

From PHYSIOLOGY AND PHARMACOLOGY
Karolinska Institutet, Stockholm, Sweden

CRITICAL ILLNESS MYOPATHY: UNDERSTANDING DIFFERENT EFFECTS ON MUSCLE FIBRE FUNCTION

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**Karolinska
Institutet**

Stockholm 2015

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Printed by Eprint AB 2015

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ISBN 978-91-7676-019-2

Critical Illness Myopathy: Understanding different
effects on muscle fibre function
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“After all muscle moves the world”

Sir Charles Sherrington

ABSTRACT

Skeletal muscle is an essential component of the human body, being one of the most dynamic and plastic tissues. As such it can be altered by numerous confounding factors. Critical illness myopathy is a common and complex manifestation seen in the intensive care unit and it has over the past decade become an increasing problem that will only escalate further with time. Understanding the processes and underlying mechanisms of muscle regulation with respect to critical illness myopathy is an area of great research focus and aims to improve scientific knowledge and patient care. My thesis has provided further insight into this highly orchestrated process. The overall goal of my PhD project was to investigate the importance of two potential contributors, nutrition and ageing, in the development of muscle fibre dysfunction in response to the intensive care unit condition; critical illness myopathy, and evaluate the efficiency of one potential therapeutic intervention (BGP-15) using a rodent model mimicking the intensive care unit environment.

Nutritional feeding of a eucaloric vs low caloric diet was found not to differ in the preferential myosin loss, decline in specific force and muscle fibre atrophy of the limb muscles after a period of controlled mechanical ventilation of up to 14 days. The term “specific force” has been accepted as an indicator of muscle quality and is the generated force by a muscle adjusted for its size. In both experimental groups, passive mechanical loading had a sparing effect of muscle weight independent of nutritional status. We observed in both young and old rats an unexpected response of the diaphragm fibres to 5 days controlled mechanical ventilation, an ineffective compensatory hypertrophy, in conjunction with a decreased maximum force in both age groups compared with controls, resulting in an age related dramatic loss of specific force. Administration of the pharmacological intervention BGP-15 demonstrated that after 10 days controlled mechanical ventilation the specific force of diaphragm muscle fibres increased by more than 100% compared to untreated muscle fibres. Furthermore when BGP-15 was administered to young and old rats, after 5 days controlled mechanical ventilation the age dependent significant drop in diaphragm fibre force production was restored, however only in the young. This concomitant increase in force in the young was observed also in the expression of heat shock protein 72. Thus, it is suggested that the increased Hsp72 expression, induced by BGP-15 is an indicator of the inhibition of the atrophy pathway (UPS) that as such attributes to an increased cross sectional area and specific force in only the young.

It is of crucial importance to comprehend in more depth the effect of mechanical ventilation on limb and diaphragm function in the intensive care setting in order to ascertain any further age- related differences. We have determined here that BGP-15 is a possible intervention strategy that needs to be explored further to investigate the underlying mechanisms of action in order to implement the possible highly clinical significance of this research.

LIST OF SCIENTIFIC PAPERS

- I. The Effect of Nutritional Status in the Pathogenesis of Critical Illness Myopathy (CIM).
Ogilvie H, Larsson L. *Biology* (Basel). 2014 May 30; 3(2):368-82. doi: 10.3390/biology3020368

- II. Age related differences in diaphragm muscle fiber response to mid/long term controlled mechanical ventilation.
Cacciani N*, **Ogilvie H***, Larsson L. *Exp Gerontol*. 2014 Nov; 59:28-33. doi: 10.1016/j.exger. 2014.06.017. Epub 2014 Jun 25
*contributed equally

- III. The chaperone co-inducer BGP-15 alleviates mechanical ventilation induced diaphragm muscle dysfunction.
Heba Salah, **Hannah Ogilvie**, Nicola Cacciani, Hazem Akkad, Gabor Balogh, Yvette Hedström, Jorge Ruas, Leonardo Salviati, Lars Larsson
Manuscript

- IV. Targeting heat shock proteins mitigates ventilator induced diaphragm muscle dysfunction in an age-dependent manner.
Ogilvie H, Cacciani N, Akkad H, Larsson L
Manuscript

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LIST OF ABBREVIATIONS

| | |
|------------------|--|
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| ASPEN | American Society for Parenteral and Enteral Nutrition |
| C | Controls |
| Ca ²⁺ | Calcium |
| CIM | Critical illness myopathy |
| CIP | Critical illness polyneuropathy |
| CMV | Controlled mechanical ventilation |
| CSA | Cross-sectional area |
| Ct | Threshold cycle |
| EC | Eucaloric |
| ECG | Electrocardiogram |
| EDL | Extensor digitorum longus |
| EEG | Electroencephalogram |
| EPaNIC | Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients |
| ESPN | European Society for Clinical Nutrition and Metabolism |
| F344-BN | Fisher 344-Brown Norway |
| FoxO | Forkhead Box O |
| Hsp | Heat shock protein |
| ICU | Intensive care unit |
| ICU-AW | Intensive care unit acquired muscle weakness |
| IGF | Insulin growth factor |
| IMF | Intermyofibrillar |
| IV | Intravenous |
| LC | Low calorie |
| MAFbx | Muscle atrophy F-box |
| Mfn1 | Mitofusin1 |
| Mfn2 | Mitofusin 2 |

| | |
|----------------|--|
| MuRF1 | Muscle-specific ring finger protein 1 |
| MV | Mechanical ventilation |
| MyHC | Myosin heavy chains |
| MyLC | Myosin light chains |
| NF- κ B | Nuclear Factor κ B |
| NIA | National Institute of Aging |
| NMB | Neuromuscular blockade |
| PARP | poly ADP ribose polymerase |
| P ₀ | Absolute force |
| P _i | Inorganic phosphate |
| PN | Parenteral nutritional |
| PVDF | Polyvinylidene fluoride |
| RNA | Ribosomal nucleic acid |
| ROS | Reactive oxygen species |
| SD | Sprague Dawley |
| SEM | Standard error of the means |
| SS | Subsarcolemmal |
| TEM | Transmission electron microscopy |
| UPS | Ubiquitin proteasome system |
| VIDD | Ventilator-induced diaphragm dysfunction |
| VILI | Ventilator-induced lung injury |

1 INTRODUCTION

1.1 SKELETAL MUSCLE: STRUCTURE AND FUNCTION

Skeletal muscle is the largest tissue in the human body, comprising about 40% of the body's weight and is further considered an organ. It is one of the most dynamic and plastic tissues of the human body. The main function of skeletal muscle is to generate force and power, maintain the body's posture and produce movement allowing us to perform everyday tasks and activities. More specifically from a mechanical point of view its function is to convert chemical energy into mechanical energy; from a metabolic perspective, the role of skeletal muscle includes a contribution to basal energy metabolism, serving as storage for important substrates such as amino acids and carbohydrates, the production of heat for the maintenance of core body temperature and the consumption of the majority of oxygen and fuel used during physical activity and exercise. As such skeletal muscle is a highly complex and orchestrated tissue.

1.1.1 Muscle: from protein to movement

1.1.1.1 Muscle Proteins

Within skeletal muscle, each individual muscle fibre divides into thousands of myofibrils, which contains billions of myofilaments (see figure 1). The two most abundant myofilaments (proteins) are actin and myosin, with myosin being the molecular motor protein. It is the myofilaments when assembled together in a characteristic (cross-striated) pattern that form sarcomeres. Muscle contraction is achieved by shortening of the sarcomeres caused by the interaction between the contractile myosin and actin filaments, as described by the "sliding filament model" (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954).

It is the numerous proteins that contribute to the structure of the cytoskeleton, coupling of the excitation and contraction processes, energy release and the generation of force and power (Gordon et al., 2000), all of which play important and individual roles. Of particular importance are the regulatory proteins such as the calcium-dependent troponin complex and tropomyosin, which are associated with the actin filament and play very important roles in the activation process that leads to myofilament sliding and thus force generation.

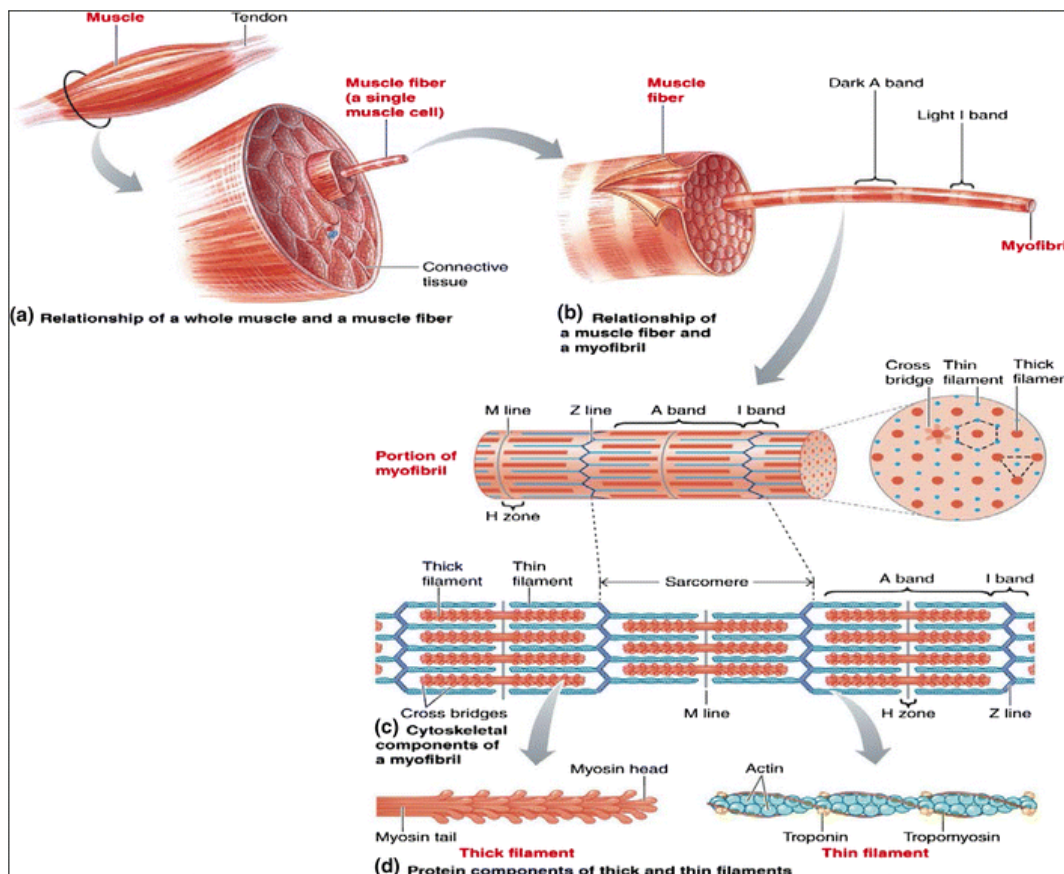


Figure 1. Structure of skeletal muscle (Sherwood, 2010)

1.1.2 Excitation–Contraction Coupling: Physiology of Muscle Activation

In order for muscle to generate force, thus allowing us to move our bodies, a process must first occur, Excitation–contraction (EC) coupling. This is the coordination of two processes; the transmission of the nerve stimulus followed by the depolarization of the sarcolemma and the consequent release of calcium from the sarcoplasmic reticulum and the resultant interaction between the two proteins actin and myosin that form cross-bridges. Briefly, when an action potential arrives at the muscle fibre membrane and is conducted into the interior of the muscle cell via the transverse tubular (T tubule), a voltage-gated sensor on the T tubule opens, allowing a flow of calcium inwards causing the opening of the ryanodine receptors in the sarcoplasmic reticulum. This allows calcium to be released in large amounts into the sarcoplasm. The calcium released into the sarcoplasm then binds to the regulatory protein troponin C on the actin thin myofibril, causing troponin to change shape, which in turn initiates a series of molecular events. Tropomyosin moves away from the myosin binding sites on actin allowing the myosin head to bind actin and form a cross bridge. The detailed structure of the myosin head was described for the first time in 1993 (Rayment et al., 1993) and contributed significantly to our understanding of the mechanics and physiology of this process. As mentioned before, the end-result of this sequence of events is the sliding of the actin and myosin filaments and the generation of force. The force generated by individual actin–myosin cross-bridges is transmitted longitudinally and laterally within the fibre.

1.1.3 Generating Force and Movement

The sliding filament theory (1954) (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954) explains the basic molecular mechanisms by which muscle fibres generate force via the cross bridge cycle (see figure 2).

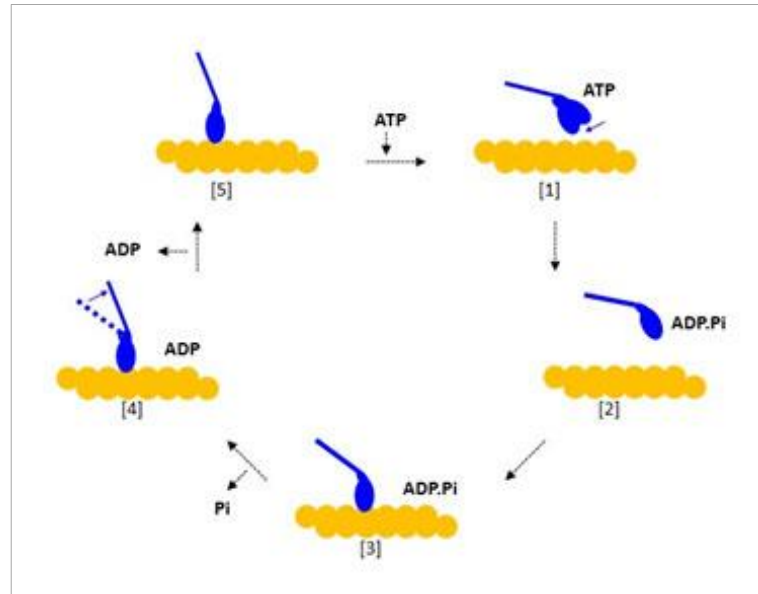


Figure 2. Cross bridge cycle. Actomyosin interaction during contraction.

This cycle begins when adenosine triphosphate (ATP) is made available to an existing actin-myosin cross bridge and binds to the myosin head resulting in the dissociation of myosin from actin and the detachment of the existing cross-bridge (see figure 2 [1]). ATP is hydrolysed to adenosine diphosphate (ADP) and inorganic phosphate (Pi), the energy generated activates the myosin head to be moved (see figure 2 [2]) and binds weakly to a new actin molecule, forming a new cross bridge (see figure 2 [3]). Release of Pi triggers a power stroke and myosin pushes the actin filament past it towards the centre of the sarcomere. Force is generated during this power stroke (see figure 2 [4]). At the end of the power stroke, ADP is released and myosin remains bound to actin in rigor (see figure 2 [5]). As long as the binding sites on actin remain exposed the cross bridge cycle will repeat. As the cycle repeats the thin myofilaments are pulled towards each other and the sarcomere shortens. This shortening causes the whole muscle to contract. Cross bridge cycling ends when calcium (Ca^{2+}) ions are actively transported back into the sarcoplasmic reticulum.

The generation of force is dependent on many factors, the muscle size, the number of actin-myosin cross-bridges formed, the force generated by each cross-bridge, the degree of activation by the nervous system, its architecture (the angle at which muscle fibres insert into the tendon known as pennation angle) and the quality of the interaction between the cellular elements. The term “specific force” has been accepted as an indicator of muscle quality and is the generated force by a muscle adjusted for its size.

1.1.4 Muscle Fibre Organelles

There are other cellular elements in the sarcoplasm of muscle fibres; these include a trans T-tubule, the sarcoplasmic reticulum, and a mitochondrial network. In order for muscle movement and the mechanisms of action talked about previously to occur, energy (ATP) needs to be generated to react with the oxygen made available to the muscle fibre (Dahl et al., 2015). It is the job of the mitochondria, which forms a three-dimensional network throughout the cell to generate the energy needed for muscle actions. Mitochondria can be found located closer to the sarcolemma, which reduces the diffusion distance for oxygen transport by the capillary supply. Another population of mitochondria is located in the inter-myofibrillar space. Mitochondria ultrastructure damage has been described in hind-limb skeletal endotoxemic rats more than 40 years ago, with frequent distortion of inner and outer mitochondrial membranes and the presence of large vacuole areas (Schumer et al., 1971). It is known that various stimuli as well as various neuromuscular pathologies can induce significant changes in the structure and function of the mitochondria. Several biophysical mechanisms may contribute to the mitochondrial dysfunction among which are disruption of mitochondrial membrane integrity, modification of protein side-groups, increased degradation or reduced synthesis of the pathway components and desegregation of the multiprotein complexes.

1.1.5 Muscle Tissue Heterogeneity

1.1.5.1 Muscle Fibre Types

The human body is required to undertake various activities with different metabolic and mechanical demands, thus the human body has different muscles with differing predominance of the various fibre types, classified according to their myosin heavy chain (MyHC) composition. The most frequently used classification for adult human limb muscles includes three fibre types: type I (slow, oxidative, fatigue-resistant), IIa (fast, oxidative, intermediate metabolic properties) and IIx (fastest, glycolytic, fatigable) MyHCs. Humans also express, under various conditions and in specific muscles, other types of myosin such as embryonic, neonatal, and extra ocular (Schiaffino and Reggiani, 2011). Similarly in rats and mice there are four MyHC isoforms that are expressed; type I, IIa, IIx and IIb. Studies using isolated muscle fibres have demonstrated an association between force-generating capacity, velocity of shortening and MyHC isoform composition (Geiger et al., 2000; Geiger et al., 1999; Larsson and Moss, 1993; Sieck and Prakash, 1997). These characteristics are very plastic and can easily be modified in response to changes in workload, activity or pathological conditions. For example, prolonged periods of skeletal muscle inactivity such as intensive care unit (ICU) stay or ageing, have been seen to alter MyHC isoform composition and force production of single muscle fibres (Gea et al., 2000; Klitgaard et al., 1990; Llano-Diez et al., 2012; Ochala et al., 2011a).

1.1.5.2 Limb versus Respiratory

The diaphragm is the most important inspiratory muscle in mammals and is essential for normal ventilation (Poole et al., 1997). Muscle fibres in the diaphragm are classified in the same way as in the limb muscle, as either type I or type II MyHC. However the activity of the muscles somewhat differ from one another. During normal conditions, the continuous rhythmic activation of the diaphragm makes it among one of the most active muscles in the body and thus is different to the peripheral muscles. The specific type of loading which the diaphragm endures is quite different from that of the postural and locomotor muscles. The diaphragm is non-weight bearing and thus in contrast to limb muscles is not exposed to gravitational effects.

1.1.6 Skeletal Muscle Plasticity

Skeletal muscle function plays an important role in daily life, but it can also be affected in several ways that can impede on our daily lives; in one such case muscle disease. The causes of muscle disease are heterogeneous, from alteration in nerves, mitochondria, sarcoplasmic reticulum, to nuclei and other intracellular components. Specifically here I address the ICU and the associated intensive care unit acquired muscle weakness (ICU-AW), critical illness myopathy (CIM).

1.2 THE INTENSIVE CARE UNIT

1.2.1 Intensive care unit-acquired weakness and critical illness myopathy

Perhaps one of the most debilitating environments for muscle is that of the critically ill patient in the ICU, with the loss of lean body mass and eventual loss of muscle function being key features. There are numerous confounding factors influencing the body and more specifically the skeletal muscle. As such, muscle wasting can be triggered by multiple conditions, including disuse, denervation, sepsis, fasting, cancer, cardiac failure, renal dysfunction and multiple organ failure. It is the exposure to multiple stressors such as fluid and electrolyte changes, catabolic stresses, nutritional deficiencies and medications that can act in combination to produce damage to the motor unit. Muscle biopsies taken from ICU patients with multiple organ failure show a range of histological changes, including severe muscle fibre atrophy, degeneration and necrosis (Puthuchery et al., 2013).

At the cellular level, a reduction in myofibre size reflects an imbalance between proteolysis and protein synthesis. This is regulated by complex changes in signalling pathways and gene products that are involved in the regulation of protein breakdown (discussed later). The loss

of muscle mass and a reduction in force generating capacity in an ICU patient can be termed Intensive care unit-acquired weakness (ICU-AW). Whereas ICU-AW is usually accompanied by muscle wasting, muscle wasting does not necessarily lead to neuromuscular dysfunction, since overall muscle strength depends both on total muscle mass and force generating capacity (force per cross-sectional area), which is affected in ICU-AW but not necessarily in muscle wasting syndromes (Callahan and Supinski, 2009). As mentioned before, muscle force capacity may remain stable in muscle wasting syndromes.

ICU-AW is a severe complication in critically ill patients that has been increasingly recognised over the last two decades. By definition ICU-AW is caused by distinct neuromuscular disorders, namely critical illness polyneuropathy (CIP) and critical illness myopathy (CIM) (Latronico and Bolton, 2011; Visser, 2006). These conditions are the primary cause of muscle weakness and paralysis during and following admission to the ICU, irrespective of the underlying conditions. In my thesis the focus lies with CIM, being the most frequent neuromuscular disorder underlying muscle weakness in ICU patients (Lacomis et al., 2000). Initially, CIM was thought to be a rare event in the ICU; we now know that neuromuscular dysfunction is found in up to 30% of the general ICU population and in 70%–80% of certain sub-groups (Lacomis et al., 2000), thus it is becoming a major problem. Its prevalence influences patient prognosis but also bears threats for secondary complications (infection, embolism), prolongs ICU treatment and rehabilitation and greatly raises the costs of intensive care medicine worldwide.

MacFarlane and Rosenthal were the first to describe CIM, (MacFarlane and Rosenthal, 1977), with it being characterised by a reduced muscle membrane excitability, loss of force production and a preferential loss of the molecular motor protein myosin (Larsson et al., 2000; MacFarlane and Rosenthal, 1977). The exact causes of CIM are unknown and are thus an area of ongoing research. Immobilisation, prolonged mechanical ventilation, use of neuromuscular blocking agents, multi-organ failure and sepsis have all been postulated as possible triggering factors. Immobilising patients by deep sedation and mechanically ventilating them during the acute phase of critical illness is suggested to contribute to the muscle weakness, however by itself, the immobilisation (pure bed rest) may be insufficient to explain the degree of muscle atrophy and weakness observed in acutely ill patients (de Jonghe et al., 2009). We have previously shown that it is the complete loss of mechanical stimuli, both external (loss of weight bearing) and internal mechanical load (activation of contractile proteins) i.e. mechanical silencing of skeletal muscle that is a dominating factor triggering the preferential myosin loss, atrophy and loss of force-generating capacity (specific force - SF) in fast- and slow-twitch skeletal muscles in a rat model (Larsson et al., 2000; Ochala et al., 2011a) which results in this severe and specific muscle wasting condition, CIM. This complete mechanical silencing is unique to ICU patients and different from other unloading conditions, i.e. bed rest, hind limb suspension, microgravity (Zhang et al., 2007).

This weakness can undoubtedly complicate the course of a significant number of ICU patients. The associated muscle weakness with CIM can affect both the limb and the

respiratory musculature and more rarely both facial and extra-ocular muscles (Akkad et al., 2014). This can reduce the chances of patient survival or of a full recovery, by complicating the weaning process from the ventilator; increasing the length of stay in the ICU and delay mobilisation and physical rehabilitation of the patient (Ali et al., 2008; Hiesmayr, 2012). Understanding the mechanisms underlying CIM is an on-going and complex web.

1.2.2 Mechanical ventilation and Ventilator Induced Diaphragm Dysfunction

Diaphragm function preservation is of critical importance to overall health in the process of ageing and in the critically ill. Mechanical ventilation (MV) is a life-saving intervention used in approximately 40% of adult ICU patients with respiratory failure for a median duration of 5 to 7 days. MV, although lifesaving is also associated with a variety of major complications, these include pneumonia, cardiovascular compromise, barotrauma, ventilator-induced lung injury (VILI) and an increased risk of morbidity and mortality (Esteban et al., 2000; Winkelmann, 2013; Wunsch et al., 2010). In the late 1970s, ventilator-induced lung injury became recognised as a serious complication of mechanical ventilation (Dreyfuss and Saumon, 1998). Our understanding of prolonged MV resulting in diaphragmatic dysfunction has increased dramatically. The first study to document the impact of MV on rodent diaphragm atrophy and contractile function was in 1994, (Le Bourdelles et al., 1994) it revealed that 48 hours of controlled mechanical ventilation (CMV) (i.e., full ventilator support of breathing) resulted in significant loss of diaphragm mass and a large reduction in maximal diaphragmatic specific force production. This detrimental impact of prolonged rest/inactivity on the diaphragm, induced by CMV triggering diaphragm dysfunction has been termed ventilator-induced diaphragm dysfunction (VIDD) (Vassilakopoulos and Petrof, 2004). VIDD is defined as a loss of diaphragmatic force-generating capacity that is specifically related to the use of mechanical ventilation (Vassilakopoulos and Petrof, 2004) and is supplementary to an increased difficulty in weaning patients from the respirator. Both animal and human experiments consistently demonstrate that prolonged MV promotes diaphragmatic atrophy resulting in a reduction in diaphragm mass, and furthermore diaphragmatic weakness (Corpeno et al., 2014; Haitzma, 2011; Levine et al., 2008; Powers et al., 2002). The mechanisms causing VIDD are considered intrinsic to diaphragm muscle fibres, rather than related to alterations in the lungs, thorax/abdominal compliance or neural input (Jaber et al., 2011; Powers et al., 2013; Radell et al., 2002). Since diaphragmatic dysfunction (in part) determines the ability of patients to be successfully weaned from the ventilator, it is therefore of critical importance to understand the effect of mechanical ventilation on diaphragm function.

1.2.3 Nutrition in the Intensive Care Unit

Nutritional support is an integral component of our general health maintenance, conservation and restoration and thus if combined with the ICU and the critically ill population of patients, it is perhaps of even greater importance. As such the administration of artificial nutrition has been advocated to attenuate muscle loss, however the success of which remains to be demonstrated (Casaer et al., 2013; Streat et al., 1987).

1.2.3.1 Nutritional guidelines

Nutritional support is generally considered an essential component in the management of critically ill patients, however current guidelines regarding nutritional support in the ICU are mainly based on physiological statements (Singer et al., 2009). Furthermore malnutrition is commonly observed among patients admitted to the ICU. Large audits including thousands of patients worldwide have shown that in daily practice calorie and protein administration are well below current recommendations, varying between 1200 and 1450kcal/day and 0.5 – 0.8g/kg/day of protein (Alberda et al., 2009). As such this problem can be exacerbated by a prolonged hospital stay resulting in a reduced energy and protein intake, increased energy expenditure and protein catabolism (Kim et al., 2010; O'Leary-Kelley et al., 2005; Schetz et al., 2013b). Perhaps the key question to ask is whether artificial nutrition administered during critical illness can prevent, improve or correct this nutritional deficit and thereby also the associated adverse outcomes, or if in fact it can worsen the outcome. This impact has thus far not been adequately addressed. The more specific question that is addressed here is the role that the nutritional deficit may play in specifically triggering CIM.

Administration of artificial nutrition without doubt, improves energy and protein intake, however on the contrary the un-physiological intervention of artificial nutrition may evoke complications and side effects. As such aggressive nutritional support in critically ill patients, instead of preventing muscle loss or reducing proteolysis and gluconeogenesis, merely results in fat gain (Hart et al., 2002; Streat et al., 1987). Furthermore it has been seen in rabbits that there is a direct link between nutrition and organ failure. Critically ill rabbits showed that early parenteral nutritional (PN) feeding, suppressed the ubiquitin–proteasome pathway, thereby contributing to the preservation of muscle mass, but also evoked a phenotype of autophagy deficiency in liver and skeletal muscle, suggesting that the maintenance of muscle mass might come at the price of accumulation of toxic protein aggregates, thus compromising function (Derde et al., 2012). Thus, whether artificial nutritional feeding prevents accelerated muscle catabolism in immobilised critically ill patients with systemic inflammation and to what extent it can prevent/attenuate muscle wasting/weakness or accelerate recovery specifically in CIM patients, remains as yet unclear.

The overall aim of nutritional care in the ICU is to minimise ‘acute disease-related malnutrition’; adequate nutritional care takes into consideration the acute disease itself, the interactions of chronic diseases, pharmacological interventions and organ dysfunction

(Hiesmayr, 2012). However it is debated as to the type of feeding that is most appropriate for ICU patients, enteral or parenteral (Casaer et al., 2011a; Hermans et al., 2013). The existing guidelines advocate early enteral nutrition, with the optimal timing for the addition of parenteral nutrition to insufficient enteral feeding being the subject of transatlantic controversy. Several guidelines, observational studies and meta-analysis support an early start of artificial nutrition within 24-48 hours of ICU of admission, of which the severely ill patients appear to benefit most. However there are some discrepancies that exist between different guidelines. Both the European Society for Clinical Nutrition and Metabolism (ESPEN) (Singer et al., 2009) and American Society for Parenteral and Enteral Nutrition (ASPEN) (McClave et al., 2009) guidelines support an early start of feeding. However a large interventional study has been carried out comparing these two methods and have found there to be no benefit of early parenteral nutrition with regards to mortality and a clear disadvantage in resource use due to the duration of artificial ventilation and length of stay in the ICU (Casaer et al., 2011a). Furthermore Herman's et al (2011) carried out a sub analysis to the Early Parenteral Nutrition Completing Enteral Nutrition in Adult Critically Ill Patients (EPaNIC) trial and found that tolerating a substantial macronutrient deficit early during critical illness did not affect muscle wasting, but allowed more efficient activation of autophagic quality control of myofibres and reduced weakness and in fact that nutritional interventions may have an adverse effect on muscle whilst suppressing autophagy (Hermans et al., 2013). Summarizing the clinical trials cited above, there is no evidence for increased energy/protein provision during ICU week 1 in protecting muscle mass, muscle force or long-term physical function. A potential explanation for the pattern of on-going muscle wasting despite increased energy and protein intake may be found in its pathogenesis (Casaer et al., 2013; Owais et al., 2014). The mechanisms underlying muscle atrophy and weakness in critical illness are extremely complex (Llano-Diez et al., 2011; Sandri, 2008), and thus expecting the atrophy and weakness to be reversible by simply providing calories and protein is perhaps an oversimplification of the situation.

1.2.4 Ageing and the Intensive Care Unit

The general population is an ageing one, with life expectancy and the number of men and women in older age groups increasing dramatically in the last century. Ageing and chronic disease reduces whole skeletal muscle performance that in turn impacts on the individual's ability to undertake everyday tasks. Furthermore ageing is associated with an increased incidence of chronic health conditions as well as an increase in the prevalence of impairment and disability. Visual and hearing impairments, cognitive decline, musculoskeletal disorders, frailty and sarcopenia all reduce activity and restrict participation in personal, work associated and social activities. Thus as a consequence it has been estimated that the number of older people requiring long-term care due to loss of functional independence will quadruple by 2050, undoubtedly having a financial impact on society (WHO, 2015). Our physical functional capacity is strongly dependent upon skeletal muscle power output, which

is calculated as the product of the force generating capacity and contractile velocity of the motor unit (Reid and Fielding, 2012). As such with ageing and disease this has been seen to decrease.

One of the most distinctive features of ageing is the presence of muscle weakness and atrophy; atrophy is defined as a decrease in size due to a loss of organelles, cytoplasm, and/or protein. This specific loss of lean body mass associated with ageing was termed “*sarcopenia*” by Rosenberg (Rosenberg, 1989, 1997). Cross sectional studies of men and women have shown significant reductions in muscle mass, strength and alterations in body composition with advancing age (Frontera et al., 1991; Jubrias et al., 1997). Indeed, ageing is associated with a constant decrease in strength of approximately 1-3% per year resulting in qualitative and quantitative changes at the single muscle fibre level. Furthermore an ageing related decline in the specific force of single muscle fibres is observed (Frontera et al., 2000; Larsson et al., 1997). A significant portion of the age-related decrease in muscle force production is undoubtedly caused by the concomitant reduction of muscle mass (atrophy), however it cannot solely be attributed to this. Alterations in the muscles intrinsic functionality, such as reduced force generation and/or contractile speed per muscle size (*i.e.*, muscle quality) may also play a role (Raj et al., 2010). The molecular mechanisms underlying these changes remain unclear and as such many molecular mechanisms have been proposed to explain such dysfunction, including a reduction in myosin protein content. The latter may be related to gene transcription with reductions in translation and protein synthesis, leading to a lower myosin concentration per unit of muscle cell area. In addition, posttranslational modifications of myosin via mechanisms such as oxidation and glycosylation may result in myofibril dysfunction and reduce the number of actin-myosin cross-bridges in the strong bound state and therefore limit the force and power generation (Lowe et al., 2002; Ramamurthy et al., 2001).

Age can also play an important factor in influencing the impact of prolonged MV on diaphragmatic function. In fact the negative effects of MV could be compounded further by the age-related decrease in diaphragmatic contractile performance. In general, compared with young adult animals, ageing results in a reduction in diaphragmatic maximal specific tension (Criswell et al., 1997). The collective effect of both ageing and MV-induced diaphragmatic contractile dysfunction could mean that patient age is a predictor of difficulties in patient weaning. Age-related muscle changes are very complex and involve multiple features and mechanisms influenced both by intrinsic and extrinsic environmental conditions. These changes to muscle are different to those associated with injuries, chronic diseases and immobilisation, so the therapeutic approach must be tailored to the individual case considering the changes at the muscle cell level.

1.3 MOLECULAR MECHANISMS CONTROLLING MUSCLE MASS AND CONTRACTILE DYSFUNCTION

As we have seen thus far skeletal muscle is a highly plastic component of the human body and one that can be affected and manipulated by many factors. Muscle mass, like that of any other tissue, depends on protein turnover. It can adapt to different pathophysiological conditions via activating pathways that regulate protein turnover. The maintenance of skeletal muscle mass is determined by a fine balance between protein synthesis and protein degradation. Increased protein synthesis and a simultaneous decrease in protein degradation results in muscle gain (hypertrophy), in contrast the loss of muscle mass (muscle atrophy) is mainly due to a loss of contractile proteins. Changes in protein synthesis result in part from modulations of the mechanisms involved in mRNA translation, which involves the initiation, elongation and termination steps, the initiation process appears to be a major regulatory event (Nader et al., 2002). Thus the cell's proteolytic mechanisms must be highly selective and tightly regulated, since the accelerated destruction of an essential protein or the failure to degrade a short-lived regulatory protein could drastically alter cell function. By understanding the hypertrophy and atrophy signalling pathways and identifying the process by which either or none are up or down-regulated and/or interact together, could allude to the process by which atrophy occurs and is stimulated in ICU patients. The focus for my thesis will be on the signalling pathways involved in muscle atrophy.

1.3.1 Atrophy signalling pathways

Muscle atrophy is an active process controlled by specific signalling pathways and transcriptional programs. There are three major proteolytic systems contributing to skeletal muscle protein loss: the cytosolic calcium-dependent calpain system, the lysosomal proteases and the ATP-dependent ubiquitin proteasome system (UPS). The UPS pathway is responsible for 80–90% of intracellular protein turnover (Solomon and Goldberg, 1996). This ATP-dependent process requires sequential interaction among three families of enzymes: ubiquitin activating enzymes (E1 proteins), ubiquitin conjugating enzymes (E2 proteins), and ubiquitin ligases (E3 proteins). There are several hundred E3's that have been identified and it is likely that each modulates the ubiquitination of a distinct set of substrates. Animal research would suggest that in particular two E3s are uniquely expressed in skeletal muscle: muscle atrophy F-box (MAFbx/atrogen-1) and muscle RING finger 1 (MuRF1). The functional importance of these gene products in atrophy processes was demonstrated by the generation of *MAFbx*^{-/-} and *MuRF1*^{-/-} mice. Mice lacking either gene showed sparing of muscle mass following denervation (Bodine et al., 2001; Gomes et al., 2001). These ubiquitin ligases (MAFbx/atrogen-1 and MuRF1) are instrumental to the processes of muscle protein degradation observed during muscle atrophy conditions (Glass, 2003), thus this pathway will be the focus here.

Critical illness and MV have both been seen to attribute to an increased expression of MAFbx/atrogen-1 and MuRF1 in both human and rat limb and diaphragm muscle (DeRuisseau et al., 2005; Glass, 2005; Hussain et al., 2010; Llano-Diez et al., 2011; Nordquist et al., 2007; Norman et al., 2006; Ochala et al., 2011a). Importantly, recent findings suggest that MyHC's are ubiquitinated and degraded by MuRF1, perhaps in part explaining myofibril disassembly (Clarke et al., 2007; Cohen et al., 2009). It has been established that it is the preferential loss of myosin that can attribute to the loss of force specifically in CIM patients (Larsson et al., 2000; Llano-Diez et al., 2012). The inhibition of MuRF1 could be a novel mechanism to prevent or reverse muscle wasting associated with various pathologies. The levels of MAFbx and MuRF1 have been described to be controlled by the insulin-like growth factor, insulin growth factor (IGF)-I/Akt/Forkhead Box O (FoxO) pathway in which phosphorylation and activation of Akt leads to phosphorylation and retention in the cytoplasm of FoxO transcription factors, thereby decreasing gene expression and subsequent protein abundance of MAFbx and MuRF-1 and attenuating muscle proteolysis (Glass, 2005). It is established that both protein synthesis and degradation are a highly orchestrated processes that are tightly regulated within the cells, requiring an interacting complex cascades of events, thus invariably things can go wrong, both in the healthy population and in the critically ill.

1.4 POTENTIAL INTERVENTION STRATEGIES TO ALLEVIATE CRITICAL ILLNESS MYOPATHY

At present there is no specific treatment for CIM, however there have been different therapies which have been suggested to reduce the severity of the myopathy. Recently, early mobilisation has been proposed to have a positive effect in slowing the development of CIM (Brahmbhatt et al., 2010; Griffiths et al., 1995; Llano-Diez et al., 2012; Martin et al., 2005). Furthermore pharmacological interventions have been put forward as possible strategies. The chaperone co-inducer BGP-15 [O-(3-piperidino-2-hydroxy-1-propyl) nicotinic amidoxime dihydrochloride], a known pharmacological co-inducer of heat shock protein (Hsp) 70, was recently shown to have a muscle sparing effect and improve muscle architecture, strength and contractile function in severely affected diaphragm muscles in a rodent Duchenne muscular dystrophy model (Gehrig et al., 2012). The stress-inducible isoform of Hsp70 (Hsp72) is associated with the atrophy of muscle fibres and plays an important role in regulating muscle fibre size during conditions of muscle atrophy. The first evidence of Hsp72's involvement in regulating skeletal muscle plasticity was through the use of muscle specific Hsp70 transgenic mice (McArdle et al., 2004). Hsp72 has been seen to negatively regulate the FoxO and Nuclear Factor κ B (NF- κ B) pathways, which are activated during numerous conditions of muscle atrophy (Hunter and Kandarian, 2004; Sandri et al., 2004; Senf et al., 2010). Furthermore it has been seen that Hsp's play an important protective role of myofibrillar proteins specifically during CMV and immobilisation (Aare et al., 2011; Banduseela et al., 2009). The mechanisms by which critical illness contributes to muscle weakness and atrophy

are multifaceted and involve several inter-related processes, which can act synergistically. Thus with regards to possible intervention strategies it is perhaps not a simple case of “one shoe fits all”, which is important to remember with regards to potential intervention strategies.

1.5 A HETEROGENEOUS POPULATION; THE NEED FOR AN ANIMAL MODEL

The critically ill ICU group of patients is a complex cohort and one that has many independent and complex confounding factors, such as underlying disease state, different modes of mechanical ventilation, medications and newly acquired complications, which can also impair skeletal and diaphragmatic muscle function (Laghi and Tobin, 2003). To consider all of these factors when trying to study and understand the underlying mechanisms of CIM is complex. Given the difficulty of a controlled clinical observation, there is a compelling need for an experimental animal model. There are very few experimental models where the long-term effects of MV and immobilisation of skeletal muscle can be studied in detail. The porcine model has been for many years been used to study the effects of sepsis on organ function (Offner et al., 1995). This was therefore thought to be an ideal candidate for an animal model of CIM, due to the well known similarity in metabolism between humans and pigs (Norman et al., 2006). One major benefit to the porcine model is the ability to use the same ventilators that are used in ICU patients. However experimental rodent ICU models offer a cost-efficient alternative to the porcine model, as well as improved logistical advantages. Mouse models allow for efficient genetic engineering, but there is increasing evidence that the rat is a better model for studies of muscle disease and ageing (Ibebunjo et al., 2013). In addition the ability to carry out long term MV in small rodents poses further problems, as the majority of commercially available ventilators are unable to maintain life support for longer than a few days, which adds a further confounding factor; time, with ICU-AW phenotypes taking longer than 24 hours to develop. Furthermore the rat and human diaphragm are anatomically alike and contain a similar fibre type composition (Powers et al., 1997), the rat has become the most commonly used animal model to study MV-induced changes in diaphragm fibre size and function. An important issue is dissecting the different entities of ICU-AW and their pathophysiological mechanisms. The definition of proper animal models that as closely as possible mimic the phenotype of the muscle weakness seen in ICU patients is therefore imperative. The model that is used in Lars Larsson lab and is used for the work in this thesis is an experimental rat model, developed by Barry Dworkin and co-workers (Dworkin and Dworkin, 1990, 2004). This model mimics the basic ICU conditions of long-term mechanical ventilation, sedation and muscle unloading: i.e. absence of mechanical loading related to muscle contraction or weight bearing (mechanical silencing) (see figure 3). It allows for the unique possibility to conduct time-resolved analyses with a high temporal resolution on the effects of the ICU condition on muscle, muscle specific difference as well as in the design and evaluation of specific intervention strategies (Larsson, 2007).

2 AIMS

The overall goal of my PhD project is to investigate the importance of potential contributors in the development of muscle fibre dysfunction in response to the ICU condition; CIM and evaluate the efficiency of one potential therapeutic intervention.

Paper I

The aim of this study is to investigate the effects of nutritional status in the pathogenesis of CIM. By using a unique experimental animal model mimicking the ICU condition we want to determine whether nutritional status is driving the preferential myosin loss, fibre atrophy and loss of specific force in skeletal muscle, all of which are key characteristics of CIM.

Paper II

The main aim of this study is to investigate the effect of ageing on rat diaphragm muscle fibre structure and function in response to five days of controlled mechanical ventilation.

Paper III

In this study we have investigated the impact of a new class of ‘membrane lipid therapy’ pharmaceuticals (BGP-15) in restoring the heat shock proteins stress response in the diaphragm, induced by controlled mechanical ventilation. By using an experimental rat model, allowing time-resolved studies for durations varying between 6h and 10 days we specifically focus on the effects on mitochondrial structure and function and regulation of muscle contraction at the muscle cell level.

Paper IV

The main aim of this pilot study was to investigate the impact of BGP-15 on diaphragm dysfunction in response to 5 days CMV in young adult vs. old rats using a unique experimental rat model.

3 MATERIALS AND METHODS

Here I will present the methods that are relevant to my personal work, thus the focus will remain on the fibre contractility methods. The other methods used in the individual papers can be found in the paper references.

In all papers the same experimental rat model was used, mimicking the intensive care unit setting. The rats were anaesthetized, mechanically ventilated and treated with α -cobra-toxin for durations varying from 0 days (control) up to 14 days. Female rats were chosen for ease of handling and insertion of the urinary catheter. In no experiments did animals show any signs of infections, septicemia, or systemic inflammation.

3.1 ETHICAL CONSIDERATIONS: PAPER I, II, III AND IV

Animal experiments were carried out at Uppsala University. The Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine and the Ethical committee at Uppsala University approved all aspects of these studies.

3.2 EXPERIMENTAL RAT MODEL

Paper's I, II, III, and IV

The experimental model has previously been described in detail (Dworkin and Dworkin, 1990, 2004) and modified to optimise studies of skeletal muscle. Briefly; (1) Electrocardiogram (ECG) electrodes were implanted subcutaneously. (2) An aortic catheter (28-gauge Teflon) was inserted via the left carotid artery to record arterial blood pressure. (3) A 0.9 mm Renathane catheter was threaded into the left jugular vein to administer parenteral nutrition. (4) Three subcutaneous electroencephalogram (EEG) needle electrodes were placed into the skull above the right and left temporal lobes, and a third reference electrode was placed on the neck. (5) The rat was placed on a heating pad to maintain body temperature; temperature was measured by a vaginal thermistor and servo-regulated at 37°C. (6) A silicone cannula was inserted in the urethra to continuously record urine output. Neuromuscular block (NMB) was induced on the first day (100 μ g intravenous (IV). α -cobra-toxin) and maintained by continuous IV infusion (250 μ g/day). Rats are ventilated through a per os coaxial tracheal cannula at 72 breaths/minute with an inspiratory and expiratory ratio of 1:2 and a minute volume of 180–200ml and gas concentrations of 49.5% O₂, 47% N₂, and 3% CO₂, delivered by a precision (volume drift <1%/wk) volumetric respirator (see figure 3). Intermittent hyperinflations (6 per hour at 15cm H₂O), positive end-expiratory pressure (1.5 cm H₂O), and expiratory CO₂ monitoring is continuous. The rat's vital signs were continuously monitored

3.4 ANIMALS

In paper I and III the rat strain Sprague Dawley (SD) was used.

Paper I

Nine female 300g \pm 5g Sprague-Dawley rats were included in this study. A total of four rats were euthanized after a period of 0 days (serving as controls (C), no mechanical ventilation). Five animals underwent surgery to induce the experimental ICU condition and remained in the mimicked ICU setting; anaesthetized, mechanically ventilated and treated with α -cobra toxin (post-synaptic neuromuscular blockade) for a 10–14 day period. Two animals were administered with the EC nutrition and three with LC nutrition.

Paper III

Twenty-four adult 369 \pm 58.5g female Sprague Dawley rats were included in this study. The rats were divided into one control group (n=4) and 5 experimental groups exposed to CMV, NMB and deep sedation for 0.25-1 day (n=5), 1-5 days (n=4), 5- 10 days (n=4), 1-5 days with BGP-15 treatment (1-5d+BGP, n=3), and 5-10 days with BGP-15 treatment (5-10d+BGP, n=4).

In paper II and IV the focus was on the effect ageing may have, thus the rat strain Fisher 344-Brown Norway (F344-BN) hybrid was used. The F344-BN hybrid rat is recommended for age-related studies by the National Institutes of Aging (NIA) because this hybrid rat lives longer and has a lower rate of pathological conditions than inbred rats. The rats were obtained from the National Institute of Aging (NIA (Bethesda, MD).

Paper II

Diaphragm samples were collected from 11 female F344-BN hybrid rats, 7-8 months (young, n=7) and 28-32 months (old, n=4). A total of 13 rats were intended for the study, but two of the old animals were not included (one had died spontaneously and the other had developed a tumour). The rats were randomly classified into two subgroups, 4 young rats and 2 old rats, at time 0 days and 3 young, 2 old rats at time 5 days. Soleus and EDL muscles were collected from young (6 months, n = 2), middle-aged (18 months, n = 2) and aged (28 months, n = 2) rats.

Paper IV

Diaphragm samples were collected from 17 female F344-BN hybrid rats, 7-8 months (young, n=10) and 28-32 months (old, n=7). Experiments were terminated at durations 0 days and 5 days. The rats were randomly classified into three subgroups within young and old. 4 young rats and 2 old rats, at time 0 days, 3 young, 2 old rats at time 5 days and 3 young, 3 old rats at time 5 days with BGP-15.

3.5 PASSIVE MECHANICAL LOADING

Paper I

The left leg of all experimental groups were activated 12 hours per day, using a mechanical lever arm that produced a continuous passive maximal ankle joint flexion-extension of 180° at a speed of 13.3 cycles per minute. The sham-operated control animals underwent the same interventions as the experimental rats, but were not pharmacologically paralyzed with α -cobra-toxin. That is, sham operated-controls were anesthetized (isoflurane), spontaneously breathing, given intra-arterial and intra-venous solutions and sacrificed within two hours after the initial anaesthesia and surgery.

3.6 MUSCLE BIOPSIES AND PERMEABLISATION OF FIBRES

Paper I and II

The extensor digitorum longus (EDL) and soleus muscles were dissected from the loaded and unloaded leg immediately after euthanasia. One half of the soleus and EDL muscles were quickly frozen in liquid propane cooled by liquid nitrogen and stored at -180°C for future analyses. The other half of the soleus and EDL muscle were dissected into bundles.

Paper II, III, IV

The diaphragm muscle was dissected immediately after euthanasia. One half of the mid-costal diaphragm muscle was frozen in liquid propane cooled by liquid nitrogen and stored at -180°C for further analyses. The other half was dissected into bundles.

Specimens were placed in relaxing solution at 4°C and bundles of approximately 50 fibres were dissected free and tied with surgical silk to glass capillary tubes at slightly stretched lengths. To permeabilise the membrane of the fibres they were 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 bundles were detached from the capillary tubes and treated with sucrose, (a cryoprotectant for long-term storage), snap frozen in liquid nitrogen-chilled propane and stored at -180°C .

3.7 SINGLE MUSCLE FIBRE EXPERIMENTAL PROCEDURE AND SPECIFIC FORCE MEASUREMENTS

Paper's I, II, III, and IV

On the day of experiment, bundles were de-sucrosed; transferred to a 2.0M sucrose solution for 30 minutes and subsequently incubated in solutions of decreasing sucrose concentrations (1.5–0.5M) and finally kept in a skinning solution at -20°C . A single fibre is removed from the muscle bundle and placed between two connectors, at a length of 1 to 2mm long (see figure 6A). One connector leads to a force transducer (model 400A, Aurora Scientific), and

the other to a lever arm system (model 308B, Aurora Scientific) (see figure 4B). The two extremities of the fibre were tightly attached to the connectors.

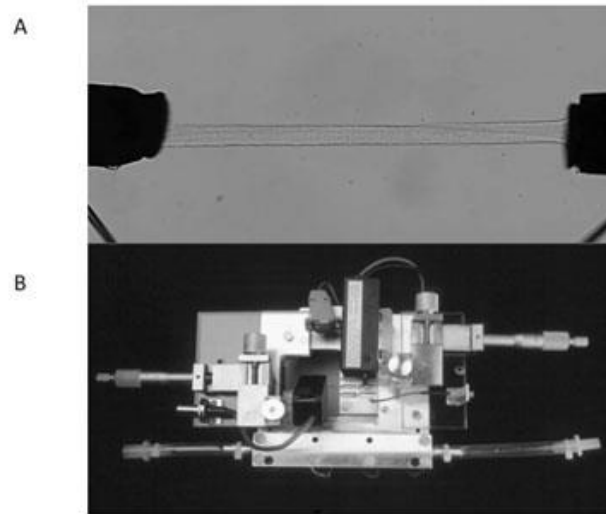


Figure 4. A) Single soleus fibre attached between two connectors B) Single fibre set- up

The apparatus was mounted on the stage of an inverted microscope (model IX70; Olympus). The sarcomere length was set to 2.65-2.75 μm by adjusting the overall segment length and controlled during the experiment using a high-speed video analysis system (model 901A HVSL, Aurora Scientific). The diameter of the fibre between the connectors was measured through the microscope with an image analysis system prior to the mechanical experiments. Fibre depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the fibre. Cross sectional area (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 (Moss, 1979). Diameter and depth were measured at three different locations along the length of each fibre and the mean was considered a representative of cell dimensions. For the mechanical recordings, relaxing and activating solutions (in mM) contained 4 Mg-ATP, 1 free Mg^{2+} , 20 imidazole, 7 EGTA, 14.5 creatine phosphate and KCl to adjust the ionic strength to 180mM and pH to 7.0. The concentrations of free Ca^{2+} were 9.00M (relaxing solutions) and 4.50M (activating solution), expressed as pCa (i.e., $-\log_{10} [\text{Ca}^{2+}]$). Apparent stability constants for Ca^{2+} -EGTA are corrected for temperature (15°C) and ionic strength (180mM). At 15°C, immediately preceding individual activations, the fibre is immersed for 10–20 seconds in a solution with a reduced Ca^{2+} -EGTA buffering capacity. This solution was identical to the relax solution except that the EGTA concentration is reduced to 0.5mM, which results in a more rapid attainment of steady-state force during subsequent activations. Force was measured by slacking the fibre once steady-state isometric force was reached at pCa 4.5. Seven different slacks were performed (7-13%) for each fibre and maximum force was calculated as the difference between the maximal steady-state isometric force in activating solution and the resting force measured in the same segment while in relaxing solution (see figure 5). Maximal force production was normalised to CSA (specific force P_0/CSA).

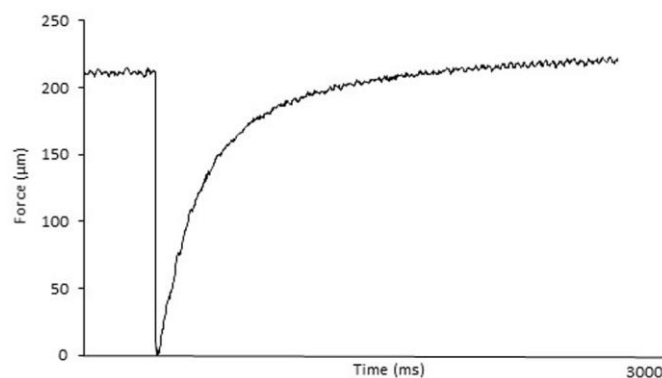


Figure 5. Original single fibre contraction of rat soleus muscle. Showing residual passive force and maximal activate force

For all the contractile measurements a strict acceptance criteria is applied. A muscle fibre was only accepted and included in the analyses if the sarcomere length of a single muscle fibre changed by $<0.10\mu\text{m}$ between relaxation and maximum activation and maximal force changed by $<10\%$ from first to seventh activation.

3.8 MYOSIN HEAVY CHAIN ISOFORM EXPRESSION

Paper's I, II, IV

After mechanical recordings each skinned fibre was placed in sample buffer (7.43ml distilled water, 2.1ml Glycerol, 1.4ml 10% SDS, 1.75 of 0.5M Trisbuffer pH6.8, 0.32ml bromophenol blue, 32.4mg Dithiothreitol, 1ml Leupeptin) in a plastic micro-centrifuge tube and stored at -180°C for subsequent electrophoretic analysis. MyHC isoform composition of fibres was then determined by 6% SDS-PAGE (see figure 6). The acrylamide concentration was 4% (wt/vol) in the stacking gel and 6% in the running gel, the gel matrix included 30% glycerol. Sample loads were kept small (equivalent to approximately 0.05mm of fibre segment) to improve the resolution of the myosin heavy chain bands (types I, IIa, IIx and IIb).

Electrophoresis was performed at 120 V for 20-22 hours with a Tris–glycine electrode buffer (pH 8.3) at 10°C . The gels were silver-stained and subsequently scanned in a soft laser densitometer with a high spatial resolution ($50\mu\text{m}$ pixel spacing) and 4096 optical density levels.

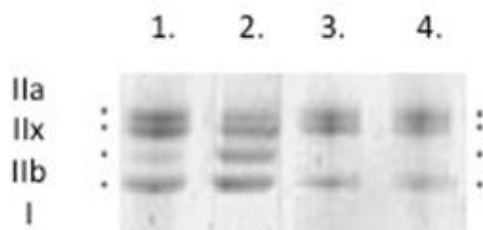


Figure 6. Myosin heavy chain (MyHC) isoform expression was determined by 6% SDS-PAGE. Types IIa, IIx, IIb and I.

3.9 MYOSIN AND ACTIN RATIOS

Paper I

The myosin and actin ratio were measured on 12% SDS-PAGE. The total acrylamide concentration was 4% (w/v) in the stacking gel, 12% in the running gel, the gel matrix included 10% glycerol. Gel electrophoresis was performed at a constant current of 16mA for 5 h 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.

3.10 IMMUNOBLOTTING

Paper IV

Ten sections from the medial part of the diaphragm were dissolved in SDS buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 2% β mercaptoethanol, (pH 8.8), heated for 2 minutes at 95°C. Total protein was quantified using the Pierce 660nm Protein Assay (Thermo Scientific). Equal amounts of protein were separated on SDS-polyacrylamide gels (Mini-PROTEAN 3 Cell, Bio-Rad Laboratories) at constant 120 volts for 90 minutes. Acrylamide concentrations were 4% and 12% (w/v) in stacking and running gels, respectively, and the gel matrix included 10% glycerol. Separated proteins were transferred to poly (vinylidene difluoride) membranes (Immobilon-P, Millipore). Membranes were blocked with 3% non-fat milk powder in Tris-buffered saline containing Tween 20 for 1 hour and incubated overnight at 4°C with Hsp72 (SMC-100B, StressMarq) and actin (sc-1616, Santa Cruz Biotechnology Inc.) primary antibodies and then with HRP secondary antibodies (anti-mouse, GE Healthcare, and anti-goat, Santa Cruz Biotech). Membranes were digitized with ECL 500 Western Blotting Detection Agent Kit (Amersham Biosciences) and imaging system (Odyssey, LI-COR Biosciences). Band densities were quantified with Image Studio Lite analysis software (LI-COR Biosciences).

3.11 STATISTICAL ANALYSIS

In all paper means and standard error of the mean was calculated according to standard procedures. According to statistical criteria the appropriate statistical tests were selected, refer to individual papers.

4 SUMMARY OF RESULTS

For the purpose of my thesis the focus will remain on the fibre contractility results.

4.1 PAPER I - THE EFFECT OF NUTRITIONAL STATUS IN THE PATHOGENESIS OF CRITICAL ILLNESS MYOPATHY (CIM).

The results from this study (paper I) show that the preferential myosin loss, decline in specific force and muscle fibre atrophy did not differ between the low caloric (LC) versus eucaloric (EC) group in Sprague Dawley rats in the soleus muscle over a period of 10-14 days.

Body and muscle weight

In both experimental groups, passive mechanical loading had a sparing effect of muscle weight compared to the unloaded leg, independent of nutritional status, in the LC and EC groups (see paper I Table 2). In parallel with the decline in body weight, significantly lower muscle weights were observed between controls and the two experimental (LC and EC) groups.

Muscle fibre size and specific force in muscle fibres expressing fast and slow myosin heavy chain (MyHC) isoforms

Loading

In both LC and EC groups, passive loading had a significant positive effect on both muscle fibre size and force generating capacity (see figure 7) irrespective of muscle fibre MyHC isoform expression. Loading appeared to have a stronger effect on both muscle fibre size and specific force in the soleus muscle fibres compared with the EDL fibres in accordance with the stronger effect of the passive loading on the myosin:actin ratio in the slow-twitch soleus than in the fast twitch EDL.

Eucaloric Vs low caloric

In both the loaded and unloaded soleus and EDL muscle, no significant difference was observed between the EC and LC group in the muscle fibre CSA and SF (see figure 7).

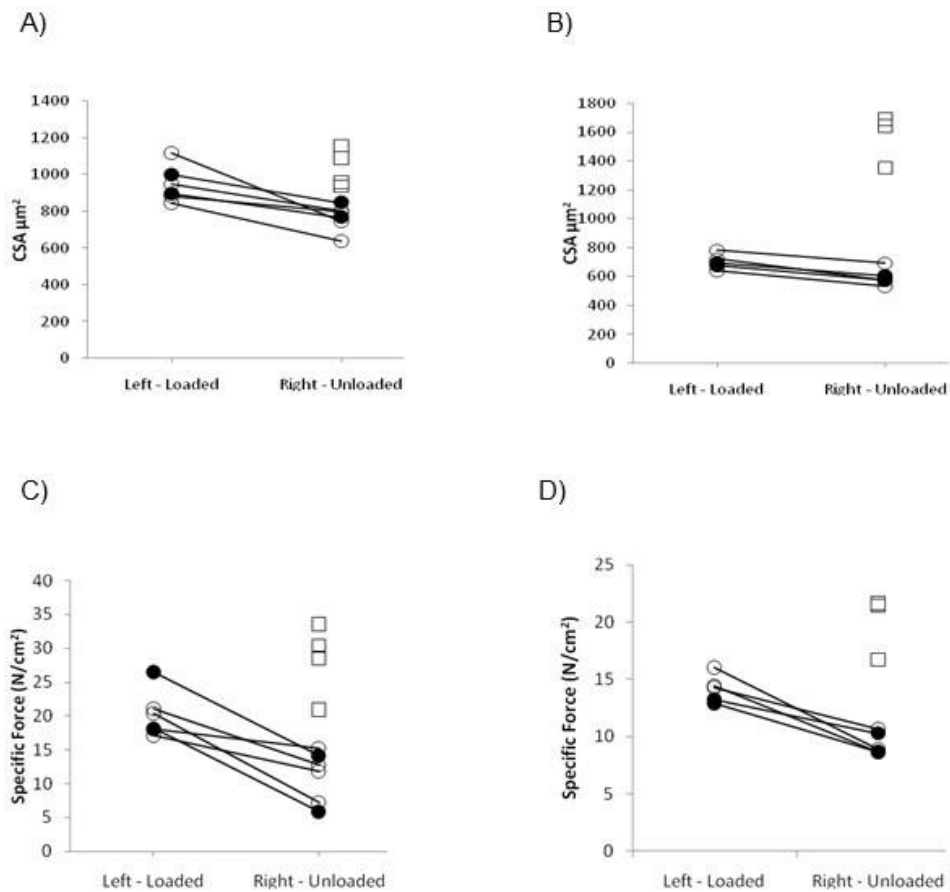


Figure 7. Average single muscle fibre cross sectional area in the soleus (A) and the EDL (B) in the loaded and unloaded limbs in the control (squares), LC (open circles) and EC groups (filled circles). Average single muscle fibre specific force in the soleus (C) and the EDL (D) in the loaded and unloaded limbs in the control (squares), LC (open circles) and EC groups (filled circles).

4.2 PAPER II - AGE RELATED DIFFERENCES IN DIAPHRAGM MUSCLE FIBRE RESPONSE TO MID/LONG TERM CONTROLLED MECHANICAL VENTILATION.

This study showed an unexpected response of diaphragm fibres to 5 days CMV in F344-BN hybrid rats, in both the young (6 months) and old (28-32 months).

Effects of 5 days CMV on diaphragm muscle fibre CSA

Before any intervention the fibre CSA from the old control animals was significantly larger than in young adult controls (see figure 8), furthermore after five days deep sedation, neuromuscular blockade (NMB) and CMV this resulted in a compensatory hypertrophy in both young and old animals, resulting in a maintained age-related difference in diaphragm muscle fibre CSA.

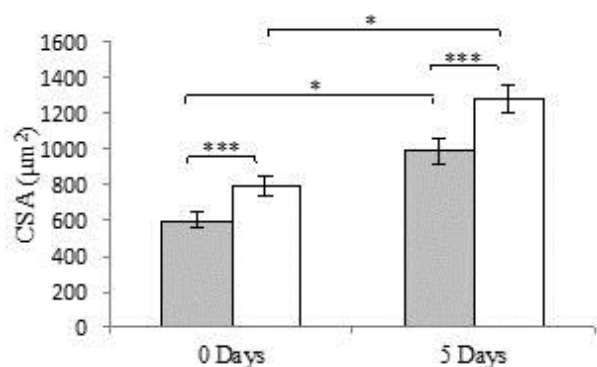


Figure 8. Single diaphragm muscle fibre cross-sectional area (CSA) measured at fixed sarcomere length in control animals compared with the age-matched animals exposed to CMV for 5 days (grey bars: young; white bars: old). Values are means \pm SEM. Significance level: * $p < 0.05$, *** $p < 0.001$.

Effects of 5 days CMV on diaphragm muscle fibre absolute (P_0) and specific force

We see that the absolute force upon maximum Ca^{2+} activation did not differ significantly between young and old animals, however a decline in absolute force was observed in response to five day's CMV irrespective of age (see figure 9A). The age related decline was also observed with specific force (see figure 9B), with five days CMV resulting in a 39.8 - 45.2% decline in young and old respectively, compared to controls.

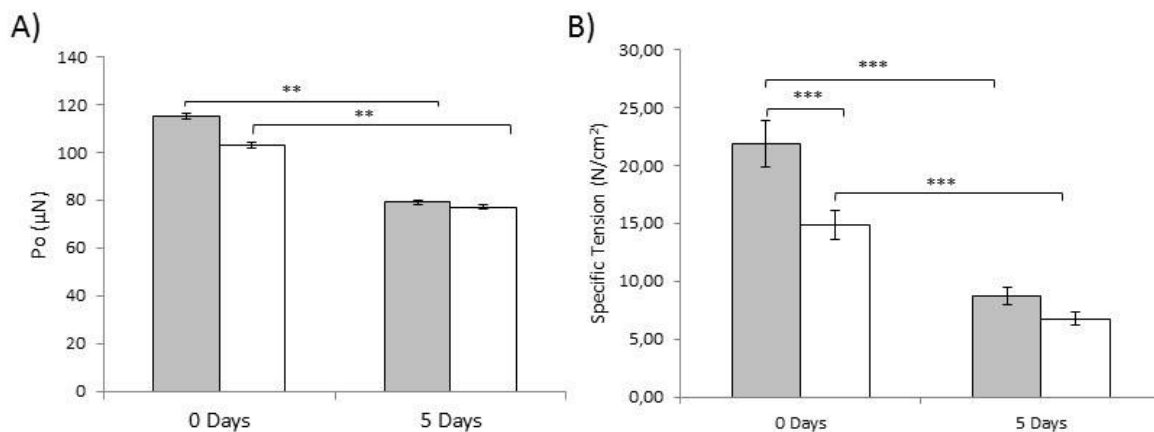


Figure 9. A) Absolute force B) Specific force of diaphragm fibres measured at fixed sarcomere length in control animals compared with the age-matched animals exposed to CMV for 5 days (grey bars: young; white bars: old). Values are means \pm SEM. Significance level: ** $p < 0.01$, *** $p < 0.001$.

Accordingly we looked into the dominating factor and ascertained that there was an interaction between CMV and age (two-way ANOVA $p < 0.03$), furthermore we can suggest that there is a dominance of CMV over age from the statistics carried out by the F-test (CMV F-value = 66.67; Age F-value = 12.74). During contractile recordings, single diaphragm muscle fibres from old animals were frailer than from young, resulting in a larger number of

fibres that broke during maximum activation than in the young (0% young control; 15% old control; 21% young 5 day-CMV; 35% old 5 day-CMV). The decrease in absolute and specific force and a compensatory muscle fibre hypertrophy in response to CMV were observed independent of muscle fibre MyHC isoform expression (see paper II table 1 and 2). Further, the age-related decline in specific force and increase in CSA was also observed in the different fibre types, but statistical difference was not always reached due to the smaller number of fibres investigated.

Determining biological age: - Effects of age on CSA and specific force of soleus and EDL muscle fibres

To establish that we looked at biologically aged animals, more specifically aged diaphragm, we studied muscle fibre CSA and specific force in female F344-BN hybrid rats in three age groups (young, middle-aged and old) to see if muscle fibre size and function differ in the same way as in humans and other mammalian species. Single muscle fibres have been studied in the fast-twitch EDL and slow-twitch soleus from 6 months, 18 months, and 28 months old animals. Muscle fibre size did not differ between the 6 and 18-month-old animals, but declined in the 28-month group, reaching a statistical significance in the soleus muscle (see figure 10A). Specific force was lower in the 28-month group compared with the 6- and 18-month groups in both the EDL and soleus fibres (see figure 10B). Thus, the changes we observed in muscle fibre size and function is in agreement with previous observations when comparing young, middle-aged and old individuals.

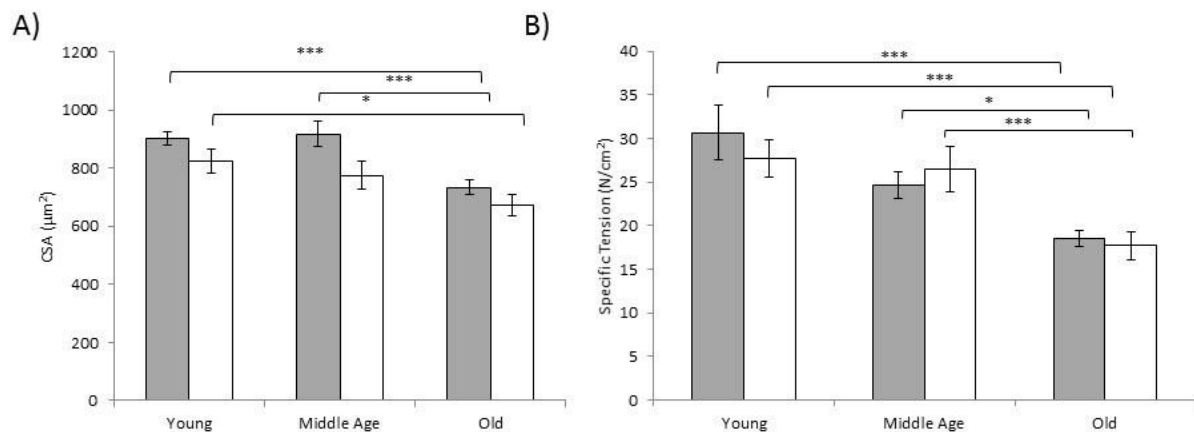


Figure 10. A) Cross-sectional area B) Specific force (P_0/CSA) measured at fixed sarcomere length in single fibres of soleus muscle (grey bars) and of EDL muscle (white bars) from animal of different age (individual fibres: young n = 27, middle aged n = 28 and old n = 30). Values are means \pm SEM. Significance level: * $p < 0.05$; *** $p < 0.001$.

4.3 PAPER III - THE CHAPERONE CO-INDUCER BGP-15 ALLEVIATES MECHANICAL VENTILATION INDUCED DIAPHRAGM MUSCLE DYSFUNCTION

Paper III was a continuation from a previous study (Corpeno et al., 2014), to determine the effect of the pharmacological intervention BGP-15 on 10 days CMV on the SD rat diaphragm. The chaperone co-inducer BGP-15 alleviated the negative effects of CMV on diaphragm function by improving diaphragm muscle fibres specific force, and by improving mitochondrial respiratory complexes III and IV functions (see paper III).

The effect of CMV and BGP-15 on diaphragm muscle fibre CSA and force generating capacity

The results show that after ten days deep sedation, neuromuscular blockade and CMV a significant decrease (~50%) in single diaphragm fibre CSA compared with the controls ($p < 0.001$) was observed (see figure 11 A). Furthermore the systemic administration of BGP-15 during the experimental period did not significantly influence the decline in CSA during the ten days CMV. The specific force of the diaphragm fibres declined ($p < 0.001$) approximately 70% compared with control values (see figure 13 B). In the rats treated with BGP-15, specific force increased ($p < 0.001$) by more than 100% compared with the 10 days untreated rats, being approximately 24.2% lower than control values. For comparison, CSA and specific force measurements from a previous study from our group has been added to figure 13 (Corpeno et al., 2014), demonstrating good correspondence with present control and 10-day untreated animals as well as adding information on the time-course of changes in diaphragm muscle fibre CSA and specific force.

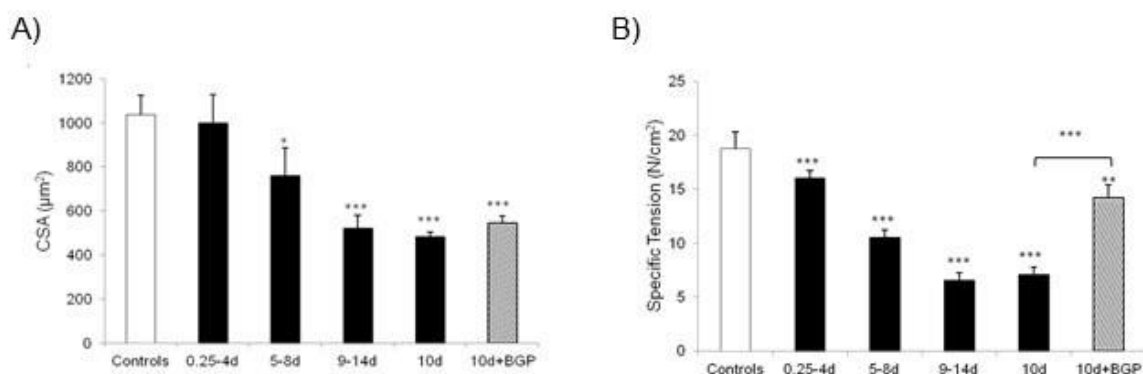


Figure 11. Single diaphragm fibre CSA measured at fixed sarcomeric length A) and specific force B), in control animals (white), animals exposed to CMV for varying durations (black bars), animals treated with BGP-15 and exposed to CMV for 10 days (fine striated). Values are presented as means + SEM. * $p < 0.05$. ** $p < 0.01$ (significant difference compared with controls). *** $p < 0.001$ (significant difference compared with controls).

4.4 PAPER IV - TARGETING HEAT SHOCK PROTEINS MITIGATES VENTILATOR INDUCED DIAPHRAGM MUSCLE DYSFUNCTION IN AN AGE-DEPENDENT MANNER

In paper IV we progressed from paper II by exploring the idea that could function be restored by use of a pharmacological intervention in our rat model. We looked at the impact of BGP-15 on diaphragm dysfunction in response to CMV in young adult vs. old F344-BN hybrid rats. Our preliminary results show that after 5 days CMV an age dependent significant drop in force production is observed, which, by administration of BGP-15, after 5 days CMV demonstrates a significant positive effect on diaphragm fibre function in only young rats, in conjunction with an increased expression of heat shock protein 72 (Hsp72).

Effects of 5 days CMV with and without BGP-15 on diaphragm single muscle fibre cross sectional area (CSA) and diaphragm muscle myosin heavy chain (MyHC) isoform composition

As previously shown in paper II old control individuals were seen to have a larger CSA than young, and, after 5 days CMV, deep sedation and NMB a compensatory hypertrophy as seen in only the young (see paper II figure 2). With the administration of BGP-15 there was no additional effect after 5 days without the drug.

The IIX MyHC isoform was dominant in all groups and there were no statistically significant differences between groups in response to age, CMV or BGP-15 administration (see figure 12).

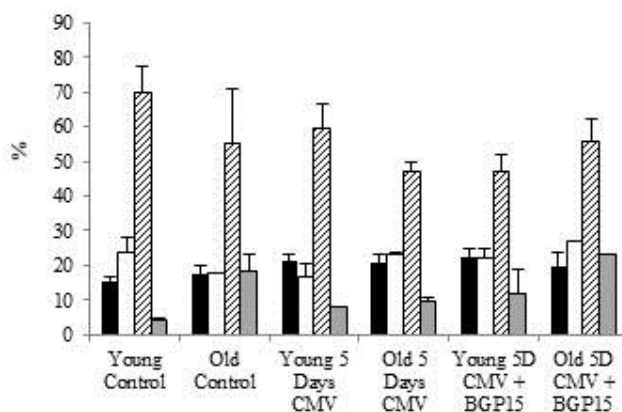


Figure 12. Myosin heavy chain isoform expression (%) from cross sections in young and old rats in control, 5 days CMV and 5 days CMV+BGP-15 groups. (Black: type I, white: type IIA, stripe: type IIX, grey: type IIB). Values are % + SEM.

Effects of 5 days CMV with and without BGP-15 on diaphragm single muscle fibre specific and absolute (P_0) force

The systemic administration of BGP-15 had a significant positive effect on the absolute and specific force in the young, but not in the old animals (see figure 13).

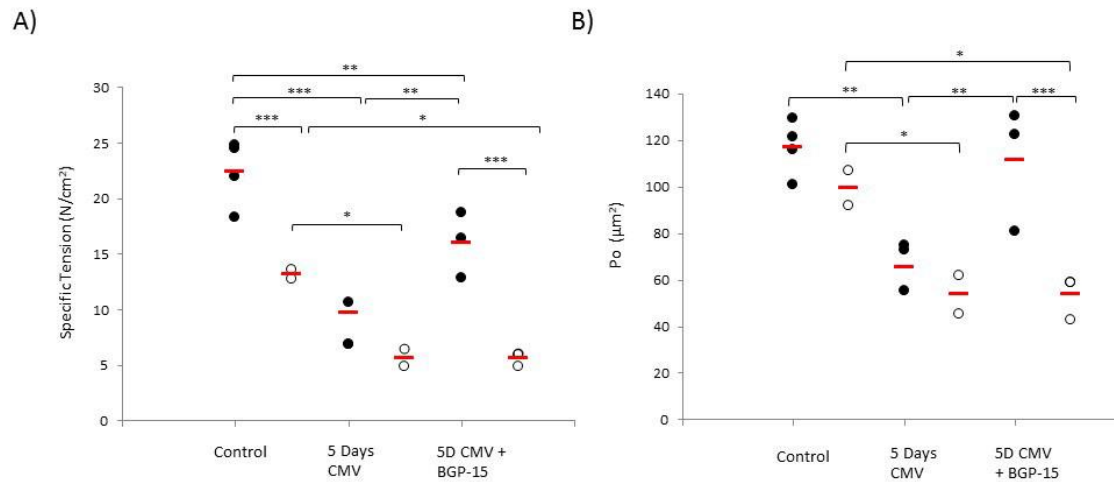


Figure 13. Individual rat single diaphragm muscle fibre: A) specific force (P_0/CSA) and B) absolute force (P_0) measured at fixed sarcomere length in control animals compared with age-matched animals exposed to CMV for 5 days without and with BGP-15. Values are averages of individual data from the different animals and the red line indicates the average for each group. (Black circles: young; white circles: old; red line: group average). Significance level: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In paper II we reported that the effects of 5 days CMV on specific force, absolute force and muscle fibre CSA were independent of the MyHC isoform expressed in the fibre (see paper II table 1 and 2). The effects of BGP-15 administration on muscle fibre size, specific and absolute force were also independent of MyHC isoform expression (see paper IV table 1).

Effects of 5 days CMV with and without BGP-15 on Hsp72 expression in diaphragm muscle

In addition to force measurements we looked at the protein expression of Hsp72. Hsp72 protein levels did not differ between young and old animals in the control group or after 5 days CMV. However, we see a significant increase in Hsp72 levels in response to 5 days CMV+BGP-15 administration, which was solely restricted to the young animals (see figure 14).

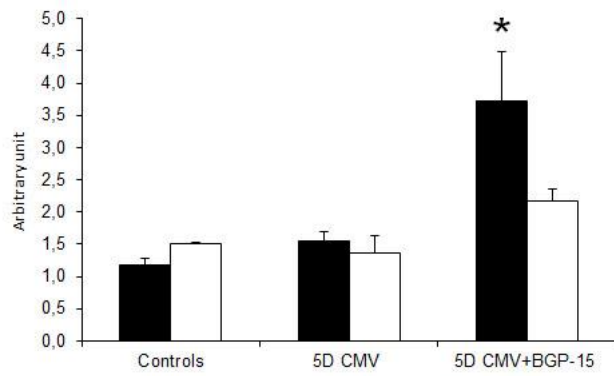


Figure 14. Hsp72 protein expression in the diaphragm of control animals (individual rats: young n = 4 and old n = 2) compared with the age-matched animals exposed to CMV for 5 days with (individual rats: young n = 3 and old n = 2) and without BGP-15 (individual rats: young n = 3 and old n = 3). (Black bars: young; white bars: old). Values are means + SEM. Significance level: *p < 0.05.

5 DISCUSSION

In this thesis the overall aim was to investigate the importance of potential contributors in the development of muscle fibre dysfunction in response to the intensive care unit condition; critical illness myopathy and evaluate the efficiency of one potential therapeutic intervention. It is well established that during prolonged critical illness muscle strength and function are compromised, which in turn can have a detrimental effect on recovery and more specifically in weaning patients from the ventilator (Derde et al., 2010). There are numerous factors and mechanisms that can play a part in the process and is thus an area of on-going research and the topic of my thesis. In my thesis I look in depth at the specific impact of nutrition (paper I), ageing (paper II) and one pharmacological intervention, BGP-15 (papers III and IV). The mechanisms behind their methods of action are somewhat of a complicated web and one which needs to be better investigated and further understood.

During the last decade, the seemingly simple task of feeding critically ill patients has become exceedingly complex with much controversy, thus the question as to whether nutritional therapy in the ICU can prevent or alleviate the associated complications still remains. Nutritional factors have been reported to have an impact on skeletal muscle in ICU patients, (Derde et al., 2010; Schetz et al., 2013a) suggesting that an insufficient nutrition/caloric intake is a possible factor that could be involved in triggering CIM and play a substantial role in overall muscle loss and weakness observed in critically ill patients (Beck et al., 2001; Friedlander et al., 2005; Griffiths et al., 1997; Plank and Hill, 2000; Schetz et al., 2013b; Winkelman, 2013). However it remains unclear and of great debate as to whether artificial nutritional support can benefit specifically the outcome of critically ill patients with CIM.

5.1 NUTRITION

In paper I we investigated the impact of nutritional feeding in a rat model mimicking the ICU condition, with the aim to study the effects of nutritional status in the pathogenesis of CIM, determining whether nutritional status is driving the preferential myosin loss, fibre atrophy and loss of specific force in skeletal muscle. The main finding in this study was that nutritional feeding of a EC diet did not in fact have a positive effect on the fibre size or force generating capacity of the single muscle fibres in the soleus or EDL muscles compared with LC feeding. However this study did further confirm the strong impact of mechanical silencing associated with the ICU condition in triggering CIM, overriding any potential effects of caloric intake in triggering CIM. We saw that irrespective of feeding, the animals developed the phenotype considered characteristic of CIM i.e., a preferential myosin loss, fibre atrophy and decreased specific force. Thus, we suggest an indication that caloric intake is of less importance compared with mechanical silencing in the pathogenesis of CIM in ICU patients. Furthermore, neither the myosin:actin nor the positive effect of passive mechanical

loading of the hind limb was further effected by feeding, with no additive effect to the loss in myosin or in the improvement in muscle fibre size or force generation after a period of 10-14 days. In accordance with previous data from our group we observed the same reduced preferential myosin loss in response to passive mechanical loading, irrespective of caloric intake (Renaud et al., 2013).

5.1.1 Current evidence and trials

There is at present an on-going and somewhat controversial debate over the how, what and when of feeding ICU patients and even that perhaps the question that should be first addressed is whether feeding per se is beneficial. There is a severe lack of consensus at present as to what is considered to be the optimal feeding regime in critically ill ICU patients (Casaer, 2014). Clinical trials have been undertaken and again the consensus remains unclear. The EPaNIC RCT compared early parenteral nutrition supplementing insufficient enteral nutrition versus withholding parenteral nutrition during the first ICU-week. It was found that early parenteral nutrition provoked more infections and delayed organ function recovery, prolonging ICU dependency and hospital stay (Casaer et al., 2011b). Furthermore late parenteral nutritional feeding meant patients performed similar 6 minute walking distances and activities of daily living despite a shorter hospital stay. Moreover the early parenteral nutrition could not prevent the important loss of femoral muscle volume observed in a subgroup of 15 neurosurgical patients (Casaer et al., 2013). Despite the provision of important amounts of early intravenous energy, protein and insulin, early parenteral nutrition was unable to attenuate catabolism, stimulate muscle protein synthesis or prevent the loss of myofibre size (Casaer et al., 2011a; Casaer et al., 2013). In summary the clinical data from clinical trials, (EDEN, EPaNIC, The Australian Early-PN trial), suggests that there is no evidence for an increased energy/protein provision during the first week in the ICU. Furthermore, Streat and co-workers found that although malnutrition in the critically ill is a recognised predictor of a poor patient outcome, nutritional support in the ICU has proved powerless in preventing a loss of lean body tissue (Streat et al., 1987). There is little evidence to suggest that muscle mass, muscle force or long-term physical function is protected (Casaer, 2014, 2015). A potential explanation for the pattern of on-going muscle wasting despite increased energy and protein intake (Casaer et al., 2013) may be found in its pathogenesis.

5.1.2 Explanation, ideas and mechanisms of why it may not work

An increased energy/protein administration within the first ICU week does not seem to prevent muscle wasting, of which there may be several mechanisms that might underlie this anabolic resistance. The precise mechanisms of action by which this phenomenon occurs can be alluded to but it is still an on-going area of research. Anabolic resistance, accumulation of

intracellular damage, lipogenesis, infectious complications, water accumulation and suppression of autophagy might help to explain these findings (Casaer et al., 2013).

In animal experiments, the driving force behind up-regulated muscle catabolism in critical illness has been shown to be linked to multiple processes, non-less so than immobilisation and mechanical silencing. In previous experimental and clinical data from our group (Llano-Diez et al., 2012; Ochala et al., 2011a; Renaud et al., 2013), we have shown that removal of both weight bearing and activation of contractile proteins, i.e., ‘mechanical silencing’ of skeletal muscle is a dominating factor triggering the specific myopathy (CIM) associated with the ICU condition. Furthermore, the loss of muscle mass and function can be attenuated by early mechanical loading (Llano-Diez et al., 2012; Renaud et al., 2013) supporting early physical therapy in immobilised ICU patients (Bailey et al., 2007). The results from this study demonstrate that the loading effect was similar in the LC and EC groups, supporting the strong effect of mechanical silencing in the development of CIM and being of greater importance compared with caloric intake. In addition to immobilisation, other frequently encountered factors such as systemic inflammation in critically ill patients compound this loss in muscle mass and function (De Jonghe et al., 2002). It is perhaps becoming clearer now that it is the dose of nutrient administration that may be more important than the route, with regard to autophagy and eventually in the modulation of clinical outcomes (Casaer and Van den Berghe, 2014; Derde et al., 2012; Harvey et al., 2014; Singer et al., 2011). Furthermore perhaps what is required within this cohort is a follow up study on the impact of nutritional therapy on a long-term patient-centred outcome, particularly on interventions beyond the acute phase of critical illness (first week).

The clinical relevance of nutritional feeding is paramount and should be researched and understood further in combination with the premise of early mobilisation.

5.2 AGEING

Mechanical ventilation although being a supportive therapy used in the ICU, comes with major complications, compounded further in an aged patient. An increasing proportion of the critically ill population are elderly, frail, sarcopaenic patients who start from a compromised position with a lower muscle mass and are therefore at a high risk of developing further muscle wasting during critical illness. Thus, the main aim of paper II was to investigate the effect of ageing on rat diaphragm muscle fibre structure and function in response to five days of CMV. An aside to this aim was to better determine the biological age of the animals and ascertain whether any differences were present between muscles.

In paper II, this study showed a significant age related decline in diaphragm muscle fibre specific force, with the further addition of the experimental condition of deep sedation, neuromuscular blockade and mechanical ventilation, this resulted in an added dramatic decline in specific force in both young and old animals after 5 days (paper II and IV). We

have previously observed the same decrease in diaphragm and limb specific force in a different rat strain (SD) (paper III) and using young rats over a period of 6 hours to 14 days (Corpeno et al., 2014) and in a pig model over 5 days (Ochala et al., 2011b). The altered diaphragm muscle fibre function in response to mechanical ventilation is suggested to have a strong negative clinical effect on the weaning process in ventilated ICU patients. It is established that the weaning of elderly patients from the ventilator after a period of time in the ICU becomes increasingly problematic with age (Epstein et al., 2002). However in our study this negative effect on fibre function was observed in both young and old animals and therefore in this instance cannot help to explain the more difficult weaning process in old age. However, it is important to mention that in addition to the impaired muscle force generating capacity in response to the CMV, we observed that with age the individual muscle fibres were frailer and that more fibres broke upon activation, i.e., an additional factor that may contribute to the age-related problems in weaning old patients from the ventilator. Furthermore we observed an unexpected increased CSA in both young and old animals, thought to demonstrate an ineffective compensatory diaphragm muscle fibre hypertrophy in response to the 5 days CMV. This was in contrast to our previous data seen in the SD rat and pig model (Corpeno et al., 2014; Ochala et al., 2011b) where CSA was maintained for the period up to 4 days CMV and then a progressive decline in CSA pursued. This data although not as expected and contrary to our previous findings, gives us yet a further insight into this complex cohort.

As a secondary methodological aim to this study we wanted to ascertain that the muscle we were using from old female F344-BN hybrid rats were in fact considered “old” rats. According to the National Institute of Ageing (NIA) 28–36 month old F344-BN hybrid rats correspond roughly to humans in their eighth decade of life. Thus, in an attempt to determine the biological age of these rats, we investigated characteristics typical of old age, i.e., muscle fibre atrophy and reduction in specific force in the soleus and EDL muscle. We looked at three different age groups, representing young (6 months), middle-aged (18 months) and old (28 months). A significant age-related decline in muscle fibre size and specific force was observed in the 28-month F344-BN hybrid female rats, justifying this age as representing biologically old in our studies on the diaphragm.

Understanding the mechanisms and the aetiology behind this phenomenon is no mean feat and one that is the focus of many researchers. The most complicating factor is the cohort themselves, with critically ill patients having a whole multitude of confounding factors, further identifying the need for a more “clean” animal model.

In critically ill MV patients the loss of muscle force generating capacity has been established and seen to be exacerbated further with age. However the cause of the decrease and the underlying mechanisms have only been alluded to in this context and are likely to be multifactorial. It is postulated that it may involve the excitation-contraction coupling apparatus (Howell et al., 1997; Zhan and Sieck, 1992) with or without the contractile machinery; a reduced myofibril protein (Geiger et al., 2001) or possible structural changes

may have a detrimental effect on the contractile machinery. Structural abnormalities of different subcellular components of diaphragmatic fibres have been observed after 48 hours of CMV (Sassoon et al., 2002). The abnormalities include disrupted myofibrils, abnormal swelling of mitochondria, lipid droplets, and vacuoles (Corpeno et al., 2014; Picard et al., 2012; Sassoon et al., 2002). In a previous study carried out in our group using a different rat strain (SD) there was an observed early loss of subsarcolemmal neuronal nitric oxide synthase activity, onset of oxidative stress, intracellular lipid accumulation and post-translational protein modifications, which strongly argue for significant qualitative changes in contractile proteins causing the severely impaired residual function in diaphragm fibres after long-term mechanical ventilation (Corpeno et al., 2014). The mechanisms for the damage are unclear but may involve activation of ubiquitin- proteasome proteolysis, calpain proteolysis, and oxidative stress (Corpeno et al., 2014; Mitch and Goldberg, 1996; Sandri, 2013; Shanely et al., 2002). It has become clearer that specific ubiquitin ligases play a prominent role (Sandri, 2008) with the ubiquitin proteasome system being seen to demonstrate the degradation of the myosin heavy chain (a major contractile protein). Clarke et al. identified the myosin heavy chain as a MuRF-1 partner protein (Clarke et al., 2007). It has further been established that FoxO factors are required for the transcriptional regulation of the ubiquitin ligases atrogin-1, also called muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1), leading to the ubiquitination of myosin and other muscle proteins and their degradation via the proteasome (Schiaffino and Mammucari, 2011).

The surprising increase in CSA is suggested to be an ineffective compensatory hypertrophy. Although different to our previous results (paper I and III), in this instance we are using a different rat strain, F344-BN hybrid, the preferred strain for ageing studies. We can suggest that there are clear differences in rat strains and in the diaphragms response to CMV (paper II and IV). We saw the same hypertrophic response in paper IV using the same rat strain.

The premise that inactivity induces muscle fibre atrophy is widely accepted and has essentially become physiological dogma in the literature. However there is suggestion that hypertrophy is evident in the diaphragm (Sieck and Mantilla, 2013). Hypertrophic diaphragm muscle fibres in response to 5 days CMV has previously been reported in a porcine ICU model and further associated with a decline in *in vivo* diaphragm muscle function (Radell et al., 2002). Furthermore in an animal model using male F344-BN rats an age-related increase in diaphragm fibre size was reported (van Lunteren et al., 1995). It has been seen that there is an increase in the muscular water content, which would explain the decrease in muscle protein concentration and the increase in CSA, which is consistent with the increased water content that occurs in conjunction with muscular injury (Shanely et al., 2002). Moreover a study undertaken in cardiac tissue has demonstrated a similar pattern (atrophy and hypertrophy) in senescent hearts of F344-BN rats (Wanagat et al., 2002). This can perhaps suggest to us and be matched to our similar finding in the diaphragm, being consistent with the idea that this pattern occurs in muscles that remain highly activated with ageing. The molecular mechanisms underlying the hypertrophy remain unknown, but are speculated to be related to age and CMV changes in mechanosensing and mechanotransduction pathways.

This is supported by a decrease in muscle stretch and age related activity of FoxO1 and FoxO3a. Thus, the age-related increase in muscle stiffness might be responsible for the altered response of the Akt and IKK signalling pathways to the mechanical stimuli, which in turn affect the FoxO1 and FoxO activities (Pardo et al., 2008).

5.3 PHARMACOLOGICAL INTERVENTION

Diaphragmatic dysfunction has a strong impact on the ability of patients to be successfully weaned from the ventilator, it is therefore of critical importance to understand the effect of mechanical ventilation on diaphragm function and explore the possible intervention strategies which could be implemented.

In papers III and IV, we investigated the impact of a pharmacological intervention, BGP-15, on restoring diaphragm function in response to CMV. In paper III, SD rats were MV for durations between 6 hours and 10 days. We specifically focussed on the effects on the regulation of muscle contraction at the muscle cell level and on the effect on mitochondrial structure and function. In paper IV, a different rat strain was used (F344-BN hybrid) allowing us to investigate the impact of BGP-15 on ageing after 5 days CMV. It was hypothesised that the chaperone co-inducer BGP-15 would have a positive effect on diaphragm muscle fibre function after varying durations of CMV (paper III and IV) and that the positive effects would be more pronounced in the old animals due to a possible compromised heat shock protein protection in old age (paper IV). In accordance with our hypothesis it was found that the chaperone co-inducer BGP-15 reduced the negative effects of 10 days CMV (paper III) and 5 days CMV (paper IV) on diaphragm muscle function. However contrary to the original hypothesis the effect after 5 days CMV was primarily observed in the young adult and not in the old animals (paper IV).

5.3.1 BGP-15 had a positive effect on SD and F344-BN hybrid young rat diaphragm force production (paper III and IV)

Ten days BGP-15 administration to SD rats during CMV exposure doubled specific force compared with untreated animals and restored the force generating capacity of diaphragm fibres to approximately 75% of its normal capacity. The molecular mechanisms underlying this process are unclear and need to be further investigated. The restored function of the diaphragm in only the young F344-BN hybrid rats is of significant importance for gaining further knowledge into this highly complex cohort and can help to unravel the possible underlying mechanisms, more specifically with the ageing process. At present the exact mechanisms are unclear but are likely to be related to the effects of BGP-15 on Hsp70. The age-related effect of the chaperone co-inducer is consistent with the increased levels of the induced form of Hsp70; Hsp72. Since, it has previously been reported that the up-regulation

of Hsp72 contributes to the maintenance of muscle fibre integrity and facilitates muscle regeneration and recovery. Conversely Hsp72 expression is decreased during muscle inactivity and ageing, with evidence supporting the loss of Hsp72 as a key mechanism that may drive muscle atrophy, contractile dysfunction and reduced regenerative capacity associated with these conditions (Dodd et al., 2009; Singh et al., 2006).

5.3.2 BGP-15 effect on diaphragm CSA (paper III and IV)

BGP-15 administration during 10 days did not restore the fibre size reduction observed in response to CMV exposure in the diaphragm of the SD rats. We have previously shown that the early activation of the ubiquitin proteasome degradation pathway and followed by later activation of calcium-activated and autophagy degradation pathways play important roles in muscle atrophy during CMV (Corpeno et al., 2014). Furthermore in contrast to this in F344-BN hybrid rats a hypertrophy of fibres was observed, as previously seen in paper II (Cacciani et al., 2014), however we see in paper IV that BGP-15 had no additive effect to hypertrophy over 5 days CMV in either young or old. This further validates a specific species difference and supports the manifestation that different mechanisms underlie muscle atrophy and loss of specific force in the diaphragm during mechanical ventilation. Furthermore it is established that Hsp70 has an intracellular effect on the inhibition of nuclear factor κ B (NF- κ B) activation, which has profound implications for immunity, inflammation, cell survival and apoptosis. The inhibitory potential of Hsp70 over apoptosis occurs via many different intracellular downstream pathways (e.g. JNK, NF- κ B and Akt), which are directly and indirectly blocked by Hsp70. Collectively, these mechanisms underlie Hsp70 anti-apoptotic effects in cells under stress conditions (Thiago Gomes Heck, 2012). The inhibition of these transcriptional pathways may explain the prevention of muscle-fibre atrophy by Hsp70, since NF- κ B and FoxO are independently sufficient to cause skeletal muscle atrophy (Sandri et al., 2004). These evidences suggest that Hsp70 inhibits key signalling pathways for muscle atrophy and could potentially be an explanation for the hypertrophy demonstrated in response to BGP-15.

5.3.3 The mitochondria (paper III)

In paper III we also looked at the impact that BGP-15 treatment had on the mitochondria in the diaphragm muscle after exposure to CMV. BGP-15 has been seen to improve mitochondrial efficacy and reduce reactive oxygen species (ROS) production (Henstridge et al., 2014). We found that the BGP-15 treatment improved the function of two specific mitochondrial respiratory complexes, III and IV, however it did not improve the structural changes that occurred in response to the CMV.

The improved function of complex III and IV can help us to understand further the increased force production of the diaphragm. Among BGP-15's multiple targets, its action as a poly ADP ribose polymerase (PARP) inhibitor is of specific interest since PARP inhibition reduces mitochondrial ROS production and ROS-related oxidative damages during stress (Bowes et al., 1999; Halmosi et al., 2001; Szabados et al., 2000). The strong negative impact of post-translational modifications of myosin on the dramatically impaired diaphragm muscle fibre function in response to long-term CMV (Corpeno et al., 2014) and the reduction in ROS production by BGP-15 resulting in decreased oxidation of contractile proteins is forwarded as a critical mechanisms underlying the approximate 75% increase in diaphragm muscle fibre force generating capacity.

5.4 STUDY LIMITATIONS

5.4.1 Sample size

I think it is important to make clear that we are aware of the low number animals per groups. However it is also of importance to point out that for our studies of up to 14-day, significant time and resources are needed to make these studies possible. However in-spite of this we felt that the knowledge that could be gained from the studies would provide vital information to the community and enable us to use the data from these studies to design future studies with correctly powered groups.

6 CONCLUDING REMARKS

Critical illness myopathy is a common and complex manifestation seen in ICU patients and it has over the past decade become an increasing problem that will only escalate further.

Understanding the processes and underlying mechanisms of muscle regulation with respect to CIM is an area of great research focus. My thesis has provided some further insight into this highly orchestrated process using a rodent model mimicking the ICU environment.

We have ascertained here (paper I) that the impact of mechanical silencing associated with the ICU condition overrides any potential effects of eucaloric feeding in triggering CIM in the limb muscle of SD rats. Furthermore, we observed that eucaloric feeding had no greater effect on muscle fibre size or force generating capacity over that of passive mechanical loading of the limb muscles. We can in this instance suggest that perhaps alternative feeding strategies may have a beneficial effect.

Ageing is a process that we will inevitably all experience and one that requires in-depth research and understanding. Combine the complex process of ageing muscle with that of the ICU environment and CIM and we confound the task in hand. A major problem in the ICU is weaning patients from the ventilator; a problem that is exacerbated in the elderly. Thus, having a better understanding of the effect of MV on the young and elderly population can help. Using the rat strain F344-BN hybrid we observed an ineffective compensatory hypertrophy in the diaphragm in response to 5 days CMV with a concomitant decrease in the force generating capacity in both the young and old (paper II and IV). This does not offer us an explanation as to why the problem of weaning is more problematic in the old, however we did observe in the old an increased number of frail diaphragm muscle fibres in both controls and after mechanical ventilation. This can perhaps lead us to believe that there is another possible mechanism that may be of significant clinical importance.

With the premise that things can go wrong with the human body, research is ongoing in both trying to understand the possible mechanisms and taken one step further in finding possible preventative and intervention strategies. As such in the context of my thesis we evaluated a pharmacological intervention with the desire to assuage diaphragmatic dysfunction in an ICU mimicking model. The drug BGP-15 was found to alleviate the negative effects of CMV on diaphragm function by improving muscle fibre specific force and by improving mitochondrial respiratory complexes III and IV functions (paper III). In addition we wanted to investigate further the age related differences seen in paper II and thus administered young and old F344-BN hybrid rats with BGP-15 (paper IV). We observed a further significant age-specific difference with BGP-15 having a strong positive effect on diaphragm muscle fibre function in only the young rats with a matched increase in Hsp72 expression after 5 days CMV. Thus it is suggested that the increased Hsp72 expression, induced by BGP-15 is an indicator of the inhibition of the atrophy pathway (UPS), that as such attributes to an increased CSA and specific force in only the young.

This research has made it more apparent the complexity of understanding this field, with differences observed between the diaphragms of different rat strains. Muscle heterogeneity is an important point to remember with regards to the differences that are elicited between muscle types, fibre types, species and age. Force production, fibre size and the time course over which changes can occur are all elements which can differ and which are seen to be intensified further by the process of ageing. This heterogeneity factor is an aspect that has meant that in the literature you can find contradicting and confusing results, thus making it difficult to ascertain the exact underlying mechanisms. Therefore it has become clearer that in fact a multifactorial process may proceed and is something that needs careful consideration when planning future research.

As I have alluded to previously it is important for us to be aware that the group sizes in these experiments are low and thus in this instance a follow up study should be undertaken to further investigate the possible highly clinical significance of this research.

7 ONGOING AND FUTURE WORK

To further the knowledge in relation to this work there are ongoing studies to investigate the underlying mechanisms of the muscle in the ICU environment, by investigating the potential signalling mechanisms involved. Having established that BGP-15 has a positive influence on muscle force production after exposure to CMV in the young F344-BN rats with a concomitant increase in Hsp72 expression (paper IV) we would like to continue to investigate the associated atrophy pathway.

As it is discussed the inhibitory potential of Hsp70 over apoptosis occurs via many different intracellular pathway (e.g. JNK, NF- κ B and Akt), which are directly and indirectly blocked by Hsp70. This and further evidences suggest that Hsp70 inhibits key signalling pathway for muscle atrophy (Thiago Gomes Heck, 2012). Muscle atrophy results primarily from accelerated protein degradation and is associated with increased expression of two muscle-specific ubiquitin ligases (E3s): atrogin-1 and MuRF1 (Sacheck et al., 2004; Sandri et al., 2004), as such gene expression of MuRF1 and atrogin-1 will be investigated.

8 ACKNOWLEDGEMENTS

Thank you to my family and friends all around the world for keeping me in touch with reality and putting things in perspective throughout my PhD. Without you all it would have all been a lot more of a challenge.

I would like to thank all of those people in the group that I have been working with. We have been on a journey together, in both the physical sense (from Uppsala to “the other side”) and in the scientific sense (from understanding a little, to understanding a little bit more and being a little more confused!). It has been a stimulating, eventful and fika filled journey! Good luck to you all in the future!

A special mention goes to my partner’s in fibre crime, my co-supervisor’s past and present Julien Ochala and Nicola Cacciani, who made running fibres even more fun than one could think! Our deep intellectual conversations will be missed!

I would like to mention my supervisor Lars Larsson; you have provided me with a rollercoaster ride of a PhD. Thank you for the experience and for exposing me further to the academic world of science.

“Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning”.

Winston Churchill

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