Fatigue mechanisms of shortening skeletal muscle:

Protein phosphorylation and O-GlcNAcylation

with emphasis on regulatory myosin light chain (MLC2)

Kristin Halvorsen Hortemo, cand.med.

Institute for Experimental Medical Research,

Oslo University Hospital and University of Oslo

Dissertation for the degree of Philosophiae Doctor (PhD)

University of Oslo, Oslo, Norway

2016

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo

ISBN 978-82-8333-317-6

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

ACKNOWLEDGEMENTS

The work for my PhD thesis was carried out at the Institute for Experimental Medical Research (IEMR), University of Oslo and Oslo University Hospital Ullevål, under the supervision of Professor Ole M. Sejersted and Senior Researcher Per Kristian Lunde.

I have been privileged to have Professor Ole M. Sejersted as my main supervisor, and I would like to thank him for his valuable guidance and feedback throughout my PhD. His extensive knowledge of physiology has been truly inspiring, and I appreciate the opportunities he has given me to run a research project in a strong and professional environment.
My co-supervisor Per Kristian Lunde introduced me to the surgical techniques of the experimental model and has later been involved in almost every part of my research. He has provided exceptional help in methodological matters and contributed to the daily discussions of new hypotheses or findings, with admirable patience and high spirits. Thanks to both Ole and Per Kristian for the fruitful meetings during which we drew up the fundamental lines of investigation and shaped the manuscripts. Learning from their skills in experimental physiology has been a privilege.

The contributions of my co-authors have also been indispensible in the work for my thesis. I appreciate their support and the exciting scientific discussions we have had over the years, in addition to their participation in the experimental work. Magnus Aronsen put great effort into the treadmill exercise protocol, and he expertly performed the coronary artery ligation surgeries and echocardiography in collaboration with Professor Ivar Sjaastad. In the molecular lab both Ida Gjervold Lunde and Heidi Kvaløy contributed with great expertise and enthusiasm. The experience of Morten Munkvik in the *in situ* model was highly appreciated, and Morten also performed the human muscle biopsies together with Tommy Aune Rehn. Thanks to Jan Haug Anonsen at the Department of Biosciences who thought me about proteomics and performed the mass spectrometry analyses.

I also want to thank Hilde Dishington, Marianne Lunde Sneve, Marita Martinsen and Per Andreas Norseng for their valuable biochemical analyses and technical work, and Ståle Nygård for his advise in statistical analysis. Thanks to Morten Eriksen and the animal facility for providing high-quality animal care, to Lisbeth Winer and Jo-Ann Fabe Larsen for excellent administration, and to all other colleagues and friends at the IEMR that contributed to a great working environment.

I was introduced to the IEMR as a medical student, when I took a year off medical school to do research. This was made possible by administration from the Medical Student Research Program at the University of Oslo, with a scholarship from the Norwegian Research Council, and I am grateful for this possibility to conduct research early in my medical career. Also, I thank my fellow student researchers and the "FLF Club" for great friendships in science and life. Later, after finishing the years as a medical intern, I got the opportunity to come back to the IEMR as a PhD student in 2013. Financial support was provided from Anders Jahre's Fund for the Promotion of Science, University of Oslo, and Stiftelsen Kristian Gerhard Jebsen. During my years at the IEMR, I have been privileged to attend international research conferences to present and discuss new findings, and I appreciate these valuable experiences.

Finally, I would like to thank my family and friends. Thanks to my parents for their love and support and for stimulating my curiosity. It was my mom, the cardiologist, that first introduced me to the world of research. I am fortunate to have a large family and good friends that inspire me and cause a lot of laughter, also when experiments are not working out. Most of all, thanks to my husband and best friend, Olav, for his never-ending encouragement, love and patience throughout the entire process. Our wonderful son Magnus was born during my final year as a PhD student. The smiles of the two of you daily remind me of what is most important in life. Thank you.

Oslo, Norway, June 2016

Kristin Halvorsen Hortemo

TABLE OF CONTENTS

1 LIST OF PAPERS

This thesis is based on the following original research papers, which are referred to in the text by their Roman numerals.

Paper I

Multiple causes of fatigue during shortening contractions in rat slow twitch skeletal muscle

Kristin Halvorsen Hortemo, Morten Munkvik, Per Kristian Lunde, Ole M. Sejersted (2013) *PLoS ONE, 8(8), e71700*

Paper II

Exhausting treadmill running causes dephosphorylation of sMLC2 and reduced level of myofilament MLCK2 in slow twitch rat soleus muscle

Kristin Halvorsen Hortemo, Jan Magnus Aronsen, Ida Gjervold Lunde, Ivar Sjaastad, Per Kristian Lunde, Ole M. Sejersted (2015) *Physiological reports*, *3(2), e12285*

Paper III

Exercise training increases protein O-GlcNAcylation in rat skeletal muscle

Kristin Halvorsen Hortemo, Per Kristian Lunde, Jan Haug Anonsen, Heidi Kvaløy, Morten Munkvik, Tommy Aune Rehn, Ivar Sjaastad, Ida Gjervold Lunde, Jan Magnus Aronsen, Ole M. Sejersted *Submitted, 2016*

2 SELECTED ABBREVIATIONS

AP	action potential
ATP	adenosine triphosphate
CrP	creatine phosphate
ECC	excitation contraction coupling
EDL	extensor digitorum longus muscle
fMLC2	fast isoform of myosin light chain 2
FT	fast twitch
GFAT	glutamine-fructose-6-phosphate amidotransferase
GlcNAc	N-acetylglucosamine
HBP	hexosamine-biosynthetic pathway
HF	heart failure
MLC1	myosin light chain 1 (essential myosin light chain)
MLC2	myosin light chain 2 (regulatory myosin light chain)
MLCK2	myosin light chain kinase 2 (skeletal muscle MLCK)
MLCP	myosin light chain phosphatase
MS	mass spectometry
MYPT2	myosin light chain phosphatase targeting protein 2
OGA	O-GlcNAcase
OGT	O-GlcNAc transferase
P_i	inorganic phosphate
PTM	post-translational modification
RyR	ryanodine receptor
Ser	serine
SERCA	sarcoplasmic reticulum Ca^{2+} ATPase
sMLC2	slow isoform of myosin light chain 2
SOL	soleus muscle
SR	sarcoplasmic reticulum
ST	slow twitch
Thr	threonine
VO_{2max}	maximal oxygen uptake

3 INTRODUCTION

General introduction

Shortening contractions of skeletal muscles enable your body to move, allowing a myriad of actions from the gentle cuddling of a baby to vigorous uphill running. The uphill running can however not continue infinitely. This is because you will experience skeletal muscle fatigue, which can be defined as a decline in muscle performance associated with muscle activity (8). If you perform systematic exercise training, before you repeat the same uphill run, fatigue will probably develop at a later stage. On the contrary, a person that suffers from heart failure (HF) might not be capable to join the uphill run at all, because he experiences fatigue just by climbing the stairs in his hallway.

Although numerous studies have investigated skeletal muscle fatigue, the intricate interplay that mediates fatigue is yet to be established (5, 8, 36, 89). Especially the regulation of shortening contractions, as opposed to isometric contractions where the muscle does not shorten, is poorly understood. Identifying basic fatigue mechanisms during shortening contractions could help to improve skeletal muscle performance in sports and exercise. At least as important, improved understanding of these fatigue mechanisms could be essential to alleviate the increased fatigability in HF and several other chronic diseases (97, 111, 113, 143, 195).

In this thesis, we have explored the fatigue development of shortening skeletal muscle using experimental animal models. We have investigated potential molecular fatigue mechanisms with emphasis on two essential post-translational modifications (PTMs) of skeletal muscle proteins, i.e. phosphorylation and O-GlcNAcylation. PTMs of a small protein called regulatory myosin light chain (MLC2) could be essential in the regulation of shortening contractions, and were investigated specifically. The primary muscle examined was the durable soleus muscle. This muscle is located on the back part of the calf, and is active during standing, walking and running (28).

Skeletal muscle

Structure

Most of the approximately 640 skeletal muscles in the human body are attached to the skeleton by tendons, producing movement of the body under voluntary control (196). Skeletal muscles have a complex structure (Fig. 1), and several of the components in the substructure of skeletal muscle are involved in the fatigue studies of this thesis. In brief, the structure of each muscle comprise bundles (fascicles) of long, cylindrical muscle cells (myofibers), surrounded by connective tissue, capillaries and a motor-neuron. Inside the muscle cells, the largest component is contractile proteins in parallel rods called myofibrils, attached to the cell membrane at each end.

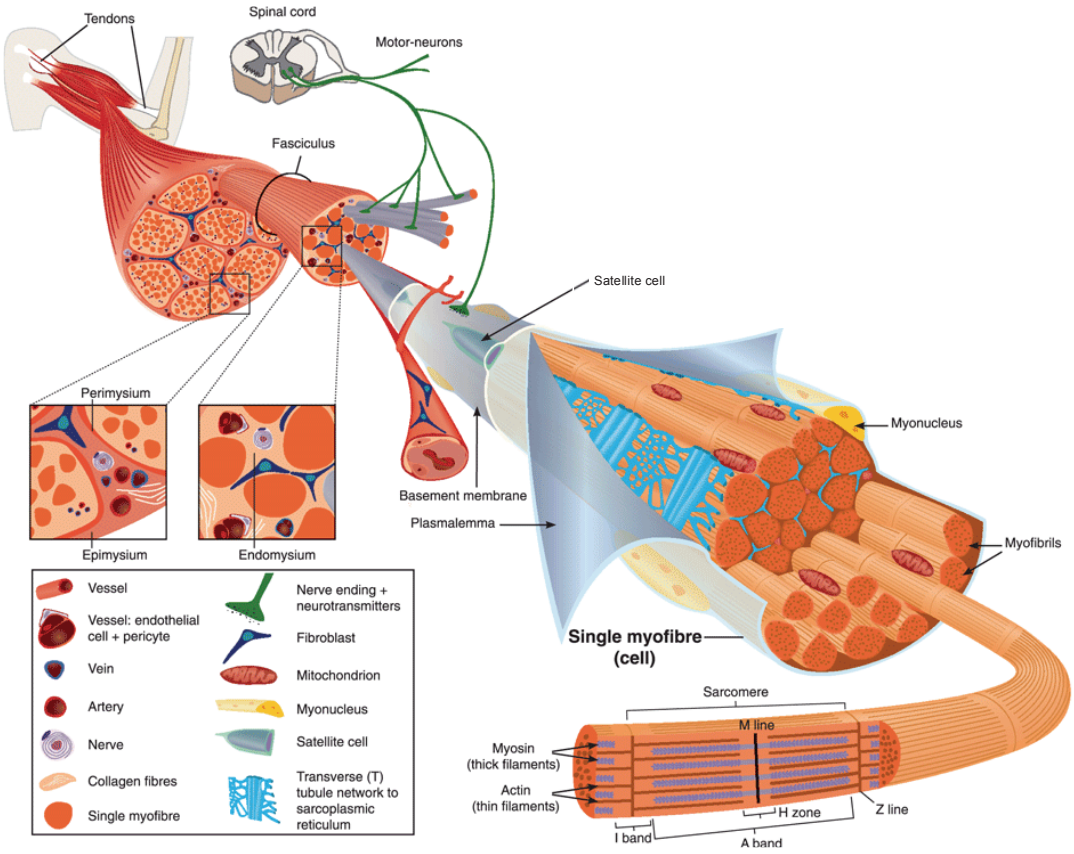

Fig. 1. Skeletal muscle structure. *From Tajbakhsh, S. (2009) Journal of Internal Medicine, 266: 372–389. Reprinted with permission from John Wiley and Sons.*

Around each of these myofibrils, a network is formed by the sarcoplasmic reticulum (SR). (26). The SR is the intracellular calcium (Ca^{2+}) store, and has terminal cisternae adjacent to deep invaginations of the cell membrane called transverse tubules (t-tubules). The t-tubules

enable the action potential (AP) to propagate into the interior of the muscle cells. In between the myofibrils are the mitochondria, responsible for respiration and energy production. Skeletal muscle cells are multinucleated, resulting from merging of several primitive myoblasts in prenatal development. Just outside the muscle fibers there are quiescent multipotent satellite cells that that can be activated in growth and repair (43, 64).

The sarcomere

In further detail, the myofibrils are composed of the thick myosin filaments and the thin actin filaments, collectively named myofilaments. They are arranged in repeated functional units called sarcomeres, shown in Fig. 1 (as part of the overall skeletal muscle structure), and in Fig. 2 (at higher magnification). In this thesis we have specifically investigated a small contractile protein called regulatory myosin light chain (= myosin light chain 2, MLC2, 18 kDa). This protein is part of the complex substructure of myosin filaments that regulates sarcomere structure and function (163). The MLC2 wraps around the neck of myosin heavy chain (MHC, 220 kDa) (Fig. 2, *right insert*), together with the essential MLC (= myosin light chain 1, MLC1, 23 kDa), providing mechanical support (96). The MHC constitutes the head (subfragment 1, S1) of myosin that is responsible for the interaction with actin, possessing the ATP binding site (the nucleotide binding pocket) and the myosin ATPase. Cyclic interactions between myosin and actin mediate the muscle contraction, as described later. The giant protein titin (3-4 MDa) (92) and the myosin binding protein-C (140 kDa) (178) are other important components associated with the thick myosin filament.

Each myosin filament is surrounded by six actin filaments arranged in a hexagonal array (Fig. 2, *cross-sectional view*), corresponding to the myosin heads sticking out from the myosin filament. This maximizes the opportunities for myosin-actin interactions (196). The actin filaments are primarily composed of actin (43 kDa) (Fig. 2, *left insert*), helically coiled with nebulin (6-900 kDa). Further, tropomyosin (TM, 66 kDa) in complex with troponin T (TnT, 33 kDa), I (TnI, 21 kDa) and C (TnC, 18 kDa) are bound to actin via TnI. At rest, the TM /Tn complex covers the myosin binding site on actin. However, when Ca^{2+} binds to TnC, the other subunits bind tighter to TnC and the TnI-actin interaction is weakened (74), and TM moves away from the myosin binding site.

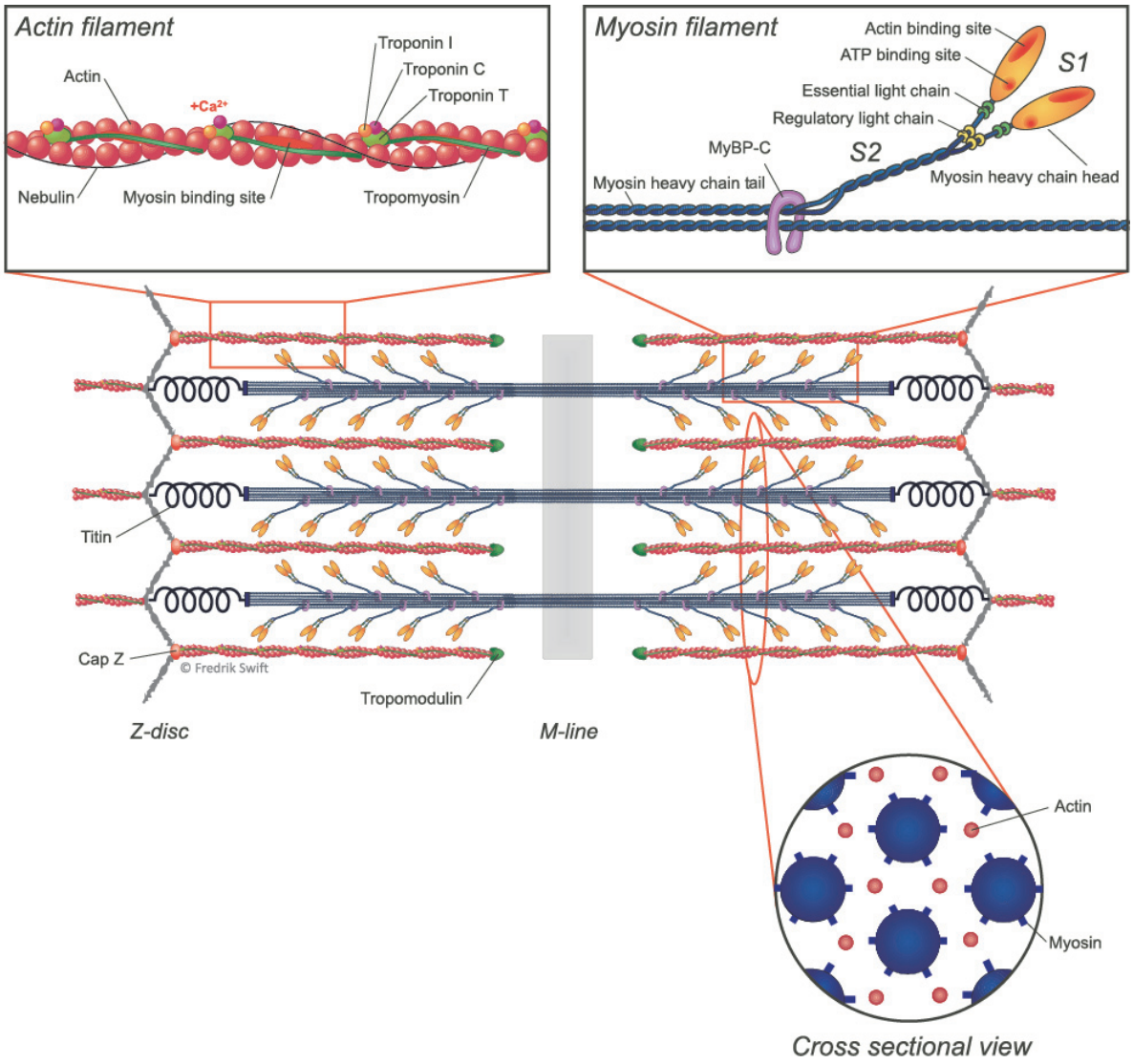

Fig. 2. Selected proteins of the sarcomere. The myosin filament (*right insert*) and the actin filament (*left insert*) both have a complex substructure of regulatory proteins. The myosin and actin filaments are arranged in a hexagonal array (*cross-sectional view*) that maximizes the opportunities for interaction. *Modified with permission from M. Munkvik and F. Swift.*

Skeletal muscle fiber types

The myofibers that comprise the skeletal muscles are heterogeneous with respect to size, metabolism and contractile function. More than a decade ago, Ranvier (140) reported that the red parts of a rabbit skeletal muscle showed slower contraction rate, but was more endurable, than the white parts. The redness of the muscle was later shown to correlate with the level of the oxygen transporter myoglobin (42), closely related to the oxidative capacity of the muscle.

Table I. Properties of different fiber types

MHC	I	IIa	IIx/b
Contraction speed	Slow	Moderately fast	Fast
Force production	Low	Medium	High
Metabolic activity	Oxidative	Oxidative-Glycolytic	Glycolytic
Mitochondrial density	High	High	Medium/low
Capillary density	High	Intermediate	Low
Major storage fuel	Triglycerides	CrP, glycogen	CrP, glycogen
Fatigue resistance	High	Intermediate	Low/very low
SR pump activity	Low	High	High
Type of activity	Aerobic	Long-term anaerobic	Short-term anaerobic

Today, muscle fibers are characterized as slow oxidative (type I) (slow twitch, ST), fast oxidative-glycolytic (type IIa) or fast glycolytic (type IIb/x) (156) (IIb not expressed in human muscle) (fast twitch, FT), with typical properties as outlined in Table I. MHC I has low myosin ATPase activity and slow contraction speed, while MHC II has high myosin ATPase activity and fast contraction speed (15, 146). The fast contraction speed in MCH II fibers is also influenced by a high content of Ca^{2+} in SR and high SR pump activity compared to MCHI fibers (52, 100). Importantly, the neural stimulation pattern of the muscle is considered the primary modulator of its contractile properties, with almost complete reversal of contractile properties in cross-innervated or cross-stimulated slow and fast skeletal muscles (34, 47, 95). In rats, the ST muscle soleus contains > 90 % type I fibers, while the FT muscle extensor digitorum longus (EDL) contains > 90 % type II fibers (162).

In the present thesis, the analyses were performed on soleus and EDL muscles, representing the extremes of muscle phenotypes.

Skeletal muscle fatigue

Shortening skeletal muscle

Prolonged use of skeletal muscles leads to a deterioration of muscle function known as fatigue (8, 36, 50, 89). Although numerous studies have investigated skeletal muscle fatigue, the intricate interplay that mediates fatigue is yet to be established. Especially the regulation of shortening contractions, as opposed to isometric contractions, is poorly understood.

The majority of experimental studies on skeletal muscle fatigue have investigated isometric contractions, and fatigue has traditionally been defined as the failure to maintain force output, leading to reduced performance (45). In isometric (static) contractions, the muscle generates force without changing length (Fig. 3A). Although there is energy expenditure, no work is done because there is no change in length (work = force x shortening). On the contrary, shortening (dynamic, concentric) contractions (Fig. 3B) produce force that overcomes the external load, resulting in muscle shortening, work and power.

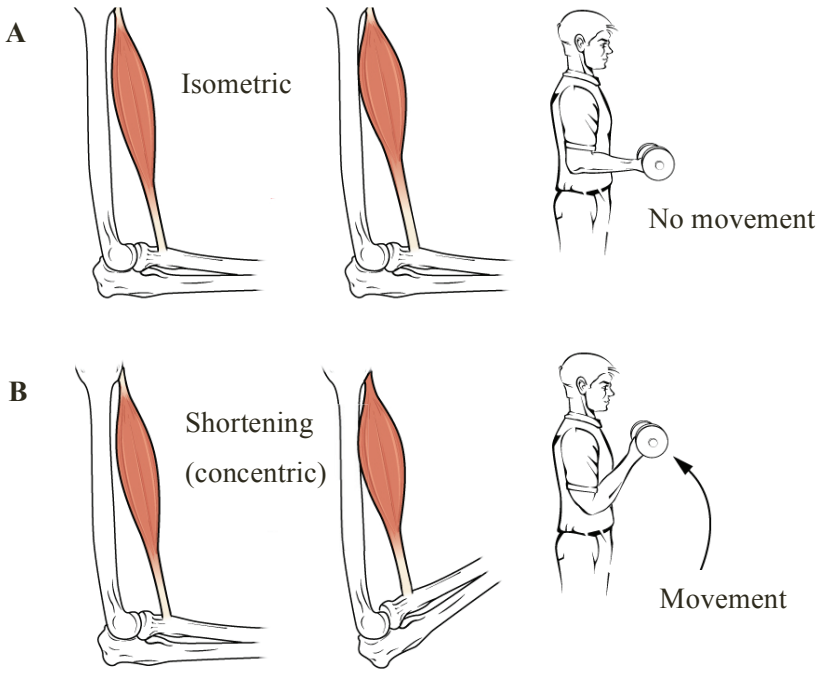

Fig. 3. Different types of muscle contractions. A, In isometric contractions, the muscle generates force but does not shorten. **B**, In shortening (concentric) contractions, the force produced by the muscle overcomes the external load, resulting in muscle shortening. *Download for free at http://cnx.org/contents/e4f804ec-103f-4157-92e1-71eed7aa8584@1, modified with permission.*

Fatigue *in vivo* typically develops during shortening contractions of locomotive skeletal muscles. Hence, the investigation of muscle function only in the isometric setting may not be sufficient to understand *in vivo* muscle function (5, 119, 148). Studies that have included shortening contractions have reported larger fatigue development during shortening vs. isometric contractions (10, 11, 108, 148, 159). Further, measuring fatigue only by the loss of force development underestimates the fatigue during shortening contractions (119, 181). Including muscle shortening (S_{max}) and velocity in the test parameters could provide a more comprehensive fatigue analysis.

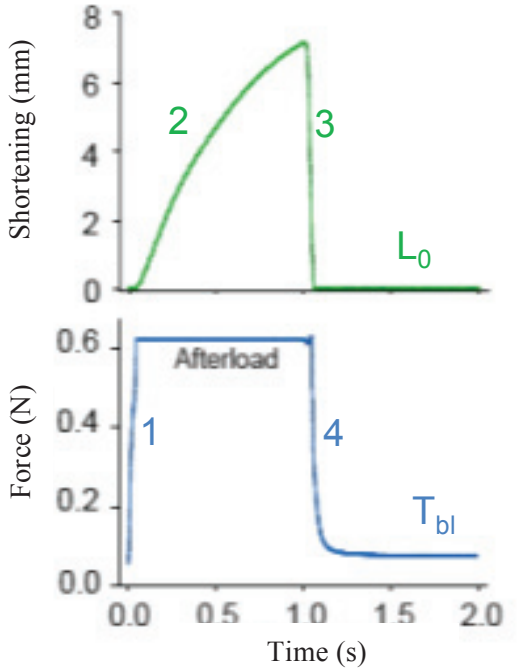

Fig. 4. Contraction-relaxation cycle of shortening muscle

Isometric (blue) and isotonic (green) parts.
1, isometric force development;
2, isotonic shortening;
3, isotonic relenghtening;
4, isometric relaxation.

L_0, resting length. T_{bl}, baseline tension.

The contraction-relaxation cycle of a shortening muscle can be divided into four sequential events, here exemplified by a cycle from the *in situ* model used in this thesis (Fig.4). Stimulation of the muscle initially evokes a rapid rise in force (isometric force development, Fig. 4, *part 1*) until the force produced exceeds the external load (afterload). Subsequently, the muscle starts to shorten (isotonic shortening, Fig. 4, *part 2*) and produce work. When the stimulation ceases, the muscle is lengthened (isotonic relenghtening, Fig. 4, *part 3*) to resting length (L_0), followed by a decline in force (isometric relaxation, Fig. 4, *part 4*) towards resting tension (baseline tension, T_{bl}).

Because the fatigue development during shortening contractions is incompletely understood, we performed a detailed analysis of the fatigue development in the four different parts of the contraction-relaxation cycle during in situ shortening contractions. Further, we investigated potential causes of the observed fatigue development (next section).

Potential sites of fatigue

Fatigue can result from failure at different levels, and a model of eight potential sites of fatigue were put forward by Bigland-Ritchie (18) (Fig.5). The four central sites represent nervous components (Fig. 5, *site 1-4*), while the four peripheral sites represent processes in the muscle cell (Fig. 5, *site 5-8*). This thesis investigates some of the peripheral sites of fatigue in the context of shortening contractions, i.e. excitation-contraction coupling (ECC), contractile mechanisms (cross-bridge cycling) and metabolic energy supply and metabolite accumulation.

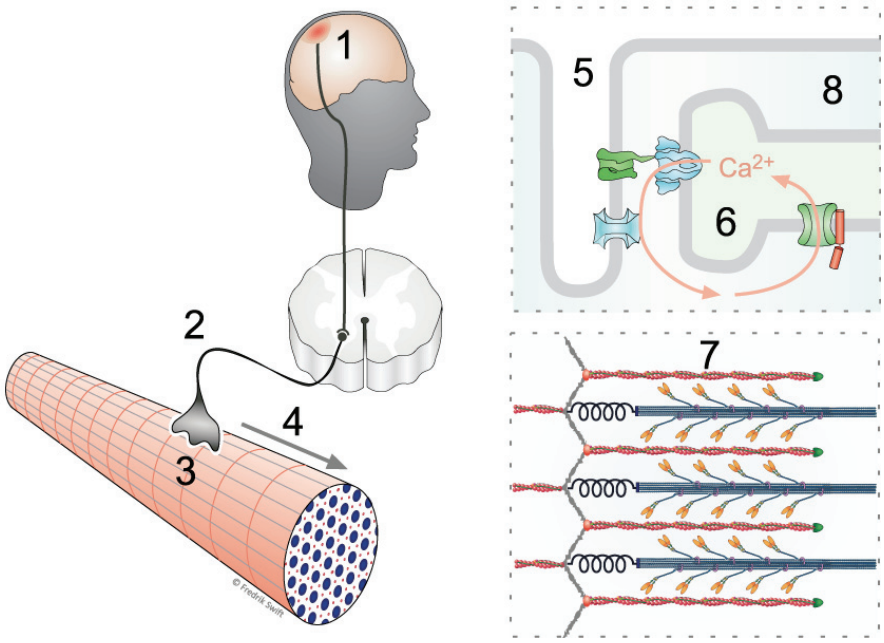

Fig. 5. Potential sites of central (1-4) and peripheral (5-8) fatigue.
1: Excitatory input to higher motor centers 2: Excitatory drive to lower motor neurons
3: Motor neuron excitability 4: Neuromuscular transmission 5: Sarcolemmal excitability
6: Excitation-contraction coupling 7: Contractile mechanisms (cross-bridge cycling)
8: Metabolic energy supply and metabolite accumulation. *Courtesy of M. Munkvik and F. Swift.*

Excitation-contraction coupling (ECC)

The term excitation–contraction coupling (ECC) was introduced in 1952 to describe the process of converting an electrical stimulus to a mechanical response (155), i.e. an AP to muscle contraction. Today, the ECC is known to involve several cellular components (Fig. 6) that tightly regulate the transient rise and fall of cytoplasmic Ca^{2+}.

In brief (26), following excitation in motor cortex, an AP in a motor neuron leads to depolarization of the muscle cell membrane and the t-tubuli. The depolarization causes a conformational change in L-type Ca^{2+} channels in the t-tubules, the dihydropyridine receptors (DHPR). This conformational change mechanically induces opening of the adjacent ryanodine receptors (RyR) in the SR membrane. The open RyRs release Ca^{2+} into cytoplasm: Ca^{2+} binds to TnC on the actin filaments and, by this activation, cross-bridge cycling and force production/shortening can occur (described in next section). High stimulation frequencies allow more Ca^{2+} to be released from SR into cytoplasm, resulting in more activation of the myofilaments than at lower frequencies. Ca^{2+} is removed from the cytoplasm by the SR Ca^{2+} ATPase (SERCA), pumping two Ca^{2+} into the SR for each ATP hydrolyzed. When the motor neuron activity ceases, the DHPR returns to resting configuration, RyR closes, Ca^{2+} is removed by SERCA, Ca^{2+} dissociates from TnC, and cross-bridge cycling stops.

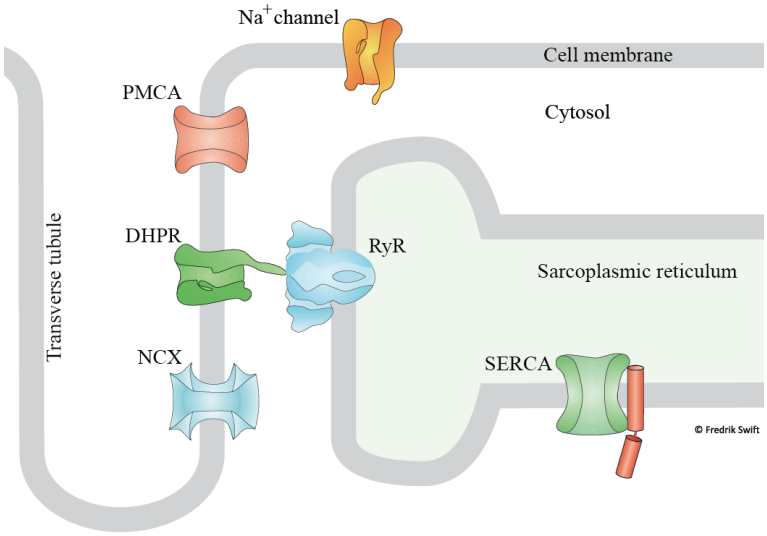

Fig. 6. Selected structural components involved in Ca^{2+} handling in skeletal muscle. DHPR, dihydropyridine receptor; RyR, ryanodine receptor; PMCA, plasmalemmal Ca^{2+} pump; NCX, Na^{+}/Ca^{2+} exchanger; SERCA, SR ATP-dependent Ca^{2+} pump. *Modified with permission from M. Munkvik and F. Swift.*

An impaired Ca^{2+} handling would affect the transient rise and fall of cytoplasmic Ca^{2+}, which again could affect both the contraction and relaxation parts (Fig. 4) of dynamic muscle activity (8). At low frequencies, a small reduction in cytoplasmic Ca^{2+} can reduce the force output greatly, because the force/Ca^{2+} curve is steep (184). Impaired Ca^{2+} release from the SR has been recognized as an important cause of fatigue during isometric contractions in isolated muscles, but the exact mechanism is not established (7, 127, 184). Further, reduced Ca^{2+} uptake by SERCA is considered another cause of fatigue, also in dynamic exercise (81).

In the present thesis, we investigated if altered Ca^{2+} handling contributed to the initial phase of fatigue during in situ shortening contractions.

Contractile mechanisms (cross-bridge cycling)

The sliding filament theory was developed in 1954 by two independent research groups (79, 80), and describes how muscle contraction results from relative sliding between actin and myosin filaments coupled with ATP hydrolysis. The theory has been modified several times, and is today widely accepted as the model for skeletal muscle contraction (35, 40, 50). However, several molecular mechanisms of the myofilament sliding are not fully understood (40, 56, 78).

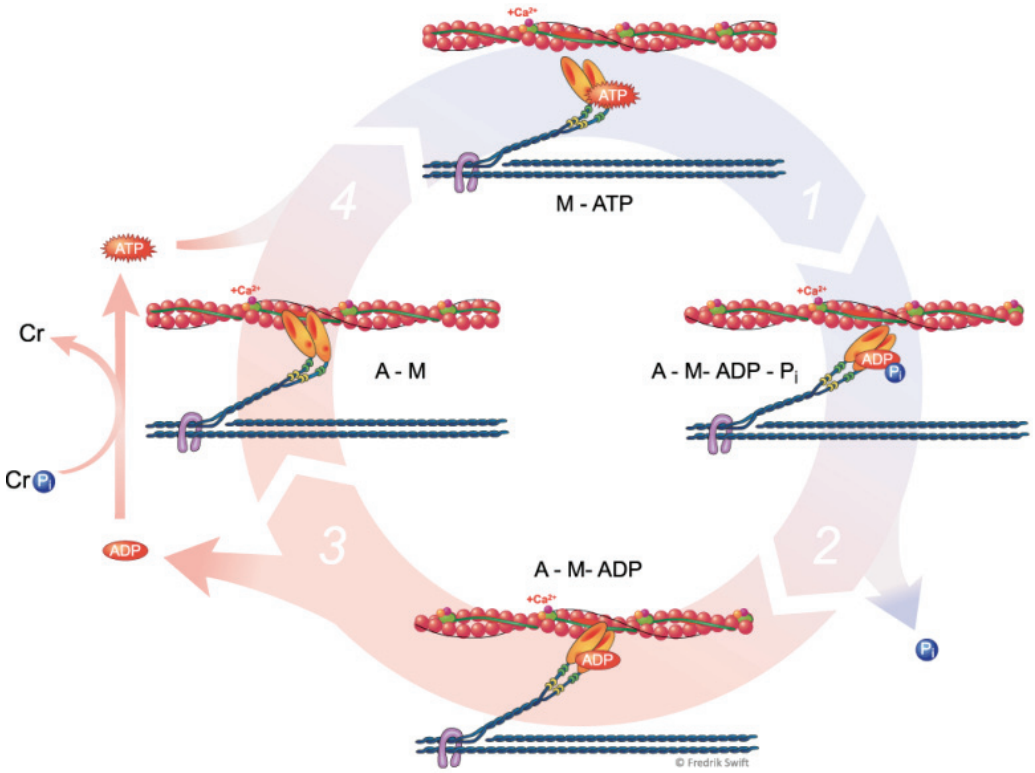

Fig. 7. The cross-bridge cycle. M – Myosin, A- Actin. P_i – inorganic phosphate. Blue and red shaded areas of the circle indicate weakly and strongly bound states of the cross-bridge, respectively, and the white numbers mark the different states as referred to in the text. *Courtesy of M. Munkvik and F. Swift.*

In a simplified view of the cross-bridge cycle (Fig. 7) based on Cook (36), cross-bridge cycling starts when Ca^{2+} binds to TnC and TM uncovers the binding site on actin. As soon as the binding site is exposed, the myosin head (with ATP in the nucleo tide binding pocket) binds weakly to actin in a pre-power stroke configuration (Fig. 7, *state 1*). ATP hydrolysis rotates the myosin neck, which places the myosin head in position for the power stroke (Fig. 7, *state 2*). Subsequently, the release of inorganic phosphate (P_i) creates a high-force generating

state (Fig. 7, *state 3*) and initiates the power stroke that pulls the actin filament towards the M-line of the sarcomere. Towards the end of the power stroke the ADP is released from the myosin head, leaving myosin tightly bound to actin, known as the rigor state (Fig. 7, *state 4*). On binding of a new ATP, the rigor state dissociates (Fig. 7, *state 1*) and ATP hydrolysis rotates the myosin neck in position for the next power stroke. The cycle repeats as long as Ca^{2+} and ATP are present.

The power stroke is believed to involve conformational changes in the region between the head and neck of the myosin molecule, also known as the lever arm (82, 141). Since the myosin backbone is fastened only to the midline (M-line) of the sarcomere, while the actin filaments are anchored to the opposing Z-disks, the result of force-producing interactions between the filaments is a longitudinal contraction of the system. The number of cross-bridges acting in parallel determines the maximal force generating capacity of the muscle (51).

The actomyosin interaction can be directly modulated during fatigue by the changing metabolites, altered pH, free radicals and PTMs of contractile proteins (36, 40, 50). The relative importance of the different modulators, and whether additional factors contribute, is not fully understood. In isometric contractions, the high-force generating state (after the release of P_i) (Fig. 4, *state 3*) is considered the dominant configuration of the cross-bridge (170), and the release of Pi is thought to be the rate-limiting step of the ATPase cycle (94). During shortening contractions, the reaction velocity of the cross-bridge cycling (attachment, force development, detachment) is higher compared to during isometric contractions (36), with only 5 % of cycle time is spent in the high-force generating state (170). Hence, the relative importance of the factors affecting cross-bridge function could be different. The higher reaction velocity during shortening contractions demands more ATP for hydrolysis by the myosin ATPase, i.e. a muscle that shortens utilizes more energy than a muscle that performs isometric contractions (49).

In this work, we investigated the potential roles of Ca^{2+} handling, metabolites and PTMs in the modulation of contractile function during fatiguing shortening contractions.

Metabolic energy supply and metabolite accumulation

With the transition from rest to strenuous exercise, the energy demand (ATP) of the muscle can increase more than 100-fold (151). The myosin ATPase in cross-bridge cycling is one of the most energy demanding cellular processes, in addition to the SERCA pumping Ca^{2+} into the SR and the Na^{+}/K^{+} ATPase maintaining sarcolemmal excitability (196).

The energy supply varies depending on the duration (Fig. 8) and intensity of exercise. Only a small amount of ATP and CrP is stored close to the contractile proteins and is readily available at onset of muscle activity, approximately enough to perform a 10-20 s sprint (151). To continue exercise, the muscle cell must resynthesize ATP through glycolytic (anaerobic) or oxidative (aerobic) pathways. Breakdown of stored glycogen-glucose via the glycolytic pathway is performed without oxygen and can provide energy to perform a 400-800 m sprint, and results in accumulation of lactate/H^{+}.

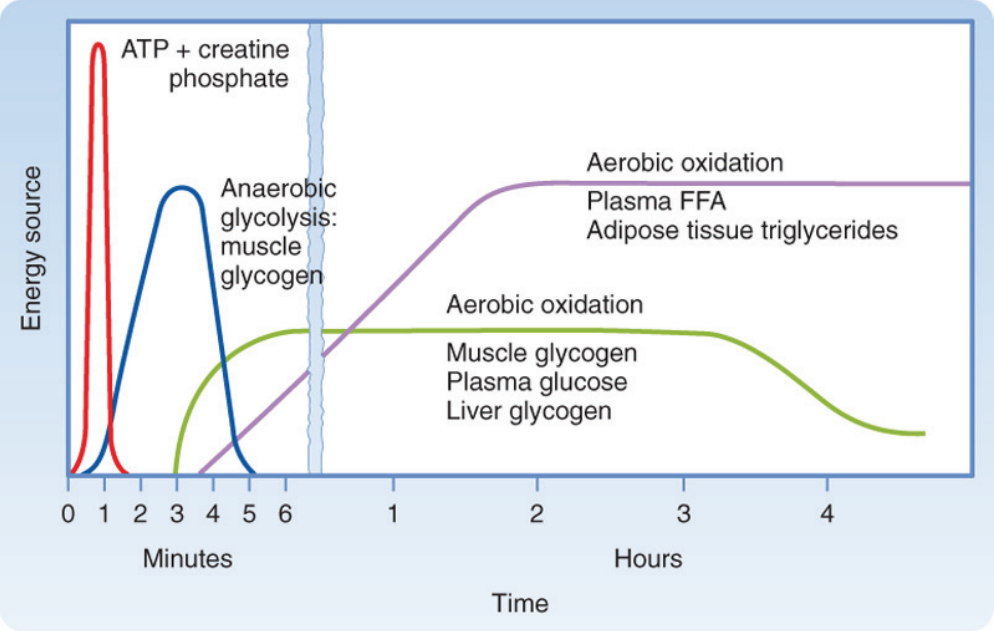

Fig. 8. Energy supply of working skeletal muscle. *Koeppen & Stanton; Berne and Levy Physiology, 6th Edition. Reprinted with permission from Elsevier.*

Activities lasting longer than 2-3 min depend primarily on oxidative metabolism, or a combination of glycolytic and oxidative metabolism, and require steady supply of oxygen. In oxidative metabolism, complete oxidation of glucose provides about 30 molecules of APT, in contrast to only 2 ATP in anaerobic glycolysis (166). Fat and amino acids can also enter oxidative metabolism, and fat is the dominant energy source during long-lasting exercise. In

addition to the intracellular energy stores, the exercising muscle utilizes glucose and fatty acids transported with the blood.

There is a lag in oxidative metabolism during the initial phase of muscle activity (Fig. 8), resulting in parallel activation of non-oxidative ATP delivery. The lag in oxidative metabolism could reside at the level of oxygen delivery or mitochondrial enzymes, or possibly both (61). However, warm-up exercise has been reported to overcome this lag in oxidative metabolism (27, 62, 65), and could possibly improve contractile function in subsequent exercise.

In the present thesis, we investigated the dynamic changes in muscle metabolites during fatigue from in situ shortening contractions. Further, we investigated whether a preceding (1st) bout of exercise (warm-up) attenuated the metabolic changes and the fatigue development in a subsequent (2nd) exercise bout.

Adaptions to exercise

Skeletal muscle has a remarkable ability to respond to environmental stimuli. In the contracting muscle, biochemical, electrical and mechanical stimuli (e.g. depletion of high-energy metabolites, mechanical stress, increased Ca^{2+} and accumulation of reactive oxygen species (ROS)) trigger activation of several signaling pathways that mediate the training responses (46). Eventually, repeated exercise training results in improved contractile function, substrate delivery and mitochondrial respiration (19, 46, 76, 126, 153), collectively enhancing the ability to resist fatigue.

However, the mechanisms that cause improved contractile function after exercise training remain unclear (51, 53, 54, 72, 144). A fiber-type shift from slow to fast or from fast to slow seems difficult to obtain with regular exercise training (51), suggesting that fiber type shift cannot explain the improved contractile function. PTMs of skeletal muscle proteins have been suggested as important modulators in the training response (121, 144), but the extent and impact of PTMs in exercise remains to map.

Therefore, we studied endurance training in addition to acute fatiguing exercise, to investigate the potential role of skeletal muscle PTMs in the adaptions to exercise training.

Post-translational modifications of skeletal muscle proteins

Post-translational modifications (PTMs) of proteins are essential in skeletal muscle, serving to control and dynamically modulate a myriad of complex cellular processes. By covalent, enzymatic modifications, the PTMs can inhibit or activate specific functions of proteins involved in ECC, cross-bridge cycling and metabolic energy supply (8, 30, 36, 40, 50, 72). Hence, unraveling the function of specific PTMs could be essential for the understanding of fatigue development during shortening contractions.

In this thesis, we explored the two important PTMs phosphorylation and O-GlcNAcylation in skeletal muscle. We specifically investigated the phosphorylation dynamics of the small regulatory protein MLC2 during shortening contractions, both in acute fatiguing exercise and after long-term endurance training.

Phosphorylation of regulatory myosin light chain (MLC2)

The observation that MLC2 could be phosphorylated in striated muscle (132) suggested direct modulation of myofilament function at the cross-bridge level, in addition to the ECC mechanism. In resting skeletal muscle, the myosin heads are highly ordered with close proximity to the thick filament (Fig.9A, *indistinct part*), and may minimize myosin head interaction with inactivated actin filaments (83). Phosphorylation of MLC2 is thought to disrupt this ordered orientation, resulting in movement of the myosin heads towards the actin filament (Fig.9A, *distinct part*) (4, 93, 169, 174, 189).

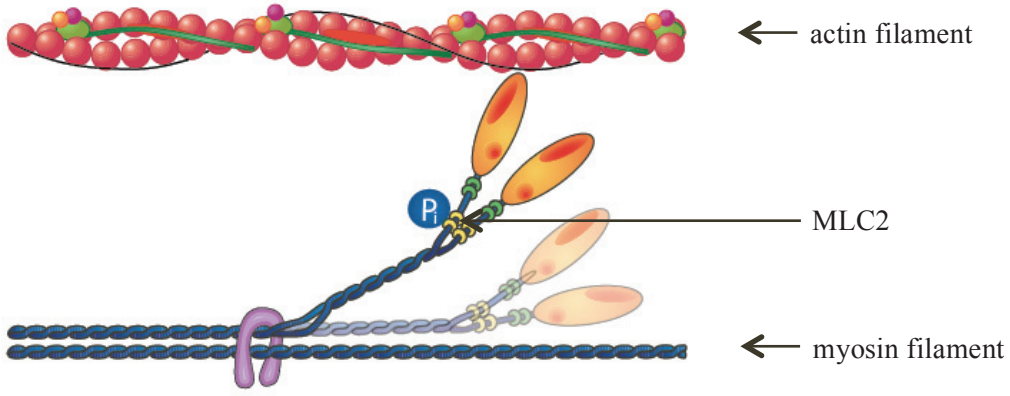

Fig. 9. Structural consequences of MLC2 phosphorylation at the cross-bridge level. Schematic illustration. Phosphorylation of MLC2 moves the myosin heads from the configuration close to the myosin filament (*indistinct part*) towards the actin filament (*distinct part*). P_i, inorganic phosphate. *Modified with permission from M. Munkvik and F. Swift*

It is suggested that MLC2 phosphorylation increases the transition of the cross bridges from non-force to force-generating states (172) and increases the Ca^{2+} sensitivity of the contractile apparatus at submaximal Ca^{2+} (112, 133, 167, 171-173, 179). Further, the rate of force development is increased at all stimulation frequencies (180), consistent with increased Ca^{2+} sensitivity in proportion to the myosin ATPase activity (172). Thus, a greater fraction of cross-bridges can contribute to force generation and filament sliding. In smooth muscle, phosphorylation of MLC2 is an obligatory event that initiates contraction (84, 85). This is different from in striated muscle, where contraction is initiated by binding of Ca^{2+} to TnC. Phosphorylation of MLC2 in striated muscle is hence not an obligatory mechanism for contraction, but a modulatory mechanism at the cross-bridge.

Persechini (133) showed that addition of skeletal muscle myosin light chain kinase (MLCK2, ~90 kDa) to permeabilized FT fibers resulted in phosphorylation of MLC2 and increased Ca^{2+} sensitivity of the contractile apparatus. Later, models of MLCK knockout and -overexpression elegantly confirmed this causative relationship in FT fibers (58, 150, 194). MLCK2 is substrate specific for MLC2 (136, 167, 168), recognizing the sequence NVF after the phosphorylatable Ser14/15 in ST/FT MLC2 (167), respectively. The opposing dephosphorylation of MLC2 is performed by myosin light chain phosphatase (MLCP). The MLCP enzyme complex consists the catalytic subunit of protein phosphatase 1 beta (PP1c β, sometimes referred to as PP1cδ, ~36 kDa), the myosin phosphatase targeting protein (MYPT2, ~110 kDa) and the small unit M20 of unknown function (116).

Most results about MLC2 phosphorylation arise from studies in FT skeletal muscle. In FT muscle, MLC2 phosphorylation is associated with post-tetanic force potentiation, i.e. a tetanic isometric contraction enhances force production in subsequent submaximal isometric contractions (167, 179). In contrast, tetanic stimulation of ST muscle does not produce force potentiation *in vitro* or *in situ*, and there is no change or only a small increase in MLC2 phosphorylation (38, 114, 115). This fiber-type difference between FT and ST seems in part to be explained by differences in activitity and expression of MLCK and MLCP/MYPT. The ratio of MLCK to MLCP is highest for FT fibers and markedly lower in ST fibers (115, 150). Further, there is different expression of MLC2 isoforms, with predominantly fast MLC2 (fMLC2) expressed in FT and slow MLC2 (sMLC2) in ST (22). Lastly, chronic low-frequency stimulation, typically of ST soleus, induced fast-to-slow transition of MLC2 isoforms and reduced MLC2 phosphorylation and the level of MLCK in EDL (22). Hence,

force potentiation by MLC2 phosphorylation has been shown to be part of the FT, but not the ST muscle phenotype.

The different regulation of MLC2 in FT and ST muscle could possibly be important to understand the fatigue development during dynamic activity. In FT muscle, MLC2 phosphorylation has been reported to enhance not only isometric force, but also the rate and extent of muscle shortening (2, 60, 106, 179). MLC2 phosphorylation in ST muscle during dynamic activity has, to the best of our knowledge, only been investigated by our group (119). Further, the role of MLC2 phosphorylation in the adaptions to endurance training has not been investigated in either ST or FT skeletal muscle. Since the regulation of MLC2 phosphorylation is fundamentally different in ST compared to FT muscle during isometric contractions, we hypothesized that the regulation during shortening contractions would also be divergent between these muscle types.

Hence, we investigated the dynamics of MLC2 phosphorylation during in situ shortening contractions of ST soleus. Subsequently, we analyzed MLC2 phosphorylation after in vivo acute exercise and long-term endurance training on the treadmill, both in soleus and EDL. Further, we investigated whether there was interplay with MLC2 O-GlcNAcylation.

Protein O-GlcNAcylation

Intracellular protein O-GlcNAcylation has emerged as an important intracellular signaling system analogous to protein phosphorylation, first discovered in 1984 (176). O-GlcNAcylation is the cycling of a monosaccharide, O-GlcNAc, on serine or threonine groups of nuclear and cytoplasmic proteins, serving to regulate signaling, transcription and processes in cytoplasm (20, 31, 69, 71, 121). Different from phosphorylation, which is regulated by hundreds of kinases and phosphatases, O-GlcNAcylation is controlled exclusively by two reciprocal enzymes that are highly conserved among species (70). O-GlcNAc transferase (OGT) catalyzes the addition of the O-GlcNAc to Ser/Thr residues of proteins (Fig. 10), and O-GlcNAcase (OGA) hydrolyzes the removal of the moiety. However, the mechanisms by which OGT and OGA modify specific proteins at certain times remain elusive. Different splice variants, PTMs, protein interactions and subcellular localization are among the mechanisms at play (122).

A small fraction of glucose entering the cell is diverted into the hexosamine-biosynthetic pathway (HBP) (Fig. 10) as an alternative route to glycolysis (110). The end product of the

HBP is uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which is the substrate for O-GlcNAcylation. The first step is controlled by the rate-limiting enzyme glutamine-fructose-6-phosphate amidotransferase (GFAT). Intriguingly, the synthesis of UDP-GlcNAc via the HBP is also regulated by several other metabolic pathways, i.e. glucose, amino acid, fatty acid and nucleotide metabolism (68, 110).

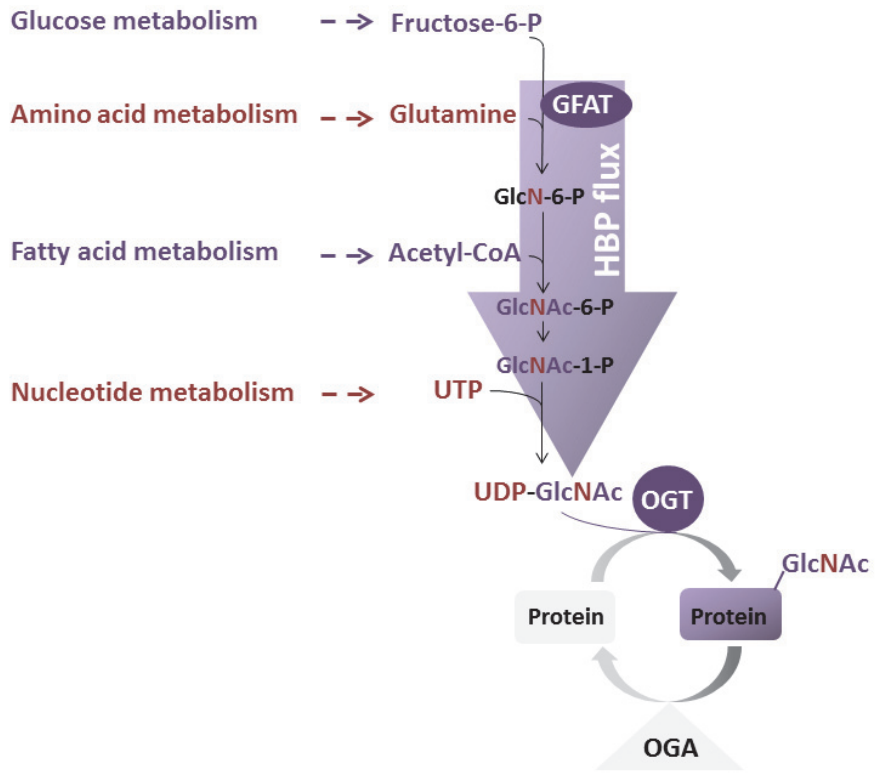

Fig. 10. Schematic illustration of the HBP and protein O-GlcNAcylation.
HBP, hexosamine-biosynthetic pathway; GFAT, glutamine-fructose-6-phosphate amidotransferase; OGT, O-GlcNAc transferase ; OGA, O-GlcNAcase.

Functionally, the role of O-GlcNAc as a metabolic sensor has been highlighted, linking metabolic status to various signaling pathways in the cell (20, 68, 70, 121). O-GlcNAcylation regulates several cellular processes and nearly every step in protein synthesis and stability, with aberrant regulation linking O-GlcNAc to the etiology of metabolic and neurodegenerative diseases and cancer (20, 68, 69, 103, 160, 193). In addition to the effect on transcriptional and epigenetic regulators, O-GlcNAcylation can directly modify enzymatic function and interact with phosphorylation and other PTMs (20, 25, 68, 71).

In skeletal muscle, the role of O-GlcNAc modifications is poorly mapped. However, several contractile proteins in skeletal muscle have been identified as O-GlcNAcylated (29, 32, 73), suggesting that O-GlcNAcylation could be involved in important regulatory processes in skeletal muscle contraction. Especially MLC2 has been suggested to be regulated by O-GlcNAcylation in cross-talk with phosphorylation (30, 32, 165), but the extent and the functional consequences of this regulation is not established. Further, several skeletal muscle metabolic enzymes have been identified as O-GlcNAc modified (29). Exercise increases muscle glucose uptake (147), and acute exercise has been shown to increase UDP-GlcNAc (124), the substrate for O-GlcNAcylation. Given its role as a sensor of metabolic status, O-GlcNAc signaling could be central in the response to exercise training (121).

Lastly, several chronic diseases are linked to aberrant O-GlcNAc signaling, including cardiovascular disease (109, 193). A recent study from our research group reported increased O-GlcNAc signaling in cardiac pressure overload and HF (98). Several other studies support that a sustained increase in protein O-GlcNAcylation is detrimental to the heart (48, 109, 139, 193). HF patients typically experience reduced exercise tolerance that in part is attributed to skeletal muscle dysfunction (195). Previous work from our group reported skeletal muscle dysfunction in HF both in rats (99, 100, 102) and humans (120). Whether altered protein O-GlcNAcylation contributes to the skeletal muscle dysfunction in HF, parallel to the aberrant O-GlcNAcylation in the heart, is not known.

In this thesis, we investigated the potential regulatory role of skeletal muscle protein O-GlcNAcylation in acute exercise, in long-term endurance training and in post-infarction HF.

4 AIMS

The general aim of this thesis was to study fatigue during shortening contractions in skeletal muscle, to expand the knowledge of regulatory mechanisms at play.

The specific research aims were as follows:

1. To study the fatigue development in the different parts of the contraction-relaxation cycle, during *in situ* shortening contraction of ST skeletal muscle (*paper I*)

2. To investigate the effect of prior exercise (warm-up) on the fatigue development in a subsequent bout of *in situ* shortening contractions (*paper I*)

3. To elucidate the dynamics of sMLC2 phosphorylation, metabolite alterations and Ca^{2+} handling in the regulation of *in situ* shortening contractions of ST skeletal muscle (*paper I*)

4. To investigate if *in vivo* treadmill running induces dephosphorylation of sMLC2 in ST skeletal muscle, and if there is interplay with sMLC2 O-GlcNAcylation (*paper II*)

5. To study the enzymatic regulation of MLC2 phosphorylation and O-GlcNAcylation during shortening contractions (*paper II*)

6. To explore the effects of acute and long-term treadmill running on protein O-GlcNAcylation in ST- and FT skeletal muscle (*paper III*)

7. To investigate if skeletal muscle protein O-GlcNAcylation is altered in post-infarction HF (*paper III*)

5 METHODOLOGICAL CONSIDERATIONS

This section discusses key aspects of the methods used in the thesis. For the detailed description of each method, please see the individual papers.

Experimental animal models

We used Wistar rats in most experiments, and this animal model was chosen for several reasons. One important factor is that the fiber type distribution of soleus and EDL in Wistar rats is well defined (reviewed by Soukup *et al.* (162)), confirmed by in house analysis (102). The soleus consists of > 90 % type I fibers (slow, oxidative) and < 10 % type IIa fibers (fast, oxidative-glycolytic), while the EDL consists of ~5 % type I, ~20-40 % type IIa and ~55-80 % type IIb fibers (fast, glycolytic). This high percentage of slow and fast fibers in soleus and EDL, respectively, allows investigation of the prototypical ST- and FT properties of skeletal muscle. Further, our research group has many years of experience with rats in experimental animal models, and the *in situ* model was custom made for rat experiments. Compared to larger animals, rats can be reared in short time at a low expense and they are easily handled (91). Mice are even faster to breed, but their relatively smaller physical proportions can be a disadvantage in regard to experimental equipment and tissue harvesting. Considering the evolving possibilities in genetic engineering (3), rats will probably remain one of the most important animals in laboratory research.

In some of the experiments in *paper II and III* we used Sprague Dawley (S.D.) rats, and the above factors are equally valid for S.D. rats. Importantly, the fiber type distribution in soleus and EDL from S.D. rats is by large similar to in Wistar rats, albeit the percentage of type I in soleus is slightly lower (80-90%) (162). We acknowledge that including two strains could be a limitation when interpreting the results. However, a comparison of control muscles from Wistar and S.D. rats showed that the patterns of protein O-GlcNAcylation were similar (*paper III*). Further, all experiments had same-strain control groups for all analyses. Lastly, the use of S.D. rats in these experiments allowed a parallel study of cardiac tissue (unpublished results) in which S.D. rats were required. Hence, by using S.D. rats we reduced the total number of animals used, as we continuously strive to in the animal experiments.

It was essential to use a general anesthetic with low cardio-depressive effect, to ensure that cardiac output was not compromised during the *in situ* exercise and the echocardiographic examinations. Further, the concentration and the duration of anesthesia should be easy to

adjust. The gas anesthetic Isoflurane seemed to meet these requirements (57). To prevent hypothermia, the animals were kept on a heated (37° C) surface while anesthetized.

In situ shortening contractions

The reductionist approach of dissecting biological systems has provided valuable knowledge of many biochemical processes. However, the integrative biology of exercise and fatigue is complex, and cannot be explained or predicted by solely studying individual components. To allow controlled investigation of loaded muscle shortening in close to physiological conditions, we utilized the *in situ* model (*paper I and II)* established at our institute (119). This model has several advantages in the study of skeletal muscle fatigue.

Firstly, the model enables investigation of fatigue development in an intact, blood perfused muscle (Fig. 11), without contributions from *central* components of fatigue. In the *in situ* model, membrane depolarization is achieved by replacing endogenous neural stimulation with direct electrical stimulation. Hence, mechanisms of *peripheral* fatigue can be isolated in a physiological setting. Importantly, the blood supply needs to be sufficiently maintained during exercise, to deliver oxygen and to remove waste products. Earlier experiments involving near-infrared spectroscopy (119) reported preserved muscle O_2 saturation in soleus throughout the protocol of *in situ* shortening contractions. Further, in contrast to *in vitro* studies, the *in situ* model preserves the structure of the muscle from the level of the whole muscle to the level of the individual sarcomere. The recovery of contractile parameters after rest underscores that the *in situ* protocol did not damage the muscle.

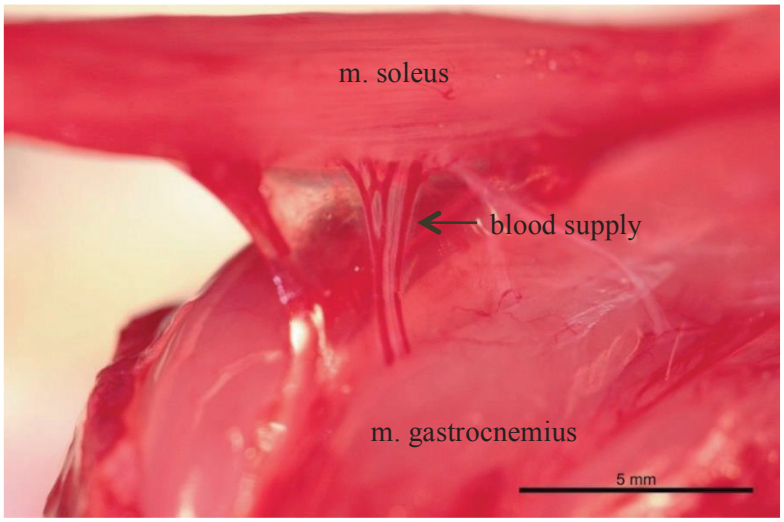

Fig. 11. Soleus muscle *in situ.* Note the intact blood supply. *Photo: Morten Munkvik.*

Secondly, skeletal muscles *in vivo* normally shorten on stimulation, and the present *in situ* model enables the extension of experimental fatigue studies to involve shortening contractions. Great effort was made to obtain relevant and reproducible *in situ* experiments, both in terms of the experimental design and the surgical training. The muscle was stimulated intermittently towards a preset load of 1/3 of maximal isometric force (F_{max}). At this load the muscle produce maximum power and fatigue most rapidly (159, 181). The stimulation pulse duration was set to 1 ms to mimic single APs, and the stimulation frequency was set to 30 Hz, i.e. in the upper physiological range of rat soleus muscle (75). The muscle was stimulated intermittently 1 s on/off to resemble dynamic activities with repeated contraction/relaxation cycles. The surgery to prepare the soleus muscle *in situ* was performed by one dedicated operator (K. H. Hortemo) in all experiments.

Finally, the *in situ* protocol allows investigation of fatigue at physiological temperature (37 °C). The choice of temperature is crucial when interpreting results from experimental studies, as many factors involved in skeletal muscle fatigue are temperature dependent (131, 142, 148, 183). As an example, harmful effects of high H^+ and P_i on force production diminishes when the temperature rise (183). Also the effects of MLC2 phosphorylation in FT fibers have been shown to change with temperature (reviewed by Vandenboom *et al.* (179)).

Treadmill running

High intensity interval training has been shown to improve skeletal muscle function and maximal oxygen consumption (VO_{2max}) more efficiently than moderate exercise training (67, 182). The six weeks of interval training on the treadmill (*paper II and III*) was modified from Wisløff et al (186). In accordance with this previous study we found a robust training effect after six weeks, evident by increased running speed and distance, increased skeletal muscle citrate synthase and cardiac hypertrophy. We did not measure VO_{2max} in this study, but similar exercise protocols demonstrated a solid (~70 %) increase in VO_{2max} compared to sedentary controls (88, 182, 186). The treadmill running was performed at 25° inclination, and a grid at the end of the lane supplied an electrical stimulus (0.25 mA, 1 stimulus of 200 ms every second) to keep the rats running on the lane. An inclined treadmill recruits a larger muscle mass than a level treadmill (196), and when comparing different inclinations of the treadmill, VO_{2max} in rats was reached at 25° inclination (186).

Wild rats typically perform several shorter runs over a long time period (149) rather than running continuously for 2 h, however, the rats managed to fulfil the exercise training every

day. In the six weeks of interval training, the distance run gradually increased from ~1.6 km/day in the first week to ~2.5 km/day in the last week. It has been estimated from voluntary wheel running that wild rats can run ~8 km/day (149), indicating that the running distance was within the physiological range.

Treadmill running is the preferred option for controlled studies of the effects of exercise training (182). In contrast to voluntary treadmill running, it allows precise control of exercise duration, speed, inclination and distance. A potential limitation of treadmill running is however that the animals could experience a stress response from imposed running (23, 117). Because also voluntary exercise training activates the stress response (41), the contribution of treadmill running to overall stress in unclear. During the six weeks of training in the present study, the rats were well acclimatized to treadmill running, and we consider that any effects due to "treadmill stress" would be minor.

Coronary artery ligation as a model of HF

Coronary artery ligation in rats was used as a model of human myocardial infarction (MI), to induce post-infarction HF in rats (*paper III)*. Sham operated rats (sham) underwent the same procedure except the ligation of the coronary artery, and served as controls. The coronary artery ligation model to induce HF is clinically relevant, considering that coronary artery disease is the most frequent cause of HF in humans (13). However, a known limitation of this experimental HF model is that MI is induced acutely, in young animals. This is different from in humans where MI often is preceded by a gradual cardiac dysfunction, in the elderly. Hence, the compensatory mechanisms related to HF could be different (143). On the other hand, the animal model has the advantage that neither coexisting diseases nor drugs confound the pathophysiology, while elderly humans often are multimorbid and use several drugs.

The echocardiographic criteria for inclusion of rats in the HF group was the presence of a large anterolateral myocardial infarction and increased left atrial diameter (LAD) >5.0 mm (161). Because inter-observer variability can be considerable in echocardiographic examinations, all the echocardiographic examinations on rats were performed by the same two operators and with standardized recording modes. In addition to the echocardiographic examination, congestive HF was confirmed post-mortem by increased lung and heart weight.

Human skeletal muscle biopsies

The muscle biopsies from human vastus lateralis were obtained from post-infarction HF patients and healthy controls subjects (HS). The included participants represent a subset from a previous study from our laboratory, and details about the methodology, testing regime and inclusion/exclusion criteria have been described (120). In the present study, the biopsies were analyzed for protein O-GlcNAcylation in HF patients compared to HS. Cardiac and skeletal muscle dysfunction in HF patients was evident by reduced cardiac ejection fraction, lower maximal oxygen uptake and lower skeletal muscle peak power compared to HS.

As discussed in the above section, there are apparent differences between HF rats and HF patients. However, in the present thesis, both the rat model of HF and the human patients represent the same type of HF, i.e. post-infarction HF with reduced ejection fraction (HFrEF). If a common O-GlcNAcylation pattern could be detected in skeletal muscles from both HF rats and HF patients, this could indicate robust mechanisms inherent to the pathophysiology of post-infarction HFrEF.

Methods of studying proteins

The analyses on frozen muscle samples were conducted with the assumption that the procedures involved in freezing, storage, homogenization and analysis of muscle tissue reflect the true conditions at the time of freezing. We managed to rapidly snap-freeze the muscle samples at the end of the experiment, as this is of great importance especially when studying protein phosphorylation and O-GlcNAcylation (192).

Western blotting and gel staining

Western blotting (immunoblotting) is an invaluable and widely used technique to identify and quantify proteins (107). The procedure involves many steps that must be performed with great accuracy to obtain reliable and high quality blots. In brief, gel electrophoresis separates proteins according to molecular weight, followed by transfer to a membrane (blotting). Target proteins are identified by incubating the membrane with specific primary antibodies and subsequent appropriate secondary antibodies. The secondary antibodies often bear an enzyme (e.g. horseradish peroxidase (HRP)) that modifies an appropriate substrate (e.g. luminol) so that it emits light. This allows visualization of the identified protein bands by chemiluminescence imaging. The intensity of the band corresponds to the amount of protein, and the amount of protein can be quantified relative to the other samples on the same blot.

Optimization of protein input, acrylamide concentration of the gel, electrophoresis and blotting times are critical to obtain high quality immunoblots. Furthermore, the sensitivity and specificity of primary- and secondary antibodies are important, and must be considered when interpreting the results.

In this thesis, western blotting allowed mapping of numerous skeletal muscle proteins and their phosphorylation and O-GlcNAcylation modifications (*paper I, II, III)*. Importantly, data obtained by western blotting are semi-quantitative: it provides a relative comparison of protein levels, but not an absolute measure of quantity. Reasons for this include variations in loading, differences in transfer from gel to membrane between individual blots, and non-linear signal strength across the concentration range of samples (107). However, there can also be variations in loading and transfer across an individual blot, and one should therefore strive to apply the different samples alternately, and not grouped.

The analysis of MLC2 phosphorylation was central in the present work, and in *paper I* we verified the MLC2 phosphorylation pattern by two different methods. Firstly, MLC2 phosphorylation was analyzed by sequential ProQ Diamond (phosphorylated proteins) and Sypro Ruby (total proteins) fluorescent gel staining. In gel staining, different from in western blotting, the proteins are not transferred to a membrane after gel electrophoresis. Instead, the gel is further processed and stained, allowing direct in-gel detection of protein bands, visualized by e.g. a laser scanner. ProQ Diamond and Sypro Ruby gel staining are sensitive methods (188) to measure protein phosphorylation and total protein expression, respectively. It is also advantageous that Sypro Ruby staining can be performed subsequently to ProQ Diamond staining, i.e. MLC2 phosphorylation (ProQ) could be calculated relative to the MLC2 protein level (Sypro Ruby) in the exact same samples.

Subsequently, the results obtained by ProQ Diamond were verified by western blotting using the phospho-specific MLC2 antibody, and reprobing with total MLC2 antibody. These MLC2 antibodies showed high specificity, with no additional bands appearing when visualizing the entire blot (*paper I and II*). In *paper II*, we decided to use the western blotting method to analyze MLC2 phosphorylation. This allowed analyzing MLC2 O-GlcNAcylation (see below) in the exact same samples after stripping of membranes. Further, the enzymes modulating MLC2 phosphorylation and O-GlcNAcylation could also be analyzed by western blotting.

For analysis of protein O-GlcNAcylation level, we used the O-GlcNAc antibody CDT 110.6 that is thought to recognize the widest range of O-GlcNAc-modified proteins and is the most

thoroughly characterized (192). It is however known that different O-GlcNAc antibodies do not recognize entirely the same O-GlcNAc modifications, and in a test run we compared CDT 110.6 with another O-GlcNAc antibody named RL2 (MA1-072) (*paper III)*. As expected, this comparison showed that many protein bands were detected by both antibodies, while there were also some differences in the O-GlcNAcylation patterns. A recent publication from our research group (98) successfully used the CTD 110.6 antibody to obtain high quality O-GlcNAcylation blots, and we decided to use this antibody for all analyses.

In addition to phosphatase and protease inhibitors, 40 mM glucosamine was added to the lysis buffers to preserve the O-GlcNAcylation level present at muscle harvest (*paper II and III*). Glucosamine is structurally similar to GlcNAc, with high concentration of glucosamine providing excess substrate for OGA, and we chose this method of OGA inhibition based on a recent publication with high-quality O-GlcNAc blots (98). We also performed a brief comparison of addition of glucosamine vs. the addition of the OGA inhibitor PUGNAc (*paper III*), showing similar O-GlcNAcylation levels by the two approaches.

Mass spectrometry

The mass spectrometry (MS) analyses were performed in collaboration with the Department of Biosciences, University of Oslo. We attempted to identify the proteins with strongly increased O-GlcNAcylation signal after the six weeks of exercise training (as assessed by western blotting with the CTD 110.6 antibody), and the protocol was described in detail in *paper III*. By the MS analyses, we identified the proteins present in the specific gel slice of interest, i.e. comprising the ~50 kDa protein band with increased O-GlcNAcylation signal. Unfortunately, we did not succeed to detect the O-GlcNAc moiety by the MS analyses. Hence, the identity of the O-GlcNAcylated proteins could not be confirmed.

Indeed, the identification of O-GlcNAc modifications is a major challenge to proteomics (103, 130). Firstly, when assessed by gel electrophoresis, the isoelectric point is not changed by O-GlcNAc, and the molecular weight is rarely altered. Secondly, in most mass spectrometers, the O-GlcNAc modification is labile. Further, peptides modified by O-GlcNAc generate a relatively lower signal than unmodified peptides, and this likely complicated the detection of the O-GlcNAc moiety by MS in the present study. It could seem that specific methods to enrich O-GlcNAc modified proteins and peptides, and specially equipped mass spectrometers, are necessary to detect the O-GlcNAc moiety (103, 130). This technology is currently not widely available, but limited to a few dedicated laboratories. In the coming years, application

of novel technologies will likely improve the identification of O-GlcNAc modified proteins and sites.

Metabolites

Metabolite content in soleus muscles from rats was analyzed at different time points in the *in situ* exercise protocol and in resting control muscles (*paper I*), by established methods. High Performance Liquid Chromatography (HPLC) was used to quantify muscle ATP, ADP, AMP and CrP (158). In short, HPLC separates and quantifies substances by pumping the sample in a solvent (mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). Substances bind to the stationary phase with different affinity and are eluted as the mobile phase flow through the column, and ultimately the different metabolites are represented by individual peaks on the chromatography. Lactate was determined using a fluorometric enzymatic coupled assay: By incubating the sample with excess amount of lactate dehydrogenase and NAD^+, the increase in NADH fluorescence can be measured (lactate + NAD^+ → pyruvate + NADH + H+).

The metabolite analyses provide valuable data in the analysis of fatigue in the *in situ* protocol, as discussed later. However, since the analyses provide the total amount of the various metabolites in the analyzed muscle tissue, potential effects of metabolite compartmentalization within the muscle cells (128) will not be detected. Further, several putative contributors to the fatigue development were not analyzed in the *in situ* model, e.g. oxidative stress (164) and glycogen depletion (128). Also, Pi was not directly measured. Tissue availability limited the number of analyses performed, but it future *in situ* studies, these putative contributors should indeed be considered.

Ca^{2+}transients

Sarcoplasmic reticulum (SR) function was measured in SR vesicles from whole muscle homogenates (*paper I*), based on a method from O'Brien (125). SR function at different time points in the *in situ* exercise protocol was compared to the resting control muscle. In brief, experiments were performed at 37 °C, and a spectrophotometer allowed analysis of SR function by measuring the intensities of the calcium binding dye fura-2. MgATP was added to initiate SR Ca^{2+} uptake. When the uptake had leveled off, thapsigargin was added to block the uptake, and SR leak could be analyzed. Addition of 4-chloro-m-cresol initiated SR Ca^{2+} release through RyR. Lastly, EGTA and $CaCl_2$ were sequentially added to measure minimal

and saturating signal, respectively. The method allows detailed *in vitro* investigation of changes in SR Ca^{2+} handling induced by *in situ* fatiguing shortening contractions. Importantly, since the *in vitro* set up provides controlled, optimized metabolic conditions, potential effects of metabolites and pH on SR Ca^{2+} handling during *in situ* contractions will not be detected by this method.

Statistics

Data are presented as mean ± standard error of the mean (SEM), and a significance level of 0.05 was chosen for all statistical analyses. The statistical tests assumed independence of sampling and observation, homogeneity of variance between groups, and normal distribution of data. The assumptions were tested using procedures and corrections of the statistical software (SigmaPlot or Excel).

When comparing two different groups, paired or unpaired t-test was used when the data were normally distributed and showed equal variance, and this applied to most analyses. In *paper I*, Welch's correction, an adaptation of the t-test, was applied if there was unequal variance between two groups. If normal distribution was not confirmed, Mann-Whitney Rank Sum Test was performed instead of the t-test. One-way ANOVA was used to compare multiple groups to a control group. One-way repeated measures ANOVA was used to compare repeated measures of the same animals, i.e. the analysis of contractile parameters.

Ethical considerations

Importantly, with an animal model follows an ethical obligation to *refine* the methodology, *reduce* the number of animals used and *replacing* animals with other modeling systems when possible (RRR). Through years of experience at our institute, continuous refinements of surgical procedures and animal handling have resulted in high reproducibility and low mortality. Great effort was made to minimize suffering of animals. All experiments were reviewed and approved by the Norwegian Animal Research Authority and conformed to the NHI Guide for the Care and Use of Laboratory Animals. In the human study on HF patients and healthy controls, the study protocol was reviewed and approved by the Norwegian regional ethics committee and conformed to the Declaration of Helsinki. Written informed consent was obtained from all participants.

6 SUMMARY OF RESULTS

Paper I

Multiple Causes of Fatigue during Shortening Contractions in Rat Slow Twitch Skeletal Muscle

In this study, we identified that fatigue developed in three distinct phases during *in situ* shortening contractions of ST skeletal muscle, and that the different rates and velocities of the contraction-relaxation cycle did not deteriorate in parallel. In the first phase, the regulatory protein sMCL2 was rapidly dephosphorylated in parallel with reduced muscle shortening. Secondly, there was degradation of high-energy phosphates and accumulation of lactate, and these changes were related to slowing of muscle relenghtening and relaxation, culminating at 100 s. This slowing was also associated with increased leak of Ca^{2+} from the SR. In the third phase of exercise, there was restoration of high-energy phosphates and elimination of lactate, and the slowing of relaxation disappeared. On the contrary, dephosphorylation of sMLC2 and reduced muscle shortening prevailed. Preceding exercise (warm-up) improved relaxation parameters in a subsequent exercise bout. The improvement correlated with less accumulation of lactate, and we propose that this effect was due to a more rapid onset of oxidative metabolism after warm-up. The correlation between sMLC2 phosphorylation and muscle shortening was confirmed in various experimental settings, and the combination of load and shortening seemed necessary to trigger sMLC2 dephosphorylation in ST muscle.

Paper II

Exhausting treadmill running causes dephosphorylation of sMLC2 and reduced level of myofilament MLCK2 in ST rat soleus muscle

A single bout of exhausting treadmill running induced dephosphorylation of sMLC2 in shortening ST muscle, extending our findings from the *in situ* protocol (*paper I*) to *in vivo* treadmill running. The dephosphorylation of sMLC2 was paralleled by reduced levels of the dedicated kinase MLCK2 associated to myofilaments. Dissociation of MLCK2 could cease the phosphorylation of sMLC2 so that dephosphorylation dominated, suggesting dissociation of MLCK2 from myofilaments as a novel mechanism to rapidly reduce the phosphorylation level of sMLC2. Both the sMLC2 phosphorylation and the level of MLCK associated to myofilaments were restored after 24 h rest. However, after six weeks of treadmill running, the

dephosphorylation of sMLC2 persisted in soleus along with reduction in MLCK2 both in myofilament and total protein fraction. In EDL, contrary to in soleus, phosphorylation of MLC2 was not altered after one exercise bout or after six weeks of treadmill running. O-GlcNAcylation of MLC2 did not change significantly in soleus or EDL, indicating that this modification was of minor importance in the regulation of MLC2 phosphorylation during *in vivo* running. Thus, in contrast to FT muscle, MLC2 dephosphorylation occurred in ST muscle during *in vivo* treadmill running and may be linked to reduced myofilament associated MLCK2 and reduced muscle shortening capacity.

Paper III

Exercise training increases protein O-GlcNAcylation in rat skeletal muscle

In this paper, we reported extensive protein O-GlcNAcylation in skeletal muscle, with higher levels of O-GlcNAcylation in ST soleus compared to FT EDL. The high level of protein O-GlcNAcylation in soleus was associated with greater level of the enzymes regulating the O-GlcNAc signaling, i.e. OGT, OGA, GFAT1 and GFAT2, compared to EDL. Six weeks of exercise training by treadmill running, but not an acute exercise bout, increased protein O-GlcNAcylation in soleus and EDL. There was a striking increase in O-GlcNAcylation of cytoplasmic proteins ~50 kDa in size, that judged from MS analysis could represent O-GlcNAcylation of one or more key metabolic enzymes. This suggests that O-GlcNAcylation of cytoplasmic proteins could be part of the training response. In contrast to exercise training, post-infarction HF in rats and humans did not affect skeletal muscle O-GlcNAcylation level. This indicates that aberrant O-GlcNAcylation cannot explain the skeletal muscle dysfunction in HF. Protein O-GlcNAcylation in human skeletal muscle was by large similar to the O-GlcNAcylation pattern in rat soleus and EDL, suggesting important functional roles of this signaling system also in human muscle.

7 DISCUSSION

The results from this thesis emphasize that fatigue development during shortening contractions is complexly regulated. As outlined by Allen (5), approaches that allow investigation of muscle function close to physiological conditions are necessary to define the mechanisms of fatigue. By employing the *in situ* model of shortening contractions in combination with *in vivo* treadmill running, we believe we have contributed a small step in this direction. In the following, some central topics from this thesis are discussed.

Fatigue development in shortening ST muscle *in situ*

A detailed analysis of contractile parameters per contraction-relaxation cycle, like we performed in the *in situ* protocol (*paper I*), allows characterizing of the temporal development of fatigue. The analysis of contractile parameters showed that the fatigue development is not linear. Obviously, these non-linear dynamics would not have been detected if only the initial and end values had been considered, as previously demonstrated in a model of human bicycling (87). Further, since we analyzed the fatigue development in the different parts of the contraction-relaxation cycle, we observed that the various rates and velocities of the contraction-relaxation cycle did not deteriorate or recover in parallel. For example, isometric contraction rate and isotonic shortening velocity fell rapidly during the first 100 s of exercise, and thereafter they stabilized and remained depressed throughout the exercise protocol. On the contrary, the slowing of isometric relaxation rate was only transient, culminating at 100 s of exercise, with subsequent restoration to initial values by the end of the exercise. This indicated that muscle shortening was regulated by other mechanisms than muscle relaxation.

Ca^{2+} handling

Slowing of muscle relaxation is a hallmark of fatigue, and may limit performance during dynamic exercise with alternating movements (9). We found that the slowing of isometric relaxation rate was strongly correlated to muscle lactate throughout the *in situ* exercise protocol, both in the 1st and 2nd bout. The role of intracellular lactic acidosis is controversial, and its effects on several fatigue parameters have been shown to decrease as the temperature increases towards the physiological range (8, 183). However, our *in situ* experiments performed at physiological temperature supports a slowing of relaxation with lactic acidosis (14, 24, 185). The mechanism could be that lactic acidosis reduces the affinity of Ca^{2+} binding to the SERCA pump (187), and this reduction in affinity is thought to be greater than the decreased Ca^{2+} affinity of the contractile apparatus at low pH (14). Following warm-up

exercise (1st bout), there was an attenuated slowing of relaxation in the subsequent exercise bout (2nd bout), which correlated with less accumulation of lactate. We suggest that an enhanced oxidative ATP-delivery following warm-up exercise (177) could explain this difference between the 1st and 2nd bout. By overcoming the lag in oxidative ATP-delivery by warm-up exercise, there could be less anaerobic ATP-delivery, and hence less accumulation of lactate as observed in the 2nd bout in *paper I.*

Further, the increased SR Ca^{2+} leak observed at 100 s exercise in the *in situ* protocol could also contribute to the slowing of isometric relaxation. A continuous leak from SR would cause a futile cycling of Ca^{2+} and increased demand on SERCA, and the decay of the cytosolic Ca^{2+} transient could be slower. The cause of the increased leak was not directly accessed in this thesis, but we speculate that increased sympathetic signaling could result in increased RyR phosphorylation and hence increased SR Ca^{2+} leak (17, 145). Alternatively, a recent study reported that high-intensity interval training of short duration resulted in ROS-induced RyR fragmentation and SR Ca^{2+} leak (137) in mice and humans. In the latter study, the Ca^{2+} imbalance reduced skeletal muscle contractile function acutely, but seemed important to trigger beneficial training effects. It would be of great interest to analyze whether such ROS-dependent RyR modifications play a role also in the slowing of isometric relaxation in the *in situ* protocol.

Metabolic energy supply

Shortening contractions utilize more energy compared to isometric contractions (49), explained in part by the higher rate of ATP consumption by the myosin ATPase. In the initial phase of the *in situ* exercise, the considerable breakdown of CrP and ATP would be expected to cause a proportional increase in P_i. Accumulation of P_i during muscle activity has been suggested as a major cause of fatigue (8, 26, 183), also during shortening contractions at physiological temperatures (148). A high level of P_i may decrease the Ca^{2+} sensitivity of the myofilaments and inhibit the transition from low-force to high-force generating states (36). Increased P_i could also affect the release of Ca^{2+} from SR (7), possibly due to calcium phosphate precipitation inside the SR (6, 44). Hence, accumulation of P_i seems important in the fatigue development. However, Pi and other metabolic changes could not fully explain the reduction in isometric force development in the *in situ* protocol.

Also in regard to $S_{max,}$ this parameter did not correlate with any metabolic change throughout the protocols, but remained attenuated despite normalization of lactate and recovery of high

energy phosphates. Further, reduced Ca^{2+} release from SR seemed not to be a major cause of the reduction in S_{max}, because the reduction of S_{max} after 15 min exercise was independent of the stimulation frequency (*paper I)*. S_{max} was however strongly correlated with MCL2 phosphorylation, at all measured time points during exercise and recovery in the *in situ* model.

What are the dynamics of MLC2 phosphorylation in shortening ST muscle?

One of the main objectives of this thesis was to investigate the dynamics of MLC2 phosphorylation in ST muscles during shortening contractions, to elucidate its potential role in muscle shortening and fatigue. From our experiments (*paper I and II)* it is evident that MLC2 is dephosphorylated in response to fatiguing shortening contractions in ST muscle, both *in situ* and *in vivo*.

The *in situ* model of loaded shortening contractions (*paper I*) revealed a strong correlation between MLC2 phosphorylation level and muscle shortening (S_{max}) in ST soleus muscle, both during fatigue and during recovery from fatigue. The reduction in S_{max} was paralleled by a corresponding reduction in maximal isotonic shortening velocity. We could not directly assess the molecular basis for the effect of MLC2 dephosphorylation in our experiments. However, our results are well compatible with the theory that MLC2 phosphorylation moves the myosin heads towards the actin filament, increasing the number of force-producing cross-bridges (172). Dephosphorylation could cause less myosin heads to move towards the actin filament, thereby reducing the recruitment of active cross-bridges to produce force and filament sliding (172). Interestingly, in cardiac muscle, dephosphorylation of MLC2 demonstrated depression of load-dependent contractility and shortening velocity (154, 175). In FT muscle, MLC2 phosphorylation not only improved isometric function, but also enhanced dynamic function (1, 2, 60, 105, 106, 179). Hence, the MLC2 phosphorylation level seems to affect loaded shortening across different muscle types.

The effect of MLC2 phosphorylation on *unloaded* shortening velocity (V_0), an indicator of maximum cross-bridge turnover rate, is however not clarified. From studies performed in FT fibers and muscles, there are ambiguous result to whether MLC2 phosphorylation increases the duty cycle of the cross-bridges (by limiting the rate of cross-bridge detachment and V_0) (55, 63, 86), or whether MLC2 phosphorylation does not influence these parameters (58, 112, 133, 172). In ST muscle, one previous study from our laboratory (119) investigated V_0 during fatigue from *in situ* shortening contractions in ST muscle, and did not find MLC2

phosphorylation to correlate with V_o. Differences in temperature and interactions with fatigue could possibly explain the discrepant results (86, 179).

We found that the amount of myofilament associated MLCK2 was rapidly reduced in ST muscle in response to loaded muscle shortening, both *in situ* and *in vivo* (*paper II)*. The level of myofilament associated MLCK2 correlated strongly with the MLC2 phosphorylation level, and we suggest dissociation of MLCK2 from myofilaments as a novel mechanism to reduce MLC2 phosphorylation in ST muscle. The dissociation of MLCK2 could rapidly cease the phosphorylation of MLC2, so that dephosphorylation would dominate.

Interestingly, another recent study also identified MLCK2 at the sarcomere, in a multienzymatic complex located preferentially at the Z-disk (30). Possibly, mechanical stress could be sensed at the Z-disk during loaded shortening contractions, triggering MLCK2 dissociation from myofilaments. However, the specific binding site for MLCK2 on myofilaments, and the upstream regulation of MLCK2 in ST muscle, remains to establish. Because there was only a small reduction in MLC2 phosphorylation and S_{max} when the work load was lowered towards zero, one might expect that maximal load (100 % of F_{max}) would produce maximal MLC2 phosphorylation. However, at maximal load the muscle produced only isometric contractions, and MLC2 phosphorylation remained unchanged in line with previous literature (179). Hence it seems that the combination of load and shortening is necessary to trigger MLC2 dephosphorylation in ST skeletal muscle.

MLC2 phosphorylation and energy expenditure

Increased MLC2 phosphorylation in FT muscle has been shown to increase the amount of work performed in a given time period (60, 105, 106). Because MLC2 phosphorylation increases the number of active cross-bridges, with correspondingly increased myosin ATPase activity (169), energy consumption is also thought to be increased. Oppositely, as MLC2 is dephosphorylated, recruitment of fewer cross-bridges, less work performed and less energy consumption could be expected. In support of this theory, dephosphorylation of MLC2 in the *in situ* protocol (*paper I*) was associated with reduction in S_{max} and hence less performed work. Further, there was partial restoration of high energy phosphates in the muscle during ongoing work, possibly due to reduced total energy consumption.

We suggest in *paper II* that the persistent dephosphorylation of MLC2 observed in the exercise trained soleus muscles could provide a beneficial restraint during long-lasting

exercise. By limiting the initial work performed and hence energy consumption, dephosphorylation of MLC2 could postpone the fatigue development. As recently highlighted (21), fatigue is the result of evolution through natural selection, and several of the restrictions in physical activity caused by fatigue could be protective mechanisms to avoid detrimental exhaustion. In FT muscle (2), MLC2 phosphorylation increased work performed compared to the non-phosphorylated muscle during *in situ* concentric contractions, but the increase in energy cost was relatively more enhanced than work output (i.e. the contraction economy was reduced). This suggests that during prolonged activity, MLC2 phosphorylation could possibly cause adverse metabolic changes that contribute to increased fatigue development. It has been suggested that as the number of active cross-bridges is increased, cross-bridge efficiency is reduced (39), which would lead to less power output from each ATP.

Hence, it seems that the different regulation of MLC2 phosphorylation between FT and ST muscle could amplify the muscles´ fast and slow contractile properties, respectively: MLC2 phosphorylation in FT muscle is associated with post-activation potentiation of force and shortening, at the cost of increased energy consumption. This could possibly enhance performance in high-intensity activities of short duration. On the contrary, the MLC2 phosphorylation level in ST muscle is generally low, it is not increased with isometric contractions and it is reduced during loaded shortening contractions. This could limit the work performed and energy consumed in ST muscle, postponing fatigue during prolonged activity.

However, the findings of Abbate *et al* (2) needs to be confirmed in different muscle types and contraction modes, as the energetic consequences of MLC2 phosphorylation have important ramifications for how we view its physiological role. The possibility remains that MLC2 dephosphorylation in ST soleus after six weeks of exercise training (*paper II*) is a persistent sign of fatigue rather than a training effect. Considering previous reports of increased myosin ATPase activity and shortening velocity after endurance training, in rat ST muscles *in situ* (53) and *in vitro* (157), our finding of persistent MLC2 dephosphorylation after endurance training seems surprising. MLC2 phosphorylation and loaded muscle shortening (S_{max}) was however not analyzed in these previous studies, and it remains to analyze MLC2 phosphorylation and contractile performance *in situ* after endurance training.

Is MLC2 phosphorylation relevant to human skeletal muscle?

Ultimately, the most important objective might be to better understand how MLC2 phosphorylation modulates human movement. Post-activation potentiation of isometric twitch

force has been reported in human skeletal muscle, first shown by Houston and co-workers (77). Like in animal models, the potentiation is observed primarily in FT muscle (66). This potentiation could possibly affect human performance during brief, explosive muscle activities, like jumping and sprinting that depends on fast fibers (16, 152).

To the best of our knowledge, MLC2 phosphorylation during shortening contractions in human ST muscle has not been investigated. Like rat soleus, the human soleus muscle is predominantly ST with > 80% type I fibers (59). The human soleus is activated during standing, walking and running (28), and has the ability to contract economically over prolonged periods (37). Hence, it seems plausible that MLC2 dephosphorylation could contribute to the fatigue resistant phenotype of soleus also in humans. It would indeed be interesting to analyze MLC2 phosphorylation in human soleus in acute treadmill running as well as after long-term exercise training (i.e. equivalent to the animal experiments performed in this thesis). The majority of human skeletal muscles are, however, mixed with regard to fiber-type composition (156), and to understand the net outcome of dynamic MLC2 phosphorylation in human muscles remains a challenge to the field.

Is protein O-GlcNAcylation a novel system to regulate skeletal muscle function?

O-GlcNAcylation has emerged as an important signaling system in several tissues (20, 68, 71), but the role of protein O-GlcNAcylation in regulation of skeletal muscle contractile function remains elusive (31, 121, 124, 134, 165). In this thesis (*paper II, III)* we show widespread protein O-GlcNAcylation in ST and FT rat skeletal muscle that was dynamically modulated by exercise training. Human skeletal muscle displayed protein O-GlcNAcylation that by large mirrored the O-GlcNAcylation pattern in rat, suggesting conserved functional roles.

Phospho-GlcNAc interplay of MLC2?

In *paper II*, we investigated the potential interplay between phosphorylation and O-GlcNAcylation of MLC2 in rat skeletal muscle. We found that, contrary to MLC2 phosphorylation, MLC2 O-GlcNAcylation level was unchanged after exercise both in soleus and EDL. This could indicate that MLC2 O-GlcNAcylation is of minor importance in the regulation of MLC2 during shortening contractions. However, there was a trend towards increased MLC2 O-GlcNAcylation in soleus after acute exercise, reciprocal to the decrease in MLC2 phosphorylation (*paper II)*. A recent study reported a small decrease in MLC2 O-GlcNAcylation after inactivity (hindlimb unloading) (30), reciprocal to a relatively larger

increase in MLC2 phosphorylation. Hence, the possibility remains that minor, but significant dynamics in O-GlcNAcylation of MLC2 are present also during exercise. More sensitive, site specific methods are warranted to reach a final conclusion about this potential interplay.

Functionally, increased O-GlcNAcylation of MLC2 has been suggested to increase the Ca^{2+} sensitivity of skeletal muscle myofilaments (31, 32, 73). However, this seems to contradict the decreased Ca^{2+} sensitivity observed with increased O-GlcNAcylation of contractile proteins in cardiac muscle (138, 139). Further, removal of the increased O-GlcNAcylation in diabetic hearts restored the Ca^{2+} sensitivity, while removing the endogenous O-GlcNAcylation from non-diabetic hearts did not affect Ca^{2+} sensitivity (139). Hence, it seems that there is no linear relationship between O-GlcNAcylation and Ca^{2+} sensitivity, but rather a complex interplay between O-GlcNAc level and other factors that remains to be elucidated.

Protein O-GlcNAcylation as a sensor of metabolic status in exercise and disease

The striking increase in skeletal muscle O-GlcNAcylation after six weeks of endurance exercise on the treadmill (*paper III)*, suggested that dynamic O-GlcNAc signaling is part of the training response. Interestingly, inactivity that cause muscle atrophy, as the counterpart to exercise, has been associated with decreased protein O-GlcNAcylation in rat soleus muscle (33). Similarly, the O-GlcNAcylation level in human vastus lateralis was reduced after 60 days of bed rest (118), and the reduction was prevented by light exercise. Together with our findings of increased O-GlcNAcylation after exercise training, it seems that protein O-GlcNAcylation could be dynamically modulated according to the activity level of the muscle.

The increased O-GlcNAcylation seemed to be a result of repeated exercise, since an acute exercise bout was not found to alter the O-GlcNAcylation level. One other group recently investigated O-GlcNAc in skeletal muscle after one acute exercise bout (134), and in accordance with our results, they did not detect changes in protein O-GlcNAcylation in soleus. However, the authors reported a small increase in protein O-GlcNAcylation in white gastrocnemius muscle (mainly FT), in some contrast to our finding of unaltered O-GlcNAcylation in EDL. They proposed that increased oxidative stress triggered this O-GlcNAcylation in FT fibers (134). If this finding holds true, measures of oxidative stress should be included in future experiments concerning O-GlcNAcylation and exercise.

In regard to muscle type, we found (*paper III)* that soleus muscle that consists > 90 % ST (type I) fibers showed a different pattern of O-GlcNAcylation in rats compared to EDL that

consists > 90 % FT (type II) fibers (162). Further, human vastus lateralis, which consists ~ 40% ST and ~60 % FT fibers (120), showed a pattern of O-GlcNAcylation that mirrored the O-GlcNAcylation levels from both soleus and EDL in rats. Hence, our results suggest a fiber-type dependent protein O-GlcNAcylation in skeletal muscle in rats and humans.

Excessive protein O-GlcNAcylation in skeletal muscle has been linked to the development of insulin resistance and diabetes (12, 104, 190, 191). In this light, the increase in O-GlcNAcylation after long-term exercise might be unexpected, because exercise training is an established tool to prevent insulin resistance and diabetes (121). However, in acute glucose flux, an increase in O-GlcNAcylation has been shown to protect against glucotoxity by regulating uptake and metabolism (193). Since the activity of OGT is enhanced with increased concentration of UDP-GlcNAc (90), protein O-GlcNAcylation is highly sensitive to the nutritional state. Possibly, balanced O-GlcNAcylation may provide optimal kinetics of metabolic pathways in exercise, while energy excess in the sedentary cause overshoot of O-GlcNAc signaling which is detrimental. Further studies are warranted to elucidate the regulation of protein O-GlcNAcylation in exercise, and how exercise potentially interferes with aberrant skeletal muscle O-GlcNAcylation in metabolic disease.

Further, there are several reports of abnormal skeletal muscle dysfunction in post-infarction HF, both in humans and in animal models (101, 135, 195). Therefore, we investigated whether aberrant skeletal muscle O-GlcNAcylation was part of this dysfunction. However, the protein O-GlcNAcylation level was not different in skeletal muscle from post-infarction HF compared to healthy controls, neither in rats nor humans (*paper III*). This indicates that modification of skeletal muscle protein O-GlcNAcylation cannot explain the reduced exercise tolerance in HF. There could however be subtle O-GlcNAc modifications of specific proteins, not detected by the analysis, but the global O-GlcNAcylation pattern seemed to be preserved.

Candidate proteins of the strongly O-GlcNAc modified ~50 kDa band

An interesting finding in the analysis of skeletal muscle protein O-GlcNAcylation was the protein band at ~50 kDa with markedly increased O-GlcNAcylation after six weeks of treadmill running (*paper III*). These modified proteins were located mainly in cytoplasm, and we hypothesized that they could represent one or more key metabolic enzymes that respond to changes in exercise status. In support of this hypothesis, MS analysis of the O-GlcNAc modified band at ~50 kDa identified several metabolic enzymes with known (29, 123, 129) or predicted (OGlcNAcScan, YinOYang) O-GlcNAc modifications. Some of these enzymes, i.e.

creatine kinase, PP2A and beta-enolase, were previously reported to be O-GlcNAc modified specifically in rat skeletal muscle (29). Current literature suggests that O-GlcNAc signaling can act as a direct regulator of enzyme activities in response to changing metabolic status (25, 48, 69). We propose that the band with increased O-GlcNAcylation at ~50 kDa represents one or more of the identified key metabolic enzymes, functionally regulated by dynamically O-GlcNAcylation after exercise training. Because the MS analysis did not succeed in detecting the O-GlcNAc moiety, the enzyme identities as well as the specific O-GlcNAc sites remain to be clarified. Hopefully, the mapping of skeletal muscle O-GlcNAcylation performed in this thesis can set the stage for future studies, to reveal the functional roles of O-GlcNAcylation in skeletal muscle.

Lastly, the integrative nature of muscle fatigue represents a challenge to integrate new knowledge across different disciplines where fatigue is studied. Future research collaborations, combining various models and disciplines, could allow identification of the most robust fatigue mechanisms that are conserved across species and models.

8 CONCLUSIONS

The present thesis adds knowledge about the fatigue development during shortening contractions in skeletal muscle. The experiments were performed *in situ* at 37°C or *in vivo* on the treadmill, enabling investigation of fatigue mechanisms at physiological conditions.

Referring to the specific aims of the thesis, the conclusions are:

1. Fatigue during *in situ* shortening contractions developed in three distinct phases in ST skeletal muscle, and the different rates and velocities of the contraction-relaxation cycle did not deteriorate in parallel. There was a prominent reduction in muscle shortening that persisted throughout the protocol, contrary to slowing of muscle relaxation which was only transient (*paper I*).
2. Warm-up exercise improved muscle relaxation parameters in a subsequent exercise bout (*paper I*).
3. Dephosphorylation of MLC2 correlated strongly with the reduced muscle shortening during *in situ* shortening contractions in ST muscle. In contrast, accumulation of lactate and increased SR Ca^{2+} leak correlated with slowing of relaxation (*paper I*).
4. A single exercise bout of treadmill running induced significant dephosphorylation of MLC2 in ST muscle *in vivo* that was rapidly reversible by rest, while long-term exercise training resulted in persistent dephosphorylation of MLC2. Dephosphorylation of MLC2 was not accompanied by significant changes in MLC2 O-GlcNAcylation (*paper II*).
5. Dephosphorylation of MLC2 in ST muscle was associated with reduced levels of the dedicated kinase MLCK2 associated to myofilaments, suggesting dissociation of MLCK2 from myofilaments as a novel mechanism to rapidly regulate MLC2 phosphorylation level during shortening contractions in ST muscle (*paper II*).
6. There was extensive protein O-GlcNAcylation in skeletal muscle, with the highest O-GlcNAcylation level in ST compared to FT muscle. Long-term exercise training by treadmill running, but not an acute exercise bout, increased O-GlcNAcylation of cytoplasmic proteins in skeletal muscle (*paper III*), suggesting that O-GlcNAc signaling participates in the training response.
7. Skeletal muscle protein O-GlcNAcylation was not different in post-infarction HF compared to healthy controls, neither in rats nor humans (*paper III*), suggesting that aberrant O-GlcNAcylation cannot explain the skeletal muscle dysfunction in HF.

REFERENCES

1. **Abbate F, Sargeant AJ, Verdijk PW, and de Haan A**. Effects of high-frequency initial pulses and posttetanic potentiation on power output of skeletal muscle. *J Appl Physiol* 88: 35-40, 2000.
2. **Abbate F, Van Der Velden J, Stienen GJ, and De Haan A**. Post-tetanic potentiation increases energy cost to a higher extent than work in rat fast skeletal muscle. *J Muscle Res Cell Motil* 22: 703-710, 2001.
3. **Abbott A**. Laboratory animals: the Renaissance rat. *Nature* 428: 464-466, 2004.
4. **Adhikari BB, Somerset J, Stull JT, and Fajer PG**. Dynamic Modulation of the Regulatory Domain of Myosin Heads by pH, Ionic Strength, and RLC Phosphorylation in Synthetic Myosin Filaments. *Biochemistry* 38: 3127-3132, 1999.
5. **Allen DG**. Fatigue in working muscles. *J Appl Physiol* 106: 358-359, 2009.
6. **Allen DG, Clugston E, Petersen Y, Röder IV, Chapman B, and Rudolf R**. Interactions between intracellular calcium and phosphate in intact mouse muscle during fatigue. *Journal of Applied Physiology* 111: 358-366, 2011.
7. **Allen DG, Lamb GD, and Westerblad H**. Impaired calcium release during fatigue. *J Appl Physiol* 104: 296-305, 2008.
8. **Allen DG, Lamb GD, and Westerblad H**. Skeletal Muscle Fatigue: Cellular Mechanisms. *Physiol Rev* 88: 287-332, 2008.
9. **Allen DG, Lännergren J, and Westerblad H**. Muscle cell function during prolonged activity: cellular mechanisms of fatigue. *Exp Physiol* 80: 497-527, 1995.
10. **Ameredes BT, Brechue WF, Andrew GM, and Stainsby WN**. Force-velocity shifts with repetitive isometric and isotonic contractions of canine gastrocnemius in situ. *J Appl Physiol* 73: 2105-2111, 1992.
11. **Ameredes BT, and Clanton TL**. Increased fatigue of isovelocity vs. isometric contractions of canine diaphragm. *J Appl Physiol* 69: 740-746, 1990.
12. **Arias EB, Kim J, and Cartee GD**. Prolonged incubation in PUGNAc results in increased protein O-Linked glycosylation and insulin resistance in rat skeletal muscle. *Diabetes* 53: 921-930, 2004.
13. **Authors/Task Force M, McMurray JJV, Adamopoulos S, Anker SD, Auricchio A, Böhm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Køber L, Lip GYH, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Rønnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A, Guidelines ESCCfP, Bax JJ, Baumgartner H, Ceconi C, Dean V, Deaton C, Fagard R, Funck-Brentano C, Hasdai D, Hoes A, Kirchhof P, Knuuti J, Kolh P, McDonagh T, Moulin C, Popescu BA, Reiner Ž, Sechtem U, Sirnes PA, Tendera M, Torbicki A, Vahanian A, Windecker S, Document R, McDonagh T, Sechtem U, Bonet LA, Avraamides P, Ben Lamin HA, Brignole M, Coca A, Cowburn P, Dargie H, Elliott P, Flachskampf FA, Guida GF, Hardman S, Iung B, Merkely B, Mueller C, Nanas JN, Nielsen OW, Ørn S, Parissis JT, and Ponikowski P**. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012. *Eur J Heart Fail* 14: 803-869, 2012.
14. **Baker AJ, Brandes R, and Weiner MW**. Effects of intracellular acidosis on Ca^{2+} activation, contraction, and relaxation of frog skeletal muscle. *Am J Physiol* 268: C55-63, 1995.
15. **Barany M**. ATPase activity of myosin correlated with speed of muscle shortening. *J Gen Physiol* 50: Suppl:197-218, 1967.
16. **Baudry S, and Duchateau J**. Postactivation potentiation in a human muscle: effect on the rate of torque development of tetanic and voluntary isometric contractions. *J Appl Physiol* 102: 1394-1401, 2007.
17. **Bellinger AM, Reiken S, Dura M, Murphy PW, Deng SX, Landry DW, Nieman D, Lehnart SE, Samaru M, LaCampagne A, and Marks AR**. Remodeling of ryanodine receptor complex causes "leaky" channels: a molecular mechanism for decreased exercise capacity. *Proc Natl Acad Sci U S A* 105: 2198-2202, 2008.
18. **Bigland-Ritchie B**. Muscle fatigue and the influence of changing neural drive. Clinics in chest medicine, 1984, p. 21-34.

19. **Bogdanis GC**. Effects of physical activity and inactivity on muscle fatigue. *Front Physiol* 3: 142, 2012.
20. **Bond MR, and Hanover JA**. A little sugar goes a long way: The cell biology of O-GlcNAc. *J Cell Biol* 208: 869-880, 2015.
21. **Boullosa DA, and Nakamura FY**. The evolutionary significance of fatigue. *Front Physiol* 4: 309, 2013.
22. **Bozzo C, Spolaore B, Toniolo L, Stevens L, Bastide B, Cieniewski-Bernard C, Fontana A, Mounier Y, and Reggiani C**. Nerve influence on myosin light chain phosphorylation in slow and fast skeletal muscles. *FEBS Journal* 272: 5771-5785, 2005.
23. **Brown DA, Johnson MS, Armstrong CJ, Lynch JM, Caruso NM, Ehlers LB, Fleshner M, Spencer RL, and Moore RL**. Short-term treadmill running in the rat: what kind of stressor is it? *J Appl Physiol* 103: 1979-1985, 2007.
24. **Bruton JD, Lännergren J, and Westerblad H**. Effects of CO_2-induced acidification on the fatigue resistance of single mouse muscle fibers at 28°C. *J Appl Physiol* 85: 478-483, 1998.
25. **Bullen JW, Balsbaugh JL, Chanda D, Shabanowitz J, Hunt DF, Neumann D, and Hart GW**. Crosstalk between two essential nutrient-sensitive enzymes: O-GlcNAc transferase (OGT) and AMP-activated protein kinase (AMPK). *J Biol Chem* 2014.
26. **Calderón JC, Bolaños P, and Caputo C**. The excitation–contraction coupling mechanism in skeletal muscle. *Biophysical Reviews* 6: 133-160, 2014.
27. **Campbell-O'Sullivan SP, Constantin-Teodosiu D, Peirce N, and Greenhaff PL**. Low intensity exercise in humans accelerates mitochondrial ATP production and pulmonary oxygen kinetics during subsequent more intense exercise. *J Physiol* 538: 931-939, 2002.
28. **Cappellini G, Ivanenko YP, Poppele RE, and Lacquaniti F**. Motor patterns in human walking and running. *J Neurophysiol* 95: 3426-3437, 2006.
29. **Cieniewski-Bernard C, Bastide B, Lefebvre T, Lemoine J, Mounier Y, and Michalski JC**. Identification of O-linked N-acetylglucosamine proteins in rat skeletal muscle using two-dimensional gel electrophoresis and mass spectrometry. *Mol Cell Proteomics* 3: 577-585, 2004.
30. **Cieniewski-Bernard C, Dupont E, Richard E, and Bastide B**. Phospho-GlcNAc modulation of slow MLC2 during soleus atrophy through a multienzymatic and sarcomeric complex. *Pflugers Arch* 466: 2139-2151, 2014.
31. **Cieniewski-Bernard C, Lambert M, Dupont E, Montel V, Stevens L, and Bastide B**. O-GlcNAcylation, contractile protein modifications and calcium affinity in skeletal muscle. *Front Physiol* 5: 2014.
32. **Cieniewski-Bernard C, Montel V, Berthoin S, and Bastide B**. Increasing O-GlcNAcylation level on organ culture of soleus modulates the calcium activation parameters of muscle fibers. *PLoS One* 7: e48218, 2012.
33. **Cieniewski-Bernard C, Mounier Y, Michalski JC, and Bastide B**. O-GlcNAc level variations are associated with the development of skeletal muscle atrophy. *J Appl Physiol* 100: 1499-1505, 2006.
34. **Close R**. Dynamic properties of fast and slow skeletal muscles of the rat after nerve cross-union. *J Physiol* 204: 331-346, 1969.
35. **Cooke R**. Actomyosin interaction in striated muscle. *Physiological Reviews* 77: 671-697, 1997.
36. **Cooke R**. Modulation of the actomyosin interaction during fatigue of skeletal muscle. *Muscle & Nerve* 36: 756-777, 2007.
37. **Cronin NJ, Avela J, Finni T, and Peltonen J**. Differences in contractile behaviour between the soleus and medial gastrocnemius muscles during human walking. *J Exp Biol* 216: 909-914, 2013.
38. **Crow MT, and Kushmerick MJ**. Phosphorylation of myosin light chains in mouse fast-twitch muscle associated with reduced actomyosin turnover rate. *Science* 217: 835-837, 1982.
39. **Curtin NA, and Woledge RC**. Power and efficiency: how to get the most out of striated muscle. *Adv Exp Med Biol* 332: 729-733; discussion 733-724, 1993.
40. **Debold E**. Recent insights into muscle fatigue at the cross-bridge level. *Front Physiol* 3: 2012.

41. **Droste SK, Chandramohan Y, Hill LE, Linthorst AC, and Reul JM**. Voluntary exercise impacts on the rat hypothalamic-pituitary-adrenocortical axis mainly at the adrenal level. *Neuroendocrinology* 86: 26-37, 2007.
42. **Dubowitz V, and Pearse AG**. A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle. *Z Zellforch Microsk Anat Histochem* 2: 105-117, 1960.
43. **Dumont NA, Bentzinger CF, Sincennes MC, and Rudnicki MA**. Satellite Cells and Skeletal Muscle Regeneration. *Compr Physiol* 5: 1027-1059, 2015.
44. **Dutka TL, Cole L, and Lamb GD**. Calcium phosphate precipitation in the sarcoplasmic reticulum reduces action potential-mediated Ca^{2+} release in mammalian skeletal muscle. *Am J Physiol Cell Physiol* 289: C1502-C1512, 2005.
45. **Edwards RH**. Human muscle function and fatigue. In: Human Muscle Fatigue: Physiological Mechanisms . R.Porter and J. Whelan. London: Pitman Medical, p. 1-18: 1981.
46. **Egan B, and Zierath Juleen R**. Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. *Cell Metab* 17: 162-184, 2013.
47. **Eken T, and Gundersen K**. Electrical stimulation resembling normal motor-unit activity: effects on denervated fast and slow rat muscles. *J Physiol* 402: 651-669, 1988.
48. **Erickson JR, Pereira L, Wang L, Han G, Ferguson A, Dao K, Copeland RJ, Despa F, Hart GW, Ripplinger CM, and Bers DM**. Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. *Nature* 502: 372-376, 2013.
49. **Fenn WO**. The relation between the work performed and the energy liberated in muscular contraction. *J Physiol* 58: 373-395, 1924.
50. **Fitts RH**. The cross-bridge cycle and skeletal muscle fatigue. *J Appl Physiol* 104: 551-558, 2008.
51. **Fitts RH**. Effects of regular exercise training on skeletal muscle contractile function. *Am J Phys Med Rehabil* 82: 320-331, 2003.
52. **Fitts RH, Courtright JB, Kim DH, and Witzmann FA**. Muscle fatigue with prolonged exercise: contractile and biochemical alterations. *Am J Physiol* 242: C65-73, 1982.
53. **Fitts RH, and Holloszy JO**. Contractile properties of rat soleus muscle: effects of training and fatique. *Am J Physiol Cell Physiol* 233: C86-C91, 1977.
54. **Fitts RH, and Widrick JJ**. Muscle mechanics: adaptations with exercise-training. *Exerc Sport Sci Rev* 24: 427-473, 1996.
55. **Franks-Skiba K, Lardelli R, Goh G, and Cooke R**. Myosin light chain phosphorylation inhibits muscle fiber shortening velocity in the presence of vanadate. *Am J Physiol Regul Integr Comp Physiol* 292: R1603-R1612, 2007.
56. **Geeves MA, and Holmes KC**. Structural mechanism of muscle contraction. *Annu Rev Biochem* 68: 687-728, 1999.
57. **Gentry-Smetana S, Redford D, Moore D, and Larson DF**. Direct effects of volatile anesthetics on cardiac function. *Perfusion* 23: 43-47, 2008.
58. **Gittings W, Huang J, Smith IC, Quadrilatero J, and Vandenboom R**. The effect of skeletal myosin light chain kinase gene ablation on the fatigability of mouse fast muscle. *J Muscle Res Cell Motil* 31: 337-348, 2011.
59. **Gollnick PD, Sjodin B, Karlsson J, Jansson E, and Saltin B**. Human soleus muscle: a comparison of fiber composition and enzyme activities with other leg muscles. *Pflugers Arch* 348: 247-255, 1974.
60. **Grange RW, Cory CR, Vandenboom R, and Houston ME**. Myosin phosphorylation augments force-displacement and force-velocity relationships of mouse fast muscle. *Am J Physiol* 269: C713-724, 1995.
61. **Grassi B**. Oxygen uptake kinetics: Why are they so slow? And what do they tell us? *J Physiol Pharmacol* 57 Suppl 10: 53-65, 2006.
62. **Gray SC, Devioto G, and Nimmo MA**. Effect of active warm-up on metabolism prior to and during intense dynamic exercise. *Med Sci Sports Exerc* 34: 2002.

63. **Greenberg MJ, Mealy TR, Jones M, Szczesna-Cordary D, and Moore JR**. The direct molecular effects of fatigue and myosin regulatory light chain phosphorylation on the actomyosin contractile apparatus. *Am J Physiol Regul Integr Comp Physiol* 298: R989-R996, 2010.
64. **Gundersen K**. Muscle memory and a new cellular model for muscle atrophy and hypertrophy. *J Exp Biol* 219: 235-242, 2016.
65. **Gurd BJ, Scheuermann BW, Paterson DH, and Kowalchuk JM**. Prior heavy-intensity exercise speeds VO_2 kinetics during moderate-intensity exercise in young adults. *J Appl Physiol* 98: 1371-1378, 2005.
66. **Hamada T, Sale DG, MacDougall JD, and Tarnopolsky MA**. Postactivation potentiation, fiber type, and twitch contraction time in human knee extensor muscles. *J Appl Physiol* 88: 2131-2137, 2000.
67. **Haram PM, Kemi OJ, Lee SJ, Bendheim MO, Al-Share QY, Waldum HL, Gilligan LJ, Koch LG, Britton SL, Najjar SM, and Wisloff U**. Aerobic interval training vs. continuous moderate exercise in the metabolic syndrome of rats artificially selected for low aerobic capacity. *Cardiovasc Res* 81: 723-732, 2009.
68. **Hardiville S, and Hart GW**. Nutrient regulation of signaling, transcription, and cell physiology by O-GlcNAcylation. *Cell Metab* 20: 208-213, 2014.
69. **Hart GW**. Three Decades of Research on O-GlcNAcylation - A Major Nutrient Sensor That Regulates Signaling, Transcription and Cellular Metabolism. *Front Endocrinol (Lausanne)* 5: 183, 2014.
70. **Hart GW, Housley MP, and Slawson C**. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* 446: 1017-1022, 2007.
71. **Hart GW, Slawson C, Ramirez-Correa G, and Lagerlof O**. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annu Rev Biochem* 80: 825-858, 2011.
72. **Hawley J, Hargreaves M, Joyner M, and Zierath J**. Integrative Biology of Exercise. *Cell* 159: 738-749, 2014.
73. **Hedou J, Cieniewski-Bernard C, Leroy Y, Michalski JC, Mounier Y, and Bastide B**. O-linked N-acetylglucosaminylation is involved in the Ca^{2+} activation properties of rat skeletal muscle. *J Biol Chem* 282: 10360-10369, 2007.
74. **Heeley DH, Golosinska K, and Smillie LB**. The effects of troponin T fragments T1 and T2 on the binding of nonpolymerizable tropomyosin to F-actin in the presence and absence of troponin I and troponin C. *J Biol Chem* 262: 9971-9978, 1987.
75. **Hennig R, and Lomo T**. Firing patterns of motor units in normal rats. *Nature* 314: 164-166, 1985.
76. **Holloszy JO, and Booth FW**. Biochemical adaptations to endurance exercise in muscle. *Annu Rev Physiol* 38: 273-291, 1976.
77. **Houston ME, Green HJ, and Stull JT**. Myosin light chain phosphorylation and isometric twitch potentiation in intact human muscle. *Pflugers Arch* 403: 348-352, 1985.
78. **Huxley AF**. Cross-bridge action: present views, prospects, and unknowns. *Journal of Biomechanics* 33: 1189-1195, 2000.
79. **Huxley AF, and Niedergerke R**. Structural Changes in Muscle During Contraction: Interference Microscopy of Living Muscle Fibres. *Nature* 173: 971-973, 1954.
80. **Huxley H, and Hanson J**. Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature* 173: 973-976, 1954.
81. **Inashima S, Matsunaga S, Yasuda T, and Wada M**. Effect of endurance training and acute exercise on sarcoplasmic reticulum function in rat fast- and slow-twitch skeletal muscles. *Eur J Appl Physiol* 89: 142-149, 2003.
82. **Irving M, St Claire Allen T, Sabido-David C, Craik JS, Brandmeier B, Kendrick-Jones J, Corrie JE, Trentham DR, and Goldman YE**. Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle. *Nature* 375: 688-691, 1995.
83. **Jung HS, Komatsu S, Ikebe M, and Craig R**. Head-head and head-tail interaction: a general mechanism for switching off myosin II activity in cells. *Mol Biol Cell* 19: 3234-3242, 2008.

84. **Kamm KE, and Stull JT**. Dedicated Myosin Light Chain Kinases with Diverse Cellular Functions. *J Biol Chem* 276: 4527-4530, 2001.
85. **Kamm KE, and Stull JT**. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol* 25: 593-620, 1985.
86. **Karatzaferi C, Franks-Skiba K, and Cooke R**. Inhibition of shortening velocity of skinned skeletal muscle fibers in conditions that mimic fatigue. *Am J Physiol Regul Integr Comp Physiol* 294: R948-R955, 2008.
87. **Karatzaferi C, Giakas G, and Ball D**. Fatigue profile: a numerical method to examine fatigue in cycle ergometry. *Eur J Appl Physiol Occup Physiol* 80: 508-510, 1999.
88. **Kemi OJ, Haram PM, Loennechen JP, Osnes JB, Skomedal T, Wisloff U, and Ellingsen O**. Moderate vs. high exercise intensity: differential effects on aerobic fitness, cardiomyocyte contractility, and endothelial function. *Cardiovasc Res* 67: 161-172, 2005.
89. **Kent-Braun JA, Fitts RH, and Christie A**. Skeletal muscle fatigue. *Compr Physiol* 2: 997-1044, 2012.
90. **Kreppel LK, and Hart GW**. Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats. *J Biol Chem* 274: 32015-32022, 1999.
91. **Krinke GJ**. *The laboratory rat*. Academic press, 2000.
92. **Kruger M, and Linke WA**. The giant protein titin: a regulatory node that integrates myocyte signaling pathways. *J Biol Chem* 286: 9905-9912, 2011.
93. **Levine RJ, Kensler RW, Yang Z, Stull JT, and Sweeney HL**. Myosin light chain phosphorylation affects the structure of rabbit skeletal muscle thick filaments. *Biophys J* 71: 898-907, 1996.
94. **Lionne C, Brune M, Webb MR, Travers F, and Barman T**. Time resolved measurements show that phosphate release is the rate limiting step on myofibrillar ATPases. *FEBS Letters* 364: 59-62, 1995.
95. **Lomo T, Westgaard RH, and Dahl HA**. Contractile properties of muscle: control by pattern of muscle activity in the rat. *Proc R Soc Lond B Biol Sci* 187: 99-103, 1974.
96. **Lowey S, and Trybus KM**. Common structural motifs for the regulation of divergent class II myosins. *J Biol Chem* 285: 16403-16407, 2010.
97. **Lundberg IE**. Idiopathic inflammatory myopathies: why do the muscles become weak? *Curr Opin Rheumatol* 13: 457-460, 2001.
98. **Lunde IG, Aronsen JM, Kvaloy H, Qvigstad E, Sjaastad I, Tonnessen T, Christensen G, Gronning-Wang LM, and Carlson CR**. Cardiac O-GlcNAc signaling is increased in hypertrophy and heart failure. *Physiol Genomics* 44: 162-172, 2012.
99. **Lunde PK, Dahlstedt AJ, Bruton JD, Lannergren J, Thoren P, Sejersted OM, and Westerblad H**. Contraction and Intracellular Ca^{2+} Handling in Isolated Skeletal Muscle of Rats With Congestive Heart Failure. *Circ Res* 88: 1299-1305, 2001.
100. **Lunde PK, Sejersted OM, Thorud HMS, Tonnessen T, Henriksen UL, Christensen G, Westerblad H, and Bruton J**. Effects of Congestive Heart Failure on Ca^{2+} Handling in Skeletal Muscle During Fatigue. *Circ Res* 98: 1514-1519, 2006.
101. **Lunde PK, Sjaastad I, Schiøts-Thorud HM, and Sejersted OM**. Skeletal muscle disorders in heart failure. *Acta Physiol Scand* 171: 277-294, 2001.
102. **Lunde PK, Verburg E, Eriksen M, and Sejersted OM**. Contractile properties of in situ perfused skeletal muscles from rats with congestive heart failure. *J Physiol* 540: 571-580, 2002.
103. **Ma J, and Hart GW**. O-GlcNAc profiling: from proteins to proteomes. *Clin Proteomics* 11: 8, 2014.
104. **Ma J, and Hart GW**. Protein O-GlcNAcylation in diabetes and diabetic complications. *Expert Rev Proteomics* 10: 365-380, 2013.
105. **MacIntosh BR, and Bryan SN**. Potentiation of shortening and velocity of shortening during repeated isotonic tetanic contractions in mammalian skeletal muscle. *Pflugers Arch* 443: 804-812, 2002.
106. **MacIntosh BR, Taub EC, Dormer GN, and Tomaras EK**. Potentiation of isometric and isotonic contractions during high-frequency stimulation. *Pflugers Arch* 456: 449-458, 2008.

107. **Mahmood T, and Yang P-C**. Western Blot: Technique, Theory, and Trouble Shooting. *N Am J Med Sci* 4: 429-434, 2012.
108. **Mardini IA, and McCarter RJ**. Contractile properties of the shortening rat diaphragm in vitro. *J Appl Physiol* 62: 1111-1116, 1987.
109. **Marsh SA, Collins HE, and Chatham JC**. Protein O-GlcNAcylation and cardiovascular (patho)physiology. *J Biol Chem* 289: 34449-34456, 2014.
110. **Marshall S, Bacote V, and Traxinger RR**. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem* 266: 4706-4712, 1991.
111. **Maughan D, and Toth M**. Discerning primary and secondary factors responsible for clinical fatigue in multisystem diseases. *Biology (Basel)* 3: 606-622, 2014.
112. **Metzger JM, Greaser ML, and Moss RL**. Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. Implications for twitch potentiation in intact muscle. *J Gen Physiol* 93: 855-883, 1989.
113. **Miller MS, Callahan DM, and Toth MJ**. Skeletal muscle myofilament adaptations to aging, disease, and disuse and their effects on whole muscle performance in older adult humans. *Front Physiol* 5: 369, 2014.
114. **Moore RL, Houston ME, Iwamoto GA, and Stull JT**. Phosphorylation of rabbit skeletal muscle myosin in situ. *J Cell Physiol* 125: 301-305, 1985.
115. **Moore RL, and Stull JT**. Myosin light chain phosphorylation in fast and slow skeletal muscles in situ. *Am J Physiol Cell Physiol* 247: C462-C471, 1984.
116. **Moorhead G, Johnson D, Morrice N, and Cohen P**. The major myosin phosphatase in skeletal muscle is a complex between the beta-isoform of protein phosphatase 1 and the MYPT2 gene product. *FEBS Lett* 438: 141-144, 1998.
117. **Moraska A, Deak T, Spencer RL, Roth D, and Fleshner M**. Treadmill running produces both positive and negative physiological adaptations in Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1321-1329, 2000.
118. **Mounier Y, Tiffreau V, Montel V, Bastide B, and Stevens L**. Phenotypical transitions and Ca^{2+} activation properties in human muscle fibers: effects of a 60-day bed rest and countermeasures. *J Appl Physiol* 106: 1086-1099, 2009.
119. **Munkvik M, Lunde PK, and Sejersted OM**. Causes of fatigue in slow-twitch rat skeletal muscle during dynamic activity. *Am J Physiol Regul Integr Comp Physiol* 297: R900-910, 2009.
120. **Munkvik M, Rehn TA, Slettalokken G, Hasic A, Hallen J, Sjaastad I, Sejersted OM, and Lunde PK**. Training effects on skeletal muscle calcium handling in human chronic heart failure. *Med Sci Sports Exerc* 42: 847-855, 2010.
121. **Myslicki JP, Belke DD, and Shearer J**. Role of O-GlcNAcylation in nutritional sensing, insulin resistance and in mediating the benefits of exercise. *Appl Physiol Nutr Metab* 39: 1205-1213, 2014.
122. **Nagel AK, and Ball LE**. O-GlcNAc transferase and O-GlcNAcase: achieving target substrate specificity. *Amino Acids* 46: 2305-2316, 2014.
123. **Nandi A, Sprung R, Barma DK, Zhao Y, Kim SC, Falck JR, and Zhao Y**. Global identification of O-GlcNAc-modified proteins. *Anal Chem* 78: 452-458, 2006.
124. **Nelson BA, Robinson KA, Koning JS, and Buse MG**. Effects of exercise and feeding on the hexosamine biosynthetic pathway in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 272: E848-855, 1997.
125. **O'Brien PJ**. Calcium sequestration by isolated sarcoplasmic reticulum: real-time monitoring using ratiometric dual-emission spectrofluorometry and the fluorescent calcium-binding dye indo-1. *Mol Cell Biochem* 94: 113-119, 1990.
126. **Ortenblad N, Lunde PK, Levin K, Andersen JL, and Pedersen PK**. Enhanced sarcoplasmic reticulum Ca(2+) release following intermittent sprint training. *Am J Physiol Regul Integr Comp Physiol* 279: R152-160, 2000.
127. **Ortenblad N, Sjogaard G, and Madsen K**. Impaired sarcoplasmic reticulum Ca(2+) release rate after fatiguing stimulation in rat skeletal muscle. *J Appl Physiol (1985)* 89: 210-217, 2000.

128. **Ortenblad N, Westerblad H, and Nielsen J**. Muscle glycogen stores and fatigue. *J Physiol* 591: 4405-4413, 2013.
129. **Park J, Kwon H, Kang Y, and Kim Y**. Proteomic analysis of O-GlcNAc modifications derived from streptozotocin and glucosamine induced beta-cell apoptosis. *J Biochem Mol Biol* 40: 1058-1068, 2007.
130. **Paruchuri VD, and Zachara NE**. Defining the heart and cardiovascular O-GlcNAcome: a review of approaches and methods. *Circ Cardiovasc Genet* 4: 710, 2011.
131. **Pedersen TH, Clausen T, and Nielsen OB**. Loss of force induced by high extracellular [K^+] in rat muscle: effect of temperature, lactic acid and beta2-agonist. *J Physiol* 551: 277-286, 2003.
132. **Perrie WT, Smillie LB, and Perry SB**. A phosphorylated light-chain component of myosin from skeletal muscle. *Biochem J* 135: 151-164, 1973.
133. **Persechini A, Stull JT, and Cooke R**. The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers. *J Biol Chem* 260: 7951-7954, 1985.
134. **Peternelj TT, Marsh SA, Strobel NA, Matsumoto A, Briskey D, Dalbo VJ, Tucker PS, and Coombes JS**. Glutathione depletion and acute exercise increase O-GlcNAc protein modification in rat skeletal muscle. *Mol Cell Biochem* 400: 265-275, 2015.
135. **Piepoli MF, and Crisafulli A**. Pathophysiology of human heart failure: importance of skeletal muscle myopathy and reflexes. *Exp Physiol* 99: 609-615, 2014.
136. **Pires E, Perry SV, and Thomas MAW**. Myosin light-chain kinase, a new enzyme from striated muscle. *FEBS Letters* 41: 292-296, 1974.
137. **Place N, Ivarsson N, Venckunas T, Neyroud D, Brazaitis M, Cheng AJ, Ochala J, Kamandulis S, Girard S, Volungevicius G, Pauzas H, Mekideche A, Kayser B, Martinez-Redondo V, Ruas JL, Bruton J, Truffert A, Lanner JT, Skurvydas A, and Westerblad H**. Ryanodine receptor fragmentation and sarcoplasmic reticulum Ca^{2+} leak after one session of high-intensity interval exercise. *Proc Natl Acad Sci U S A* 112: 15492-15497, 2015.
138. **Ramirez-Correa GA, Jin W, Wang Z, Zhong X, Gao WD, Dias WB, Vecoli C, Hart GW, and Murphy AM**. O-linked GlcNAc modification of cardiac myofilament proteins: a novel regulator of myocardial contractile function. *Circ Res* 103: 1354-1358, 2008.
139. **Ramirez-Correa GA, Ma J, Slawson C, Zeidan Q, Lugo-Fagundo NS, Xu M, Shen X, Gao WD, Caceres V, Chakir K, DeVine L, Cole RN, Marchionni L, Paolocci N, Hart GW, and Murphy AM**. Removal of Abnormal Myofilament O-GlcNAcylation Restores Ca^{2+} Sensitivity in Diabetic Cardiac Muscle. *Diabetes* 64: 3573-3587, 2015.
140. **Ranvier L**. Propriétés et structures différentes des muscles rouges et des muscles blancs chez les lapins et chez les raies. *CR Hebd Acad Sci (Paris)* 1030-1043, 1873.
141. **Rayment I, Rypniewski W, Schmidt-Base K, Smith R, Tomchick D, Benning M, Winkelmann D, Wesenberg G, and Holden H**. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science* 261: 50-58, 1993.
142. **Reardon TF, and Allen DG**. Time to fatigue is increased in mouse muscle at 37°C; the role of iron and reactive oxygen species. *J Physiol* 587: 4705-4716, 2009.
143. **Rehn TA, Munkvik M, Lunde PK, Sjaastad I, and Sejersted OM**. Intrinsic skeletal muscle alterations in chronic heart failure patients: a disease-specific myopathy or a result of deconditioning? *Heart Fail Rev* 17: 421-436, 2012.
144. **Reidy PT, Hinkley JM, Trappe TA, Trappe SW, and Harber MP**. Protein composition of endurance trained human skeletal muscle. *Int J Sports Med* 35: 476-481, 2014.
145. **Reiken S, Lacampagne A, Zhou H, Kherani A, Lehnart SE, Ward C, Huang F, Gaburjakova M, Gaburjakova J, Rosemblit N, Warren MS, He Kl, Yi Gh, Wang J, Burkhoff D, Vassort G, and Marks AR**. PKA phosphorylation activates the calcium release channel (ryanodine receptor) in skeletal muscle: defective regulation in heart failure. *J Cell Biol* 160: 919-928, 2003.
146. **Reiser PJ, Moss RL, Giulian GG, and Greaser ML**. Shortening velocity in single fibers from adult rabbit soleus muscles is correlated with myosin heavy chain composition. *J Biol Chem* 260: 9077-9080, 1985.

147. **Richter EA, and Hargreaves M**. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93: 993-1017, 2013.
148. **Roots H, Ball G, Talbot-Ponsonby J, King M, McBeath K, and Ranatunga KW**. Muscle fatigue examined at different temperatures in experiments on intact mammalian (rat) muscle fibers. *J Appl Physiol* 106: 378-384, 2009.
149. **Russell JC, Epling WF, Pierce D, Amy RM, and Boer DP**. Induction of voluntary prolonged running by rats. *J Appl Physiol* 63: 2549-2553, 1987.
150. **Ryder JW, Lau KS, Kamm KE, and Stull JT**. Enhanced Skeletal Muscle Contraction with Myosin Light Chain Phosphorylation by a Calmodulin-sensing Kinase. *J Biol Chem* 282: 20447-20454, 2007.
151. **Sahlin K, Tonkonogi M, and Soderlund K**. Energy supply and muscle fatigue in humans. *Acta Physiol Scand* 162: 261-266, 1998.
152. **Sale DG**. Postactivation potentiation: role in human performance. *Exerc Sport Sci Rev* 30: 138-143, 2002.
153. **Saltin B, Henriksson J, Nygaard E, Andersen P, and Jansson E**. Fiber types and metabolic potentials of skeletal muscles in sedentary man and endurance runners. *Ann N Y Acad Sci* 301: 3-29, 1977.
154. **Sanbe A, Fewell JG, Gulick J, Osinska H, Lorenz J, Hall DG, Murray LA, Kimball TR, Witt SA, and Robbins J**. Abnormal Cardiac Structure and Function in Mice Expressing Nonphosphorylatable Cardiac Regulatory Myosin Light Chain 2. *J Biol Chem* 274: 21085-21094, 1999.
155. **Sandow A**. Excitation-Contraction Coupling in Muscular Response. *Yale J Biol Med* 25: 176-201, 1952.
156. **Schiaffino S, and Reggiani C**. Fiber types in mammalian skeletal muscles. *Physiol Rev* 91: 1447-1531, 2011.
157. **Schluter JM, and Fitts RH**. Shortening velocity and ATPase activity of rat skeletal muscle fibers: effects of endurance exercise training. *Am J Physiol* 266: C1699-1673, 1994.
158. **Sellevold OF, Jynge P, and Aarstad K**. High performance liquid chromatography: a rapid isocratic method for determination of creatine compounds and adenine nucleotides in myocardial tissue. *J Mol Cell Cardiol* 18: 517-527, 1986.
159. **Seow CY, and Stephens NL**. Fatigue of mouse diaphragm muscle in isometric and isotonic contractions. *J Appl Physiol* 64: 2388-2393, 1988.
160. **Singh JP, Zhang K, Wu J, and Yang X**. O-GlcNAc signaling in cancer metabolism and epigenetics. *Cancer Lett* 356: 244-250, 2015.
161. **Sjaastad I, Sejersted OM, Ilebekk A, and Bjornerheim R**. Echocardiographic criteria for detection of postinfarction congestive heart failure in rats. *J Appl Physiol* 89: 1445-1454, 2000.
162. **Soukup T, Zacharova G, and Smerdu V**. Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta Histochem* 104: 399-405, 2002.
163. **Squire JM, Al‐khayat HA, Knupp C, and Luther PK**. Molecular Architecture in Muscle Contractile Assemblies. In: *Advances in Protein Chemistry*Academic Press, 2005, p. 17-87.
164. **Steinbacher P, and Eckl P**. Impact of oxidative stress on exercising skeletal muscle. *Biomolecules* 5: 356-377, 2015.
165. **Stevens L, Bastide B, Hedou J, Cieniewski-Bernard C, Montel V, Cochon L, Dupont E, and Mounier Y**. Potential regulation of human muscle plasticity by MLC2 post-translational modifications during bed rest and countermeasures. *Arch Biochem Biophys* 540: 125-132, 2013.
166. **Stryer L, Berg J, and Tymoczko J**. Section 18.6: The Regulation of Cellular Respiration Is Governed Primarily by the Need for ATP. *Biochemistry* 2002.
167. **Stull JT, Kamm KE, and Vandenboom R**. Myosin light chain kinase and the role of myosin light chain phosphorylation in skeletal muscle. *Arch Biochem Biophys* 510: 120-128, 2011.
168. **Stull JT, Nunnally MH, Moore RL, and Blumenthal DK**. Myosin light chain kinases and myosin phosphorylation in skeletal muscle. *Adv Enzyme Reg* 23: 123-IN122, 129, 1985.
169. **Sweeney HL, Bowman BF, and Stull JT**. Myosin light chain phosphorylation in vertebrate striated muscle: regulation and function. *Am J Physiol Cell Physiol* 264: C1085-C1095, 1993.

170. **Sweeney HL, and Houdusse A**. The motor mechanism of myosin V: insights for muscle contraction. *Philos Trans R Soc Lond B Biol Sci* 359: 1829-1841, 2004.
171. **Sweeney HL, and Kushmerick MJ**. Myosin phosphorylation in permeabilized rabbit psoas fibers. *Am J Physiol* 249: C362-365, 1985.
172. **Sweeney HL, and Stull JT**. Alteration of cross-bridge kinetics by myosin light chain phosphorylation in rabbit skeletal muscle: implications for regulation of actin-myosin interaction. *Proc Natl Acad Sci U S A* 87: 414-418, 1990.
173. **Sweeney HL, and Stull JT**. Phosphorylation of myosin in permeabilized mammalian cardiac and skeletal muscle cells. *Am J Physiol Cell Physiol* 250: C657-C660, 1986.
174. **Sweeney HL, Yang Z, Zhi G, Stull JT, and Trybus KM**. Charge replacement near the phosphorylatable serine of the myosin regulatory light chain mimics aspects of phosphorylation. *Proc Natl Acad Sci U S A* 91: 1490-1494, 1994.
175. **Toepfer C, Caorsi V, Kampourakis T, Sikkel MB, West TG, Leung MC, Al-Saud SA, MacLeod KT, Lyon AR, Marston SB, Sellers JR, and Ferenczi MA**. Myosin regulatory light chain (RLC) phosphorylation change as a modulator of cardiac muscle contraction in disease. *J Biol Chem* 288: 13446-13454, 2013.
176. **Torres CR, and Hart GW**. Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *J Biol Chem* 259: 3308-3317, 1984.
177. **Tschakovsky ME, and Hughson RL**. Interaction of factors determining oxygen uptake at the onset of exercise. *J Appl Physiol* 86: 1101-1113, 1999.
178. **van Dijk SJ, Bezold KL, and Harris SP**. Earning stripes: myosin binding protein-C interactions with actin. *Pflugers Arch* 466: 445-450, 2014.
179. **Vandenboom R, Gittings W, Smith IC, Grange RW, and Stull JT**. Myosin phosphorylation and force potentiation in skeletal muscle: evidence from animal models. *J Muscle Res Cell Motil* 34: 317-332, 2013.
180. **Vandenboom R, Grange RW, and Houston ME**. Myosin phosphorylation enhances rate of force development in fast-twitch skeletal muscle. *Am J Physiol* 268: C596-603, 1995.
181. **Vedsted P, Larsen AH, Madsen K, and Sjogaard G**. Muscle performance following fatigue induced by isotonic and quasi-isometric contractions of rat extensor digitorum longus and soleus muscles in vitro. *Acta Physiol Scand* 178: 175-186, 2003.
182. **Wang Y, Wisloff U, and Kemi OJ**. Animal models in the study of exercise-induced cardiac hypertrophy. *Physiol Res* 59: 633-644, 2010.
183. **Westerblad H, Allen DG, and Lannergren J**. Muscle fatigue: lactic acid or inorganic phosphate the major cause? *News Physiol Sci* 17: 17-21, 2002.
184. **Westerblad H, Bruton JD, Allen DG, and Lannergren J**. Functional significance of Ca^{2+} in long-lasting fatigue of skeletal muscle. *Eur J Appl Physiol* 83: 166-174, 2000.
185. **Westerblad H, Lännergren J, and Allen DG**. Slowed Relaxation in Fatigued Skeletal Muscle Fibers of Xenopus and Mouse. *J Gen Physiol* 109: 385-399, 1997.
186. **Wisloff U, Helgerud J, Kemi OJ, and Ellingsen O**. Intensity-controlled treadmill running in rats: $VO_{2\ max}$ and cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 280: H1301-1310, 2001.
187. **Wolosker H, Rocha JB, Engelender S, Panizzutti R, De Miranda J, and de Meis L**. Sarco/endoplasmic reticulum Ca^{2+}-ATPase isoforms: diverse responses to acidosis. *Biochem J* 321 (Pt 2): 545-550, 1997.
188. **Wu J, Lenchik NJ, Pabst MJ, Solomon SS, Shull J, and Gerling IC**. Functional characterization of two-dimensional gel-separated proteins using sequential staining. *Electrophoresis* 26: 225-237, 2005.
189. **Yamaguchi M, Kimura M, Li ZB, Ohno T, Takemori S, Hoh JF, and Yagi N**. X-Ray Diffraction Analysis of the Effects of Myosin Regulatory Light Chain Phosphorylation and Butanedione Monoxime on Skinned Skeletal Muscle Fibers. *Am J Physiol Cell Physiol* ajpcell 00318 02015, 2016.

190. **Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, Kudlow JE, Michell RH, Olefsky JM, Field SJ, and Evans RM**. Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. *Nature* 451: 964-969, 2008.
191. **Yki-Jarvinen H, Virkamaki A, Daniels MC, McClain D, and Gottschalk WK**. Insulin and glucosamine infusions increase O-linked N-acetyl-glucosamine in skeletal muscle proteins in vivo. *Metabolism* 47: 449-455, 1998.
192. **Zachara NE**. Detection and analysis of (O-linked beta-N-acetylglucosamine)-modified proteins. *Methods Mol Biol* 464: 227-254, 2009.
193. **Zachara NE**. The roles of O-linked beta-N-acetylglucosamine in cardiovascular physiology and disease. *Am J Physiol Heart Circ Physiol* 302: H1905-1918, 2012.
194. **Zhi G, Ryder JW, Huang J, Ding P, Chen Y, Zhao Y, Kamm KE, and Stull JT**. Myosin light chain kinase and myosin phosphorylation effect frequency-dependent potentiation of skeletal muscle contraction. *Proc Natl Acad Sci U S A* 102: 17519-17524, 2005.
195. **Zizola C, and Schulze PC**. Metabolic and structural impairment of skeletal muscle in heart failure. *Heart Fail Rev* 18: 623-630, 2013.
196. **Åstrand P-O, and Rodahl K**. *Textbook of work physiology : physiological bases of exercise*. Champaign, Ill: Human Kinetics, 2003.

Errata list

Cor – correction of language
Cpltf – change of page layout or text format

Page	Line	Original text	Type of correction	Corrected text
9	4	swaddling..running uphill	Cor	cuddling..uphill running
9	24	was	Cor	were
16, 17	Figure	font 10	Cpltf	font 11
16, 18	Figure	Curtesy	Cor	Courtesy
27	18	post-infraction	Cor	post-infarction
30	26	sedate control	Cor	sedentary controls
33	32	it	Cor	is
45	5	However, contradict	Cor	However, this seems to contradict
49	20-32	Missing punctuation	Cpltf	Punctuation inserted
49	22	disassociation	Cor	dissociation
49	31	or	Cor	nor
49	32	explanation	Cor	explain