP and stays strongly bound to actin until a new ATP molecule binds, initiating a new power stroke[ii]. A calcium pumping enzyme, the sarco(endo)plasmic calcium ATPase (SERCA) actively brings calcium ions back into the sarcoplasmic reticulum to the pre-activation levels, leading to relaxation.

1.3 MUSCLE FIBER TYPES

Skeletal muscle contains fast and slow twitch fibers that differ in their content of myoglobin, mitochondria, metabolism and the kinetics of their myosin ATPase[iii]. ATP, the power source for muscle contractions, is produced by carbohydrate and fat oxidation in general but by anaerobic reactions in fast fibers. Slow twitch muscle fibers, or type I fibers, are oxidative and can generate ATP efficiently as they contain more myoglobin and mitochondria and thus can contract for longer periods of time. Slow muscles endure fatigue and are typically postural muscles, red in color (due to high concentrations of myoglobin) and densely supplied with blood capillaries. In contrast, fast twitch muscle fibers, or type II fibers, are more glycolytic/anaerobic and are sub-grouped according to ascending contractile force and speed and decreasing endurance to types IIa, and the fastest IIx (in humans), and IIb (in rodents and other mammals). Type II fibers achieve powerful but brief bursts of contraction before fatiguing. Muscles are usually made up of a blend of all these fiber types but the share of each type differs considerable from a person to another and between muscles depending on their function.

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i - One myosin head contains two binding sites; one for actin and another for an ATP molecule.
ii - Unavailability of ATP molecules leads to the state of rigor mortis, or postmortem rigidity, the third stage of death.
iii - different overlapping methodologies are usually used to classify fiber types according to their myosin ATPase activity (histochemical staining) or MyHC isoforms (immunohistochemistry for MyHC)

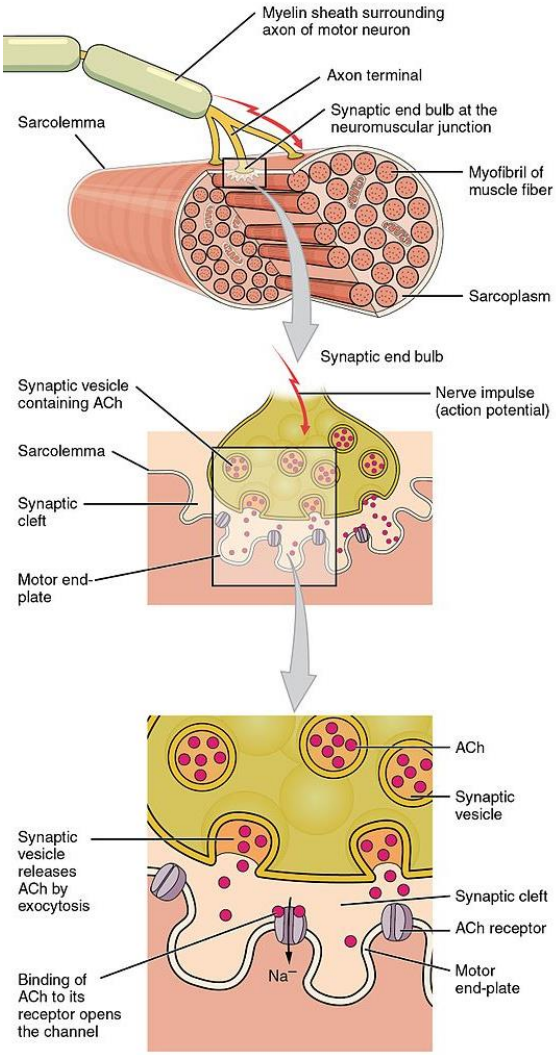

Figure 2: Muscle activation. *An action potential in a motor neuron arrives at the end plate (neuromuscular junction) that activates the release of acetylcholine into the synaptic cleft and its binding to its nicotinic receptors that help depolarize the myofiber. Image source: OpenStax Anatomy and Physiology, Published May 18, 2016 (https://cnx.org/contents/FPtK1zmh@8.25:fEI3C8Ot@10/Preface*)

1.4 MUSCLE ATROPHY AND MUSCLE ADAPTATION MECHANISMS

Skeletal muscle is a highly plastic tissue that can change its mass in response to physiological and pathological conditions including mechanical/physical, metabolic and hormonal signals. For example, muscle atrophy is a condition of reduced muscle mass that develops in response to conditions of unloading or limited movement such in bed rest. In ideal conditions, protein turnover, the rate at which a given protein is synthesized and degraded, reflects a balanced state of muscle catabolism and anabolism. However, increased muscle mass and muscle fiber size, known as hypertrophy, happens during development, mechanical stimulation and/or in response to anabolic hormones. Inversely, on a stress signal, a catabolic state leads to muscle loss or reduction in myofiber size due to loss in proteins and/or cytoplasm, known as atrophy.

Skeletal muscle atrophy leads to severe reduction in functionality. Our knowledge of the physiological changes in muscle atrophy has increased substantially, however, there is still significant need for research to elucidate the role of genetic regulation and signaling pathways. It is well established that the removal of weight bearing leads to an initial decrease in protein synthesis and increase in protein degradation but the upstream processes remain

unclear. The initial trigger can be any event that causes reduced skeletal muscle activation, such as conditions of immobilization, denervation, muscle unloading, aging, starvation, certain drugs or events secondary to diseases such as cancer or critical illness (6). This entails initiation of signaling pathways and downstream effector molecules that induce protein loss. However, irrespective of the trigger, muscle atrophy leads to reduction in protein content, size and force production. The shift of balance toward more protein degradation is largely responsible for atrophied skeletal muscle but the interplay between protein synthesis and different degradation pathways (ubiquitin-proteasome, lysosomal, and calpains) is complex and far from being understood. In addition, elevated levels of pro-inflammatory cytokines, the transcription factor NF-κB, glucocorticoids (GC), myostatin, and reactive oxygen species (ROS) are also involved in muscle mass loss.

1.4.1 Decreased protein synthesis

The rate of protein synthesis decreases rapidly after cancellation of mechanical stimulation or muscle unloading (7). Protein synthesis is affected by both the upstream signal and downstream effectors. Akt, a known protein kinase upstream activator of protein synthesis, can promote cellular growth. Akt over-expression significantly attenuates denervation-induced atrophy (8). mTOR and p70S6 are downstream effectors of the Akt pathway. The decreased translation rate observed in unloading atrophy is usually associated with the consistent decrease in both mTOR and p70S6 kinase phosphorylation. The translation of mRNA into proteins happens in three phases: initiation, elongation and termination. Downstream of the initiation phase there is the translation initiation factor binding protein (4E-BP-1) that, upon dephosphorylation, can block protein synthesis by binding the eukaryotic initiation factor (eIF)-4E. The bound portion of 4E-BP-1 increases with unloading and starvation atrophy, suggesting its involvement in decreased protein synthesis (6).

It is important to note that protein synthesis and degradation processes are interconnected and the induction of one leads to the suppression of the other. This coordinated action limits the energy loss when synthesizing new proteins while degrading others (9). However, the reversed relationship does not need to hold true in all conditions, for example, during denervation, free amino acids released from protein degradation may briefly induce protein synthesis through the mTOR pathway that inhibits the initiation of lysosomes (10).

1.4.2 Increased proteolysis

The role of increased protein breakdown is described in more detail than the decline in protein synthesis during muscle atrophies. Myofibrillar protein, being half the total muscle proteins, are lost at a faster rate than other proteins during muscle atrophy (11). Three proteolytic systems are important in this regard, specifically: the ubiquitin-proteasome system, the autophagy lysosomal system, and the calcium-dependent calpain system. Rather than one system being solely activated at a time, these systems cooperate together to accomplish proteolysis upon atrophic signals.

1.4.2.1 Ubiquitin-proteasome system

The ATP-dependent ubiquitin-proteasome pathway is responsible for degradation of the majority of myofibrillar protein during muscle atrophy (12). The ubiquitin-proteasome system is a complex and selective proteolysis mechanism that promotes protein degradation via proteasome. Blocking the proteasome has consistently shown a great reduction in proteolysis. A moiety of ubiquitin is cyclically attached to the targeted protein to form a chain that is the molecular signal for the degradation. This reaction is catalyzed in a coordinated and consecutive cascade reactions of three different sets of enzymes: the ubiquitin activating (E1), the ubiquitin conjugating (E2) and ubiquitin ligating (E3) enzymes. The rate-limiting step is the activity of E3 ligases that bind to the substrate and facilitates its attachment to ubiquitin transferred from the E2 enzyme. Once poly-ubiquinated, the substrate is transferred to the proteasome for degradation. E3 protein ligases have a significant tissue and substrate specificity. In muscle atrophy, only a handful of E3 ligases were found to be specifically up-regulated in skeletal muscle. In particular, MuRF1 (muscle RING finger 1) and MAFbx (muscle atrophy F-box) also known as atrogin-1, are the two highly muscle-specific[i] E3 ligases that increase markedly during muscle atrophies secondary to different triggers. Atrogin-1 and MuRF1 knockout mice are resistant to muscle atrophy induced by denervation (13). MuRF1 was shown to be necessary for GC-induced muscle atrophy (14) and targets a number of muscle protein substrates, such as troponin I, myosin heavy and light chains, actin, and myosin binding protein C (9). Atrogin-1, on the other hand, was shown to be necessary for fasting-induced atrophy (15) and targets proteins involved in muscle growth and survival, such as MyoD, and protein synthesis activator eIF3-f (16). When measuring E3 ligases, it is important to note that it often happens that transcriptional and protein levels do not necessarily match. This is especially true in the case of elevated MuRF1 and atrogin-1, where their upregulated transcription is in part a response to replenish the self-degraded E3 ligases due to the highly catabolic milieu (9).

1.4.2.2 Autophagy lysosomal system

Autophagy is a conserved mechanism that orderly recycles long-lived proteins and clears up damaged organisms to ensure homeostasis. The lysosome contains many enzymes such as cathepsins and can fuse with the phagosomes in order to break down their contents. The autophagy-lysosomal pathway is usually a non-selective intracellular protein degradation mechanism but can be specific in some conditions, e.g., mitophagy. Autophagic activity increases in muscle cells in a wide variety of atrophy conditions including cancer, aging, fasting, critical illness, disuse and exercise (9). Cathepsins do not degrade cytosolic proteins such as myofibrillar ones, instead, they target membrane proteins, such as receptors, ion channels and transporter proteins thus can affect muscle function (17). During different types

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i - Found in both striated and smooth muscle tissue

of muscle atrophy, various cathepsins increase transcriptionally, however, cathepsin blockers did not reduce protein degradation rate significantly (6).

FoxO is one of the first identified transcription factors responsible for initiating muscle atrophy. In muscle atrophy conditions, activation of the ubiquitin -proteasome and autophagy-lysosomal systems is coordinated by FoxO (18). This transcription factor is negatively influenced by IGF-1-Akt (protein synthesis) pathway.

1.4.2.3 Calpains and caspases

In muscle atrophies, the ubiquitin-proteasome pathway plays a dominant role, but the proteasome mechanism cannot degrade intact myofibrils. Instead, calpains help making myofibrillar proteins ready for degradation and thus play a rate-limiting role at the initiation of protein degradation process. Calpains substrates are known to be assembly protein such as titin, nebulin and C-protein but not actin or myosin per se (19). Calpains are likely to mediate muscle atrophy by disintegrating Z-bands in order to release myofilaments. However, the complex nature of calpain regulation with endogenous inhibitors and activators makes their transcriptional changes very difficult to interpret under different atrophy conditions.

Other possible proteolytic rate-limiting factors are the caspases, a group of proteases that contribute to maintaining homeostasis through regulation of programmed cell death, inflammation, cell differentiation and proliferation. Caspase activity increases in disease-related muscle atrophy (20) at the initiation phase of actomyosin cleavage in a similar fashion to calpains, thus making myofibrillar protein ready for ubiquitination.

1.4.3 Other triggering signals

Other signaling pathways can influence protein synthesis or degradation and are strongly involved in the process of muscle atrophy. For example, NF-kB and inflammatory cytokines, ROS, myostatin and GCs. The role of pro-inflammatory cytokines in inducing muscle atrophy is not clear but there is significant evidence supporting elevated cytokines levels, such as TNF-a, IL-1, IL-6 in certain muscle atrophy conditions such as cachexia (21). NF-kB is a family of transcription factors expressed in many tissues including skeletal muscle, that mediates many cellular processes depending on the context such as apoptosis, inflammation and cellular differentiation. NF-κB has a complex regulatory mechanism but, similarly to other transcription factors, needs to translocate into the nucleus in order to exert its genomic actions. NF-κB has been implicated in a number of different muscle atrophy conditions but the downstream target genes seem to vary. For example, the upstream signal of TNF-α, a proinflammatory cytokines, can induce NF-κB in the myocyte, which, in turn, can transcriptionally activate the ubiquitin proteasome proteins, leading to muscle atrophy (6).

Another widely studied pathway in muscle atrophy is the ROS signaling. ROS are usually metabolized or neutralized by protective enzymes such as glutathione peroxidase, and Mn superoxide dismutase. The transcription of these enzymes was shown to be reduced by

muscle unloading conditions (22). Increased ROS is suggested to up-regulate the transcription of the ubiquitin-proteasome proteolytic pathway, NF-κB, and FoxO and decrease myosin expression in the myocyte (6). Additionally, myostatin, a member of the TGF-β family, is a known inhibitor of muscle growth and functions as translation inhibitor (23). Myostatin knockout are clearly hypertrophic but not resistant to unloading-induced atrophy, suggesting that unloading atrophy does not require myostatin. Finally, glucocorticoids (GCs) are plausibly believed to be important triggers of muscle atrophy but their role has not been elucidated. Increasing GC levels, either endogenously and exogenously, is a commonly implemented method of inducing muscle atrophy in vitro and in vivo (24). GCs are known to be detrimental to skeletal muscle by acceleration of both protein synthesis rate reduction and protein breakdown rates. However, the use of adrenalectomy or GC receptor antagonists do not prevent unloading-induced muscle atrophy, indicating that high GC levels are not necessary.

1.5 CRITICAL ILLNESS MYOPATHY (CIM) AND VENTILATOR-INDUCED DIAPHRAGM DYSFUNCTION (VIDD)

Innovation in medical technology, pharmacology and the increased knowledge of pathophysiology of critical illnesses have contributed to the improved intensive care unit (ICU) outcomes. The elimination/reduction of invasiveness and introduction of new supportive equipment has enhanced the overall survival of the critically ill. This has prompted critical care medicine to expand at an unprecedented pace (25), however, the long-term outcomes of ICU patients are still poor and abound with complications, such as critical illness myopathy (CIM). CIM is an acquired severe muscle wasting and weakness in immobilized and mechanically ventilated ICU patients, characterized by preferential myosin loss that leads to generalized, symmetric atrophy and weakness in limb and trunk muscles while craniofacial muscles, sensory and cognitive functions are usually less affected or intact. CIM is suggested to be triggered by common ICU interventions such as mechanical ventilation (MV), neuromuscular blocking agents (NMBAs), immobilization, muscle unloading, sepsis and systemic GCs (26). The first reported case of severe myopathy was published in the Lancet in the seventies of the last century (27) where a young asthmatic patient, on prolonged MV, NMBAs and high doses of systemic GCs, developed a generalized muscle paralysis. The term CIM was recently quoted more often, replacing older terms, such as acute quadriplegic myopathy and thick filament myosin myopathy and many other names.

Nearly 30% of the overall ICU patients suffer some form of neuromuscular dysfunction. The proportion can be much higher (up 70-100%) in specific sub-groups (28, 29). A great share of mechanically ventilated patients for five or more days develops CIM in their axial (limb and trunk) skeletal muscles. We have shown previously that long-term mechanical silencing, i.e., the absence of mechanical strain both externally (weight bearing) and internally (contractile proteins activation), is the main trigger of the CIM phenotype in patients as well as in animal models. Down-regulated MyHC transcription coupled with ubiquitin proteasome-based proteolysis, in particular, were shown to be responsible for the characteristic preferential loss

of myosin and myosin-associated proteins leading to the dramatic muscle wasting and weakness in CIM patients (28, 30, 31).

The specific weakness and dysfunction in the major respiratory muscle, the diaphragm, in response to MV, has recently been termed, VIDD, or the ventilator-induced diaphragm dysfunction (33). MV is a life-saving intervention in the ICU but can nonetheless lead to severe complications. The diaphragm muscle is very sensitive to the unloading put upon it by the ventilator. Studies on organ donors showed that as early as 18-69 hours of MV are enough to halve the size of both fast and slow twitch diaphragm fibers (34). At longer durations, MV elicits the condition of VIDD, making ventilator removal, or weaning, a significant challenge. Typically, five days on the ventilator are long enough to trigger weaning requirement. Studies estimate that weaning in and of itself consumes 40% of the MV time (35). Further, delayed ventilator weaning has significant negative effects on morbidity/mortality, recovery, and staggering increases in health care economy. Mortality rate is about one quarter in ICU patients with protracted weaning (36). Further, the long periods of ICU stays are burdensome to the intensivists (37, 38) apart from being very expensive and have long-term impact on the patient's quality of life.

Studying CIM and VIDD mechanisms and the impact of each trigger on different muscles in clinical settings is difficult. This is due to several reasons: the insufficient differentiation between CIM and neuropathies or other myopathies; difficulty of obtaining timely diaphragm biopsies and the heterogenic nature of CIM patient population. The latter reason is inherent since the ICU population varies widely with respect to primary diseases, clinical histories, concurrent pharmacotherapies and pre-existing conditions. Despite this variance, CIM patients usually receive similar ICU interventions such as MV and immobilization, indicating that ICU interventions as such, are likely to be the primary trigger of CIM. In addition, the correlation between weaning difficulty and MV duration suggests a key role of MV in the development of VIDD (35, 39, 40). Experimental models that that mimic ICU interventions usually have poor records of sustaining MV for more than a day or two. Therefore, extending the MV time is obligatory in CIM/VIDD experimental studies.

There is an urgent need to develop new diagnostics, monitoring and therapeutics for skeletal muscle dysfunction in the ICU which deepens our insights into CIM pathophysiology and enables its distinction from other types of neurogenic muscle wasting in ICU patients[i] (41). Our laboratory has been investigating CIM in parallel clinical and experimental studies for more than two decades, using two animal models: the large porcine and the small rat ICU model. These models resulted in important findings in limb, diaphragm and craniofacial muscles and involved numerous analyses, including gene/protein expression, intracellular signaling, and regulation of muscle contraction at the cell and motor protein levels (42-54). In the porcine ICU model, animals were sedated, immobilized and mechanically ventilated for 5

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i - CIM is currently classified as part of the umbrella term of intensive care unit acquired muscle weakness.

days. This model has significant strengths due to the relative similarities with humans in size and drug dosages, but on the other hand, it is logistically demanding and too expensive to maintain for long-term studies (longer than 5 days). Therefore, an ICU model that can be studied at different time points and for extended periods was warranted.

The introduction of the rat ICU model allowed high temporal resolution and long-term observation periods. This ICU model was initially used for cardiovascular physiological studies (55, 56), but was subsequently modified for skeletal muscle structure and function studies as it had shown low mortality in the early phases (57, 58). In principle, the rat ICU model undergoes MV, pharmacological paralysis by inhalational general anesthesia, deep sedation, postsynaptic NMBA and is thoroughly monitored for varying periods of time. This combination of treatments and procedures could be sustained over long time periods such as the remarkable three months reported earlier (55). Using this custom-made ICU model, our laboratory has performed numerous experiments aiming at discovering the pathogenesis underlying muscle wasting and weakness associated with ICU treatment.

1.6 PHARMACOLOGY

Muscle atrophy therapeutics have a significant potential in reducing morbidity and improving quality of life, but to date, no effective treatment that counteracts muscle wasting and paralysis has made it to the clinic. There is a number of approaches but they are typically associated with significant drawbacks, such as boosting anabolic pathways via the IGF-1-Akt signaling and blocking the ubiquitin-proteasome system. IGF-1 mimetics are likely CIM drugs since IGF-1-Akt axis is quite exceptional in that it lies at the intersection of both protein synthesis and proteolysis, thus influencing both. However, this pathway is also involved in many important cellular mechanism, including survival, and can give rise to tumorigenesis (9). On the other hand, reduction of protein breakdown, e.g., via proteasome inhibitors, is also risky since proteasome is a major cellular quality control measure and reducing it can lead to protein mis-folding and increased aggregation (59). However, in this thesis, we have first investigated genomic effects of typical GCs and then studied two completely new pharmacological approaches, namely, vamorolone, a dissociative GC and BGP-15, a chaperone co-inducer.

1.6.1 Glucocorticoids

GCs are a family of endogenous steroidal hormones made in the adrenal cortex with multiple essential roles in regulating immunity, metabolism and other functions. GC hormones and their synthetic analogues, such as prednisolone and dexamethasone are used extensively in the clinic[i] to suppress immune/inflammatory reactions in many conditions such as allergy, asthma, rheumatoid arthritis, cancers and sepsis or in form of replacement therapy in adrenal insufficiency, to name a few indications. GCs metabolic effects converge at maintaining

i - Prednisolone and dexamethasone are 4 and 40 times, respectively, more potent than hydrocortisone/cortisol in anti-inflammatory action.

normal blood glucose concertation, hence, rapidly eliciting insulin resistance. Glucose levels maintenance requires reducing muscle and fat tissue uptake of glucose while enhancing glucose synthesis in the liver (gluconeogenesis) using substrates such as amino acids (mainly from skeletal muscle) and glycerol (from adipose tissue). A wide spectrum of side effects results from these metabolic changes, such as bone loss, skeletal muscle atrophy and weakness, gastrointestinal bleeding, increased infection risk, and renal insufficiency. Increased circulating levels of GCs occurs in several muscle atrophy conditions secondary to sepsis, cachexia, starvation and metabolic acidosis. GCs primarily affect fast twitch glycolytic fibers reducing their size and protein content and have almost negligible effects on slow twitch aerobic muscle fibers. Muscle atrophy caused by GC administration is characterized by decreased protein synthesis (via suppression of the anabolic IGF-I and induction of the catabolic myostatin) and most notably, activation of ubiquitin-proteasome (mainly, atrogin-1, and MuRF-1) and the lysosomal proteolysis systems (24).

1.6.2 Vamorolone

GCs have genomic and non-genomic effects but evidence suggests that the majority of GC anti-inflammatory effects is derived from the non-genomic ones (60), hence the design of "dissociative" GCs that make use of only the non-genomic effects, thus dissociating anti-inflammation from most side effects. Typically, GCs defuse through the cellular lipid membrane to bind with cytosolic GC receptors (GRs). The GC-GR complex then migrates to the nucleus and binds to the regulatory elements of the targeted genes to regulate a broad array of genes in a process called trans-activation or genomic effects (61). However, new GC mechanisms of action are emerging as non-genomic effects and gaining momentum. Recently, it was shown that the GC-GR complex directly interact with a protein rather than a DNA segment (62). Vamorolone, also known as VBP-15, is a synthetic prednisolone derivative, initially designed to replace prednisolone use in Duchenne muscular dystrophy. Vamorolone was developed as a Δ9,11 steroidal scaffold and optimized for the retention of NF-κB inhibition but also for reduced genomic activity-related side effects (63, 64). Indeed, vamorolone has shown retained GR binding with anti-inflammation efficacy equal with traditional GCs. Additionally, the chemical structure of vamorolone contributes to the potentially salubrious effect of cellular membrane stabilization, as opposed to prednisolone membrane destabilization (64) (Figure 3). Several mouse models have shown that vamorolone inhibits inflammation at the same levels as prednisolone but has an improved side effect profile including diminished stunting and bone loss. Vamorolone is presently in phase 1 clinical trials (64-67).

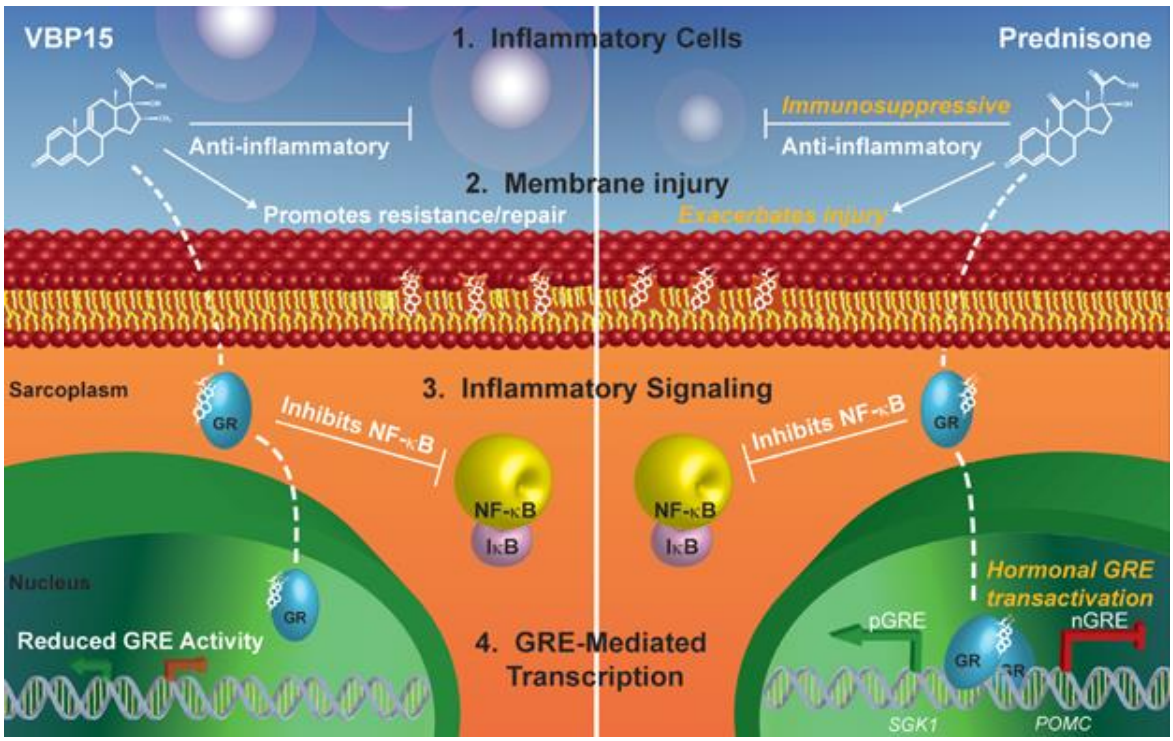

***Figure 3**: **Vamorolone vs. prednisolone mechanism of action.** Both GCs defuse across the cellular membranes and bind to their cytosolic receptors and have a certain extent of GRE (glucocorticoid response element) binding. However, vamorolone maintains the same levels of (non-genomic) NF-κB inhibition but have a reduced GRE activity (thus less side effects). Prednisolone also have a positive impact on the cellular membrane and is less immunosuppressive than prednisolone. Image source: Christopher R. Heier et al., 2013, (68) with permission from EMBO Molecular Medicine.*

1.6.3 BGP-15

Derivatives of hydroximic acid are a new class of insulin sensitizers and heat shock protein (HSP) co-inducers (69, 70). In this drug class, BGP-15[i] is an experimental agent that has various effects including insulin sensitizing (71), anti-inflammatory, anti-fibrotic (72, 73), PARP-1 inhibitor and chaperone co-inducer (74), lipid membrane stabilizer and antioxidant (75). Due to the versatility of its mechanisms, BGP-15 has been under pre-clinical studies and clinical trials for a number of conditions including diabetes mellitus type II, heart failure (due to cardiovascular protective effects), Duchenne muscle dystrophy and other muscle dysfunctions (54, 73, 76).

Impaired HSPs expression can induce insulin resistance. HSP72, the stress-induced isoform of HSP70, enhances insulin sensitivity by activating NF-κB inhibitor, IkB, thus reducing inflammation. HSP70 is required for proper protein folding and HSP72 has an important role in repairing mis-folded and damaged proteins and polypeptides during stress (77). Further, HSP72 is a mediator in inflammation and growth signal (78). In skeletal muscle, HSP72 is

i - BGP-15 chemical name is O-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime.

important for myofiber integrity and regeneration (79) and is thought to be a negative regulator of FoxO (80). Mechanistic studies indicate that BGP-15 reduces insulin resistance by activating HSPs and inhibiting the inflammatory protein JNK, that can block insulin signaling (81, 82). BGP-15 interacts directly with the heat shock factor HSF-1, responsible for inducing transcription of HSPs, by stabilizing its binding on its DNA response element (83). Further, BGP-15 improves mitochondrial biogenesis and function (81) and remodels cell membranes, improving signal transduction (79).

2 AIMS OF THE THESIS

The general aims of this thesis is to complement and extend our past investigations aiming at understanding of the molecular mechanisms underlying critical illness myopathy (CIM) and ventilator induced diaphragm dysfunction (VIDD) through a muscle-specific, time-resolved approach. A further aim of this thesis is to discover the possibilities of new pharmacological approaches through the use new drug molecules that have not been studied before in the context of CIM or VIDD, such as BGP-15 and vamorolone.

Specific aims:

Paper I: Exploring the genetic transcriptional changes (genomic effects) induced by systemically administered corticosteroids on limb muscles using the porcine ICU model.

Paper II: Understanding the temporal molecular changes that contribute to sparing of the masseter, as an example of a craniofacial muscle in response to mechanical silencing, using the rat ICU model.

Paper III: The aim of this pilot study is to test whether the diaphragm responds to BGP-15, a chaperone co-inducer drug, in an age-dependent manner, using the rat ICU model.

Paper IV: This study aims to investigate the effects of a moderate dose of a typical GC, prednisolone, in comparison with those of a prednisolone-cognate, vamorolone, that induces less genomic alterations and thus less unwanted effects. This study focuses on limb skeletal muscle function and molecular aspects in the rat ICU model.

3 MATERIALS AND METHODS

3.1 THE PORCINE ICU MODEL

In paper I, female domestic piglets (Sus scrofa), from the same farm, were randomized into a control and corticosteroid-treated groups (CS). Detailed description of the anesthesia, mechanical ventilation, infusions, drug doses and monitoring methods are detailed in paper I. Briefly, the pigs were sedated by medetomidine and zolazepam and then anaesthetized using intravenous ketamine and mechanically ventilated (volume-controlled). During the study, isoflurane was used and supplemented by intravenous bolus doses of morphine and ketamine as required for immobilization. A bolus dose of 50 mg hydrocortisone was given intravenously three times a day for 5 days. This study protocol was approved by the Ethical Committee on Animal Research at the Karolinska Institute, Stockholm, Sweden (Dnr N71/98, N54/02, and N75/04). Biopsies from the *biceps femoris* muscle were taken on experimental day 5 in both control and CS group. The biopsies were frozen and stored for later analyses.

3.2 EXPRESSION PROFILING

Total RNA from the muscle samples were extracted, from which biotin-labeled cRNA was generated and hybridized to Affymetrix Porcine Genome Array (23 937 probes of 20 201 genes). Gene expression data analysis was performed using the freely available statistical computing language R and a twofold transcript change were included for further analyses. More detailed description and references are available in paper I on methods of averaging (84), normalization (85) statistics, gene annotation (86) and clustering (87).

3.3 THE RAT ICU MODEL

In papers II and IV, adult female Sprague-Dawley rats were used, whereas Fischer 344-Brown Norway (F344-BN) hybrid rats (young, of 7–8 months and old, of 28–32 months) were used in paper III. Animals were randomized into sham-operated control, ICU intervention with and without drugs groups in papers IV and III. The latter paper included age-matched groups as well. Paper II grouped animals by experimental time (0-14 days) into four groups.

3.3.1 Control rats

Control rats were sham-operated, i.e., neck incision and catheterization of *vena jugularis* and *arteria carotis*, and received treatments similarly to the experimental rats except for α-cobratoxin. These rats were anesthetized while on spontaneous breathing, received intravenous and intra-arterial solutions identical to the intervention groups and were euthanized within 2 hours of the initial isoflurane anesthesia.

3.3.2 Intervention groups

Intervention groups received controlled MV, pharmacological paralysis using an NMBA (α-cobratoxin in continuous infusion) and deep sedation (isoflurane) for varying periods of time. Protein and fluid balance was maintained in all experimental animals throughout the duration of MV as described elsewhere (16, 17). Detailed description of the model including surgery, MV, intra-arterial and intravenous solutions, nutrients and monitoring parameters can be found elsewhere (43, 44). At the termination of the experiments, rats were euthanized by isoflurane overdose and samples were collected for analyses. All aspects of this study were carried out according to the guidelines of the Swedish Board of Agriculture and were approved by the ethical committee at Uppsala University and Karolinska Institutet (ethical permit number N263/14).

3.3.3 Drug interventions

In paper III, BGP-15 (N-gene) was administered intravenously at 40 mg/kg/day. In paper IV, prednisolone 5 mg/kg/day and vamorolone (ReveraGen Biopharma) 20 mg/kg/day (10) were given *per os* to their respective groups.

3.3.4 Samples

Masseter (paper II), diaphragm (paper III) and soleus and EDL muscles (paper IV) were dissected immediately after euthanasia. The whole masseter was snap-frozen, while all of the diaphragm, soleus and EDL were dissected in two parts; one snap-frozen and the other treated with a chemical skinning solution for single fiber contractile measurements.

3.4 SINGLE MUSCLE FIBER EXPERIMENTAL PROCEDURE (PAPERS III AND IV)

A part of the muscle was placed in relaxing solution and bundles of 50 muscle fibers were dissected free and treated with a skinning (glycerol) solution, cryo-protected by serial solutions of ascending sucrose concentrations and finally stored at -160 °C. Details on sample preparation and solution contents can be found in papers II and III and elsewhere (18, 19).

Contractile measurements protocols have been previously described in detail elsewhere (18). Briefly, on the day of contractility measurements, bundles were gradually de-sucrosed and single fibers were pulled out and connected between a force transducer and a motor lever arm. Sarcomere length was set to 2.70 ± 0.05 µm and fiber dimensions were measured in order to calculate cross-sectional area (CSA) assuming an elliptical circumference. In mechanical recordings, relaxing and activating solutions were used where force was measured by the slack-test procedure (21). Specific force (SF) was calculated as maximal force (P_0) normalized to CSA, where maximal force was considered the difference between the maximal steady-state isometric force (taken in the activating solution) and the resting force (in relaxing solution).

3.5 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

After each contractile measurement, muscle fibers were individually dissolved in sample buffer and stored at -80°C. Proteins were fractionated on a 6% acrylamide SDS-PAGE and finally silver stained for determination of the myosin isoforms composition. To measure the myosin:actin ratio samples were prepared by dissolving 10-µm cross-sections of the muscle in urea buffer and fractionated on 12% acrylamide SDS-PAGE and stained with Coomassie blue.

3.6 OTHER ANALYSES

Other analyses such as quantitative real-time PCR (including mRNA extraction, quantification and primers), immunoblotting (including buffer, antibodies and normalization methods) (in all papers), hematoxylin and eosin (H&E) (paper II) and systemic cytokines assay (paper IV) were detailed in their respective papers.

3.7 STATISTICS

Statistical analyses were performed using SigmaPlot 13 software. One-way analyses of variance (ANOVA) and *post hoc* tests were used to compare multiple groups with one variable (i.e., intervention comparisons in paper IV, and time-groups in paper II); while two-way ANOVA (age and intervention) in paper III, and Student's *t* test for two group comparisons (CS group vs. control in paper I and ICU vs. control in paper IV). $P < 0.05$ was considered statistically significant in all papers.

4 RESULTS AND DISCUSSION

4.1 HEAT SHOCK PROTEINS

In the porcine model study (paper I), adding GC treatment to the ICU intervention lead to transcriptional down-regulation in a number of heat shock proteins (HSPs), including HSP70, along with decreased fast fiber specific force (SF), thus supporting a SF-HSP70 correlation. Impaired HSPs response in skeletal muscle was found to be associated with muscle dysfunction in CIM animal models (45, 88). The SF-HSP70 correlation confirmed our findings in previous porcine studies where HSP70 levels correlated with limb SF, suggesting an HSP70 muscle protective role. In those studies, we attempted to understand the impact of each proposed triggers on CIM by ruling out one factor at a time, including the variables of NMBAs, endotoxin-induced sepsis, and systemic corticosteroids in combination with common basic interventions (immobilization and MV) for 5 days (47). We found the maintained SF in response to ICU condition including MV and deep sedation, was coupled to maintained HSP70 levels in pigs exposed to a 5-day ICU intervention regardless of NMBA treatment, however, the addition of sepsis intervention resulted in a loss of SF, muscle fiber size along with an impaired HSP70 response (44, 47).

Additionally, one of the main findings in paper II is the important role of HSPs in general and HSP70 in particular in the development of CIM (Figure 4). This is not completely a novel finding since HSPs activation in response to ICU intervention was observed previously in the ICU rat model in limb muscles [13]. However, the scale and speed of HSP70 activation in the masseter was starker and faster, suggesting an early protection of masticatory muscles. A similar masseter-limb disparity with regards to HSP activation onset and extent was seen before in the porcine ICU model where 5 days ICU intervention induced a significant up-regulation in ten HSPs in the masseter compared with the smaller activation in the biceps femoris, a limb muscle (88).

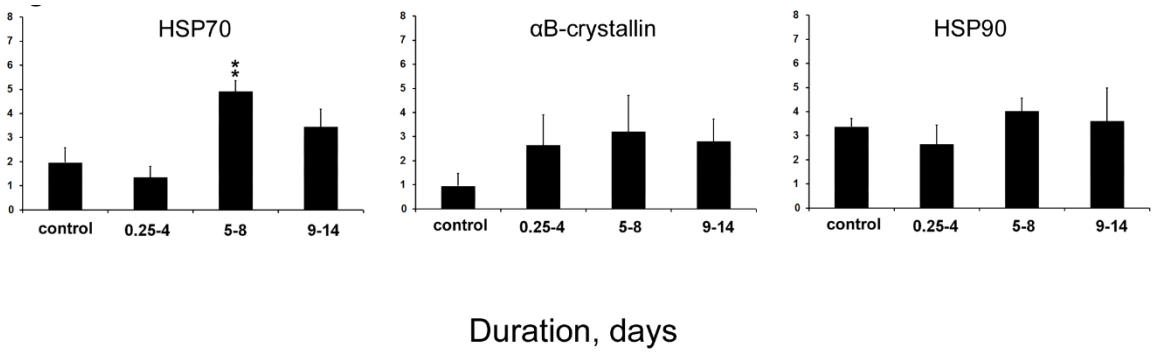

***Figure 4*: Masseter heat shock proteins protein levels.** *Immunoblotting measurement of HSP70, alpha-B crystallin, and HSP90 measured in the main masticatory muscle, the masseter, of rats exposed to ICU interventions including deep sedation, immobilization and mechanical ventilation over a period of two weeks. Source: (Paper II) Akkad et al, 2014 (89), with permission from Plos One.*

HSPs expression increases in the condition of mechanical silencing, while it decreases in other unloading conditions such as those of limb suspension, joint fixing, and denervation (90-92). HSPs are an essential part of the stress response mounted by cells in stressful conditions that helps recover homeostasis. At their basal levels, HSPs, as chaperones, play multiple house-keeping roles including proper folding of nascent proteins, protein transport, translation and regulation of redox balance and apoptosis (5). However, during stress, HSPs activation helps preventing protein aggregation and maintaining muscle fiber integrity and regeneration (93-95). HSP72 is suggested to reduce the activation of the transcription factors FoxO (30) and the cytokine transcription factor NF-κB, both of which may independently play a role in CIM underlying mechanism (95). HSPs, including HSP70 and the small protein αB-crystallin in particular contribute to cellular protection through their anti-apoptotic function and mitochondrial redox regulation (96-98) which are components of CIM underlying mechanisms.

4.2 BGP-15

Muscle wasting and old age are the strongest predictors of morbidity and mortality in the ICU. The aging process is usually accompanied by the degenerative loss in muscle mass and function known as sarcopenia, thus it is mandatory to understand the age-specific effects of muscle dysfunction related to ICU condition which help designing age-tailored therapeutics. As discussed above, impaired HSP activation may contribute to the muscle wasting and dysfunction in the ICU. The HSP chaperone co-inducer BGP-15 has shown positive effects on diaphragm function in young rats exposed to MV in animal experiments but it is not clear if the same applies to the old. In paper III, by comparing diaphragms from young and old rats, both of which received 5-day controlled MV and immobilization with or without systemic BGP-15 treatment, we found that positive BGP-15 muscle effects were limited to young rats (Figure 5). In old rats, on the other hand, BGP-15 administration had no effect on inducing HSP72 nor improving diaphragm contractility. As HSP72 protein levels did not differ between young and old animals either in controls or after the MV intervention, a probable explanation for BGP-15 inefficacy in aged rats could be the blunted HSP response is in old age due to the low-grade inflammation or oxidative stress (93, 99). Yet, a right shift (higher dose) in the dose-response curve in the old animals is not excluded.

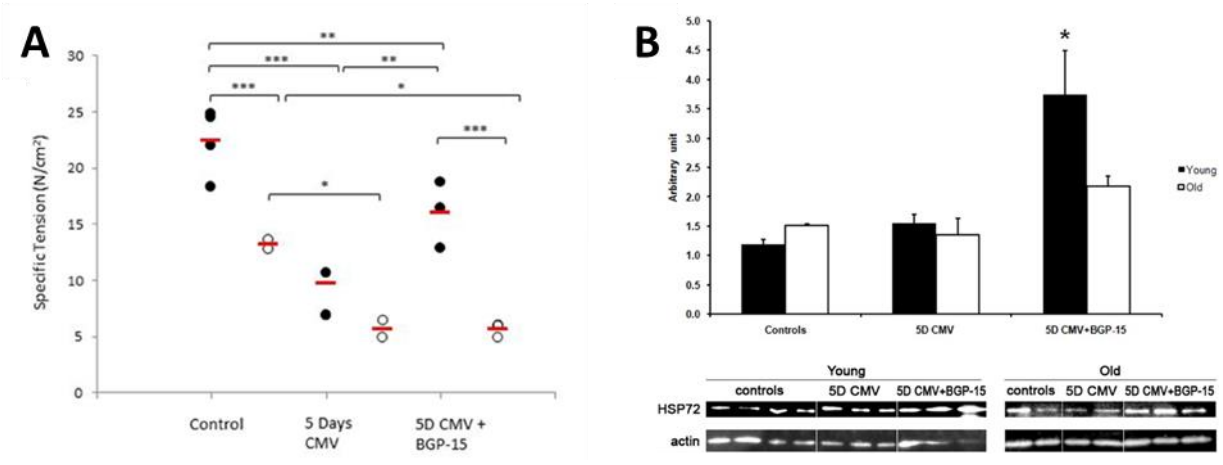

Figure 5. BGP-15 age-dependent effects on the diaphragm. *Control rats and those exposed to ICU intervention including mechanical ventilation for 5 days with and without BGP-15. A, single diaphragm muscle specific tension/force of young (filled circles) and old (open circles). B, protein levels of HSP72 induced by BGP-15 in the diaphragm in young (black bars) and old (white bars) rats. * p<0.05, ** p<0.01, *** p<0.001. Source: (Paper III) Ogilvie et al, 2016, (94) with permission from Frontiers in Physiology.*

BGP-15 positive effects on young rat diaphragms were observed in a previous study where BGP-15 not only doubled the diaphragm residual SF after 10 days of MV at the single muscle fiber level, but also improved muscle contraction at the motor protein level and mitochondrial complexes activity and mass (54). Chaperone co-inducers are a new family of drugs that include BGP-15, a hydroxamic acid derivative which amplifies HSP72 expression in response to stress and stabilize cellular membranes (83). The exact mechanism of action of these drugs is not clear and seems to be multi-faceted and a number of BGP-15 effects were documented. BGP-15 interacts with membrane lipids, alters the oxidative stress response and facilitates the expression of HSF-1, the HSP72 transcription factors. BGP-15 also increases the expression of HSPs through stabilization of the cellular membrane lipids, thus facilitating Rac1 signaling, a membrane bound protein which regulates ROS, playing a major role in the heat shock response (100). Further, BGP-15 acts as a PARP-1 inhibitor. PARP-1 is a DNA repairing transcription factor that co-activates inflammation signal of NF-κB (83, 101) and reduces the activity of SIRT-1, a deacetylase that boosts HSF-1-dependent HSP transcription. Furthermore, BGP-15 protects mitochondrial membranes, thus enhance the respiratory chain function and reduces the production of free radicals (101). Therefore BGP-15 effects can improve HSPs expression, mitochondrial function and oxidative stress response and be a potential pharmacological intervention to reduce muscle cell stress in response to the ICU condition. Our animal experiments in mechanically ventilated and immobilized rats [unpublished results and a previous study (54)] hold promise of BGP-15 positive effects in both limb and respiratory muscle functions. Currently, we are in the process of translating these experiments into the clinical phase.

4.3 GC GENOMIC EFFECTS

GCs have many deleterious unwanted effects that promote metabolic alterations and muscle loss, making them plausible triggering factors of CIM (102), however, their anti-inflammatory effects can be beneficial to muscle function as inflammation is a component of the CIM etiology. In paper I, we showed that 5-day hydrocortisone treatment in the porcine model triggered the CIM phenotype at least in the fast twitch muscle fibers. In study IV, the use of prednisolone in the rat model showed mixed result of severe functional deterioration in the fast-twitch EDL muscle while a beneficial effect was observed in the slow twitch soleus. A previous study indicated that a single daily dose of systemic GC administration for 5 days in the porcine ICU model affected muscle function negatively (Figure 6).

Paper I aimed at finding the transcriptional changes underlying the dysfunction in a limb muscle, the biceps femoris. In this paper, GC- induced significant alterations in 186 genes in

multiple pathways including cell cycle, protein synthesis, channel regulation, oxidative stress, cytoskeletal, sarcomeric proteins and HSPs. However, importantly, no additional up-regulation in proteolytic pathways. Examples of the gene microarray results include:

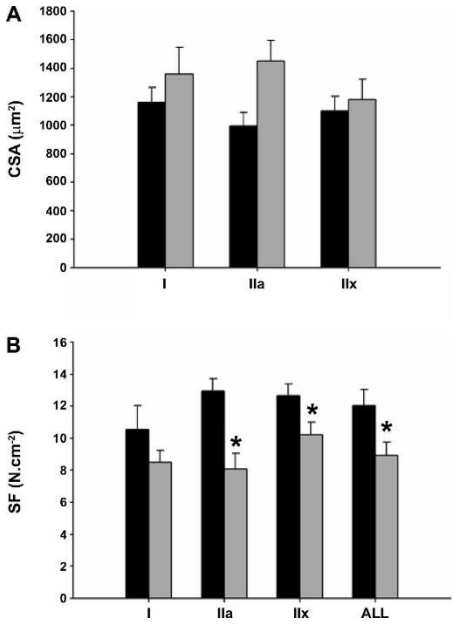

***Figure 6**: A, Cross-sectional area (CSA) and B, specific force (SF) of the biceps femoris type I, IIa, and IIx myofibers. Control at day 5 (black) and corticosteroids at day 5 (gray). Source: (Paper I) Aare et al., 2013 (43). Courtesy of physiological Genomics (originally taken and adjusted from Ochala et al. (47), with permission from Plos ONE.)*

- Cell cycle, e.g., upregulation of activin receptor; NR2F2, a nuclear receptor required for muscle proliferation and represses GR transcription; IL1RAP, involved in myogenesis and IL-1 signaling, important for the synthesis of acute phase and proinflammatory proteins; BMP4, also a part of the TGF-beta superfamily, responsible for inducing myogenic differentiation and downregulation of HDAC9, MEF2 repressor.

- Protein synthesis, e.g., upregulated translation repressors, and downregulated translation initiation factor, the rate-limiting component in the translation machinery.

- Oxidative stress response, e.g., downregulated oxidative stress defense genes such as thioredoxin reductase 1 and the mitochondrial MnSOD.

- Cytoskeletal and sarcomeric proteins, e.g., downregulated MYH7, that encodes type I myosin heavy chain isoform; TNNT1 encoding the slow skeletal muscle troponin T, and MAP1A gene, encoding microtubule-associated protein 1A responsible for microtubule assembly.

- HSPs, e.g., downregulated HSPA4 and HSP90

- No additional upregulation in proteolytic pathways such as the proteolytic atrogin-1, Fbxo33 and four cathepsins genes.

4.4 VAMOROLONE

As demonstrated in paper I, systemic GCs treatment is associated with various side effects stemming from their vast genomic effects. These side effects limit the GCs use and probably

trigger CIM phenotype. However, new generation of GCs, named dissociative GCs e.g., vamorolone, shares the potent anti-inflammatory mechanism with typical GCs but causes fewer harsh unwanted effects (Figure 3). In study IV, we compared effects of prednisolone, as a typical GC and vamorolone, as a dissociative GC on fast and slow-twitch limb muscles in the rat ICU model exposed for 5 days ICU condition. Our results showed that both drugs improved muscle fiber maximal force and size of the slow-twitch soleus while only prednisolone worsened the fast-twitch EDL force and size. Vamorolone is a novel anti-inflammatory delta 9,11 steroid that lacks many genomic-mediated side effects of GCs but preserves significant anti-inflammatory effects shown by most other GCs (64, 67). Another important effect of vamorolone is cellular membrane protection. These two mechanisms were shown to be beneficial in a muscle dystrophy animal model where vamorolone improved muscle strength in comparison to its parent molecule, prednisolone (68). Vamorolone has now cleared safety studies and forwarded to phase 1 clinical trials.

In the rat ICU model after a 5-day ICU intervention, vamorolone showed beneficial effects on survival rates, body and muscle weight loss, slow muscle fiber atrophy and maximal force, and did not affect protein levels of the atrogenes nor calpain-1. Prednisolone, on the other hand, showed positive general effects, including slightly improved survival rate, and decreased slow muscle fiber atrophy and weakness comparable to those of vamorolone, but the effect on the fast-twitch EDL muscle were deleterious (Figure 7) and coupled with activated calcium-induced calpain 1 (Figure 8). Myosin:actin ratio was not affected in either drug interventions. In the soleus, vamorolone treatment protected the muscle from the MuRF1 and atrogin-1 transcriptional up-regulation observed in non-drug-treated and prednisolone-treated animals. In the EDL, however, atrogene up-regulation was observed in all three intervention groups (ICU, PRED and VAM, see Figure 7). Protein expression of the atrogenes or the autophagy sensor LC3B was not statistically different in the either muscle among the treatments.

The drop in SF in limb muscle has been shown to be preceded by a reduction in myosin to actin ratio, also known as preferential myosin loss (32, 50, 52, 53, 103). However, in the case of prednisolone and vamorolone, the drop in SF was coupled with maintained myosin levels. Decreased SF concomitant with maintained myosin levels might be caused by myosin post-translational modifications (PTMs) that precede myosin loss as the case in the diaphragm (48) and limb muscles (unpublished data). Another explanation could be a change in sequence of proteolytic pathway activation. Our previous studies indicate that the ubiquitin-dependent E3 ligases up-regulates in an early phase of ICU stress, followed by the autophagic pathway and finally, the calcium dependent calpains increases after nine days of exposure to ICU condition. Our immunoblotting showed that calpain 1 protein levels were higher in the prednisolone and lower in vamorolone group at five days. Given that calpains facilitates myofibrils disassembly, prednisolone-induced calpain activation may compromise contractile function prior to the actual decrease in myosin levels.

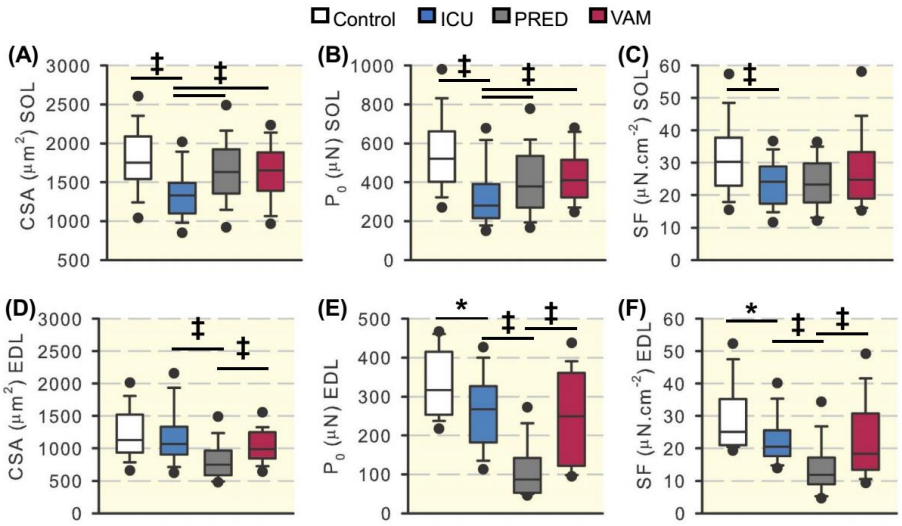

Figure 7. Single muscle fiber measurements. *Cross sectional area (CSA), maximal force (P_0), and specific force (SF) in soleus (SOL) and EDL muscles. ICU (5 days of ICU condition), PRED (5 days of ICU condition + prednisolone) and VAM (5 days of ICU condition + vamorolone). Asterisk (*) represents p<0.05, obelisk (†) p<0.01 and diesis (‡) p<0.001. Source: Paper IV (In manuscript).*

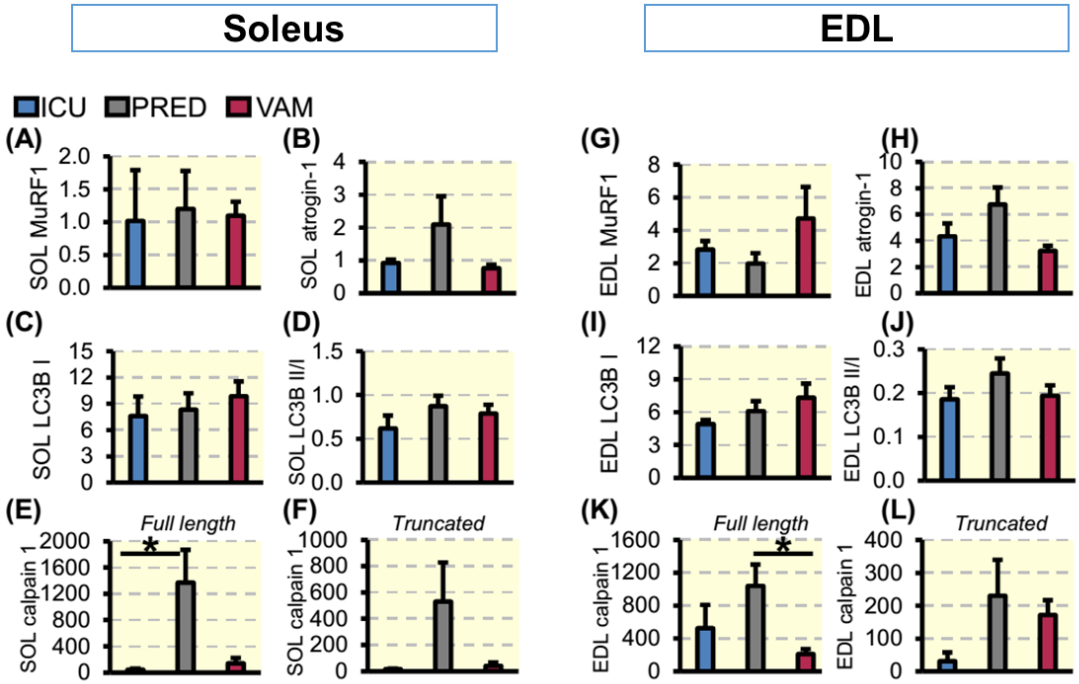

Figure 8. Western blot analysis. *Protein levels of MuRF1, atrogin-1, and LC3B I, ratio of LC3B II:I and calpain 1 full-length and truncated calpain 1 in the soleus and EDL. Asterisk (*) represents p<0.05. Source: Paper IV (In manuscript).*

In conclusion, 5-day vamorolone treatment was associated with ICU outcomes superior to those of prednisolone, including muscle and body weight loss, muscle fiber atrophy, and maximal force in slow-twitch fibers, without worsening atrophy and dysfunction in fast-

twitch fibers. Further, the use of GCs in low-moderate doses is not supported as a CIM-triggering risk factor.

4.5 MUSCLE-SPECIFICITY AND CIM PHENO- AND GENOTYPES

In papers II, III and IV, the rats showed muscle specific atrophy and dysfunction characteristic of clinical CIM and VIDD in the diaphragm after exposure to the ICU condition, but the extent, mechanisms and triggering factors varied between different muscles. In patients, CIM manifests as a progressive muscle wasting and weakness in limb and trunk muscles including the diaphragm, while craniofacial muscles are usually less affected. In the masseter muscle (paper II) clinical observations were confirmed as 14 days of exposure to ICU condition of deep sedation, NMBA and MV treatments did not affect the transcriptional regulation of myofibrillar proteins or myosin to actin ratios. In contrast, limb muscles showed rapid downregulation of myofibrillar proteins and preferential loss of myosin and myosin-associated proteins. The unaffected structure and function of cranial nerve innervated muscles was attributed to multiple mechanisms, such as slow atrogene activation and sustained motor protein synthesis (Akt phosphorylation), inhibited metalloproteinases, and early activation of the autophagosome (measured by LC3B) and HSP70 expression. In line with CIM clinical description, the porcine ICU model previously showed a clear preservation of masticatory muscle size and function and adding sepsis and GC hormone treatment did not change the outcome (88).

In study IV where limb muscles were investigated, the major geno- and pheno-type aspects of CIM patients were all reproduced after five-day exposure to the ICU condition, including preferential myosin loss in soleus and EDL muscles, transcriptional down-regulation of myosin, up-regulated ubiquitin-dependent proteolysis, muscle atrophy, and decreased SF (32). Consistently with these findings, previous time-resolved studies in limb muscles demonstrated that exposure to the ICU condition for more than 5 days lead to progressive muscle atrophy and dysfunction in both fast and slow twitch limb muscles. These findings resemble the described clinical picture with sustained proteolysis via the early up-regulation of the ubiquitin-proteasome system prior to a pronounced myosin loss and subsequently the activation of calcium-dependent and lysosomal proteolytic enzymes (52).

On the other hand, in the diaphragm, MV *per se* is the key trigger of VIDD, while other triggering factors play a smaller role. Paper III showed that exposure to five-day ICU condition of deep sedation, NMBAs and MV caused a significant loss in SF compared with the control animals independent of the MyHC isoform. These findings are in line with our earlier VIDD investigation where the rat diaphragm showed a dramatic deterioration in contractile force that left only 15% of the initial pre-MV force after two weeks. The force loss was not associated with a significant preferential loss in myosin nor down-regulation in contractile protein transcription, as was the case in limb muscles. Rather, the atrogenes were up-regulated, oxidative stress indicators increased, and intracellular lipids accumulated. A porcine study tried to determine the importance of each VIDD triggering factor such as MV, NMBAs, sepsis and GCs in inducing VIDD, reported that five days of MV, regardless of any

other factor, induced a loss of half the SF of single muscle fibers in all fiber types (I, IIa or IIx MyHC isoforms), while size was unaffected (42). These studies suggested that post-translational modifications (PTMs) were responsible for qualitative modifications of myosin leading to the myofiber weakness.

5 CONCLUSIONS

Skeletal muscle is a large and highly specialized tissue responsible for essential functions such as locomotion and breathing. Skeletal muscle can adapt its size and force via physiological and pathological signals by tipping the balance of protein synthesis and degradation, resulting in contractile protein increase or decrease. Triggers of protein loss include over-activated proteolytic pathways (especially the atrogenes, MuRF1 and atrogin-1) and decreased protein synthesis.

We focused in this thesis on the condition known as critical illness myopathy (CIM), an acquired muscle wasting and paralysis that develops rapidly in response to prolonged intensive care unit (ICU) interventions, such as mechanical ventilation (MV), unloading and certain drugs. CIM affects limb and trunk muscles and relatively spares craniofacial muscles, while the diaphragm weakness due extended MV was termed ventilator induced diaphragm dysfunction (VIDD). CIM and VIDD prolong the ICU length of stay and have direct and indirect negative consequences on survival, recovery, quality of life and the health care economy. Research into CIM and VIDD is inherently complex and the establishment of animal models that can mimic long term exposure to ICU condition is necessary. In this thesis, we used two animal ICU models to better understand the mechanisms of both CIM and VIDD: pig and rat ICU models exposed to ICU interventions such as deep sedation, immobilization and MV for varying periods of time. The rat model allows longer periods of intervention and can reveal temporal changes.

Heat shock proteins (HSPs) were activated more rapidly in the masseter in comparison to limb muscles (paper II), a finding that may underlie craniofacial muscle protection. HSPs play an important role in cellular basal regulation and in response to stress, such as regulation of redox, apoptosis and inflammatory signals. HSPs activation is triggered in muscle cells by the cellular stress induced by the state of mechanical silencing associated with the ICU condition. This confirms our previous findings that maintained muscle specific force is correlated with HSP activation in CIM animal studies.

In paper III, in order to test the effects of HSPs induction on VIDD, we used a new drug, BGP-15 that mainly induces the expression of HSP70. BGP-15 have a cellular membrane stabilizing effects and a complex mechanism that improves mitochondrial function and amplifies HSP activation. We demonstrated that BGP-15 reduced the diaphragm dysfunction in response to MV in young rats, confirming previous findings, but was ineffective in old ones. It is not clear whether the lack of efficacy in the old animals is due to age-related impairment of HSP activation or due to a shift in the dose-response curve.

The effects of GC treatment were studies in paper I, which showed that glucocorticoids (GCs) exacerbated the CIM-induced muscle fiber atrophy and weakness (in the fast twitch fiber, particularly) in the porcine ICU model. The muscle dysfunction was accompanied with

numerous transcriptional changes including suppression of protein synthesis, cell cycle and HSPs but not coupled with additional proteolysis.

Study IV, compared two GCs, vamorolone and prednisolone. Vamorolone is a new dissociative GC, designed to maintain potent anti-inflammatory effects while eliminating many unwanted effects via its reduced genomic interference. Vamorolone and prednisolone showed a comparable attenuation of atrophy and weakness in the slow-twitch soleus muscle fibers, but only prednisolone exacerbated muscle dysfunction in the fast-twitch EDL, suggesting an improved vamorolone side-effect profile.

Finally, the rat ICU model showed a great similarity with the CIM clinical phenotype in different muscles including loss in myofiber size and specific force coupled with activated proteolysis, decreased myosin heavy chain (MyHC) transcription and myosin protein loss in limb muscles. Our studies supported the notion that the main CIM trigger in limb muscles is the complete of mechanical silencing, i.e., the lack of internal or external muscle activation that severely decrease myosin content, while the diaphragm dysfunction is triggered by long-term MV through post-translational modifications (PTMs) that cause qualitative alterations in MyHC rather than myosin loss.

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