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Cellular and Molecular Mechanisms of Skeletal Muscle Atrophy after Spinal Cord Injury

Mladen Savikj

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To my mother.

“And the boy loved the tree very much.

And the tree was happy.“

Shel Silverstein,

The Giving Tree

ABSTRACT

Spinal cord injury causes intense changes in whole-body physiology, with negative consequences spanning most bodily systems. Among such consequences is a rapid and profound loss of skeletal muscle mass, which promotes metabolic disturbances and contributes to increased risk of type 2 diabetes and cardiovascular disease in spinal cord-injured individuals. Thus, further development of rehabilitative and preventive strategies for alleviating muscle wasting may improve the health of individuals with spinal cord injury. Deeper understanding of the mechanisms underlying skeletal muscle atrophy following spinal cord injury may inform these efforts.

Skeletal muscle mass is regulated, at the molecular level, through balance in protein turnover. Disturbances in this balance, favouring protein degradation over synthesis, lead to skeletal muscle atrophy. Markers of both protein synthesis and degradation decrease into the chronic phase of spinal cord injury. However, a comprehensive timeline of such changes following injury in human skeletal muscle remains unknown. Furthermore, skeletal muscle atrophy is accompanied by increased reactive oxygen species production and oxidative stress. Oxidative stress is proposed to promote muscle wasting through increased protein degradation, decreased protein synthesis and activation of apoptosis. Whether reactive oxygen species homeostasis in human skeletal muscle following spinal cord injury is disturbed remains to be determined. Additionally, satellite cells are myogenic stem cells, which are indispensable to skeletal muscle regeneration and play a role in regulating muscle mass. Spinal cord injury leads to satellite cell activation in murine models, while human skeletal muscle in the late post-injury stages contains less satellite cells per skeletal muscle fibre. However, whether spinal cord injury leads to a defect in satellite cell differentiation capacity remains unknown. The studies in this thesis attempt to elucidate some of these events, which may contribute to muscle atrophy following spinal cord injury.

The first study of this thesis examines the protein content and phosphorylation of regulators of protein synthesis and degradation in skeletal muscle at one, three and 12 months spinal cord injury. Our results suggest that protein translation and autophagy are more active in the early post-injury phase, and reduce by 12 months post-injury. Conversely, the ubiquitin-proteasome system remains more active throughout the first year. The second study investigates oxidative stress markers, enzymes involved in reactive oxygen species generation and decomposition, and apoptotic signalling in skeletal muscle at one, three, and 12 months following spinal cord injury. Our data indicate increased non-mitochondrial reactive oxygen species production, apoptotic signalling, and antioxidant defences in the early post-injury phases. Conversely, 12 months post-injury we detect decreased mitochondrial content. Finally, we studied the differentiation capacity of skeletal muscle satellite cells *in vitro* from individuals with a longstanding spinal cord injury. Our results suggest that the intrinsic differentiation capacity of satellite cells is retained following spinal cord injury and that they are able to fuse and form multinucleated myotubes with normal metabolic characteristics.

Collectively, the work presented in this thesis indicates that changes in protein turnover are most profound during the initial rapid phase of atrophy. Rehabilitative interventions during this phase may be more efficacious in alleviating skeletal muscle atrophy. Furthermore, this stage is accompanied by increased non-mitochondrial ROS production and increased antioxidant capacity. Further investigations into ROS production during these stages may lead to development of adjuvant antioxidant therapies promoting the beneficial effects of rehabilitation. Finally, satellite cells are more responsive to higher intensity exercise modalities. Hence, conserved satellite cell intrinsic differentiation capacity encourages studies into the efficacy of resistance-type electrically stimulated training in activating satellite cells and alleviating muscle atrophy following spinal cord injury. Overall, the findings presented in this thesis provide insight into some of the mechanisms underlying muscle wasting following spinal cord injury and may provide support to further development of rehabilitative strategies.

LIST OF PAPERS

The individual papers listed below will be referred to by their Roman numerals.

I. Lundell LS, **Savikj M**, Kostovski E, Iversen PO, Zierath JR, Krook A, Chibalin AV, Widegren U. "Protein translation, proteolysis and autophagy in human skeletal muscle atrophy after spinal cord injury." Acta Physiol 2018; 223:e13051.

II. **Savikj M**, Kostovski E, Lundell LS, Iversen PO, Massart J, Widegren U. "Altered oxidative stress and antioxidant defences in skeletal muscle during the first year following spinal cord injury" Submitted manuscript

III. **Savikj M**, Ruby MA, Kostovski E, Iversen PO, Zierath JR, Krook A, Widegren U. "Retained differentiation capacity of human skeletal muscle satellite cells from spinal cord-injured individuals." Physiol Rep. 2018; 6:e13739.

CONTENTS

LIST OF ABBREVIATIONS

4EBP1	eIF4E-binding protein
4HNE	4-hydroxynonenal
AB	Able-bodied
ACC	Acetyl coenzyme A carboxylase
AIS	ASIA impairment scale
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ASIA	American Spinal Injury Association
Atg7	Autophagy-related protein 7
ATP	Adenosine triphosphate
ATP5A	ATP synthase F1 subunit alpha
BMI	Body mass index
BSA	Bovine serum albumin
CSA	Cross sectional area
DMEM	Dulbecco's Modified Eagle Medium
DNP	2,4-dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
EDTA	Ethylenediaminetetraacetic acid
eIF2	Eukaryotic initiation factor 2
eIF4B	Eukaryotic initiation factor 4B
eIF4E	Eukaryotic initiation factor 4E
eIF4F	Eukaryotic initiation factor 4F
FBS	Foetal bovine serum
FoxO	Forkhead box protein O
GLUT4	Glucose transporter type 4
gp91phox	Cytochrome B-245 ß chain/NADPH oxidase 2
GPx	Glutathione peroxidase
GRx	Glutathione reductase
GSH	Glutathione (reduced)
GSK3ß	Glycogen synthase kinase 3ß
GSSG	Glutathione disulfide (oxidized)
GTP	Guanosine triphosphate
IGF1	Insulin-like growth factor 1
JNK	c-Jun N-terminal kinase
LC3	Microtubule-associated proteins 1A/1B-light chain 3
m	Month
MAFbx	Muscle atrophy F-box protein/atrogin1
MRFs	Myogenic regulatory factors
MTCO2	Mitochondrial encoded cytochrome C oxidase II
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MuRF1	Muscle RING-finger protein 1
Myf5	Myogenic factor 5
Myf6	Myogenic factor 6
Myh1	Myosin heavy chain 1
Myh2	Myosin heavy chain 2
Myh7	Myosin heavy chain 7
MyHC	Myosin heavy chain
MyHC I	Myosin heavy chain type I
MyHC II	Myosin heavy chain type II
MyoD	Myogenic differentiation protein
NAD+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCF1	Neutrophil Cytosolic Factor 1 / p47phox
NDUFB8	NADH ubiquinone oxidoreductase subunit B8
NOX	NADPH oxidases
NOX2	NADPH oxidase 2
NOX4	NADPH oxidase 4
p62	Sequestosome-1/p62
p70S6K	Ribosomal protein S6 kinase
PAGE	Polyacrylamide gel electrophoresis
Pax7	Paired box protein 7
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time polymerase chain reaction
Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
RPLP0	60S acidic ribosomal protein P0
S6	40S ribosomal protein S6
SCI	Spinal cord-injured
SDHB	Succinate dehydrogenase complex iron sulfur subunit B
SDS	Sodium dodecyl sulfate
Ser	Serine
SOD	Superoxide dismutase
SOD1	[Cu-Zn] Superoxide dismutase (cytosolic)
SOD2	[Mn] Superoxide dismutase (mitochondrial)
SOD3	[Cu-Zn] Superoxide dismutase (extracellular)
TBP	TATA-binding protein
TBS	Tris-buffered saline
TCA	Trichloracetic acid
Thr	Threonine
TRAF6	TNF receptor associated factor 6
Trim32	Tripartite motif-containing protein 32
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
TUNEL	Termina deoxynucleotidyl transferase dUTP nick end-labelled
Tyr	Tyrosine
UQCRC2	Ubiquinol-cytochrome C reductase core protein 2
XO	Xanthine oxidase
y	Year

1 INTRODUCTION

1.1 EPIDEMIOLOGY OF SPINAL CORD INJURY

The spinal cord is the major conduit for exchange of information between the central nervous system and the body. It relays efferent signals from the primary motor cortex to the effector skeletal muscle as well as afferent sensory signals to the primary sensory cortex. Additionally, the spinal cord transmits autonomic nervous system signals regulating visceral organ function. Traumatic injuries to the spinal cord cause a disruption in the transmission of these signals and carry profound impairments in whole-body physiology leading to intense changes in both the quality and style of life in affected individuals.

The annual incidence of spinal cord injury in developed countries varies widely. In the Nordic countries incidence of spinal cord injury ranges between 9.2 and 16.5 per million inhabitants per year (1-3). On the other hand, incidence rates as high as 39.4, 39.7 and 40.1 per million inhabitants per year have been reported in Japan, Estonia, and the United States, respectively (4-6). Concurrently, the prevalence of spinal cord injury ranges from 250 to 906 cases per million population (7). Spinal cord injury predominantly occurs in younger individuals (people 16–30 years of age account for ~35% of all injuries) and is mainly due to high-impact trauma events such as vehicular accidents (accounting for ~40% of all injuries) (8). While this profile of spinal cord injury remains true over time, the last decades have seen an increase in fall-related injuries (accounting for ~30% of all injuries), especially in older age groups (~25% of all injuries occurring in people 46–60 years of age). Modern advances in treatment have led to increased life expectancy following spinal cord injury which is slowly approaching that of the able-bodied population (9). Societies face substantial economic impact from spinal cord injury due to the cost of treatment as well as low return-to-work rates in the injured population (7, 10).

Spinal cord injury is defined by the level of injury and the extent of the consequential neurological deficit. The neurological level of injury is represented by the most distal segment of the spinal cord retaining bilateral sensory and antigravity motor function, with intact neurological function in more rostral segments (11). Furthermore, spinal cord injury can be classified by injury severity. Complete injury is regarded as complete absence of sensory and motor function in the distal sacral segments of the cord, while incomplete is characterized by any remaining neurological function below injury level (11). The severity of the injury can be expressed by the American Spinal Injury Association (ASIA) impairment scale (AIS) on a scale from A to E (11). Complete neurological deficit is graded as A, while normal preserved motor and sensory function is graded as E. Sensory incomplete but motor complete injuries are graded by B, while incomplete motor injuries are graded by C and D, depending on the extent of remaining motor function measured by the AIS motor score. The score grades the functional capacity in five key muscle groups on each extremity on a scale 0–5 with a maximal total score of 25 representing normal motor function of the limb (sum of 50 for upper or lower extremities) (11). If more than half of the muscle groups below injury

level have a motor score lower than three the injury is graded as incomplete C. Conversely, if at least half of the muscle groups retain a score of three or more, the injury is graded as incomplete D. Injuries to the cervical segments and tetraplegia are more common (~59% of cases) than injuries to thoracic, lumbar or sacral segments and paraplegia (8). The most common types of spinal cord injury are incomplete tetraplegia (~35% of all cases), followed by complete paraplegia, complete tetraplegia and incomplete paraplegia (~20% each) (9).

Thus, depending on the injury level and severity, spinal cord injury can result in varying degrees of functional impairments and exhibit diverse clinical presentation. Furthermore, it typically affects younger populations and leaves a profound, life-long impact on whole-body physiology. In light of the substantial functional impairments of these injuries, comprehensive rehabilitative strategies are warranted to improve the well-being and life quality of spinal cord-injured individuals.

1.2 SEQUELAE AND COMPLICATIONS OF SPINAL CORD INJURY

Although this thesis focuses on one aspect of spinal cord injury, namely the ensuing skeletal muscle atrophy, it is important to acknowledge the full extent of consequences and ensuing complications that follow the injury in order to grasp its severity. Considering the severing of sensory, motor, and autonomic connections, it is not surprising that the complications of the injury, both in the acute and chronic phase, are numerous and span most bodily systems. While acute complications can be life-threatening and important to recognize, chronic complications can be disruptive to the rehabilitative process. One third of all individuals with spinal cord injury are re-hospitalized at least once within the first year after injury due to complications (12, 13). Thus, there is an evident need for improvement in the treatment and prevention strategies aimed at alleviating complications of spinal cord injury.

1.2.1 Neurological sequelae and complications

Injuries to the spinal cord manifest acutely as spinal shock characterized by flaccid paralysis coupled to a loss of sensory function below the level of injury. Accompanying these is a loss of autonomic neurological function and a transitory areflexia (14). Recovery of neurological function is primarily achieved during the first months to a year after spinal cord injury and depends on severity of focal damage to the spinal cord, the level and extent of the original neurological deficit, as well as the age at onset of injury (15, 16). Although most recovery occurs earlier, functional improvements have been observed five years after injury (17), underscoring the importance of late rehabilitative efforts in individuals living with spinal cord injury.

Conversely, progressive neurological deficit can occur in spinal cord-injured individuals as a consequence of syringomyelia. This condition is marked by formation of fluid-filled cysts within the spinal cord, which commonly develop after three or more months post-injury. Syringomyelia can be asymptomatic or it can present as a dull or burning pain that gradually worsens, coupled with sensory loss in the affected segments, followed by a progressive loss of motor function rostral to the injury (18). The treatment of symptomatic syringomyelia is

surgical and aims to correct the original deformity of the vertebral column and reduce spinal cord compression that might have led to the development of the condition (18). However, the aetiology of the condition remains unclear and thus renders possible prevention strategies difficult. Furthermore, around 40% of individuals with spinal cord injury start experiencing burning or dull pain at or below the injury level within the first year post-injury (19). The aetiology of such neuropathic pain includes direct damage to primary sensory neurons as well as dysregulation of supraspinal centres uncoupled from sensory input (20). Pharmacological and non-pharmacological treatments of neuropathic pain have been utilized with variable efficacy (20), but as neuropathic pain is experienced as excruciating and disturbing (21), pain management after spinal cord injury is important.

1.2.2 Respiratory sequelae and complications

Respiratory complications are the leading cause of death in both the acute and chronic phases following spinal cord injury (22). The acute phase of spinal cord injury carries a risk of respiratory failure which depends on the severity and neurological level of injury. The consequential impact on the respiratory musculature is exacerbated by increased airway mucus secretion and accumulation (23). The combination of these factors often indicates intubation and ventilation-support in the acute phase of spinal cord injury (24). Over time, the paralysis of intercostal musculature leads to constrictive reduction in lung capacity (25) as well as posture-dependent strains on the diaphragm which can lead to fatigue (26). Injury-impaired respiratory function, reduced cough capacity and a blunted immune response lead to a high incidence of infectious pneumonia and mortality in spinal cord-injured patients (22, 27).

1.2.3 Cardiovascular sequelae and complications

The acute phase of spinal cord injury can be dramatic. In addition to respiratory failure, a state of neurogenic shock can result from blood loss due to trauma, pooling of blood in the paralysed muscle, and bradycardia and vascular hypotonia due the decrease sympathetic stimulation (28). Even in the chronic phase of spinal cord injury, the imbalance in autonomic signals can lead to life-threatening conditions. In individuals with high thoracic injuries (>T6), a noxious stimulus such as bladder or bowel distension can overstimulate sympathetic reflexes causing generalized vasoconstriction. The result is acute hypertension below the level of injury, while compensatory parasympathetic mechanisms above the injury level can cause bradycardia (29). The condition is termed autonomic dysreflexia and it clinically presents as pounding headaches, sweating and nausea coupled with hypertension which can be life-threatening. The treatment is removal of the stimulus, postural correction and antihypertensive therapy for persistent cases. Additionally, the lack of sympathetic and unopposed parasympathetic innervation leads to loss of vascular tone and development of orthostatic (postural) hypotension (30).

Finally, in light of the increased risk factors, including lack of mobility and disturbed glucose and lipid homeostasis (31, 32), it is no surprise that the risk of cardiac and cerebrovascular

disease is two-fold and three-fold higher in the spinal cord-injured population, respectively (33). Cardiovascular disease has approached pulmonary diseases as one of the most frequent cause of death following spinal cord injury (34). Thus, there is a need to develop therapeutic interventions and increase mechanistic understanding of underlying conditions contributing to cardiovascular disease in the spinal cord-injured population.

1.2.4 Endocrine sequelae and complications

Spinal cord injury induces changes in several major endocrine systems regulating bodily functions. Namely, spinal cord-injured individuals have higher glucose and insulin levels in response to an oral glucose tolerance test (35) and lower whole-body peripheral glucose disposal rate during a hyperinsulinemic clamp in comparison to able-bodied controls (31). The underlying cause of such disturbances is likely substantial changes in skeletal muscle mass and morphology (36), as muscle is a major site of peripheral glucose disposal (37).

Furthermore, spinal cord injury leads to an immediate decrease in plasma levels of insulin-like growth factor 1 (IGF1), followed by gradual recovery over the following weeks (38, 39). However IGF1 levels remain lower than in able-bodied individuals even into the chronic post-injury phase (40). While the resting plasma levels of growth hormone are comparable to able-bodied controls, growth hormone release in response to stimuli is blunted in spinal cord-injured individuals (41, 42). Hence, it is likely that a disturbance in hypothalamus – pituitary axis promotes the decrease in IGF1 levels.

Plasma testosterone levels have been shown to be lower in men with longstanding spinal cord injury in comparison to able-bodied controls (43, 44). Follicle stimulating hormone and luteinizing hormone levels have been reported to be unchanged or decreased by spinal cord injury (43, 44). Hypogonadism occurs during early post-injury phases and as much as ~80% of men have been reported to have low serum testosterone levels during the first four months post-injury (45). In the late post injury stage, ~40% of spinal cord-injured have low testosterone levels, with the frequency being higher in complete than incomplete injuries (46).

Decreased thyroid hormone levels in both the acute and chronic phase post-injury have been reported (47, 48). While serum levels of thyroid stimulating hormone are preserved, it has been speculated that diurnal pulse secretion is decreased and promotes hypothyroidism (49). Furthermore, increased hypercalcaemia and hypercalciuria occur in the post-acute stage (50). These increases are attributed to bone demineralization and increased release of calcium and are accompanied by depleted serum levels of parathyroid hormone and calcitriol (50). Similar observations have been made in late post-injury phases (51) indicating that calcium release from the skeletal system persists contributing to osteoporosis. However, some reports indicate that secondary hyperparathyroidism can also occur in a subset of patients with longstanding spinal cord injuries (52).

1.2.5 Immunological sequelae and complications

The onset of spinal cord injury is often accompanied by secondary immunodeficiency. The deficiency takes form of monocyte and lymphocyte depletion, macrophage dysfunction, and reduced antibody synthesis (27, 53, 54). The exact mechanisms that lead to immunodeficiency remain unclear. However, as the deficiency occurs in a neurological level-dependent manner, disruption in sympathetic stimulation of the spleen is believed to be causal (27). The importance of secondary immunodeficiency manifests as susceptibility to respiratory, urinary, gastrointestinal, and skin infections in the spinal cord-injured population (55).

1.2.6 Genitourinary and gastrointestinal sequelae and complications

Spinal cord injury can lead to loss of parasympathetic innervation to the distal segments of the bowel, or loss of voluntary control of the anal sphincter, in both cases resulting in constipation (56). The condition leads to increased risk of infections, significantly reduced quality of life and negative impacts on rehabilitative efforts, due to a tendency for lower re-integration and frequent re-hospitalization in individuals suffering from neurogenic bowel (57).

Most individuals suffer some level of urinary bladder dysfunction after spinal cord injury (9). The clinical presentation of the neurogenic bladder varies depending on the neurological level and severity of injury. Complete sacral lesions lead to urine retention due to an inability to relax bladder sphincters, while higher injuries lead to lack of voluntary control of micturition, incontinence, and residual urine volume in the bladder. Most common complications of neurogenic bladder are urinary tract infections, although more serious conditions such as renal lithiasis and failure can occur (58).

Reduced sexual function is commonly impaired in both men and women suffering from spinal cord injury. After the initial loss of reflexes, the reflexive sexual arousal can return as well as the subjective ability to experience an orgasm (59). However, 95% of men with spinal cord injury have ejaculatory problems leading to impaired fertility (59).

1.2.7 Skeletal sequelae and complications

Skeletal muscle paralysis and ensuing disuse lower the loadbearing force on the skeletal system. Furthermore, vascular hypotonia and venous retention lead to increased osseous intramedullary pressure, which promotes osteoclast activation (60). Moreover, loss of sympathetic innervation is suspected to promote bone resorption and to decrease mineralization (61). Together these factors contribute to increased frequency of osteoporosis and osteopenia following spinal cord injury which can often lead to pathological fractures (62). Furthermore, joint cartilage atrophy is observed in regions below the injury level in an injury severity-dependent manner (63).

1.2.8 Skin sequelae and complications

Decubital ulcers are a common complication that follows immobility and occur as a consequence of sheer force and pressure-obstructed blood flow to the underlying tissues. Following spinal cord injury, pressure ulcers are very common and are one of the leading causes of re-hospitalization (13). In light of susceptibility to secondary infections, decubital ulcers can become a potentially life-threatening conditions and it is therefore important to begin prevention immediately after spinal cord injury.

1.3 SKELETAL MUSCLE ATROPHY

1.3.1 Skeletal muscle morphology after spinal cord injury

Spinal cord injury leads to separation of skeletal muscle from the central nervous system. The ensuing muscle paralysis and disuse lead to a rapid and profound atrophy. By six months after complete spinal cord injury, cross sectional area (CSA) of the *quadriceps femoris* muscle is reduced to ~50% of able-bodied controls (64). Similarly, although less pronounced, individuals with incomplete spinal cord injury have a ~30% lower *quadriceps femoris* CSA than able-bodied controls (65). The loss of muscle mass after injury does not develop linearly. After the initial six weeks of complete spinal cord injury, the average fibre CSA is ~60% of able-bodied controls (36). An additional five weeks bring about a loss of ~30% of the remaining fibre CSA (~40% of able-bodied controls), while by week 24 the fibre CSA is reduced an additional ~10% (~35% of able-bodied controls) (36). Thus, the initial period immediately following injury brings about the most rapid decrease in fibre size, while in the later stages the rate of muscle atrophy decreases and the average fibre size stabilizes into the chronic phase of spinal cord injury (Fig. 1) (66).

The reduction in mass is accompanied by distinct morphological changes. Skeletal muscle after spinal cord injury accumulates substantial amounts of intramuscular fat. In the chronic phase of complete spinal cord injury, the affected skeletal muscle has four times higher intramuscular fat percentage than able-bodied controls (67). Similarly, individuals with incomplete spinal cord injury have a three-fold higher relative intramuscular fat levels than able-bodied controls (67). Additionally, blood-flow reduces concurrently with skeletal muscle mass (68). However, such reductions are proportional to the muscle atrophy, as blood-flow values corrected for muscle mass and per fibre capillarization of tissue are both comparable to able-bodied controls (31, 68).

Finally, atrophy after spinal cord injury is characterized by a change in fibre type composition of the affected skeletal muscle (Fig. 1). By the chronic phase the muscle is composed mainly of type II fast twitch fibres, which replace type I slow twitch fibres (69, 70). The fibre type switch also occurs at a non-linear rate. The initial six months post-injury are accompanied by changes in fast twitch fibres, shifting to more glycolytic type IIx fibres (increase from ~25% to ~45%) at the expense of type IIa fibres (decrease from ~35% to ~10%) (36). Despite remaining steady during the initial six months (36, 71), over time the percentage of type I

fibres decreases dramatically representing less than 25% of all fibres in the chronic phase post-injury (69, 71, 72).

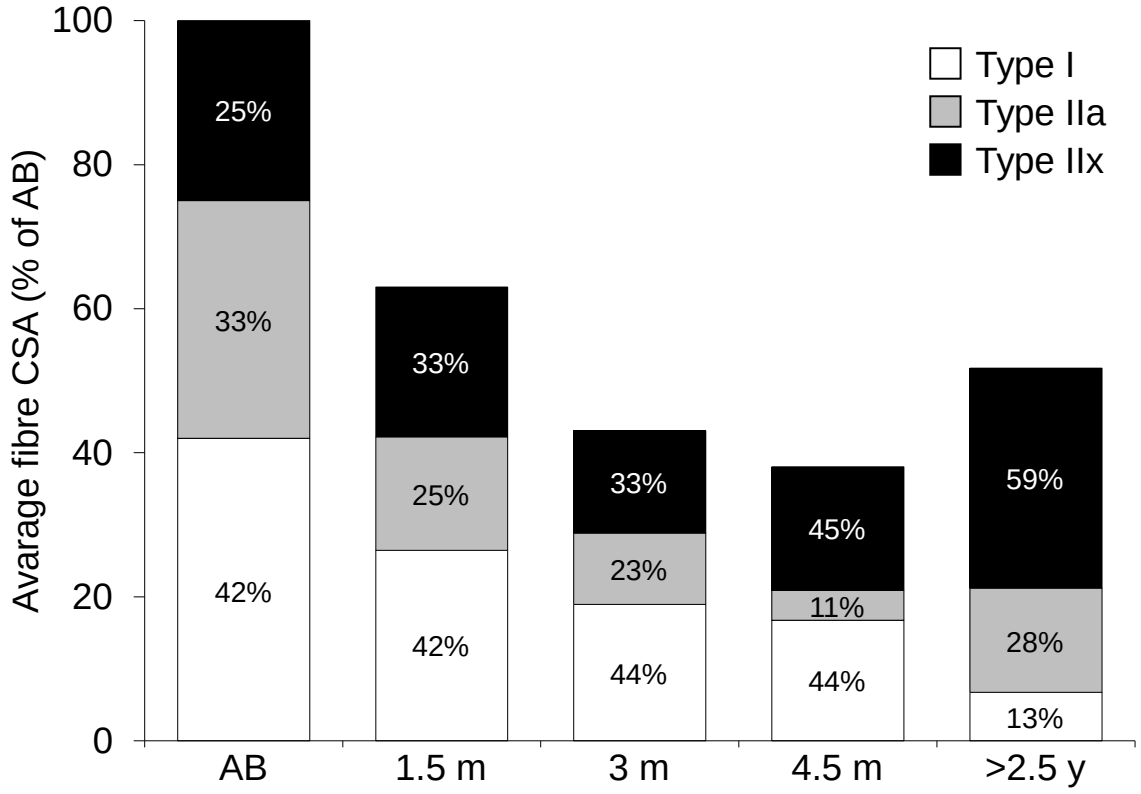

Figure 1 – Generalized model of skeletal muscle atrophy and fibre type changes after spinal cord injury. The model is based on the data from Castro et al. (1999, J. Appl. Physiol.), Gerrits et al. (2003, Pflugers Arch.) and Aksnes et al. (1996, Am J Physiol) (36, 73, 74). The bars represent the average fibre CSA of *vastus lateralis* muscle at 1.5, 3, and 4.5 months (m) and >2.5 year (y) after spinal cord injury as % of able-bodied controls (AB). Percentage of all fibres belonging to type I, type IIa and type IIx fibres is indicated in areas shaded white, grey or black, respectively.

1.3.2 Skeletal muscle metabolic properties after spinal cord injury

Skeletal muscle is not only an essential part of the locomotor system, but also a highly metabolically active tissue which plays an important role in regulating whole-body energy homeostasis. A major portion of peripheral glucose uptake occurs within skeletal muscle and decrease of skeletal muscle capacity to take in and store glucose is a first step towards development of metabolic disease (37, 75, 76). Spinal cord injury and consequential muscle atrophy lead to systemic metabolic disturbances, which contribute to the development and higher incidence of metabolic and cardiovascular disease in this population (33, 77).

Individuals with spinal cord injury display glucose intolerance, with higher plasma insulin and glucose levels than able-bodied controls in response to an oral glucose tolerance test (35). Additionally, they show signs of insulin resistance at the whole-body level, exhibited as a reduced glucose disposal rate during a euglycaemic hyperinsulinemic clamp (31). At the level of skeletal muscle, spinal cord injury leads to coordinated changes in the expression of enzymes responsible for glucose metabolism, with decreases of those responsible for glycogen synthesis and glucose storage, and increases in those involved in glycolysis (78).

However, the skeletal muscle membrane protein content of insulin-dependent glucose transporter type 4 (GLUT4) remains unaltered by spinal cord injury (31). Additionally, both resting and insulin-stimulated glucose uptake measured *ex vivo* in skeletal muscle strips are comparable to able-bodied individuals (31). As a substantial part of peripheral glucose disposal occurs in skeletal muscle (37), it is probable that the loss of muscle mass itself, rather than a defect in insulin action, promotes whole-body insulin resistance in individuals with spinal cord injury.

Furthermore, atrophying skeletal muscle after spinal cord injury shows diminished oxidative capacity and a substantial loss of mitochondrial content (78, 79). The expression of genes involved in fatty acid uptake, transport and oxidation is decreased in comparison to able-bodied controls (78). These changes lead to lower fatty acid uptake and oxidation in skeletal muscle of spinal cord-injured individuals, favouring glucose and glycogen breakdown as an energy source during electrically-stimulated exercise (32). These findings are in line with increased expression of enzymes responsible for glycolysis in skeletal muscle of spinal cord-injured individuals (78) and might be reflective of the distinct fibre type switch from oxidative type I to predominantly glycolytic type II fibres. The decreased oxidative capacity of skeletal muscle coupled with lower utilization of fatty acids as an energy source could contribute to altered blood lipid profile in spinal cord-injured individuals (80).

Metabolic disturbances that occur in skeletal muscle can be alleviated by rehabilitative interventions, such as electrically-stimulated exercise regimens. Protein content of GLUT4, hexokinase 2, and glycogen synthase are all increased in response to eight weeks of electrically-stimulated cycling, which also leads to improvements in glucose homeostasis and skeletal muscle glucose uptake (81, 82). Although fatty acid uptake increases above resting levels during electrically stimulated exercise it remains lower than able-bodied controls (32). Several mechanisms could be responsible for the preference towards glucose utilization as an energy source in skeletal muscle after spinal cord injury. The distinct fibre type change from oxidative to glycolytic likely promotes the fuel utilization preference. In addition, expression of both pyruvate dehydrogenase and carnitine palmitoyltransferase 1 and 2 are decreased following spinal cord injury and an imbalance between the two could lead to glucose breakdown preference (78, 83).

1.3.3 Molecular mechanisms regulating skeletal muscle mass

At the molecular level, muscle mass is regulated by protein turnover. Constant muscle mass is maintained through a balance between protein anabolism and catabolism. In response to resistance training skeletal muscle protein synthesis increases and overcomes protein degradation, leading to net protein accretion and hypertrophy (84). Conversely, conditions promoting protein catabolism and overwhelming the protein synthesis rate lead to net protein depletion and atrophy (85). Even though protein degradation promotes loss of muscle mass, maintaining healthy protein turnover through both synthesis and degradation is essential for skeletal muscle health (86).

1.3.3.1 Protein synthesis regulation

The protein kinase B- (PKB/Akt) mechanistic target of rapamycin (mTOR) signalling pathway is an essential driver of protein translation (Figure 2). This anabolic axis is under control of IGF1 and insulin, which, through phosphoinositide 3-kinase (PI3K) and phosphoinositide-dependent kinase 1 (PDK1) lead to Akt phosphorylation and activation (87). Downstream of Akt lies mTOR which represents the catalytic subunit of two major cellular complexes, mTOR complex 1 and 2 (mTORC1 and 2). mTORC1, characterized by its binding partner regulatory-associated protein of mTOR (raptor), is the main complex involved in protein synthesis and cell size regulation (88). Akt indirectly leads to mTORC1 activation, through phosphorylation of tuberous sclerosis complex 2 (TSC2). Phosphorylation of TSC2 by Akt lifts inhibition of Rheb GTPase, further activating mTORC1 (89, 90). Once activated, mTORC1 catalyses the phosphorylation of eukaryotic initiation factor 4E- (eIF4E) binding protein (4EBP1) and ribosomal protein S6 kinase (p70S6K), leading to increased protein translation. Before phosphorylation, 4EBP1 binds the eIF4E, inhibiting the 5'cap-dependent initiation of translation. Once phosphorylated, 4EBP1 dissociates from eIF4E, lifting its inhibitory effect (91). Furthermore, mTORC1-dependent activation of p70S6K leads to phosphorylation of eIF4B, promoting translation (92). Additionally, p70S6K phosphorylates 40S ribosomal protein S6 (S6) which does not directly promote protein translation but plays a role in ribosomal biogenesis and is a proxy measurement of p70S6K activity (93). mTORC1 promotion of protein translation is nutrient sensitive and increases or increases concurrently with nutrient availability (88).

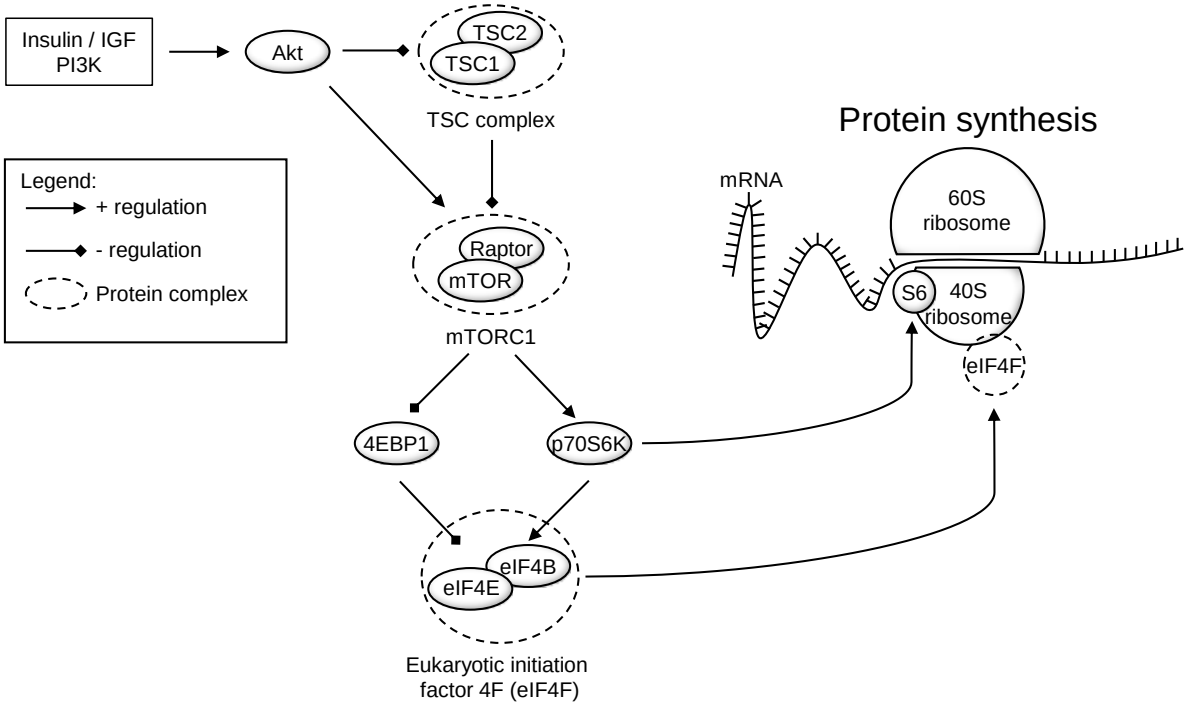

Figure 2 – A diagram of the Akt/mTOR signalling cascade. Insulin and IGF1 signals are transduced through Akt leading to activation of mTORC1. Ultimately, through inhibition of 4EBP1 and activation of p70S6K this cascade promotes protein synthesis.

The role of the Akt-mTOR signalling axis as a positive regulator of skeletal muscle mass is well established. Hypertrophy induced by synergist ablation and subsequent *plantaris* muscle overload in rats leads to Akt activation and an increase in both muscle mass and fibre cross sectional area, an effect blunted by mTOR inhibition with rapamycin (94). Additionally, expression of constitutively active Akt induces hypertrophy and is effective in alleviating atrophy induced by denervation of rat *tibialis anterior* muscle (94). Similarly, in human *vastus lateralis* muscle, eight weeks of resistance training leads to increased phosphorylation of both Akt and mTOR and this effect is reversed upon detraining (95). Conversely, atrophying skeletal muscle in individuals with amyotrophic lateral sclerosis has lower Akt phosphorylation compared to healthy controls (96). Thus, the Akt-mTOR signalling axis has been implicated in regulating skeletal muscle mass in rodents as well as humans.

1.3.3.2 Protein degradation

Protein degradation in skeletal muscle involves several different systems, namely calpains, caspases, the ubiquitin-proteasome system, and macroautophagy (Figure 3). Protein degradation is necessary for maintaining steady protein turnover and skeletal muscle health. Defects in either the proteasomal or autophagy systems in mouse models are detrimental as they lead to decreases in muscle mass, fibre CSA and force production (86, 97). However, increases in protein degradation underlie the development of skeletal muscle atrophy (85).

In large part, intracellular proteins are degraded by the ubiquitin-proteasome system (98). However, skeletal muscle myofibril protein complexes are resistant to direct degradation by the proteasome (99). Calpains and caspase-3 are two proteolytic systems implicated in deconstruction of these large structural complexes. A cysteine protease Calpain-1 (μ-calpain) localized at the Z-discs of rat *soleus* muscle is activated in the acute stages of denervation (100) while *in vitro* inhibition of calpain in rat skeletal muscle cell lines protects large structural proteins from degradation (101). Caspase-3 is a cysteine-aspartate protease responsive to a cascade of enzymes activated by apoptotic signals. Caspase-3 proteolytic activity has been implicated in disassociation of actin-myosin complexes before further degradation by the ubiquitin-proteasome system (102). Immobilization-induced atrophy in rat *soleus* muscle requires both caspase-3 and calpain activities with cross-regulation between the two systems (103). An increase in calpain expression has been observed in the *vastus lateralis* of bedridden patients following a traumatic brain injury (104), while increased protein content of caspase-3 has been noted in skeletal muscle of individuals suffering from muscular dystrophy (105).

Liberated myofibrillar and cytosolic proteins are further marked for degradation by the addition of a polyubiquitin chain in a three-step enzymatic reaction. E1 ubiquitin-activating enzyme catalyses the adenosine triphosphate- (ATP) dependant transformation of ubiquitin into its reactive form, which is then bound by an E2 ubiquitin-conjugating enzyme that transfers it further to the E3 ubiquitin ligase. E3 ubiquitin ligases catalyse the rate limiting step of ubiquitination, namely the binding of a ubiquitin molecule to a lysine residue either directly on the target protein or on the previously bound ubiquitin molecule elongating the

polyubiquitin chain. Several skeletal muscle specific ubiquitin ligases, such as muscle atrophy F-box protein (atrogin1/MAFbx) and muscle RING-finger protein 1 (MuRF1), have been implicated in the development of atrophy. Knockouts of either MAFbx or MuRF1 in mice reduces denervation-induced atrophy of *gastrocnemius* muscle by ~55% and ~35%, respectively (106). Polyubiqutinated proteins are shuttled into the 26S proteasome complex, which consists of the 20S catalytic subunit flanked on both sides by 19S regulatory subunits. The 19S subunit plays a role in polyubiquitin chain recognition, deubiquitination, and subsequent transfer of protein into the 20S catalytic cylinder for hydrolization. In human skeletal muscle, two weeks of limb immobilization and subsequent muscle atrophy in healthy young men are accompanied is increased expression of MAFbx, MuRF1, and the 20S proteasomal subunit (107). Similarly, 20 days of bedrest leads to upregulation of MAFbx concurrent with increased total protein ubiquitination (108). Considering that the ubiquitin-proteasome system is responsible for the bulk of protein degradation, upregulation of key players in this pathway likely promotes skeletal muscle atrophy.

Another system responsible for protein degradation is macroautophagy (autophagy). Autophagy is initiated by formation of a vesicle (autophagosome) that sequesters molecules poised for degradation. The molecules are then transported to the lysosome, where autophagosome-lysosome fusion occurs, allowing for hydrolysis of sequestered molecules. Although originally thought to be unselective, evidence of targeted protein degradation has emerged; the selectivity of autophagy is primarily based on the interaction of microtubule-associated proteins 1A/1B-light chain 3 (LC3) and sequestosome-1/p62 (p62) proteins. p62 recognizes and binds to ubiquitin on marked proteins, showing preference for mono-ubiquitination and lysine 63 (K63)-linked poly-ubiquitin chains over the lysine 48 (K48)-linked poly-ubiquitin chains (109, 110). LC3 protein exists in its cytoplasmic form (LC3 I), which is conjugated to phosphatidylethanolamine and recruited to the autophagosome membrane (LC3 II) (111). p62 binds to LC3 II directly leading to incorporation of proteins targeted for degradation into the autophagosome (112). Autophagy is induced in murine skeletal muscle following denervation, and autophagic deficiency attenuates muscle wasting (113). Furthermore, 24 days of bedrest induces expression and protein content of markers of autophagosome formation in healthy young men (113). Thus, even though more emphasis has been placed on the proteasome system, autophagy has emerged as an important contributor to development of skeletal muscle atrophy.

At the transcriptional level protein degradation is regulated through forkhead box protein O (FoxO) transcription factors and their cross-talk with the Akt-mTOR signalling pathway. Akt activity leads to phosphorylation of FoxO transcription factors, binding to 14-3-3 proteins, exclusion from the nucleus and inhibition of their transcriptional activity (114). In humans, the protein content of FoxO is reduced following eight weeks of resistance training in the *vastus lateralis* of healthy individuals, while an additional eight weeks of detraining lead to an opposing effect (95). Overexpression of constitutively active FoxO mutants in mouse *tibialis anterior* muscle causes marked muscle atrophy, and FoxO transcription factors are known to increase the expression of E3 ubiquitin ligases (115, 116). Furthermore, FoxO

activation leads to an increase, while FoxO inhibition leads to a decrease of autophagy and LC3 transcriptional regulation is under FoxO control (117). Thus, FoxO transcriptional activity contributes to skeletal muscle atrophy through promotion of both the ubiquitin-proteasome and autophagy systems.

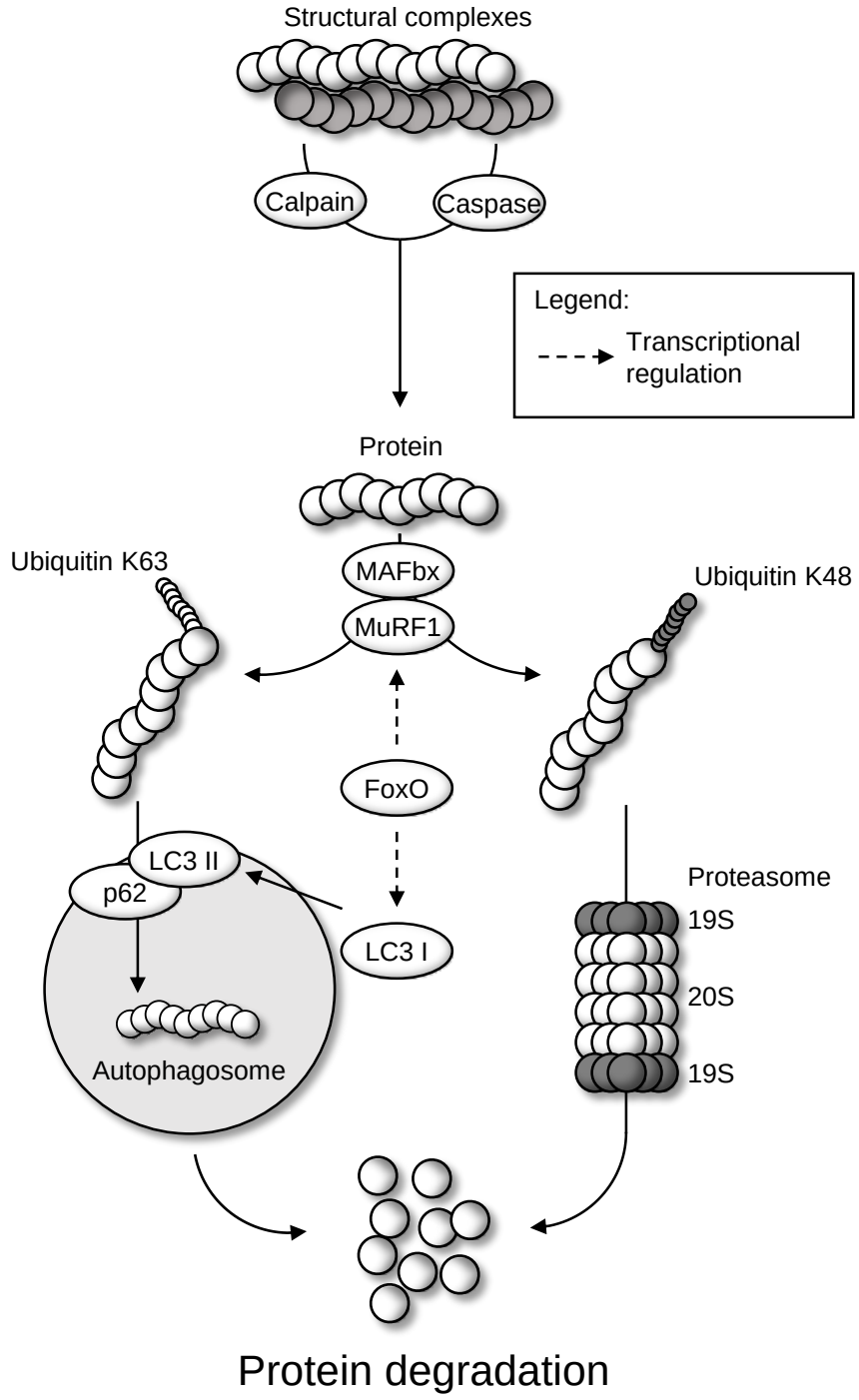

Figure 3 –A diagram of protein degradation systems. Large skeletal muscle protein complexes are broken down through the activity of calpains and caspases. Proteins poised for degradation are then marked by polyubiquitin chains by E3 ligases (MAFbx/MuRF1) and shuttled to the autophagosome or the proteasome for degradation. FoxO promote degradation by enhancing gene expression of E3 ligases, LC3 and p62.

1.3.4 Reactive oxygen species and skeletal muscle atrophy

Reduction of molecular oxygen (O_2) leads to formation of the superoxide anion (O_2^-) and occurs commonly within living cells. Superoxide can, in turn, react with a water molecule to form a hydroxyl radical (OH^-), or it can be enzymatically converted to hydrogen peroxide (H_2O_2). Furthermore, hydrogen peroxide, through the Fenton reaction with metals, can also be transformed into a more reactive hydroxyl radical. These three molecules (superoxide, hydroxyl radical, and hydrogen peroxide) are the most physiologically relevant reactive oxygen species (ROS). In skeletal muscle, ROS are abundantly produced in response to contraction and play an important role in the development of beneficial adaptations to exercise (118, 119). However, an imbalance in ROS homeostasis favouring production, leads to oxidative stress which leads to damage to cellular structures and is suspected to promote the development of skeletal muscle atrophy (120).

1.3.4.1 ROS production

ROS in skeletal muscle can be produced by several mitochondrial and non-mitochondrial sources including nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and xanthine oxidase (Figure 4). Mitochondria produce ROS through an electron transport chain "leak" at complexes I and III. The extent of mitochondrial ROS production in response to contraction has been brought into question, as specific complex I and III inhibitors do not significantly impact cytosolic superoxide content in contracting isolated mouse muscle fibres (121). However, mitochondria-targeted antioxidants substantially reduce ROS production and oxidative stress markers in skeletal muscle after 14 days of hindlimb immobilization in mice (122). Thus, mitochondrial ROS production might play a more important role in atrophying skeletal muscle.

The NOX family of enzymes catalyses the oxidation of NADPH to $NADP^+$, transferring an electron to molecular oxygen and forming superoxide. The most abundant isoforms present in the skeletal muscle are NOX2 and 4. While both are localized at the sarcolemma, NOX4 is also present in the sarcoplasmic reticulum and mitochondria (123, 124). While NOX2 is regulated by activating phosphorylation and translocation of its cytoplasmic subunit $p47^{phox}$, NOX4 is considered to be constitutively active (125, 126). Through *in vitro* studies of isolated mouse muscle fibres, NOX activity has been implicated in skeletal muscle ROS production in both resting and contracting skeletal muscle (121).

Xanthine oxidase is a subtype of xanthine oxidoreductase formed by either reversible sulfhydryl oxidation or irreversible proteolytic cleavage of xanthine dehydrogenase (127). Both enzymes catalyse the final two steps in purine degradation, converting hypoxanthine to xanthine and then uric acid, transferring an oxygen atom to the substrate at each step. While xanthine dehydrogenase preferably uses nicotinamide adenine dinucleotide (NAD+) as a substrate, xanthine oxidase uses molecular oxygen, thus producing superoxide or hydrogen peroxide. Increased xanthine oxidase activity has been noted in rat *soleus* muscle in response

to 14 days of hindlimb-unloading, thus implicating it in ROS production in atrophying skeletal muscle (128).

1.3.4.2 ROS decomposition

Cellular networks of enzymatic antioxidants are responsible for maintaining ROS homeostasis and reducing oxidative stress (Figure 4). The superoxide dismutase (SOD) family catalyses the conversion of superoxide to hydrogen peroxide or molecular oxygen utilizing a metal ion at its core for the electron transfer. SOD1 and SOD3 are copper-zinc enzymes localized in the cytoplasm or extracellular matrix, respectively. SOD2 contains manganese at its core and is localized within the mitochondrial matrix. Hydrogen peroxide produced by SOD is further detoxified through the action of catalase and glutathione peroxidase (GPx). Catalase utilizes two molecules of hydrogen peroxide, converting them to water and molecular oxygen. Similarly, GPx converts a single peroxide molecule to water, using glutathione (GSH) as an electron donor. Two oxidized GSH molecules form a disulphide (GSSG) which can be reduced back into GSH through the activity of glutathione reductase (GRx).

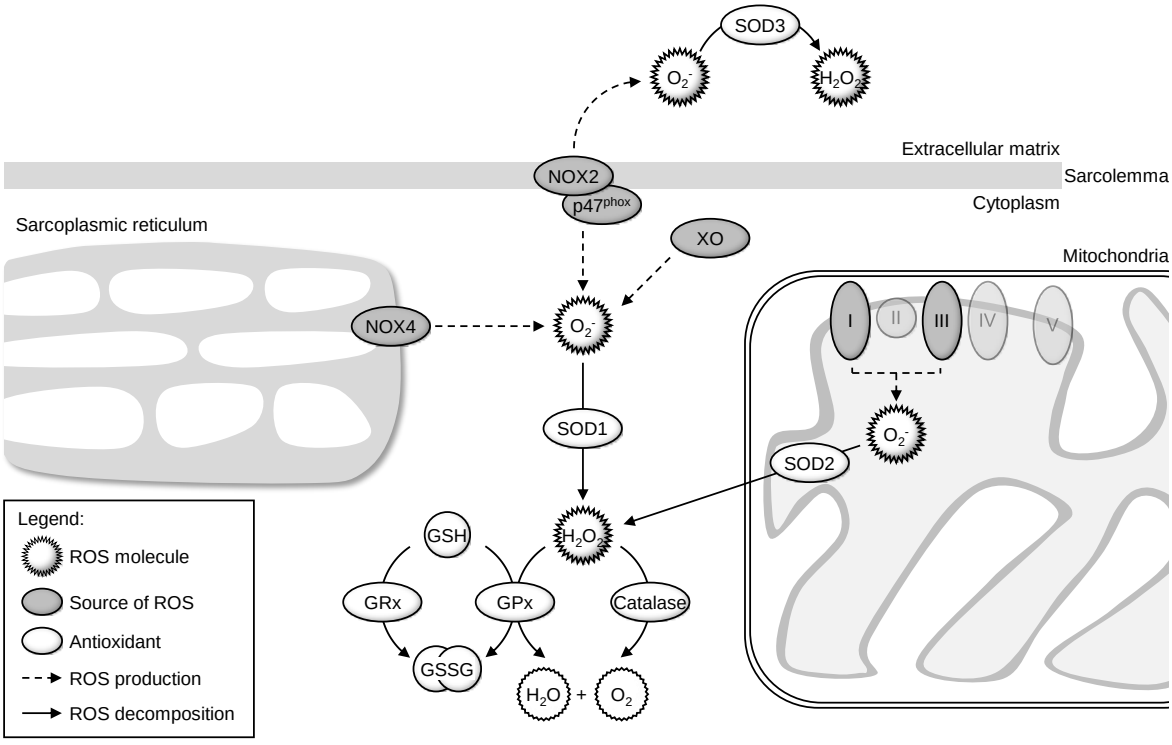

Figure 4 – A diagram of ROS homeostasis in skeletal muscle. ROS in skeletal muscle are produced through mitochondrial complexes I and III, NOX2, NOX4 and XO (xanthine oxidase). The produced superoxide (O_2^-) is converted to hydrogen peroxide (H_2O_2) by SOD1 in the cytoplasm, SOD2 in the mitochondria and SOD3 in the extracellular matrix. Catalase and GPx degrade hydrogen peroxide further into oxygen and water. GPx oxidizes GSH into GSSG, while GRx performs the reverse reaction.

Some conditions affecting skeletal muscle can lead to decreases in antioxidative defence. For example, aging reduces GPx and catalase activity, coupled with higher hydrogen peroxide content in murine skeletal muscle (129). Additionally, compensatory adaptations of antioxidant systems to increased ROS production during muscle atrophy have been reported.

Hindlimb immobilization for 12 days leads to increased SOD1, catalase and GRx activity in the rat *soleus* muscle (130). Therefore, some conditions lead to a reduction in antioxidant defences and oxidative stress, while in others antioxidant defences show compensatory increases in response to excess ROS production.

1.3.4.3 ROS as a possible cause of muscle atrophy

The first studies implicating ROS as causative in skeletal muscle atrophy examined the effects of 12 days of hindlimb immobilization on rat *soleus* muscle. Immobilization led to an increase in markers of oxidative stress accompanied by a 50% reduction in skeletal muscle mass. Importantly, this reduction in muscle mass was partially prevented by antioxidant (vitamin E) supplementation (120). Mechanistically, the increase in oxidative stress was accompanied by higher xanthine oxidase activity (131). Further studies described the involvement of other ROS producing systems in the development of skeletal muscle abnormalities. Namely, targeted inhibition of either mitochondrial or xanthine oxidase ROS production reduces the levels of oxidative stress markers and alleviates disuse-induced atrophy in murine models (122, 128), while NOX inhibition alleviates force production loss and rigidity of skeletal muscle occurring in mouse skeletal muscle dystrophy models (132). Thus, both mitochondrial and non-mitochondrial sources contribute to the development of oxidative stress and might have a causative role in muscle atrophy.

ROS are highly reactive and in excess cause damage to cellular structures. ROS lead to the addition of a carbonyl group (C=O) in direct reaction with arginine, lysine, proline or threonine on protein side-chains. Oxidatively modified proteins are preferably degraded by the ubiquitin-proteasome even in absence of ubiquitination, or form dysfunctional protein aggregates resistant to degradation (133, 134). Furthermore, ROS cause a non-enzymatic lipid peroxidation, causing direct damage to cellular and organelle membranes and forming lipid peroxides. Lipid peroxides are further detoxified into lipid aldehydes such as 4-hydroxynonenal (4HNE), which functions as a ROS-secondary messenger, with numerous effects such as induction of endoplasmic reticulum, apoptosis, and reduction of protein synthesis (135-137). Therefore, excessive exposure to ROS leads to direct and indirect interference with normal cellular functions.

The proposed mechanism by which ROS contribute to atrophy is through interaction with protein balance, favouring degradation over synthesis. Exposure to exogenous hydrogen peroxide *in vitro* leads to inhibition of eIF4E and activation of 4EBP, while lipid peroxidation aldehydes form adducts with eIF2, inhibiting the initiation of protein translation (137, 138). Exposure of murine muscle cell lines to hydrogen peroxide leads to increased FoxO transcriptional activity and higher expression of MAFbx, MurF1 and autophagy-related protein 7 (Atg7), favouring higher protein degradation (139). Conversely, overexpression of catalase and increased antioxidant defence reduce FoxO transcriptional activity in rat *soleus* during immobilization (140). Thus, skeletal muscle atrophy is coupled with increased ROS production which can lead to apoptosis, increased protein degradation, and decreased protein synthesis, — all of which contribute to muscle mass loss.

1.3.5 Skeletal muscle satellite cells and regulation of muscle mass

Satellite cells are a distinct population of myogenic stem cells, essential for skeletal muscle embryonic formation (141). Even though they hold embryonic origin and are more numerous in skeletal muscle during the perinatal period, their population still represents ~5% of nuclei within adult skeletal muscle (74, 142). Satellite cells are located on the surface of muscle fibres between the plasma membrane and the basal lamina, and protrude inwards toward the muscle fibre (143). They are dormant under normal conditions, but are activated in response to skeletal muscle damage. Once injury occurs, satellite cells exhibit typical stem cell behaviour, undergoing asymmetric division, thus giving rise to myogenic progenitors and replenishing the satellite cell pool (144). The activation of satellite cells is indispensable for skeletal muscle regeneration in response to injury (145).

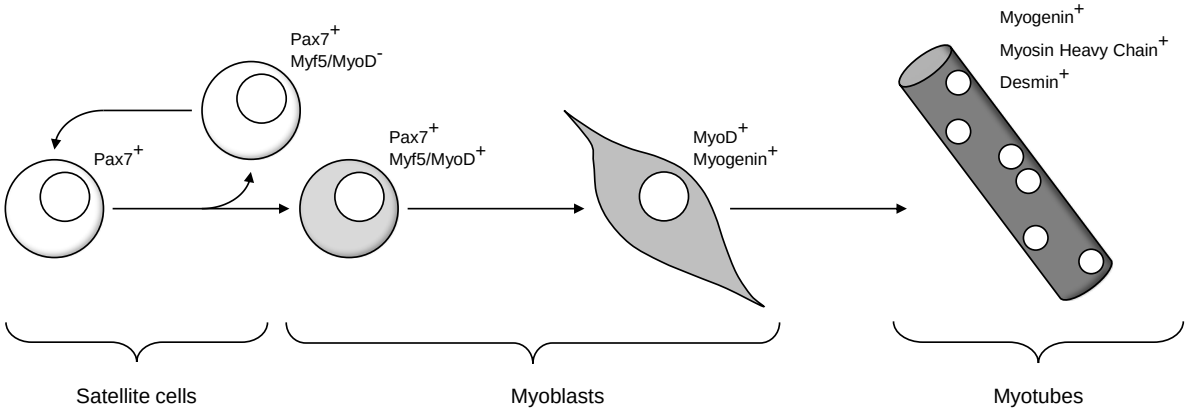

Figure 5 – A diagram of myogenic differentiation of satellite cells. Activated Pax7 positive satellite cells give rise to two distinct cell populations. Myf5/MyoD-negative cells repopulate the satellite cell pool while Myf5/MyoD-positive cells are committed to the myogenic lineage. Further down the differentiation path cells express myogenin required for terminal differentiation.

Skeletal muscle satellite cells are marked by the expression of paired box protein 7 (Pax7), which makes them distinct from the remaining non-myogenic stem cell population (141). The expression of Pax7 is high in quiescent satellite cells and decreases through differentiation. Additionally, Pax7 has a distinct role in maintaining the satellite cell pool by preserving them in an undifferentiated state (146). Once activated, the myogenic fate of satellite cells is controlled by the expression of a group of basic helix-loop-helix transcription factors named myogenic regulatory factors (MRFs), such as myogenic factor 5 (Myf5), myogenic differentiation protein (MyoD), myogenic factor 6 (Myf6), and myogenin. The early phase of differentiation is characterized by Myf5 expression. Importantly, cells expressing Myf5 (myoblasts) continue through myogenic differentiation, while Myf5 negative cells are mainly responsible for replenishing the pool (147). Additionally, in this early phase of differentiation myoblasts also express MyoD. In fact, distinct cells have been shown to express either Myf5 or MyoD upon activation (~25% each), while a certain number co-express both (~50%) (147). These transcription factors have an overlapping role and each has the ability to compensate in case there is a loss of function of the other (148). Progression further down the

myogenic path is marked by decreased expression of Myf5 and MyoD as other MRFs take control of terminal differentiation.

Expression of myogenin occurs during the terminal stages of myogenesis. In the absence of myogenin, terminal skeletal muscle fibre formation fails and knockout mice exhibit an abundance of undifferentiated myoblasts (149). Myf6 (MRF4/herculin) has originally been considered to have an overlapping role with myogenin. Lack of Myf6 is accompanied by an increase in myogenin and *vice versa*. However, myogenin expression, but not Myf6, is sufficient and necessary for myogenic differentiation (149, 150). More recent reports have indicated an opposing role of Myf6, implicating it as a negative regulator of myogenesis and muscle mass (151). Thus it is possible that the increase in myogenin observed in the absence of Myf6 is due to the removal of an inhibitory mechanism of muscle formation.

Satellite cells play an indispensable role in muscle regeneration in response to injury. Namely, formation of regenerative muscle fibres in response to a cardiotoxin injection is completely absent in the *tibialis anterior* of satellite cell-deficient mice (145). In addition satellite cells have a proposed role in regulation of muscle mass, which finds its basis within the myonuclear domain hypothesis. According to the hypothesis the cytoplasm-to-nucleus ratio within skeletal muscle is held constant. Hence, hypertrophy is presumed to be accompanied by addition of new nuclei originating from satellite cells, while atrophy leads to a loss of nuclei. However, parts of the myonuclear domain hypothesis are still controversial.

Notably, during synergist ablation and skeletal muscle overload in mice, muscle hypertrophy is preceded by addition of new myonuclei, originating from satellite cells (152). Some reports have challenged the necessity of satellite cells for hypertrophy, showing that satellite cell ablation prior to overload does not impair an increase in muscle weight (153). However, more recent studies show that, while synergist ablation and overload of satellite cell-deficient muscles leads to increased weight, the fibre CSA does not increase (154). Such discrepancies among muscle weight and fibre CSA have been explained through post-surgical adhesions after synergist ablation and/or changes in muscle morphology. Hence, satellite cells function and addition of new myonuclei are likely necessary for skeletal muscle hypertrophy.

Conversely, spinal cord transection-induced atrophy of rat *soleus* muscle is proposed to be accompanied by decreases in myonuclear number through apoptosis (155). Furthermore, such decreases occur in spite of a higher number of myogenin-positive, activated satellite cells post-injury compared to uninjured controls (155). Such decrease in myonuclei, in spite of satellite cell activation, may suggest a defect in satellite cell differentiation. Interestingly, the injury does not seem to lead to an increase in satellite cell proliferation, as the number of bromodeoxyuridine-positive, proliferating nuclei does not increase in injured compared to control rats (155). Higher expression of MRFs and unchanged proliferation may indicate that spinal cord injury favours the myogenic activation, at the expense of satellite cell pool repopulation. A depletion of the satellite cell pool is observed in the late stages after denervation of murine skeletal muscle, accompanied by inefficient differentiation of myogenic precursors (156, 157). Interestingly, human skeletal muscle following spinal cord

injury also has a lower number of satellite cells per muscle fibre (158). Activation and inefficient differentiation of satellite cells could lead to such depletion of the stem cell pool.

On the other hand, more recent reports have challenged the idea of myonuclear loss during atrophy altogether. Utilizing novel *in vivo* imaging techniques the authors show that nuclei added to mouse *extensor digitorum longus* muscle during 14 days of overload hypertrophy are not lost through subsequent 14 days of denervation (152). The same authors also examined rat *soleus* muscle after 14 days of hindlimb unloading, detecting a decrease in fibre CSA but no loss of myonucleai (159). These studies strongly suggest that myonuclear loss does not occur during atrophy. Furthermore, in both studies the detected number of TUNEL-positive, apoptotic nuclei per muscle cross section increased in response to either denervation or unloading, however not within the muscle fibres but rather within stromal or satellite cells (152, 159). Hence, apoptosis may be contributing to the depletion of the satellite cell pool observed following spinal cord injury (158). However, due to methodological limitations, assessment of satellite cell function in skeletal muscle atrophy in human muscle remains difficult.

1.4 MOTIVATION FOR THE THESIS

Improvements in treatment and care over the past decades have led to increased longevity of individuals with spinal cord injury (160). In light of these improvements, long-term care and prevention of late complications is becoming more important. Profound skeletal muscle atrophy is accompanied by metabolic changes which promote disturbances in whole-body glucose and lipid homeostasis (31, 161). Consequently, late phases of spinal cord injury carry increased risk of type 2 diabetes and cardiovascular disease (33, 77). This underscores the importance of improving strategies for alleviating muscle wasting in order to prevent development of metabolic disease in individuals with spinal cord injury.

The established molecular mechanisms leading to skeletal muscle atrophy involves an imbalance in protein metabolism. Evidence indicates a progressive decrease in both protein synthesis and degradation in skeletal muscle of individuals with spinal cord injury, reaching a new steady state of low protein turnover in the chronic post-injury phase (162). However, comprehensive information on the timing and pattern of these changes is lacking. Oxidative stress is known to occur in murine models of disuse atrophy, and has been linked to changes in protein metabolism underlying muscle wasting (120, 140). Furthermore, mitochondrial production of hydrogen peroxide has been shown to increase in response to two weeks of immobilization in human skeletal muscle (163). However, whether redox homeostasis is disturbed in human skeletal muscle after spinal cord injury remains unknown. Finally, satellite cells are regulators of muscle mass and contribute to skeletal muscle hypertrophy (152). Spinal cord injury leads to depletion of the satellite cell pool (158). Apoptosis, or activation and inefficient differentiation could drive such depletion. However, whether there is a defect in satellite cells intrinsic differentiation capacity following spinal cord injury remains unknown.

Collectively, the studies in this thesis will elucidate some changes in skeletal muscle induced by spinal cord injury which could contribute to atrophy. This thesis establishes a sequence of alterations in protein anabolism and catabolism during the first year following spinal cord injury, contributing to net protein loss and promoting muscle wasting. Furthermore, the thesis discerns whether spinal cord injury induces adaptations in ROS production and decomposition, leading to oxidative stress in skeletal muscle. Finally, these studies describe the intrinsic myogenic capacity of satellite cells following spinal cord injury. The work presented herein may inform efforts to develop preventive strategies to combat atrophy by elucidating the perturbations in skeletal muscle protein metabolism, ROS homeostasis and satellite cell intrinsic differentiation capacity.

2 AIMS OF THE STUDIES

The overall aim of this thesis was to examine the underlying molecular and cellular mechanisms of muscle atrophy and to establish a timeline of changes contributing to muscle wasting following spinal cord injury. The specific aims of the papers included in the thesis were:

Paper I: To examine the protein content and phosphorylation of regulators of protein synthesis and degradation in skeletal muscle during the first year following spinal cord injury.

Paper II: To examine markers of oxidative stress, abundance of enzymes responsible for ROS generation and decomposition, and protein content of apoptotic signalling molecules in skeletal muscle during the first year following spinal cord injury.

Paper III: To examine whether spinal cord injury leads to a defect in the intrinsic differentiation capacity of skeletal muscle satellite cells in the chronic phase of spinal cord injury.

3 MATERIALS AND METHODS

3.1 ETHICAL PERMISSIONS

All studies were approved by the Regional Committee for Medical and Health Research Ethics at Helse Sør-Øst Trust, Norway and by the Regional Ethics Committee at Karolinska Institutet, Sweden. The studies were conducted according to the ethical principles expressed in the Declaration of Helsinki. All participants gave their written informed consent.

3.2 STUDY PARTICIPANTS AND SAMPLE COLLECTION

3.2.1 Study participants (Papers I, II and III)

Individuals with spinal cord injury included in the studies were recruited from Sunnaas rehabilitation hospital in Nesodden, Norway. The participants with spinal-cord injury received upper body and postural stability exercises during rehabilitative treatment. They also received low molecular weight heparin therapy during the first 3 months after injury as well as spasmolytic therapy for individuals with complete injuries (baclofen or equivalent). They did not receive any corticosteroid treatment. Able-bodied controls consisted of non-smokers, non-athletes, with no current use of medications. Neither spinal cord-injured nor able-bodied participants had any known malignant, systemic or musculoskeletal disease, nor intercurrent infection.

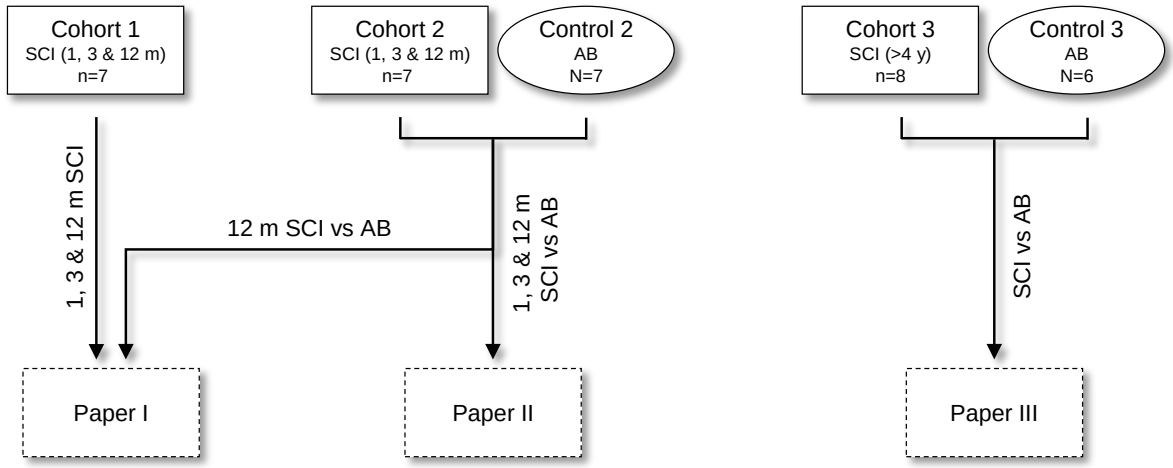

Figure 6. – Overview of study groups used in the thesis. SCI – spinal cord-injured; AB – able-bodied, m – month, y – year.

An overview of study groups used in the thesis is presented in Figure 6. A cohort of seven individuals (six men and one woman) with a complete cervical spinal cord injury was studied in Paper I at one, three and 12 months after injury (Cohort 1). The anthropometric data of participants from Paper I (Cohort 1) are presented in Table 1. An additional cohort of individuals with spinal cord injury (four men and three women) were studied at one, three and 12 months after injury (Cohort 2) in Paper II and compared to a group of seven age- and body mass index- (BMI) matched able-bodied controls (seven men). The anthropometric data of participants from Paper II (Cohort 2) are presented in Table 2. This second cohort of spinal cord-injured individuals and able-bodied controls were used for comparisons at 12 months

after spinal cord injury in Paper I as well. In Paper III we compared a group of eight individuals with spinal cord injury (Cohort 3, eight men, four with complete and four with incomplete injuries) and a group of six age-matched able-bodied controls. The anthropometric data of participants from Paper III are presented in Table 3.

Cohort 1	Spinal Cord-Injured (n=7)		
	Months since injury		
	1	3	12
Age, y	33 ± 12		
BMI, kg/m^2	24 ± 3	24 ± 3	25 ± 4
Injury level	AIS motor score (0-100)		
C5, n=6	17 ± 11	20 ± 13	23 ± 12
C6, n=1	27	27	28

Table 1 – Participant information on Cohort 1 from Paper I. Values presented are mean and standard deviation. y – year; BMI – body mass index.

Cohort 2		Able-Bodied (n=7)	Spinal Cord-Injured (n=7)		
			Months after injury		
			1	3	12
Age, y		49 ± 6	43 ± 15		
BMI, kg/m^2		26 ± 2	25 ± 4	25 ± 4	27 ± 3
Injury Level			AIS motor score (0-100)		
Cervical, n=2	C4	n/a	8	8	16
	C6	n/a	27	27	28
Thoracic, n=5	Th3-12	n/a	50	50	50

Table 2 – Participant information on Cohort 2 from Paper 2 and the age- and BMI-matched able-bodied controls. Values presented are mean and standard deviation. y – year; BMI – body mass index.

Cohort 3	Able-Bodied (n=6)	Spinal Cord-Injured (n=8)
Age, y	42 ± 9	52 ± 14
BMI, kg/m^2	22 ± 1	26 ± 5
Level of injury (n)	Motor deficiency	Time since injury, y
C7 (2)	Complete	11; 4
C7	Incomplete	8
Th4	Complete	34
Th5	Complete	20
Th12 (3)	Incomplete	6; 5; 4

Table 3 – Participant information on Cohort 3 from Paper 3 and the age-matched able-bodied controls. Values presented are mean and standard deviation. y – year.

3.2.2 Skeletal muscle biopsies (Papers I, II and III)

Able-bodied participants were instructed to abstain from strenuous physical activity the day before the biopsies were taken. Skeletal muscle biopsies were obtained in postprandial conditions from the mid-section of the *vastus lateralis* portion of the *quadriceps femoris* muscle under local anaesthesia (Lidocaine, 5 mg/ml). In papers I and II samples were obtained by a Bergström needle under suction, cleaned from visible fat and blood, and rapidly frozen in liquid nitrogen. In paper III biopsies were obtained by a semi-open biopsy procedure with a Weil-Blakesley conchotome or an open muscle biopsy procedure. These biopsies was placed in ice-cold phosphate buffered saline (PBS) supplemented with 1% PenStrep (100 IU/ml penicillin and 100 µg/ml streptomycin) and kept at 4°C until processing.

3.2.3 Sample lysis (Papers I and II)

Portions of the biopsies (25-100mg) were lysed in ice cold lysis buffer (137 mmol/l NaCl, 1 mmol/l $MgCl_2$, 2.7 mmol/l KCl, 1 mmol/l EDTA, 20 mmol/l Tris, pH 7.8, 5 mmol/l Na pyrophosphate, 10 mmol/l NaF, 1% Triton X-100, 10% glycerol, 0.2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 0.5 mmol/l Na_3VO_4 and protease inhibitor cocktail Set 1 (Calbiochem, EMD Biosciences, San Diego, CA, US)). Lysates were centrifuged at 12000 *g* for 10 minutes at 4°C to remove insoluble material and the supernatant was collected for further analysis. The protein concentration of the lysates was determined by Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, US).

3.2.4 Satellite cell isolation and culture (Paper III)

Satellite cells were isolated as previously described (164). Skeletal muscle samples were cleared of visible blood, adipose, and connective tissue and then minced. They were then

placed in a collagenase digestion-solution (1.5 mg/ml collagenase IV, 8% trypsin, 1.5 mg/ml bovine serum albumin (BSA) and 1% PenStrep in Ham's F-10 medium) and incubated for 20 minutes at 37°C under gentle agitation. The undigested tissue was allowed to settle and the supernatant containing isolated satellite cells was collected. The digestion was then repeated and the obtained supernatants pooled. The supernatant was centrifuged for 10 minutes at 350 *g* at room temperature and the pellet containing satellite cells was suspended in growth medium (20% foetal bovine serum (FBS), 1% PenStrep, 1% Amphoteracin B (250 µg/ml) in DMEM/F-12). The suspended pellet was then incubated in a petri dish for one hour to allow for attachment of non-satellite cells. The supernatant containing satellite cells was collected for further culturing.

Satellite cells proliferated at 37°C and 7.5% CO_2 until ~80% confluence. Cells were then detached using TrypLE (Thermo Fisher Scientific) and sub-cultured. Cells grown after the first sub-culturing were designated as passage 1 and each subsequent sub-culturing was assigned a progressively higher number. All experiments were performed on cells in passages 5-7.

Differentiation of cells was induced at ~80% confluence by switching to differentiation media (100 µg/ml apo-transferrin, 0.286 IU/ml insulin, 0.03µg/ml $ZnSO_4$, 1.4 mg/mL vitamin B_{12}, 1% PenStrep, 1% Amphoteracin B, 20% Medium 199 in DMEM containing 4.5 g/l glucose). Cells were kept in differentiation medium for four days, after which the cells were placed in post-differentiation medium (0.03µg/ml $ZnSO_4$, 1.4 mg/mL vitamin B_{12}, 1% PenStrep, 1% Amphoteracin B, 20% Medium 199 in DMEM containing 4.5 g/l glucose) for an additional four days.

To assess differentiation, protein and RNA was extracted from activated satellite cells (myoblasts) before the induction of differentiation and from differentiated myotubes eight days after the induction of differentiation. Cell lysis and protein extraction was performed using the same lysis buffer and protocol as described for the biopsy samples in section 3.2.3 and protein content was determined using a commercially available Pierce BCA assay (Thermo Fisher Scientific). RNA was extracted by using the TRIzol reagent (Life Technologies, Carlsbad, CA, US) according to the manufacturer's instructions. Concentration was determined by Nanodrop ND-1000 (Thermo Fischer Scientific) and the absorbance 260/280nm ratio >1.8 was considered acceptable.

3.3 ANALYTICAL METHODS

3.3.1 Western blot (Papers I, II and III)

Equal amounts of protein were diluted in Laemmli buffer and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (164) using Criterion XT precast gels (BioRad, Hercules, CA, US). Protein was then transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Burlington, MA, US) and Ponceau S staining was performed. The membranes were blocked by 7.5%

non-fat dried milk in tris-buffered saline- (TBS) Tween 20 (20 mmol/l Tris pH 7.6, 137 mmol/l NaCl, 0.02% Tween 20) and incubated overnight at 4°C with gentle agitation in primary antibody dilutions 1:1000 in TBS containing 0.1% BSA and 0.1% NaN_3. The list of primary antibodies used in all papers is presented in Table 4. Membranes were then washed in TBS-Tween 20 and incubated in species appropriate horseradish peroxidase conjugated secondary antibodies diluted 1:25000 in 5% non-fat dried milk in TBS-Tween 20. Proteins were visualized by chemiluminescence using ECL and ECL select reagents (GE Healthcare, Chicago, IL, US). Optical density of the detected bands was quantified using QuantityOne or ImageLab software (BioRad).

Antigen	Molecular Weight (kDa)	Catalog No	Manufacturer	Papers
4EBP1	15-20	#9452	Cell Signaling	I & III
p-4EBP1$^{Thr37/46}$	15-20	#2855	Cell Signaling	I & III
4HNE	n/a	#ab46545	Abcam	II
ACC	280	#3676	Cell Signaling	III
p-ACC^{Ser222}	280	#3661	Cell Signaling	III
Akt	60	#9272	Cell Signaling	I & III
p-Akt^{Ser473}	60	#9271	Cell Signaling	I & III
p-Akt^{Thr308}	60	#4056	Cell Signaling	I & III
Calpain-1	82 / 68	#ab28258	Abcam	II
Caspase-3	35 / 19	#9662	Cell Signalling	II
Caspase-9	37	#ab2324	Abcam	II
Catalase	64	#sc-50508	Santa Cruz	II
OXPHOS Cocktail	18-54	#ab110411	Abcam	II
Complex I (NDUFB8)	18	#ab110242	Abcam	II
Complex II (SDHB)	29	#ab14714	Abcam	II
Complex III (UQCRC2)	48	#ab14745	Abcam	II
Complex IV (MTCO2)	22	#ab110258	Abcam	II
Complex V (ATP5A)	54	#ab14748	Abcam	II
Desmin	55	#ab15200	Abcam	III
FoxO1	70	#ab12161	Abcam	I & III
p-FoxO1^{Ser256}	70	#9461	Cell Signaling	I & III
FoxO3	100	#ab47409	Abcam	I & III
p-FoxO3^{Ser253}	100	#13129	Cell Signaling	I & III
GPx (Glutathione Peroxidase 1)	22	#ab22604	Abcam	II
GRx (Glutathione Reductase)	58	#ab16801	Abcam	II
JNK (SAPK)	46-54	#9252	Cell Signalling	II
p-JNK$^{Thr183/Tyr185}$ (SAPK)	46-54	#9251	Cell Signalling	II
LC3A/B	16-18	#L8918	Sigma	I & III
MAFbx	42	#sc-166806	Santa Cruz	I & III
MyHC I (Myh7)	225	#A4.840	DHSB	III
MyHC II (Myh1/2)	225	#sc-53088	Santa Cruz	III

mTOR	289	#2983	Cell Signaling	I & III
p-mTOR^{Ser2448}	289	#600-401-422	Rockland	I & III
MuRF1	40	#sc-32920	Santa Cruz	I & III
Myogenin	34	#sc-12732	Santa Cruz	III
NOX2 (gp91^{phox})	65	#ab80508	Abcam	II
NOX4 (NADPH oxidase 4)	67	#ab133303	Abcam	II
p-p47$^{phox\ Ser328}$ (NCF1)	47	#ab111855	Abcam	II
p62	62	#P0067	Sigma	I & III
Proteasome 20Sα	29	#ab22674	Abcam	I & III
Raptor	150	#2280	Cell Signaling	I & III
S6	32	#2317	Cell Signaling	I & III
p-S6$^{Ser235/236}$	32	#2211	Cell Signaling	I & III
SOD1 (Superoxide Dismutase 1)	17	#ab16831	Abcam	II
SOD2 (MnSOD)	26	#ab13534	Abcam	II
TSC2	200	#4308	Cell Signaling	I
Ubiquitin linkage-specific Lys48	n/a	#ab140601	Abcam	I
Ubiquitin linkage-specific Lys63	n/a	#ab179434	Abcam	I
Xanthine Oxidase	85	#ab109235	Abcam	II

Table 4 – List of primary antibodies used in all studies. The full addresses of manufacturers are: Cell Signaling, Danvers, MA, US; Abcam, Cambridge, UK; Santa Cruz, Dallas, TX, US; DSHB, Iowa City, IA US; Rockland, Limerick, PA, US.

3.3.2 Glutathione determination (Paper II)

Glutathione content was determined using a commercially available assay kit (ab138881, Abcam, Cambridge, UK). Equal amounts of sample, as determined by protein concentration were loaded for the reaction. To avoid enzymatic interference with the assay, samples were first deproteinized by addition of 1:5 (v:v) of 100% (w:v) trichloracetic acid (TCA). Precipitated protein and TCA were removed by centrifugation at 12000 *g* for 5 minutes at 4°C. The remaining TCA in the supernatant was neutralized by addition of 1 mmol/l $NaHCO_3$. The samples were then centrifuged at 12000 *g* for 15minutes at 4°C and the supernatant collected for further analysis. The amount of GSH and total glutathione were determined, and the amount of GSSG was calculated according to manufacturer's instructions.

3.3.3 Protein carbonylation assay (Paper II)

A commercially available kit (OxyBlot, Merck Millipore) was used to determine total protein carbonylation as previously described (25). Equal amounts of protein were loaded, denatured by addition of 6% SDS (v/v) and carbonyl groups on protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH). After 15 minutes at room temperature the derivatization reaction was stopped by adding the neutralizing solution and 5% (v/v) of 2-mercaptoethanol. The samples were loaded for SDS-PAGE, and western blot analysis was performed as described in section 3.5. Optical density

of detected bands was determined (molecular weights 250, 225, 100, 80, 50, 40 and 35 kDa) and the sum of all bands was taken as total protein carbonylation.

3.3.4 Gene expression (Paper III)

cDNA synthesis was performed with the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA) with 1000 ng of RNA as template. Quantitative RT-PCR (qPCR) was performed using Fast SYBR Green Master Mix (Applied Biosystems) and Ct values were determined using StepOne software v2.1 (Applied Biosystems). The results were normalized to the geometric mean of two controls genes, TATA-binding protein (TBP) and 60S acidic ribosomal protein P0 (RPLP0), and the expression calculated by the ΔCt method. Primers used for the reactions were designed to exon to exon junctions of the targeted genes. Sequences of used primers are listed in Table 5. Each assay was accompanied by no-template and no-reverse transcription controls, as well as a melt curve step showing a single clear peak for each set of primers.

Target gene	Forward primer	Reverse primer
TBP	AGTTCTGGGATTGTACCGCA	TATATTCGGCGTTTCGGGCA
RPLP0	TGGAGAAACTGCTGCCTCAT	GATTTCAATGGTGCCCCTGG
Pax7	GAGGACCAAGCTGACAGAGG	CTGGCAGAAGGTGGTTGAA
Myf5	CCACCTCCAACTGCTCTGAT	GCAATCCAAGCTGGATAAGG
MyoD	AGCACTACAGCGGCGACT	GCGCCTCGTTGTAGTAGGC
Myf6	GGATCAGCAGGAGAAGATGC	CCTGGAATGATCGGAAACAC
Myogenin	GCTCAGCTCCCTCAACCA	GCTGTGAGAGCTGCATTCG
Desmin	CTGGAGCGCAGAATTGAATC	GGCAGTGAGGTCTGGCTTAG

Table 5 – Sequences of primers used for qPCR gene expression analyses.

3.3.5 [^{14}C] Phenylalanine incorporation into protein (Paper III)

Differentiated myotubes were incubated with 425 μmol/l phenylalanine and 0.4 μCi/mL of [^{14}C] phenylalanine (Perkin Elmer, Waltham, MA, US) in DMEM (1 g/l glucose) for 6 h at 37°C and 7.5% CO_2. The cells were then washed in ice cold PBS and lysed in 0.03% SDS for 1 hour at room temperature. The total amount of protein per well was determined by the Pierce BCA protein assay kit (Thermo Fischer Scientific). Protein from the cell lysates was precipitated in 50% TCA with 1% BSA, overnight at −20°C. The samples were centrifuged at 12000 *g* for 10 minutes at 4°C. The protein pellet was then washed in acetone, dissolved in 0.5 mol/l NaOH and the amount of [^{14}C] determined by scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac, Turku, Finland). Counts per minute were normalized to the total amount of protein per well, and the amount of phenylalanine incorporated into protein is presented as pmol/mg/hour.

3.3.6 [^{3}H] Palmitic acid oxidation (Paper III)

Production of [^{3}H]-labelled water was used to measure the oxidation of [^{3}H]-labelled palmitic acid as previously described (165). Differentiated myotubes were incubated for 6 h with 1 mCi/ml [^{3}H] palmitic acid (Perkin Elmer), 25 μmol/l palmitic acid and 0.02% fatty acid-free BSA in DMEM (1 g/l glucose). The collected medium was incubated in a charcoal-slurry (0.1 g activated charcoal per 1 ml 0.02 mol/l Tris-HCl at pH 7.5) for 30 minutes, mixing every 5 minutes. The slurry was then centrifuged at 13000 *g* for 10 minutes at room temperature, and the supernatant was collected for scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac). Cells were lysed in 0.03% SDS and the protein concentration was determined using a commercially available Bradford protein assay kit (BioRad). Results were normalized to the total protein amount per well and the oxidation rate of palmitic acid is presented as pmol/mg/hour.

3.4 STATISTICAL ANALYSES

In Paper I the statistical significance of differences between one, three and 12 months after spinal cord injury was determined by Friedman's test, followed by Dunn's post-hoc test corrected for multiple comparisons. In Paper II the differences between the skeletal muscle after one, three and 12 months of spinal cord injury and skeletal muscle from able-bodied controls was determined by Kruskal-Wallis' test followed by Dunn's post-hoc test corrected for multiple comparisons. In paper III the differences between myoblasts and myotubes from individuals with spinal cord injury and able-bodied controls was determined by a two-way ANOVA followed by Sidak's post-hoc test corrected for multiple comparisons. Other comparisons between able-bodied and spinal cord-injured groups were performed by Mann-Whitney's assigned ranks test. P values of <0.05 are reported as significant, while $p<0.1$ are reported as trends. Values in Paper I are presented as arbitrary units of optical density. Values in Paper II have been scaled to the mean of able-bodied controls, and in Paper III to the mean of myotubes from able-bodied controls unless otherwise stated. The bars in all papers represent the mean, error bars are standard deviation (SD) and the individual data points are shown.

Values in the thesis have been scaled to the mean of one month after spinal cord injury (Paper I), able-bodied control group (Paper I and II) or myotubes obtained from able-bodied donors (Paper III). The data are presented as mean ± SD. Z-scores for figures 9 and 11 have been calculated for each individual data point from the mean and standard deviation of the entire row as $z = (x-\mu_x)/\sigma_x$ where x is the data point, μ_x is the mean of all data points and σ_x is the standard deviation of all data points. The mean z-score for each group is then plotted on the heatmaps.

4 RESULTS

4.1 REGULATION OF PROTEIN METABOLISM IN SKELETAL MUSCLE DURING FIRST YEAR AFTER SPINAL CORD INJURY (PAPER I)

We aimed to examine the sequence of changes in protein metabolism regulation in human skeletal muscle during the first year after spinal cord injury. To achieve this, we determined the protein content and phosphorylation of regulators of protein anabolism and catabolism in the skeletal muscle one, three, and 12 months after spinal cord injury. Additionally, to place the detected changes in the perspective of healthy skeletal muscle, we made comparisons between a cohort of individuals 12 months after spinal cord injury and able-bodied controls.

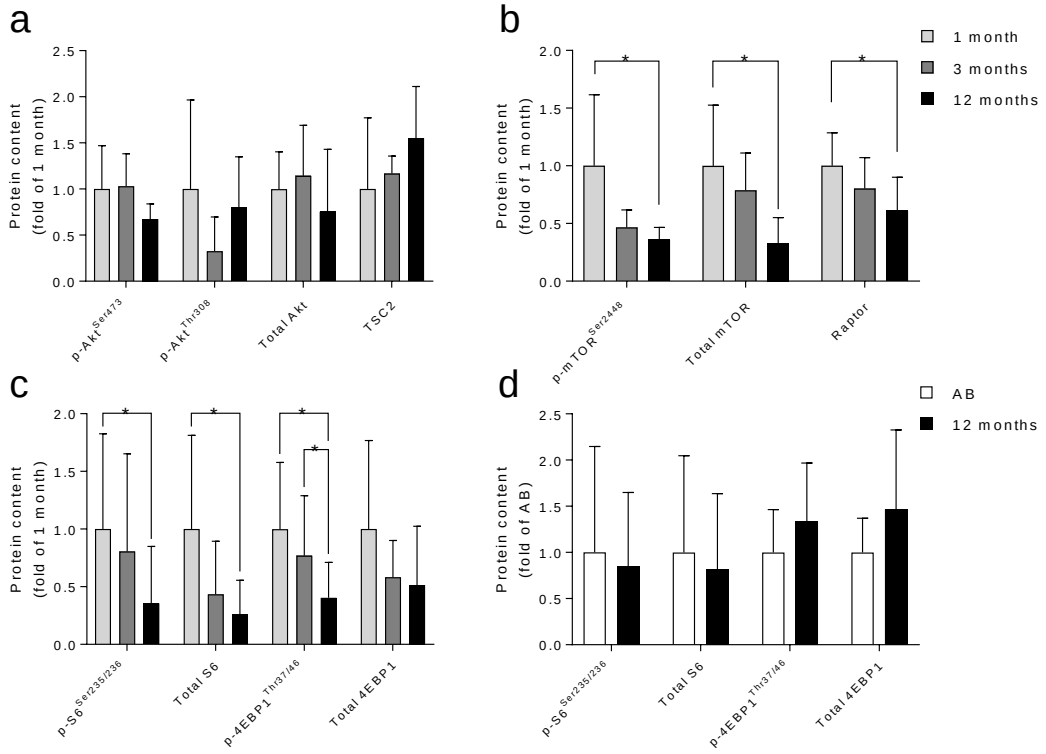

Figure 7 – Protein synthesis regulation in skeletal muscle during the first year after spinal cord injury. (a) Total and phosphorylated protein content of Akt, and TSC2, **(b)** mTOR, and Raptor, **(c)** S6, and 4EBP1 at one, three, and 12 months after spinal cord injury. **(d)** Total and phosphorylated protein content of S6 and 4EBP1 compared between able-bodied (AB) and individuals 12 months after spinal cord injury. Data are scaled to the mean of one month (panels a, b and c) and to mean of able-bodied (panel d) and presented as mean and SD. * - $p<0.05$ Friedman's test followed by Dunn's multiple comparison.

To determine changes in protein synthesis regulation, we examined the Akt-mTOR signalling axis, a driver of protein anabolism. We detected no significant changes in total Akt and TSC2 protein content, nor Akt Ser^{473} and Thr^{308} phosphorylation (Fig. 7a). On the other hand, total mTOR and Raptor protein content, and mTOR Ser^{2448} phosphorylation decreased by 12 months post-injury (Fig. 7b). Similarly, total protein content of S6, as well as S6 Ser$^{235/236}$ phosphorylation and 4EBP1 Thr$^{37/46}$ phosphorylation decreased by 12 months post-injury, while total 4EBP1 protein content remained unchanged (Fig. 7c). Total and phosphorylated protein content of S6 and 4EBP1 were not different between skeletal muscle from individuals 12 months after spinal cord injury and able-bodied controls (Fig. 7d).

Protein degradation is in part regulated by FoxO transcription factors. Total protein content of FoxO1 was not changed during first year of spinal cord injury, while FoxO1 Ser^{256} phosphorylation was decreased at three and 12 months post-injury (Fig. 8a). Conversely, FoxO3a Ser^{253} phosphorylation did not change during the first year, while the total protein content decreased by 12 months post-injury (Fig. 8a). Protein content of two E3 ubiquitin ligases, MuRF1 and MAFbx decreased by three and 12 months post-injury, respectively (Fig. 8b). On the other hand, total protein ubiquitination by both K48-linked and K63-linked poly-ubiquitin chains (Fig. 8b), as well as the protein content of the proteasomal 20Sα subunit remained unchanged during the first year of spinal cord injury (Fig. 8c). Protein content of both LC3 I and LC3 II decreased by three months, while the p62 autophagosome cargo adapter decreased by 12 months post-injury (Fig. 8c). Total protein ubiquitination by K48-linked poly-ubiquitin chains was higher in individuals 12 months after spinal cord injury, than in able-bodied controls (Fig. 8d). We detected no differences in ubiquitination by K63-linked poly-ubiquitin chains, nor the protein content of 20Sα, LC3 I, LC3 II, and p62 between individuals 12 months after spinal cord injury and able-bodied controls (Fig. 8d).

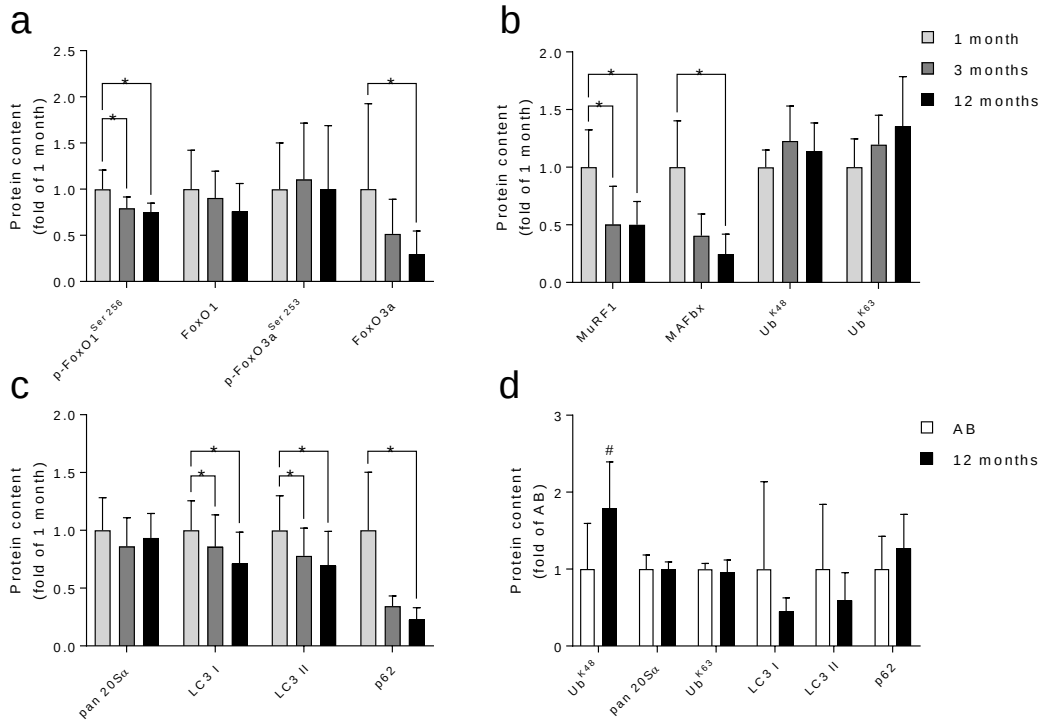

Figure 8 – Protein degradation in skeletal muscle during the first year after spinal cord injury. (a) Total and phosphorylated protein content of FoxO1 and FoxO3a, **(b)** MurF1, MAFbx, and total protein ubiquitination by K48-linked and K63-linked poly-ubiquitin chains, **(c)** proteasomal 20Sα subunit, LC3 I, LC3 II, and p62 at one, three, and 12 months after spinal cord injury. **(d)** Protein content of proteasomal 20Sα subunit, LC3 I, LC3 II, and p62 compared between able-bodied (AB) and individuals 12 months after spinal cord injury. Data are scaled to the mean of one month (panels a, b and c) and to the mean of able-bodied (panel d) and presented as mean and SD. * - $p<0.05$ Friedman's test followed by Dunn's multiple comparison. # - $p<0.05$ Mann-Whitney assigned ranks test.

In summary, we detected a coordinated decrease in total and phosphorylated content of mTOR and its downstream targets by 12 months after spinal cord injury, and no changes in the downstream targets (S6, 4EBP1) between able-bodied controls and 12 months post-injury

(Fig. 9a). Concurrently, we detected isoform specific changes in FoxO transcription factor protein content and phosphorylation (Fig. 9b). Even though we detected decreases in E3 ubiquitin ligases (MuRF1 and MAFbx) during the first year post-injury, protein ubiquitination remained stable (Fig. 9b). Additionally, we detected higher protein ubiquitination by K48-linked poly-ubiquitin chains 12 months after spinal cord injury in comparison to able-bodied controls (Fig. 9b). Markers of autophagy decreased during the first year of injury, while proteasomal catalytic subunit 20Sα remained unchanged (Fig. 9b). However, no differences were detected at 12 months after injury in comparison to able-bodied controls (Fig. 9b).

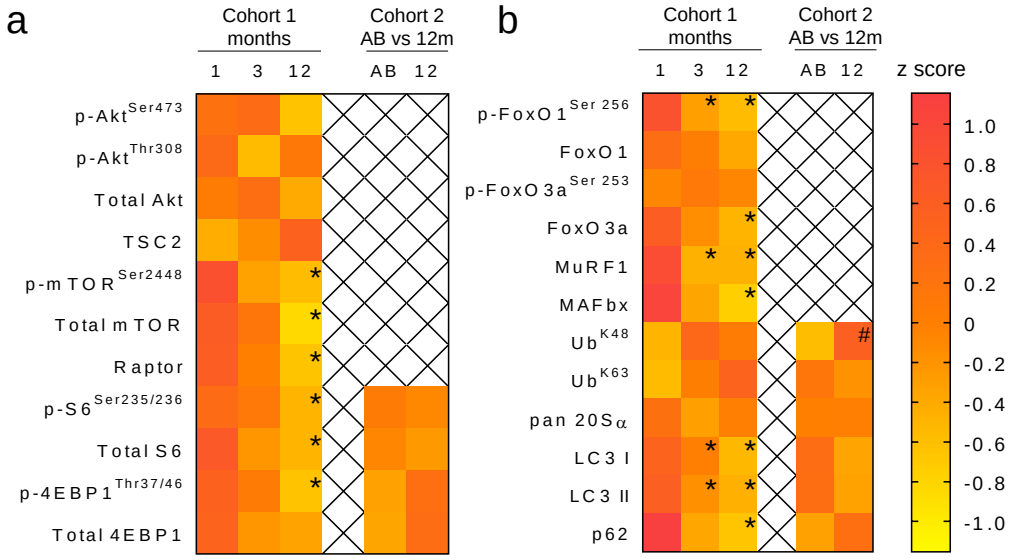

Figure 9 – Protein metabolism regulation. (a) Protein content and phosphorylation of Akt-mTOR signalling pathway during the first year after spinal cord injury (cohort 1) and comparison between 12 months post-injury to able-bodied (AB) controls (cohort 2). **(b)** Protein content and phosphorylation of protein degradation regulators and effectors during the first year after spinal cord injury (cohort 1) and comparison between 12 months post-injury to able-bodied (AB) controls (cohort 2). The heatmaps represent mean row z-scores. Positive z-scores (red) represent higher and negative z-scores (yellow) represent lower protein content. Z-scores were calculated for each individual data point separately for cohort 1 and cohort 2. * - $p<0.05$ vs 1-month, Friedman's test followed by Dunn's multiple comparison. # - $p<0.05$ vs able-bodied (AB) Mann-Whitney assigned ranks test.

4.2 ROS HOMEOSTASIS IN SKELETAL MUSCLE DURING THE FIRST YEAR AFTER SPINAL CORD INJURY (PAPER II)

We aimed to examine whether spinal cord injury leads to disturbances in human skeletal muscle ROS homeostasis and the possible sequence of these events during the first year post-injury. To investigate this, we measured the protein content of enzymatic ROS sources, antioxidant levels, and oxidative stress markers in skeletal muscle at one, three, and 12 months post-injury and compared them to skeletal muscle from able-bodied controls.

We measured the protein content of several sources of ROS, including the mitochondrial complexes, NOX2, NOX4, and xanthine oxidase. Protein content of mitochondrial complexes I, III, IV, and V was lower at 12 months after spinal cord injury in comparison to able-bodied controls (Fig. 10a). Conversely, the protein content of xanthine oxidase and

NOX2 was higher at one and three months post-injury in comparison to able-bodied controls (Fig. 10b). We detected a similar increase in NOX4 protein content at one month after spinal cord injury (Fig. 10b). Interestingly, $p47^{phox}$ Ser^{328} phosphorylation was not different between skeletal muscle from spinal cord-injured and able-bodied participants (Fig. 10b).

We determined the protein content of antioxidative enzymes responsible for maintaining ROS homeostasis. Protein content of SOD1, Catalase and GPx was similar between spinal cord-injured individuals and able-bodied controls (Fig. 10c). Protein content of SOD2 tended (p=0.08) to be lower in skeletal muscle at 12 months post-injury in comparison to able-bodied controls (Fig. 10c). Conversely, protein content of GRx was higher at three months post-injury in comparison to able-bodied controls (Fig. 10c). We detected higher total glutathione and GSH levels at one and three months post-injury in comparison to able-bodied controls, but no differences in GSSG levels (Fig. 10d).

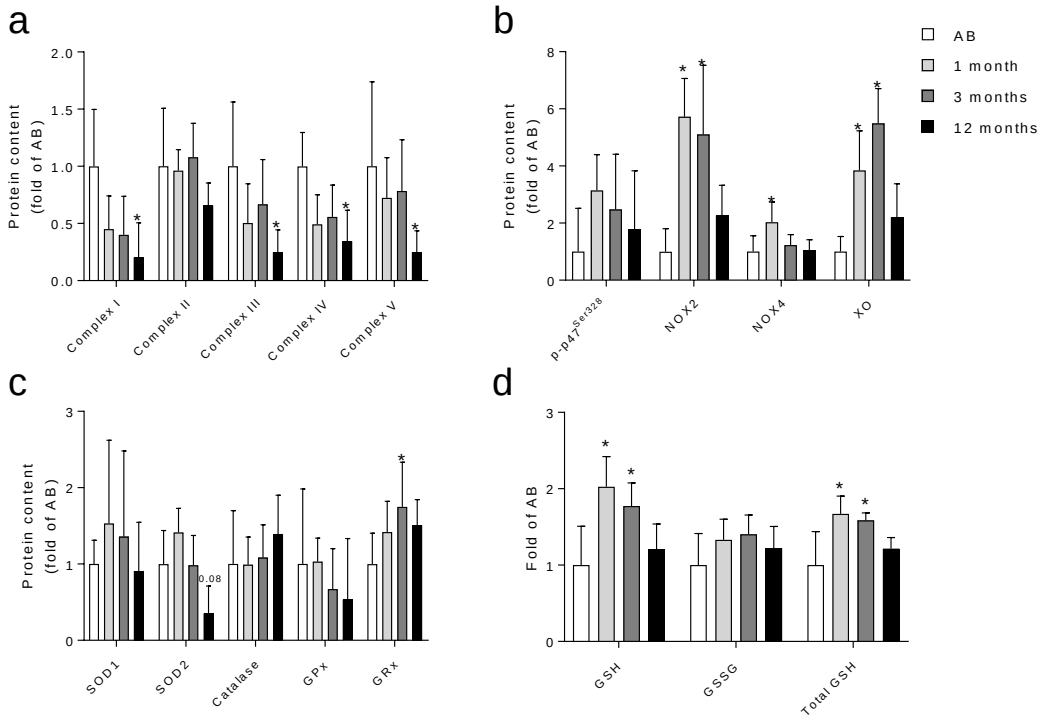

Figure 10 – Skeletal muscle content of ROS producing enzymes and antioxidants during the first year after spinal cord injury. (a) Protein content of mitochondrial complexes, **(b)** NOX2, NOX4, xanthine oxidase, and $p47^{phox}$ Ser^{328} phosphorylation, **(c)** SOD1, SOD2, Catalase, GPx, and GRx at one, three, and 12 months after spinal cord injury and in able-bodied (AB) controls. **(d)** GSH, GSSG, and total glutathione content in skeletal muscle at one, three, and 12 months after spinal cord injury and in able-bodied controls. Data are scaled to mean of able-bodied controls and presented as mean and SD. * - p<0.05 vs able-bodied controls, Kruskal-Wallis' test followed by Dunn's multiple comparison.

Overall, we show decreases in mitochondrial protein content by 12 months after spinal cord injury, while non-mitochodrial ROS producing enzymes such as NOX2, NOX4, and xanthine oxidase are increased in early phases (one and three months) post-injury (Fig. 11a). SOD2 protein content decreases concurrently with the mitochondrial content by 12 months post-injury (Fig. 11b). Conversely, GRx, reduced and total glutathione levels are increased in the early post-injury phases (Fig. 11b).

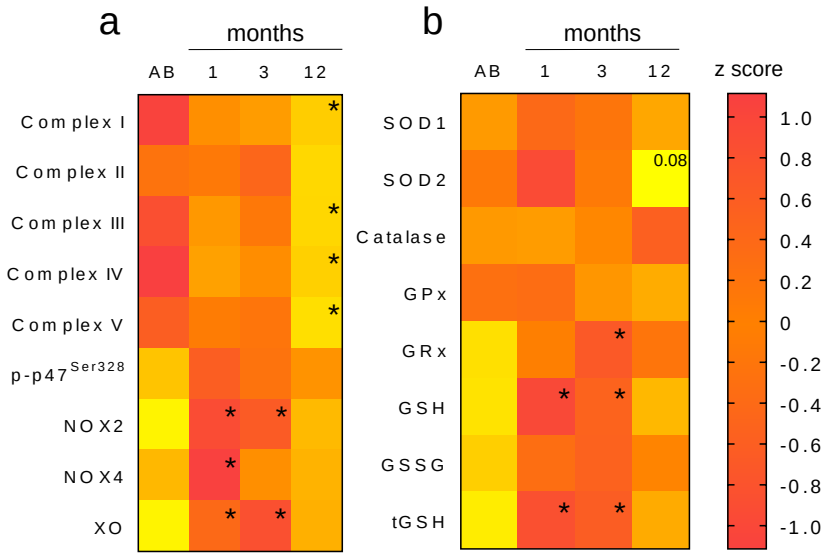

Figure 11 – Changes in ROS producing enzymes and antioxidants during the first year after spinal cord injury. (a) Protein content and phosphorylation of enzymes responsible for ROS production and **(b)** protein content of antioxidative enzymes and glutathione levels at one, three, and 12 months after spinal cord injury and in able-bodied (AB) controls. The heatmaps represent mean row z-scores. Positive z-scores (red) represent higher and negative z-scores (yellow) represent lower protein content. Z-scores were calculated for each individual data point separately. * - $p<0.05$ vs able-bodied controls, Kruskal-Wallis' test followed by Dunn's multiple comparison.

To discern whether skeletal muscle after spinal cord injury undergoes oxidative stress we measured several related markers. Amount of 4HNE protein adducts, produced by lipid peroxidation, was higher in skeletal muscle 12 months after spinal cord injury in comparison to able-bodied controls (Fig. 12a). We did not detect changes in total protein carbonylation and reduced-to-oxidised glutathione ratio in skeletal muscle from spinal cord-injured and able-bodied participants (Fig. 12a).

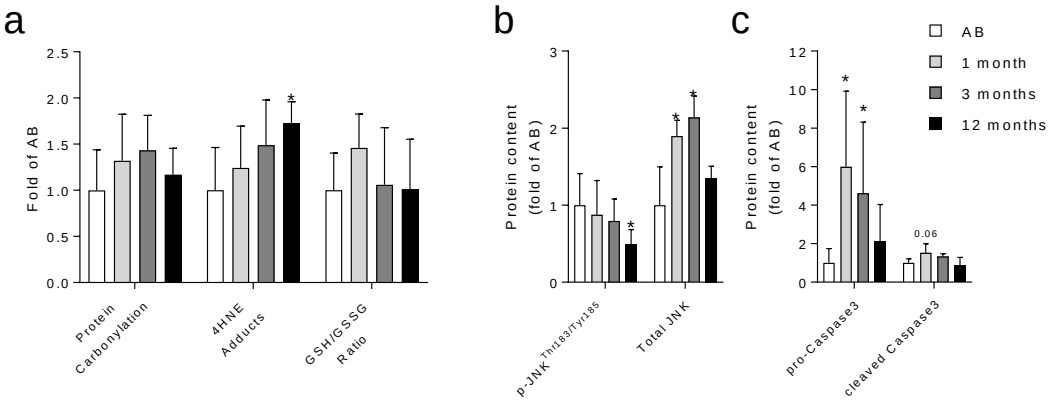

Figure 12 – Oxidative stress markers and apoptotic signalling in skeletal muscle during the first year following spinal cord injury. (a) Total protein carbonylation, 4HNE protein adducts, and reduced-to-oxidised glutathione ratio, **(b)** total and phosphorylated JNK protein content, **(c)** and cleaved and pro-Caspase3 protein content in skeletal muscle at one, three, and 12 months after spinal cord injury and in able-bodied (AB) controls. Data are scaled to the mean of able-bodied controls and presented as mean and SD. * - $p<0.05$ vs able-bodied controls (AB), Kruskal-Wallis' test followed by Dunn's multiple comparison.

As exposure to high doses of ROS leads to activation of apoptotic pathways, we investigated the protein content and phosphorylation of several apoptosis regulators in skeletal muscle

from spinal cord-injured individuals during the first year post-injury. While c-Jun N-terminal kinase (JNK) Thr^{183}/Tyr^{185} phosphorylation was lower at 12 months, the total JNK protein content was higher at one and three months post-injury in comparison to able-bodied controls (Fig. 12b). Similarly, pro-caspase3 protein levels were increased at one and three months, while cleaved-caspase3 levels tended ($p=0.06$) to be higher at one month post-injury (Fig. 12c). We did not detect changes in total calpain-1, cleaved calpain-1 nor cleaved caspase-9 protein content between skeletal muscle from spinal-cord injured and able-bodied individuals (data not shown). Thus, we observed increases in apoptotic signalling in the skeletal muscle at one and three months after spinal cord injury in comparison to able-bodied controls.

4.3 DIFFERENTIATION CAPACITY OF SATELLITE CELLS FOLLOWING SPINAL CORD INJURY (PAPER III)

To examine whether spinal cord injury leads to a defect in the intrinsic capacity for differentiation of skeletal muscle satellite cells we isolated satellite cells from spinal cord-injured and able-bodied donors and studied them *in vitro*. We confirmed that activated satellite cells (myoblasts) from both spinal cord-injured and able-bodied donors were able to fuse and form multinucleated myotubes using immunohistochemistry (data not shown).

To quantitatively assess differentiation we measured the protein content of several structural proteins and differentiation markers in myoblasts and myotubes from spinal cord-injured and able-bodied individuals. Desmin, myogenin, and both type I and type II myosin heavy chain (MyHC) increased during differentiation in cells from both groups (Fig. 13a). Myotubes from spinal cord-injured donors had higher levels of MyHC II and a similar tendency for MyHC I ($p=0.06$) (Fig. 13a). Regulation of differentiation was investigated by measuring the expression of Pax7 and myogenic regulatory factors in myoblasts and myotubes from spinal cord-injured and able-bodied individuals. Cells from both groups decreased the expression of Pax7, Myf5, MyoD, and Myf6 through differentiation (Fig. 13b). Conversely, expression of myogenin and desmin was increased by differentiation (Fig. 13b). Myoblasts from spinal cord-injured donors expressed higher MyoD levels than those from able-bodied controls (Fig. 13b). No differences between the able-bodied and spinal cord-injured groups were found in the expression of Pax7, Myf5, Myf6, myogenin, and desmin (Fig. 13b).

Consequently, we determined the metabolic properties of differentiated myotubes from spinal cord-injured and able-bodied donors. We measured protein synthesis and fatty acid ß-oxidation capacity, as well as protein degradation regulation. We determined protein synthesis by [^{14}C]-labelled phenylalanine incorporation into protein and found no differences between both groups of myotubes (Fig. 13c). Similarly, myotubes from spinal cord-injured and able-bodied donors have shown similar capacity for fatty acid ß-oxidation, measured by production of [^{3}H]-labelled water from [^{3}H]-labelled palmitic acid (Fig. 13c). To assess protein degradation regulation, we measured protein content of FoxO transcription factors and their targets by Western blot. Protein content of Ser^{256} phosphorylated FoxO1, Ser^{253} phosphorylated FoxO3a, and total FoxO3a were similar in myotubes from the two groups (Fig. 13d). Total FoxO1 protein content tended ($p=0.06$) to be lower in myotubes from spinal

cord-injured individuals than from able-bodied controls (Fig. 13d). No differences were found in the protein content of LC3 I, LC3 II, p62, MAFbx, and proteasomal 20Sα catalytic subunit between myotubes from spinal cord-injured and able-bodied donors (Fig. 13d).

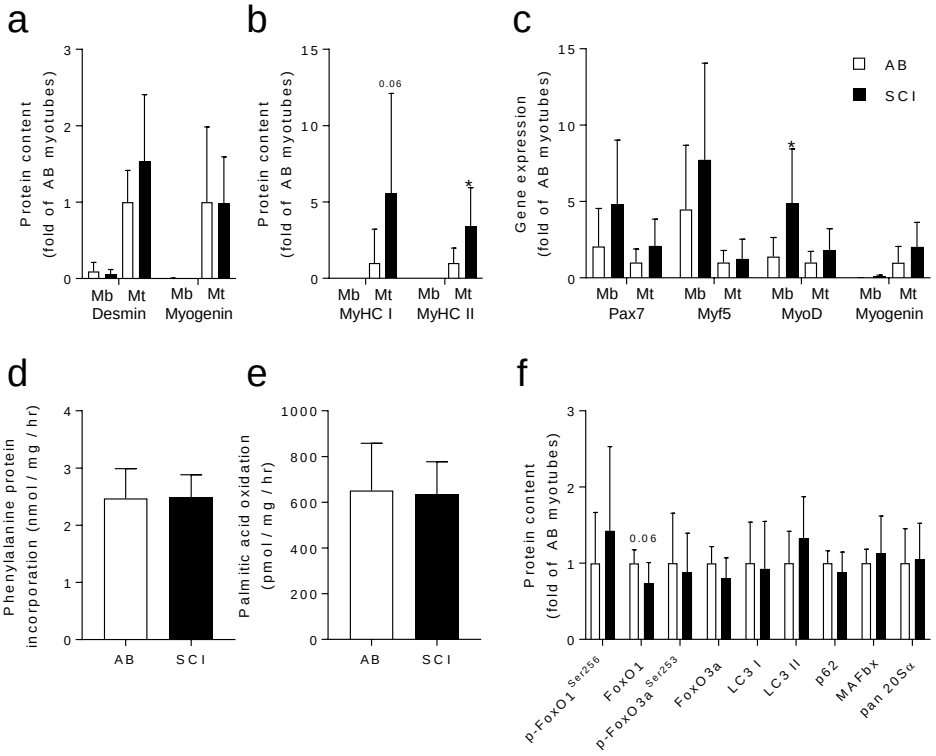

Figure 13 – Satellite cell differentiation capacity and metabolic properties of differentiated myotubes from individuals with spinal cord injury and able-bodied controls. (a) Protein content of desmin, myogenin, **(b)** MyHC I, and MyHC II in undifferentiated myoblasts (Mb) and differentiated myotubes (Mt) from spinal cord-injured (SCI) and able-bodied (AB) donors. **(c)** Gene expression of Pax7 and MRFs in undifferentiated myoblasts (Mb) and differentiated myotubes (Mt) from spinal cord-injured (SCI) and able-bodied (AB) donors. **(d)** [^{14}C] phenylalanine incorporation into protein and **(e)** [^{3}H] palmitic acid oxidation in myotubes from spinal cord-injured (SCI) and able-bodied (AB) donors. **(d)** Total protein content and phosphorylation of FoxO transcription factors and regulators of protein degradation in myotubes from spinal cord-injured (SCI) and able-bodied (AB) donors. Data are scaled to mean of myotubes from able-bodied controls and presented as mean and SD. * - $p<0.05$ vs able-bodied controls (AB). Panels a-c: two-way ANOVA followed by Sidak's multiple comparison test. Panels d-f: Mann-Whitney assigned ranks test.

5 DISCUSSION

5.1 SKELETAL MUSCLE PROTEIN METABOLISM REGULATION DURING THE FIRST YEAR FOLLOWING SPINAL CORD INJURY

We examined changes in protein content of protein metabolism regulators in skeletal muscle during the first year following spinal cord injury. We observed coordinated decreases in phosphorylation and total protein content of mTOR, and its downstream targets through the first year of spinal cord injury. Concurrently, we detected isoform-specific changes in FoxO transcription factors, coincident with a progressive decrease in protein content of their transcriptional targets MuRF1, MAFbx and LC3 I/II. Additionally, we show that proteasomal content and total protein ubiquitination by K48/K63-linked poly-ubiquitin chains remains stable through the first year following spinal cord injury. Together our data indicates that protein synthesis and autophagy are more active in the initial stage, while proteolysis remains higher throughout the first year post-injury.

5.1.1 Protein synthesis regulation

The Akt-mTOR signalling pathway is a key positive regulator of skeletal muscle mass (94). We did not find a significant decrease in total and Ser^{473} and Thr^{308} phosphorylated Akt during the first year following spinal cord injury. Unchanged Ser^{473} Akt phosphorylation has been previously described in the *vastus laterials* of individuals in chronic stages (>6 yr) after spinal cord injury (166). However, we observed a decrease in phosphorylation of Akt downstream targets from one to 12 months post-injury. Similar disconnects between the Akt protein content and phosphorylation of its downstream targets has been previously reported both in spinal cord injury (162) and type 2 diabetes (167). Thus, it is possible that the enzymatic activity of Akt in skeletal muscle after 12 months of spinal cord injury is decreased, in spite of stable protein content.

Downstream of Akt, we detected a coordinated decrease in phosphorylation of mTOR, 4EBP1 and S6 as well as total protein content of mTOR, Raptor and S6 from one to 12 months post-injury. Decreased phosphorylation of 4EBP1, together with decreased phosphorylation and total content of S6 indicate lower signal transduction through mTOR and p70S6K. Previous studies on skeletal muscle after spinal cord injury have indicated a progressive decrease in signal transduction through the Akt-mTOR axis, by showing a negative correlation between 4EBP1 as well as glycogen synthase kinase 3ß (GSK3ß) phosphorylation and duration of injury (162). Together with these findings, our data indicates that promotion of protein translation by the Akt-mTOR axis in skeletal muscle is higher at one month and decreases by 12 months after spinal cord injury.

Additionally, we did not observe differences in 4EBP1 and S6 phosphorylation and total protein content between able-bodied and individuals 12 months after spinal cord injury. In contrast, previous studies examining a cohort of eight individuals ranging two months to 30 years after spinal cord injury have detected lower 4EBP1 phosphorylation and total protein content in comparison to able-bodied controls (162). However, this decrease was mainly

driven by individuals with long-standing injuries (6.5 to 30yr). While our data indicate that Akt-mTOR promotion of protein translation is similar 12 months post-injury to able-bodied controls, it is conceivable that it decreases further into the chronic phase of spinal cord injury. Hence the previously reported decrease in 4EBP1 phosphorylation could be developing progressively, reaching levels lower than in healthy skeletal muscle further into the chronic phase post-injury.

Akt-mTOR signalling has been shown to be important for protein synthesis in human skeletal muscle, as mTOR inhibition by rapamycin lowers protein synthesis rates both in response to nutrient and exercise in healthy individuals (168, 169). Furthermore, hypertrophy and atrophy in human skeletal muscle are coincident with increased and reduced Akt phosphorylation, respectively (95, 96). Thus, even though we did not directly measure protein synthesis flux, our results indirectly estimate protein translation. We indicate a decrease in protein translation through the first year, and a transient increase in protein synthesis one month post-injury. In contrast, direct measurements of phenylalanine incorporation into protein have shown decreased protein synthesis rates in human skeletal muscle after 14 days of immobilization or after seven days of bed rest (170, 171). Additionally, these studies indicate that human muscle atrophy promotes resistance to anabolic stimuli. Namely, higher doses of amino-acid infusion exacerbate the differences in protein synthesis rate between the immobilized and non-immobilized limb (170). Similarly, ingestion of a mixture of essential amino acids causes an increase in protein synthesis and Akt-mTOR signalling in normal conditions, but not after seven days of bed rest (171). Similar changes may occur in the acute phase following spinal cord injury, which can be revealed through more direct measurements of protein synthesis.

Additionally, spinal cord injury is accompanied by an acute decline in plasma concentrations of IGF1, an important driver of protein anabolism (44). While in the first days post-injury IGF1 plasma levels decrease, the following weeks lead to partial recovery, peaking around four weeks post-injury (38, 39). It is also worth noting that muscle relaxant therapy (baclofen) in spinal cord-injured can lead to elevation of plasma IGF1 levels, which remaining below the referent values (172). The participants in our cohort have not received regular baclofen therapy, but have however taken relaxants when indicated. Interestingly, spinal cord contusion leads to increased production of IGF1 peptide within rat *soleus* muscle in the first two weeks post-injury (173). The increased autocrine effect and partial recovery of plasma IGF1 levels could lead to the transient increase in Akt-mTOR signalling.

An increase in protein synthesis rate (~25%), coupled with increased protein degradation (~85%) has been described in isolated rat *soleus* muscle after three days of denervation (85). Considering that skeletal muscle atrophy is an enzymatically driven process, this transient increase in protein synthesis might be necessary to upregulate protein degradation effectors which ultimately lead to net protein loss. In support of this hypothesis, during the acute phase post-injury transcription of genes involved in protein degradation is increased, making them more available for translation (174). Conversely, any increases in protein synthesis are not

likely to lead increased translation of structural proteins during this phase. Myofibrillar protein synthesis rate decreases after two weeks of disuse in human skeletal muscle (170). Furthermore, cumulative measurements of protein synthesis, using deuterium oxide labelling coupled to mass spectrometry, during first four days post-denervation in rat *gastrocnemius* muscle show decreased synthesis of actin, myosin light chain, troponin, and tropomyosin (175). Conversely, synthesis of certain proteins is upregulated in response to denervation (175). Similar investigations during the early stage after spinal cord injury might elucidate whether conserved Akt-mTOR signalling leads to increased synthesis of protein degradation effectors.

5.1.2 Protein degradation regulation

FoxO transcription factors are important transcriptional regulators of protein degradation effectors (115, 116). We observed a decrease in FoxO1 Ser^{256} phosphorylation by three months following spinal cord injury, without changes in total FoxO1 protein content. Conversely, FoxO3 total protein content was reduced by 12 months post-injury while Ser^{253} phosphorylation remained stable. As phosphorylation of FoxO by Akt leads to their retention in the cytoplasm (114) our data suggests increasing FoxO1 and decreasing FoxO3 activity through the first year following spinal cord injury. However, we observed a decrease in transcriptional targets of FoxOs (MuRF1, MAFbx and LC3) (115, 117) at 12 compared to one month following spinal cord injury, indicating overall reduction of transcriptional activity of FoxO factors. We did not directly compare the protein content of FoxO transcription factors to able-bodied controls. Previous comparisons show lower FoxO1 and FoxO3 content in the nuclear fraction of skeletal muscle samples from individuals two months to 30 years post-spinal cord injury, concurrent with diminished expression of FoxO transcriptional targets (MAFbx/MuRF1) (162). Conversely, other investigations of skeletal muscle >6 years post-injury have shown increased FoxO1 expression and total protein content and similar levels of MAFbx to able-bodied controls (78). While our data suggest that FoxO signalling decreases through the first year, it remains unclear whether the chronic phase post-injury brings about a further decrease or an increase of FoxO signalling.

Cellular protein degradation is mainly mediated by the ubiquitin-proteasome system (98). We detect a progressive decrease of two E3 ubiquitin ligases (MAFbx/MuRF1) through the first year following spinal cord injury. Our data are in line with previous reports which show induction of MAFbx and MuRF1 expression in the first days (2–5 days) following spinal cord injury with a decrease in their protein content in the later stages post-injury (162, 174). Conversely, protein ubiquitination by K48-linked poly-ubiquitin chains remains stable during the first year and higher at 12 months post-injury in comparison to able-bodied controls. One explanation for decreased MAFbx and MuRF1 protein content concurrent with stable protein ubiquitination, is increased activity of other E3 ligases. MAFbx and MuRF1 have been described as important for development of muscle atrophy (106). However, recent reports in mouse models have implicated other E3 ligases as well. Notably, inhibition of tripartite motif-containing protein 32 (Trim32) in *tibialis anterior* protects mouse desmin filaments

from ubiquitination and degradation induced by fasting while TNF receptor associated factor 6 (TRAF6) muscle-specific knockout models are partially protected from denervation-induced atrophy (176, 177). On the other hand, protein ubiquitination could remain high due to decreased proteasomal degradation leading to accumulation of tagged protein. However, we did not detect changes in the 20Sα catalytic subunit of the proteasome during the first year post-injury, with similar levels at 12 months compared to able-bodied controls. Together our data indicates that proteolysis by the ubiquitin-proteasome system remains stably increased through the first year following spinal cord injury.

Additionally, we examined several markers of autophagosome-mediated protein degradation during the first year following spinal cord injury. We detected a decrease in LC3 I, LC3 II protein content by three months and p62 by 12 months post-injury. A reduction in LC3 and p62 could be attributed to either decreased transcription or increased autophagy-mediated degradation of both targets (111). However, both LC3 and p62 are under transcriptional control of FoxO transcription factors (178). Considering the reduction of protein content of other FoxO targets, it is more likely that p62 and LC3 content reduction is mainly due to decreased transcription. Thus, progressive reduction of LC3 and p62 could indicate lower capacity for autophagosome formation at 12 compared to one month after spinal cord injury. Additionally, we detected similar LC3 and p62 levels when comparing skeletal muscle from spinal cord-injured individuals 12 months post-injury and able-bodied controls. In rat models skeletal muscle autophagy markers are increased seven days after denervation and 10 weeks after spinal cord transection (179, 180). In light of this, it is likely that autophagy is higher in the initial stage of spinal cord injury, and progressively decreases throughout the first year.

Collectively, our data indicate higher protein turnover in early post-injury phases, trough conserved synthesis and increased autophagy and ubiquitin-proteasome activity. Conversely, in the late stages post-injury protein turnover decreases, possibly still favouring net protein loss. It is worth noting that testosterone levels decrease following spinal cord injury and frequently remain low in motor complete injuries (45, 46). Testosterone is a known driver of skeletal muscle mass and low testosterone levels lead to decreased fibre CSA of mouse *gastrocnemius* muscle, accompanied by lower Akt-mTOR signal transduction, decreased FoxO phosphorylation, and higher expression of MuRF1 and MAFbx six weeks after castration (181). Furthermore, such effects are abolished by weekly anabolic steroid supplementation (181). Hence, hypogonadism is likely to promote skeletal muscle atrophy following spinal cord injury. Considering the protective effects of anabolic steroids, supplementation strategies during post-injury rehabilitation might prove beneficial in alleviating muscle wasting.

5.2 SKELETAL MUSCLE ROS HOMEOSTASIS DURING THE FIRST YEAR FOLLOWING SPINAL CORD INJURY

We examined whether spinal cord injury induces disturbances in ROS homeostasis and apoptotic signalling in skeletal muscle during the first year post-injury. At one and three months post-injury we found higher protein content of NADPH oxidases, xanthine oxidase

and both total and cleaved caspase-3. Concurrently, we detected increases in reduced and total glutathione levels, and increase GRx protein content. Conversely, we observed decreased mitochondrial and SOD2 protein levels at 12 months following spinal cord injury, along with increased 4HNE protein adducts. Together, these data suggest increased apoptotic signalling and non-mitochondrial ROS production during the first three months following spinal cord injury, concurrent with increases in antioxidant defences.

5.2.1 ROS production

In murine models, inhibition of mitochondrial ROS production substantially reduces oxidative stress and skeletal muscle atrophy induced by immobilization (122). Thus mitochondria have been suspected to be a major source of excess ROS production in atrophying skeletal muscle. We detected a decrease in the protein content of most mitochondrial complexes in skeletal muscle by 12 months following spinal cord injury. Studies have previously reported a progressive decrease of mitochondrial content during the first year following spinal cord injury, although without comparing it to able-bodied controls (182). However, succinate dehydrogenase activity is decreased in the chronic (>2 years) phase of spinal cord injury in comparison to able-bodied controls (183). In light of these previous reports, our findings of decreased protein content indicate a lower contribution of skeletal muscle mitochondrial ROS production in the late phase post-injury. However, we did not observe differences between spinal cord-injured and able-bodied individuals at one and three months post-injury. In contrast to the chronic phase, the early post-injury phase is accompanied by a transient increase in succinate dehydrogenase activity (36). Thus, mitochondrial ROS production could be more pronounced in the early post-injury phase in spite of unchanged protein content.

Several non-mitochondrial enzymes have been suspected to play a role in excessive ROS production during skeletal muscle atrophy (130, 184). We found that NOX2 protein content was increased one and three months, while NOX4 was higher three months after spinal cord injury. Conversely, Ser^{328} phosphorylation of $p47^{phox}$ regulatory subunit of NOX2 was unchanged. NOX2 in immune cells requires phosphorylation and translocation of its regulatory $p47^{phox}$ subunit for ROS production, and this prerequisite is conserved in murine skeletal muscle (126, 185). Therefore, unchanged phosphorylation of $p47^{phox}$ in skeletal muscle after spinal cord injury indicates comparable ROS production by NOX2 to able-bodied controls in resting conditions. However, assembly of NOX2 regulatory subunits and their translocation to the sarcolemma is an event which occurs during muscle contraction (121). Thus increased NOX2 protein content suggests a larger capacity for ROS production which could play an important role during muscular spasms or electrically-stimulated rehabilitative exercise following spinal cord injury. On the other hand, NOX4 does not require similar assembly of cytoplasmic regulators and is considered to be constitutively active (125). Hence, increased NOX4 protein content one month post-injury indicates excessive skeletal muscle ROS production even in resting conditions.

We observed an increase in xanthine oxidase protein content at one and three months following spinal cord injury. Increased xanthine oxidase activity has been reported in murine skeletal muscle in the acute phase of disuse-induced atrophy, and this increase is mainly due to the activity of the ROS producing enzyme isoform (130). Furthermore, in murine skeletal following spinal cord transection, increased NOX4 protein content leads to oxidative modification of ryanodine receptor 1 and reduced binding to calstabin 1, which may promote Ca^{2+} release (186). Proteolytic modification of xanthine oxidoreductase into its ROS producing form is dependent on a Ca^{2+}-activated protease, other than calpain (187). Therefore, it is possible that in the early phase post-injury, NOX4 and xanthine oxidase form a feed-forward loop which promotes non-mitochondrial ROS production in atrophying skeletal muscle.

Previous studies have examined mitochondrial function in permeabilized skeletal muscle fibres after 14 days of immobilization in humans. Both reports indicate increased mitochondrial hydrogen peroxide production following immobilization, although they diverge on the respiratory state during which ROS is produced (163, 188). However, these studies implicate the mitochondria as an important source of ROS during skeletal muscle atrophy in humans. Further functional studies are need to examine whether similar changes occur following spinal cord injury. Additionally, one of these reports shows unchanged NOX4 protein levels with decreased xanthine oxidase protein content in response to 14 days of disuse (188). This directly contrasts our findings of increased NOX2, NOX4 and xanthine oxidase one month following spinal cord injury. Conceivably, the duration of muscle atrophy could explain these differences and longer disuse could potentially lead to similar increases we detect at one month post-injury. On the other hand, spinal cord injury carries a profound impact on whole-body physiology and is not limited to effects on skeletal muscle. Individuals with spinal cord injury have increased resting levels of plasma angiotensin II (189), which has been shown to activate and increase protein content of NOX2 in skeletal muscle cells (190). It is thus possible that underlying differences between models are responsible for divergent findings between our studies.

5.2.2 ROS decomposition

We observed that SOD1 protein content remains stable, while SOD2 protein content decreased during the first year following spinal cord injury. Retained protein content of both SOD1 and 2 at one and three months post-injury indicates conserved superoxide scavenging capacity. The acute phase (14 days) of skeletal muscle atrophy in murine disuse models induces an increase in SOD1 and a decrease in SOD2 activity (130). It is possible that similar adaptations occur preceding our one month time point. However, decreased SOD2 content at 12 months following spinal cord injury suggests lower antioxidant capacity and closely follows the decrease in mitochondrial content. Overall, our data demonstrate retained superoxide scavenging capacity in skeletal muscle post-injury.

Hydrogen peroxide is further detoxified into molecular oxygen and water through the activity of catalase and GPx. We observed unchanged protein content of both enzymes during the

first year post-injury, indicating retained hydrogen peroxide scavenging capacity. GPx oxidizes GSH during hydrogen peroxide decomposition, converting it into GSSG, which is reduced back through the action of GRx. We detected an increased GSH and total glutathione pool at one and three months post-injury. Glutathione is synthesized from glutamate, cysteine and glycine in a two-step process catalysed by glutamate cysteine ligase and glutathione synthase and is promoted by the Akt-mTOR-p70S6K axis (191). Previous investigations of human skeletal muscle atrophy following 30 days of bed-rest have shown increased glutathione synthesis measured by stable isotope labelled precursors (192). It is possible that similar increases in biosynthesis occur in the skeletal muscle in early phases following spinal cord injury, promoted by the transient increases in signalling through the Akt-mTOR axis (Paper I). Furthermore, we detected increased protein content of GRx three months following spinal cord injury. GRx activity is responsive to increased glutathione oxidation in murine myocardium, and this increase is dependent on NOX activation (193). Conceivably, longer exposure to increased NOX-mediated ROS production could lead to an adaptive increase in GRx protein content and increased glutathione turnover. Collectively, our data suggest that early phases following spinal cord injury lead to compensatory increases in skeletal muscle antioxidant capacity.

5.2.3 Oxidative stress markers and apoptotic signalling

Total protein carbonylation levels were similar between skeletal muscle from spinal cord-injured and able-bodied individuals. One possibility is that during the early phase following spinal cord injury, increases in antioxidant capacity are sufficient to compensate for the increased ROS production thus maintaining redox balance. However, it is important to consider that carbonylated proteins are preferably degraded by the proteasome system even in absence of ubiquitination (194). We and others have suggested that the initial rapid phase of skeletal muscle atrophy is coupled with increased protein degradation and transiently increased synthesis (Paper I) (85). Therefore, it is also possible that in this state of high protein turnover, we are underestimating total protein carbonylation.

We observed an increase in 4HNE protein adducts 12 months post-injury which could suggest increased lipid peroxidation. However, decreases in mitochondrial content and normalization of NOX and xanthine oxidase protein content at 12 months suggest that ROS production is not increased at these time points. This apparent increase in lipid peroxidation in comparison to able-bodied controls in absence of increased ROS production could be attributed to changes in skeletal muscle composition and metabolic properties. Resting 4HNE protein adducts are higher in skeletal muscle from individuals with type 2 diabetes than in normal glucose tolerant individuals (195). Furthermore, skeletal muscle from obese individuals produces more 4HNE in comparison to athletes in response to exercise (196), suggesting that intramuscular lipid composition might regulate lipid peroxidation. Skeletal muscle from individuals with spinal cord injury contains substantially more intramuscular fat in comparison to able-bodied controls (67). Additionally, skeletal muscle protein turnover decreases by the late stages following spinal cord injury (Paper I) (162). Hence, it is possible

that higher lipid availability together with lower protein turnover could lead to increased lipid peroxidation and 4HNE-modified protein accumulation at 12 months post-injury.

One of the proposed mechanism by which oxidative stress interferes with skeletal muscle function is through promotion of apoptosis (197). We observed that total JNK protein content was higher at one and three months post-injury compared to able-bodied controls. This could suggest underlying transcriptional mechanisms promoting apoptosis during these early timepoints. Phosphorylation of JNK on Thr^{183}/Tyr^{185} was not increased at these time points, which may suggest unchanged signalling through upstream kinases (mitogen-activated protein kinase kinase 4 and 7) (198). Furthermore, we observed that pro-caspase-3 protein content was higher at one and three, while cleaved caspase-3 was increased at one month following spinal cord injury. Caspase-3 is an effector caspase lying downstream of most apoptotic signalling pathways and active caspase-3 is sufficient to induce apoptosis (199). Therefore an increase in caspase-3 content and cleavage suggests increased apoptosis in the early rapid phase following spinal cord injury. Furthermore, caspase-3 shows proteolytic activity and is essential for the initial degradation of myofibrillar contractile protein complexes, allowing their further degradation by the proteasome system (102). Thus, increased caspase-3 content may promote protein degradation and loss of muscle mass in the initial phase post-injury.

5.3 DIFFERENTIATION CAPACITY OF SKELETAL MUSCLE SATELLITE CELLS AFTER SPINAL CORD INJURY

We examined whether spinal cord injury leads to intrinsic disturbances in the differentiation capacity of satellite cells following long-term spinal cord injury. Our data suggests that satellite cells retain their ability to differentiate *in vitro* and form multinucleated myotubes. Furthermore, differentiated myotubes show comparable metabolic characteristics to myotubes derived from satellite cells of able-bodied controls.

Impaired intrinsic capacity for differentiation of human skeletal muscle satellite cells has been shown to occur in neuronal degeneration-induced muscle atrophy. Namely, satellite cells from *deltoid* or *vastus lateralis* muscle from individuals with amyotrophic lateral sclerosis (ALS) show aberrant differentiation when grown *in vitro* (200, 201). These cells have lower capacity for fusion and form thinner myotubes with lower MyHC content in comparison to cells from able-bodied individuals (200). Conversely, our results show that skeletal muscle satellite cells from individuals with spinal cord injury are able to fuse and form multinucleated myotubes *in vitro*, with comparable amounts of desmin and myogenin to myotubes from able-bodied controls. Surprisingly, we found that myotubes from spinal cord-injured donors had higher MyHC I and II protein content. The detected increase in MyHC II could indicate fibre type memory, as in the late phases post-injury skeletal muscle is composed mainly of fast-twitch fibres (31). However, a similar increase in MyHC I indicates different underlying transcriptional or protein stability mechanisms leading to overall increase in MyHC content. However, the ability to fuse and induce increases in skeletal muscle

structural protein indicates that the differentiation capacity of satellite cells from skeletal muscle of individuals with spinal cord injury is preserved.

Furthermore, we found similar expression levels of Pax7, Myf5 and myogenin in myoblasts and myotubes from spinal cord-injured and able-bodied donors. The high expression of Myf5 in myoblasts indicates their commitment to the myogenic lineage, while the increase of myogenin suggests normal progression through the terminal stages of differentiation. Interestingly, we found higher expression of MyoD in myoblasts obtained from the spinal cord-injured group. A similar increase in MyoD expression was found in skeletal muscle satellite cells from individuals with ALS (201). However, as these cells have shown deficient differentiation, increase in MyoD expression could be attributed to compensatory mechanisms attempting to overcome this impairment (201). Conversely, satellite cells from individual with spinal cord injury were able to fuse and form multinucleated myotubes. Hence, different mechanisms might be responsible for higher MyoD expression in myoblasts from spinal cord-injured individuals. Myf5 and MyoD show an overlapping role in early stages of differentiation (202). Studies of MRF expression in murine skeletal muscle during regeneration have shown that distinct populations of satellite cells express either Myf5 or MyoD or co-express both upon activation (147). It is possible that skeletal muscle satellite cells from spinal cord-injured individuals belong predominantly to the MyoD positive group. Collectively, our data indicate that satellite cells from spinal cord-injured individuals show commitment to the myogenic lineage and are able to undergo terminal differentiation.

Human primary skeletal muscle cells grown *in vitro* have shown to mirror the metabolic properties of donor skeletal muscle. Namely, primary cultures from obese individuals have shown lower mitochondrial oxidation in response to lipid treatment in comparison to cultures from lean controls (203). Similarly, interleukin-6-induced glucose uptake and glycogen synthesis is blunted in primary skeletal muscle cultures from type 2 diabetic compared to normal glucose tolerant individuals (204). Thus, we resolved to ascertain whether differentiated myotubes shared some of the characteristics of the skeletal muscle following spinal cord injury. Skeletal muscle in the chronic stages following spinal cord injury shows lower mitochondrial enzymatic activity (183) and measurements of the Akt-mTOR signalling pathway and FoxO transcription factor content have implied decreases in protein synthesis and degradation (162). We found similar capacity for palmitate oxidation and phenylalanine incorporation into protein between myotubes from spinal cord-injured and able-bodied donors. Furthermore, FoxO3a Ser^{253} phosphorylation and total protein content were not changed indicating similar transcriptional activity. Conversely, we observed decreased FoxO1 total protein content and conserved Ser^{256} phosphorylation, indicating lower transcriptional activity. However the protein content of FoxO transcriptional targets involved in autophagy (LC3 and p62) and ubiquitination (MAFbx) were similar to able-bodied controls. The same was true for the protein content of 20Sα proteasome subunit. Collectively, our data indicate that satellite cells from spinal cord-injured individuals are able to produce myotubes with retained FoxO signalling and regulation of protein degradation. Furthermore, they exhibit conserved capacity for fatty acid oxidation and protein synthesis.

Skeletal muscle satellite cell activation is observed in the acute phase following both complete (155) and incomplete spinal cord injury in mice (173). Similar observations of myogenic stem cell activation have been made in denervation-induced atrophy (205). The number of satellite cells following denervation increases from 3 to 9% of all muscle nuclei during the first two months (156). Following this increase, satellite cell number progressively decreases into the chronic phase reaching <1% by 25 months post-denervation (156, 157). Electron microscopy studies have shown that myogenesis during this period produces small myotubes with disorganized contractile machinery (206). In human skeletal muscle following chronic spinal cord injury the number of satellite cells per muscle fibre is reduced (158). It is possible that similar mechanisms of activation and inefficient differentiation are responsible for the depletion of the satellite cell pool following spinal cord injury. However, our data indicates that spinal cord injury does not lead to an intrinsic defect in satellite cell differentiation capacity and possible causes for impaired differentiation *in vivo* might lie in the atrophying skeletal muscle milieu.

One of the possible underlying reasons for insufficient differentiation of satellite cells during spinal cord injury-induced muscle atrophy is oxidative stress. Namely, during the initial phase of *in vitro* differentiation myogenic precursors transiently enhance their total glutathione content (207). Depletion of glutathione prior to differentiation, and subsequent oxidative stress lead to impaired myotube formation, atrophic phenotype and lower content of structural protein (207). Conceivably, early increases in non-mitochodrial ROS production in skeletal muscle following spinal cord injury, especially through NOX2 at the sarcolemma (Paper II) could contribute to oxidative stress and impaired differentiation of satellite cells in skeletal muscle *in vivo*.

5.4 STUDY LIMITATIONS

Skeletal muscle atrophy following spinal cord injury is most rapid during the initial 6 weeks, and slows down in the following months (64). For papers I and II, we have chosen the one, three and 12 month time points to conform to this timeline. It is probable that extensive changes occur prior to our one month sample collection. However, due to ethical considerations related to the severity of the acute post-injury consequences, we did not collect samples at an earlier time. It is also worth noting that the studies in this thesis are cross-sectional. While it is tempting to assume linearity of events between the sampling time points, we cannot exclude the possibility that acute changes occur in a non-linear manner.

The cohort sizes in our studies were limited and therefore might not allow for detection of more subtle changes occurring within skeletal muscle following spinal cord injury. However, considering the intense nature of the muscle atrophy, our work still provides valuable insight into the profound changes occurring in the skeletal muscle following spinal cord injury. It is limiting that samples in our studies were obtained in postprandial conditions. While signal transduction through Akt-mTOR is nutrient sensitive, it is important to note that observed phosphorylation events were accompanied by changes in total protein content, which cannot be accounted for by differences in a single meal. Another considerations is the physical

activity of able-bodied controls. Healthy participants included in our studies were not taking part in any formal training and were instructed to refrain from physical activity before biopsy collection. Hence, we eliminated possible acute effects of physical activity. However, long-term nutritional and physical habits may lead to some heterogeneity in our results.

Due to sample limitations we have opted for more indirect observations of protein synthesis, degradation and ROS homeostasis, measuring protein content by western blot. This approach allowed us to elucidate coordinated changes over several regulatory systems. However, enzymatic activity of some measured targets might not always follow the protein content. When possible, we attempted to overcome this limitation by showing coordinated changes on several levels of signalling cascades. Additionally, the nature of western blot analysis is semi-quantitative. Hence, the obtained protein content values are arbitrary and relative to the cohorts we examined.

In paper III it is important to note that we observed satellite cell function *in vitro* and are able to make conclusions only on their intrinsic differentiation capacity. In other words, we cannot exclude impaired differentiation capacity of satellite cells in the skeletal muscle *in vivo*. When establishing the primary cell cultures we did not perform a formal assessment of purity and cannot exclude presence of different cell types in addition to satellite cells. However, our satellite cell isolation protocol allows for attachment and filtering of non-satellite cells for one hour. Hence, the presence of other cell types in our cultures should be minimal. Furthermore, in paper III our cohort included participants with both incomplete and complete spinal cord injury. While none of the participants with an incomplete injury were freely ambulating, we cannot exclude differences in satellite cell function due to remaining motor activity or muscular spasms.

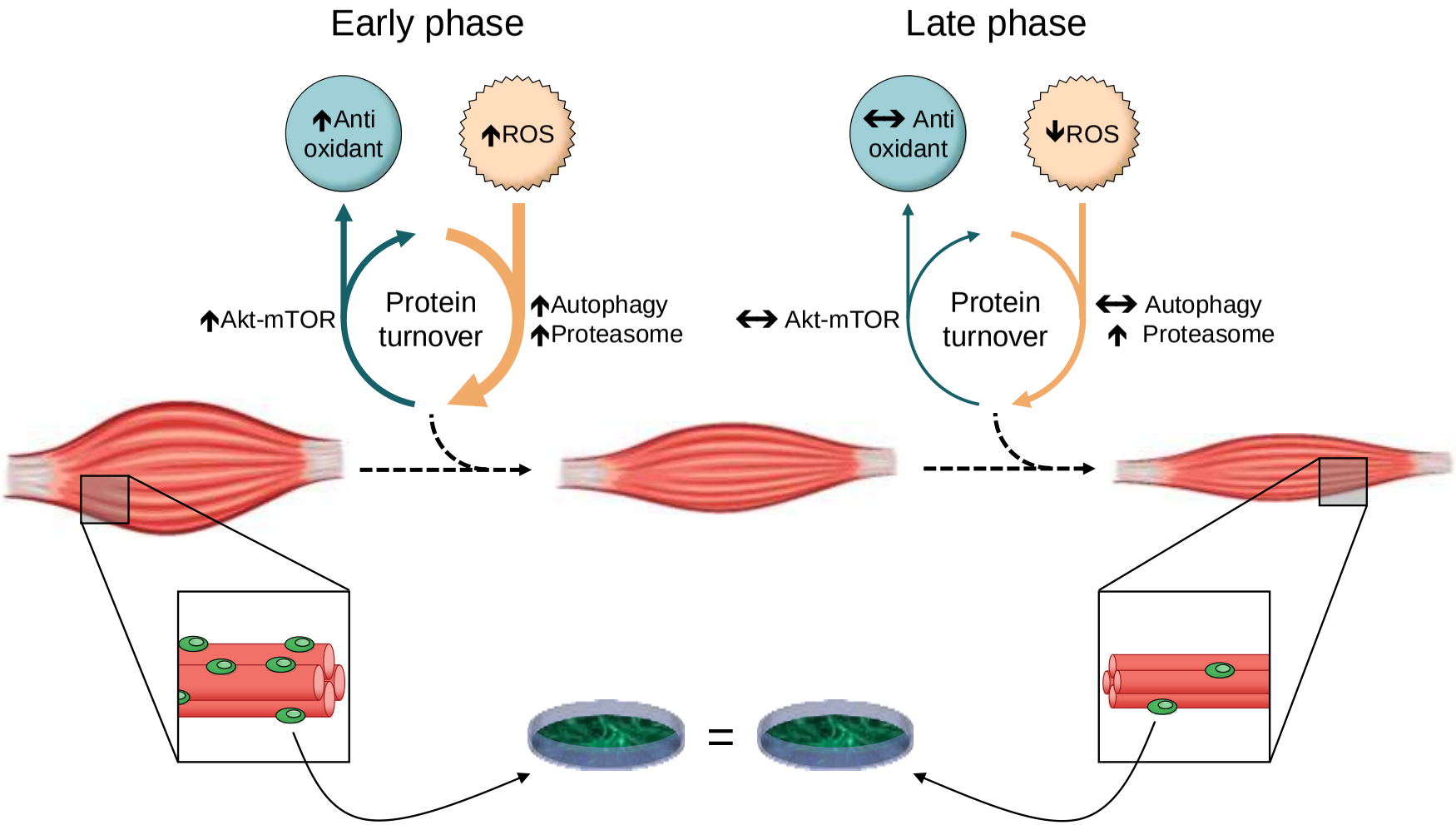

Figure 14 – Schematic of the major findings of the thesis. During the early phase post-injury protein turnover is higher, favouring net protein loss. Increased ROS production during this phase could promote such a shift in protein metabolism balance, while a transient increase in Akt-mTOR signalling may enhance antioxidant defences. By the late phase (12 months) post-injury protein turnover and ROS production decrease. Even though satellite cells beyond this phase are less numerous, their intrinsic differentiation capacity is preserved.

6 SUMMARY AND CONCLUSIONS

The findings in this thesis show that the early phase following spinal cord injury brings about most profound changes in skeletal muscle protein turnover. Protein translation and autophagy are more active, concurrently with higher ubiquitin-proteasome-dependent degradation (Paper I). Additionally, caspase-3 protein content is increased during this phase, which may promote degradation of myofibrillar complexes and muscle wasting. Furthermore, our data indicate increases in non-mitochondrial ROS production and concurrent increases in antioxidant capacity (Paper II). As ROS induce expression of genes involved in protein degradation, increased non-mitochondrial ROS production could promote protein degradation during this phase. Conversely, conserved Akt-mTOR signalling could promote glutathione biosynthesis and lead to a compensatory increase in the antioxidant capacity.

The late stage following spinal cord injury is accompanied by a return-to-baseline of skeletal muscle protein translation and autophagy, while ubiquitination and proteasomal degradation remain high (Paper I). Hence, the protein turnover in this phase decreases, possibly still favouring protein degradation and net loss. Additionally, apoptotic signalling and caspase-3 activation decline in the late stage post-injury. Concurrently, antioxidant capacity and non-mitochodrial ROS production return to baseline, while mitochondrial content decreases (Paper II). Hence, skeletal muscle ROS production is likely less pronounced in the late stage post-injury.

While ROS and the atrophying skeletal muscle milieu may lead to impaired differentiation and satellite cell pool depletion *in vivo*, observations in this thesis indicate that the intrinsic differentiation capacity of satellite cells is retained after long-term spinal cord injury. Satellite cells from skeletal muscle of spinal cord injured individuals in the late post-injury stage are able to fuse and form multinucleated myotubes *in vitro*, with similar metabolic characteristics to those from able-bodied controls (Paper III).

Overall, the findings in this thesis provide insight into the cellular and molecular mechanisms underlying skeletal muscle atrophy following spinal cord injury. Deeper understanding of such events provides valuable basis for further investigations and may inform efforts to develop novel or improve current rehabilitative and preventive strategies for alleviating muscle wasting.

7 FUTURE PERSPECTIVES

Spinal cord injury carries a profound impact on skeletal muscle. However, even in the chronic phase following spinal cord injury, rehabilitative interventions are able to partially restore metabolic properties to atrophying skeletal muscle. Namely, eight weeks of daily electrically-stimulated cycling exercise lead to increases in the protein content of transporters and enzymes involved in glucose metabolism (81). These increases are paralleled by an increase in whole-body and skeletal muscle glucose uptake (81). Thus, in spite of the profound changes occurring after spinal cord injury, the plasticity of skeletal muscle is retained.

Exercise interventions in healthy human skeletal muscle regulate protein metabolism and induce Akt/mTOR signalling (208), promoting protein anabolism over catabolism. The findings in this thesis suggest most profound changes in protein turnover, facilitating net protein loss, in the early phase post-injury. It is conceivable that rehabilitative interventions, such as electrically-stimulated exercise, during the early phase would be more effective in alleviating the loss of skeletal muscle mass following spinal cord injury. Furthermore, recent reports show that testosterone supplementation is efficacious in increasing unspecific, whole-body protein synthesis flux in hypogonadal men (209). Similar adjuvant therapies, in addition to electrically-stimulated exercise, may shift the balance in skeletal muscle protein turnover post-injury and help alleviate atrophy.

The results in this thesis suggest higher ROS production and increases in antioxidant defences in the early phase following spinal cord injury. The persistent exposure to excessive ROS production could promote protein degradation during this early stage post-injury. Furthermore, ROS production is increased during skeletal muscle contraction (121, 210). In healthy muscle such increases promote the beneficial adaptations to exercise, which are blunted by antioxidant supplementation (119). However, excessive resting ROS production in atrophying muscle, further increased by contraction during electrically-stimulated exercise could be detrimental. Hence, further investigations of ROS production during rehabilitative interventions could lead to development of adjuvant antioxidant therapies, which may support the beneficial effects of exercise.

Finally, we show that the intrinsic differentiation capacity of skeletal muscle satellite cells is retained following spinal cord injury. However, satellite cell differentiation *in vivo* may be impaired by the atrophying skeletal muscle milieu. Satellite cell activation occurs during exercise in a modality- and intensity-dependant manner. Stronger activation of satellite cells is achieved through more intense, resistance-type training (211, 212). Similar resistance-type electrical stimulation protocols have been established and shown to be efficacious in increasing the CSA of atrophying skeletal muscle (213). Hence, such rehabilitative interventions could provide the necessary stimulus to harness the conserved intrinsic differentiation capacity of satellite cells following spinal cord injury.

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Paper II

Altered oxidative stress and antioxidant defences in skeletal muscle during the first year following spinal cord injury

Savikj M[1], Kostovski E[1,2], Lundell LS[3], Iversen PO[4,5], Massart J[6], Widegren U[6]

[1]Faculty of Medicine, University of Oslo, Oslo, Norway; [2]Department of Research, Sunnaas Rehabilitation Hospital, Nesoddtangen, Norway; [3]Department of Physiology and Pharmacology, Section for Integrative Physiology, Karolinska Institutet, Stockholm, Sweden; [4]Department of Haematology, Oslo University Hospital, Oslo, Norway; [5]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; [6]Department of Molecular Medicine and Surgery, Section for Integrative Physiology, Karolinska Institutet, Stockholm, Sweden;

Address for correspondence: Ulrika Widegren, Ph.D. Department of Molecular Medicine and Surgery, Section for Integrative Physiology, Karolinska Institutet, SE-171 77 Stockholm, Sweden. Phone: +46 70 298 67 47, Fax: +46 8 33 54 36, email: Ulrika.Widegren@ki.se.

Abstract

Aim Oxidative stress promotes protein degradation and apoptosis in skeletal muscle undergoing atrophy. We aimed to determine whether spinal cord injury leads to changes in oxidative stress, antioxidant capacity and apoptotic signalling in human skeletal muscle during the first year after spinal cord injury.

Methods *Vastus lateralis* biopsies were obtained from individuals one, three and 12 months after spinal cord injury and from able-bodied controls. Protein content of enzymes involved in reactive oxygen species production and detoxification, and apoptotic signalling was analysed by western blot. Protein carbonylation and 4-hydroxynonenal protein adducts were measured as markers of oxidative damage. Glutathione content was determined fluorometrically.

Results Protein content of NADPH oxidase 2 and 4, xanthine oxidase, as well as pro- and cleaved caspase-3 was increased at one and three months after spinal cord injury compared to able-bodied controls. Furthermore, total and reduced glutathione content was increased at one and three months after spinal cord injury. Conversely, mitochondrial and superoxide dismutase 2 protein content was decreased 12 months after spinal cord injury compared to able-bodied controls.

Conclusions We provide indirect evidence of increased reactive oxygen species production through non-mitochondrial sources and increased apoptotic signalling at one and three months after spinal cord injury, concomitant with compensatory adaptations in antioxidant systems. We speculate that these adaptations indicate that redox plasticity is retained following spinal cord injury.

Keywords: atrophy, oxidative stress, reactive oxygen species, skeletal muscle, spinal cord injury

Introduction

Onset of traumatic spinal cord injury leads to extensive skeletal muscle atrophy due to neural system-decentralization and disuse.[1] The loss of muscle mass is rapid during the initial two months after injury, with a ~60% reduction in average fibre cross-sectional area compared to able-bodied controls, and an additional ~20% decrease in the following four months.[2] Muscle wasting after spinal cord injury is accompanied by decreased peripheral glucose disposal and reduced skeletal muscle mitochondrial content and fatty acid uptake,[3, 4] contributing to increased risk of cardiovascular disease and type 2 diabetes.[5, 6] Interventions targeted towards mechanisms to mitigate skeletal muscle atrophy are warranted to prevent such chronic disorders and improve the well-being of individuals with spinal cord injury.

Skeletal muscle atrophy has been linked to increased oxidative stress, defined as an imbalance between the production and detoxification of reactive oxygen species (ROS).[7] Excessive ROS production by the mitochondria, as well as non-mitochondrial sources, such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, occurs in skeletal muscle during atrophy.[8-10] Accordingly, activity of antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase and -reductase show compensatory increases in response to excessive ROS production in skeletal muscle undergoing atrophy.[9] Hence, skeletal muscle atrophy is accompanied by changes in ROS homeostasis, which may lead to oxidative stress and damage to cellular structures.[11, 12]

While ROS play a role in beneficial adaptive response to exercise in healthy skeletal muscle, ROS also have a causative role in the development of skeletal muscle atrophy.[7, 10, 13, 14] Oxidative damage makes myofibrillar proteins susceptible to degradation and exposure to exogenous ROS increases the expression of genes involved in protein catabolism, while simultaneously interfering with protein synthesis.[15-18] This imbalance between protein synthesis and degradation lies at the core of skeletal muscle atrophy.[19] Additionally, ROS exposure triggers apoptotic pathways and may contribute to the decreased mitochondrial content during muscle atrophy.[20] Conversely, inhibition of either mitochondrial or non-mitochondrial ROS production alleviates muscle wasting in murine models of disuse atrophy.[10, 13] Whether spinal cord injury leads to changes in ROS homeostasis and oxidative stress in human skeletal muscle remains unknown.

During the initial three months after spinal cord injury, plasma antioxidant levels are decreased, while both plasma and urine markers of oxidative stress are increased.[21] Our aim was to determine whether changes in ROS homeostasis and oxidative stress occur in skeletal muscle following spinal cord injury. Furthermore, based on the differing rates of muscle mass loss and the early increases in systemic oxidative stress, we hypothesized that separate phases of disturbed ROS homeostasis in skeletal muscle exist during the first year after spinal cord injury. To address this question,

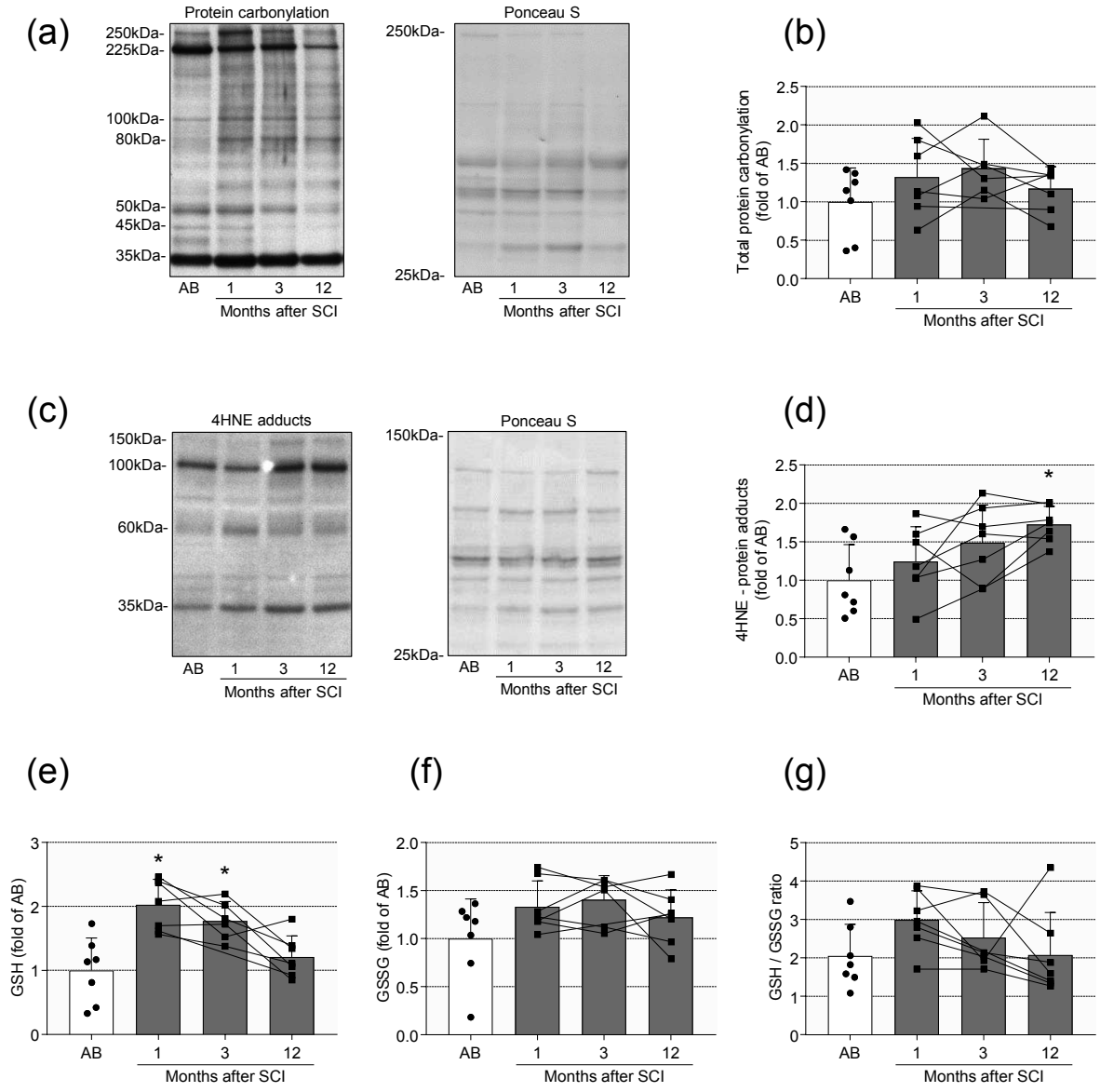

Figure 1. Oxidative stress markers in skeletal muscle following spinal cord injury. Representative western blot image and Ponceau S stain (a) and quantification for total protein carbonylation (b). Representative western blot image and Ponceau S stain (c) and quantification for total 4HNE protein adducts (d). GSH (e), GSSG (f) and GSH/GSSG ratio (g) in skeletal muscle from able-bodied (AB, white bars) and spinal cord-injured individuals (SCI, grey bars) at one, three and 12 months post-injury. Units are scaled to the mean of the able-bodied controls. Data are presented as mean and standard deviation, and individual data points are overlaid. * - $p<0.05$ vs able-bodied controls, Kruskal-Wallis followed by Dunn's multiple comparisons test.

we measured oxidative stress marker levels, as well as protein abundance of enzymes involved in ROS production and detoxification in skeletal muscle from spinal cord-injured individuals at one, three and 12 months after injury, compared with able-bodied healthy controls.

Results

Oxidative stress markers in skeletal muscle following spinal cord injury.

To assess whether spinal cord injury induces oxidative stress in skeletal muscle reduced (GSH) and oxidized (GSSG) glutathione content, as well as protein carbonylation and 4-hydroxynonenal

(4HNE) protein adducts were analysed as markers of oxidative damage. Total amount of carbonylated proteins was unaltered in skeletal muscle during the first year following spinal cord injury compared to able-bodied controls (Fig.1a and b). The level of 4HNE protein adducts was increased at 12 ($p<0.05$), but not at one and three months after spinal cord injury (Fig.1c and d). The amount of GSH (Fig. 1e), as well as of total glutathione (data not shown) was increased at one and three ($p<0.05$), but not at 12 months following spinal cord injury. There were no significant differences in the amount of GSSG (Fig. 1f) or the GSH/GSSG ratio (Fig. 1g) between skeletal muscle of spinal cord-injured individuals and able-bodied controls. Thus, our results indicate increased lipid peroxidation at 12 months, as well as augmented GSH and total glutathione content in skeletal muscle at one and three months following spinal cord injury.

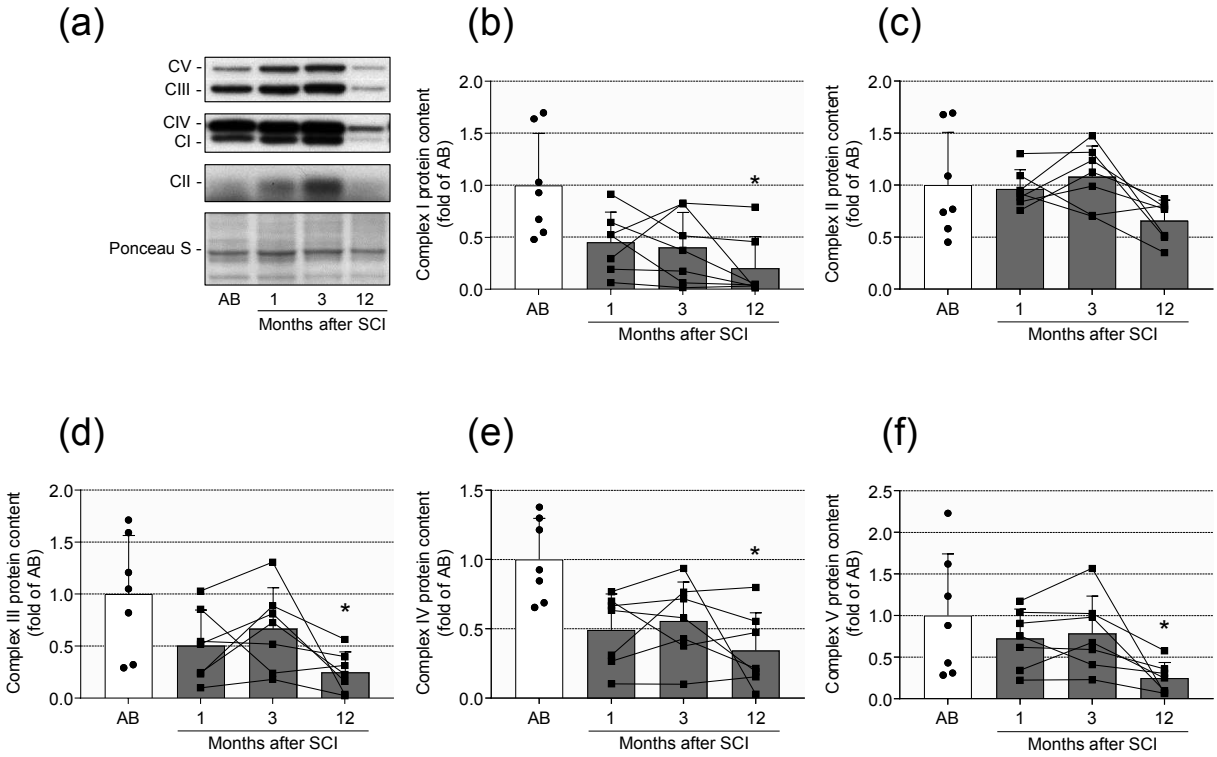

Figure 2. Mitochondrial content in skeletal muscle following spinal cord injury. Representative western blot images (a) and quantifications for mitochondrial complex I (b), complex II (c), complex III (d), complex IV (e) and complex V (f) in skeletal muscle from able-bodied (AB, white bars) and spinal cord-injured individuals (SCI, grey bars) at one, three and 12 months post-injury. Units are scaled to the mean of the able-bodied controls. Data are presented as mean and standard deviation, and individual data points are overlaid. * - $p<0.05$ vs able-bodied controls, Kruskal-Wallis followed by Dunn's multiple comparisons test.

Mitochondrial content in skeletal muscle following spinal cord injury.

Skeletal muscle mitochondrial content during the first year following spinal cord injury was determined by western blot analysis (Fig. 2a). Protein abundance of mitochondrial complexes I (Fig. 2b), III (Fig. 2d), IV (Fig. 2e) and V (Fig. 2f) were reduced at 12 months ($p<0.05$), but not at one or three months after spinal cord injury compared to able-bodied controls. Conversely, protein levels of mitochondrial

complex II were comparable between spinal cord-injured and able-bodied controls (Fig. 2c). Overall, our data indicate that skeletal muscle mitochondrial content is decreased one year after spinal cord injury.

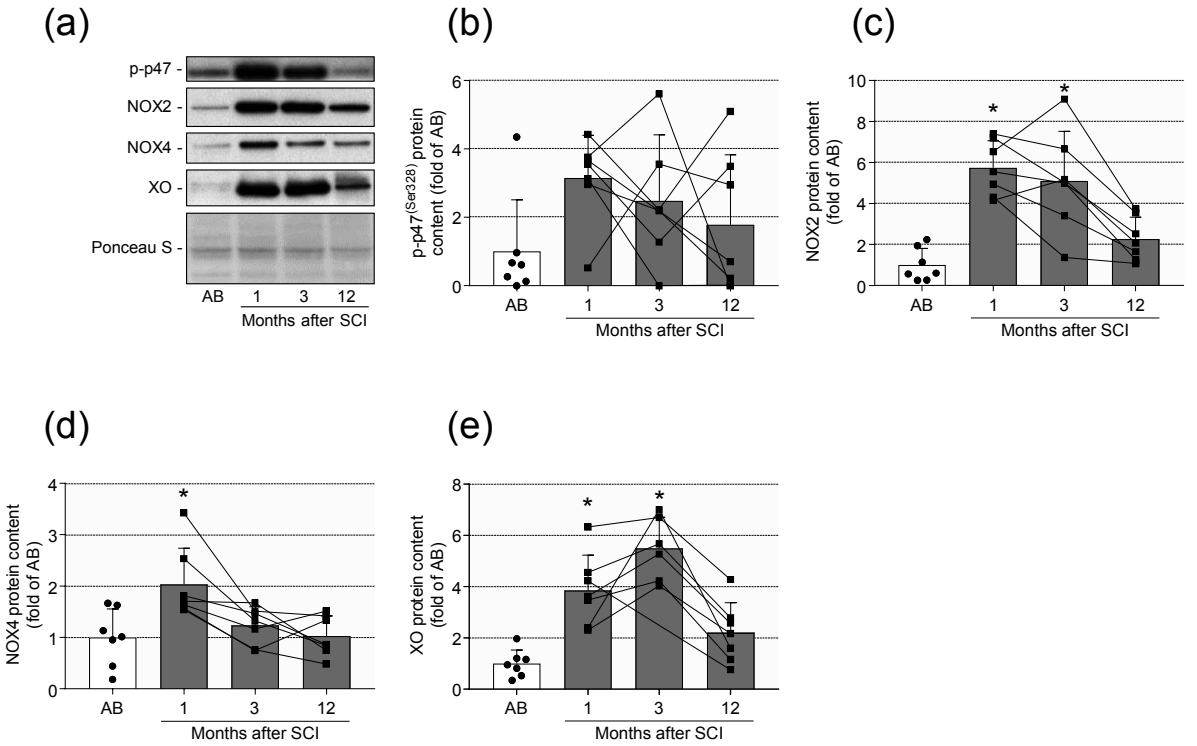

Figure 3. Non-mitochondrial sources of ROS in skeletal muscle following spinal cord injury. Representative western blot images (a) and quantifications for p-p47(Ser328) (b), NOX2 (c), NOX4 (d) and XO (e) measured in skeletal muscle from able-bodied (AB, white bars) and spinal cord-injured individuals (SCI, grey bars) at one, three and 12 months post-injury. Units are scaled to the mean of the able-bodied controls. Data are presented as mean and standard deviation, and individual data points are overlaid. * - $p<0.05$ vs able-bodied controls, Kruskal-Wallis followed by Dunn's multiple comparisons test.

Non-mitochondrial sources of ROS in skeletal muscle following spinal cord injury.

To determine whether spinal cord injury leads to changes in non-mitochondrial ROS production, protein levels of several key enzymes involved in this pathway were analysed (Fig. 3a). Phosphorylation (Ser^{328}) of the $p47^{phox}$-regulatory subunit of NADPH oxidase 2 (NOX2) was not significantly different between spinal cord-injured and able-bodied individuals (Fig. 3b). Conversely, NOX2 protein content was increased at one and three ($p<0.05$), but not at 12 months after spinal cord injury (Fig. 3c). Similarly, protein levels of NADPH oxidase 4 (NOX4) was increased at one ($p<0.05$), but not at three and 12 months after spinal cord injury (Fig. 3d). Furthermore, protein levels of xanthine oxidase (XO) were higher at one and three ($p<0.05$), but not 12 months after spinal cord injury (Fig. 3e). Thus, one and three months after the trauma, spinal cord injury leads to increased protein levels of several non-mitochondrial ROS-producing enzymes in the affected skeletal muscle.

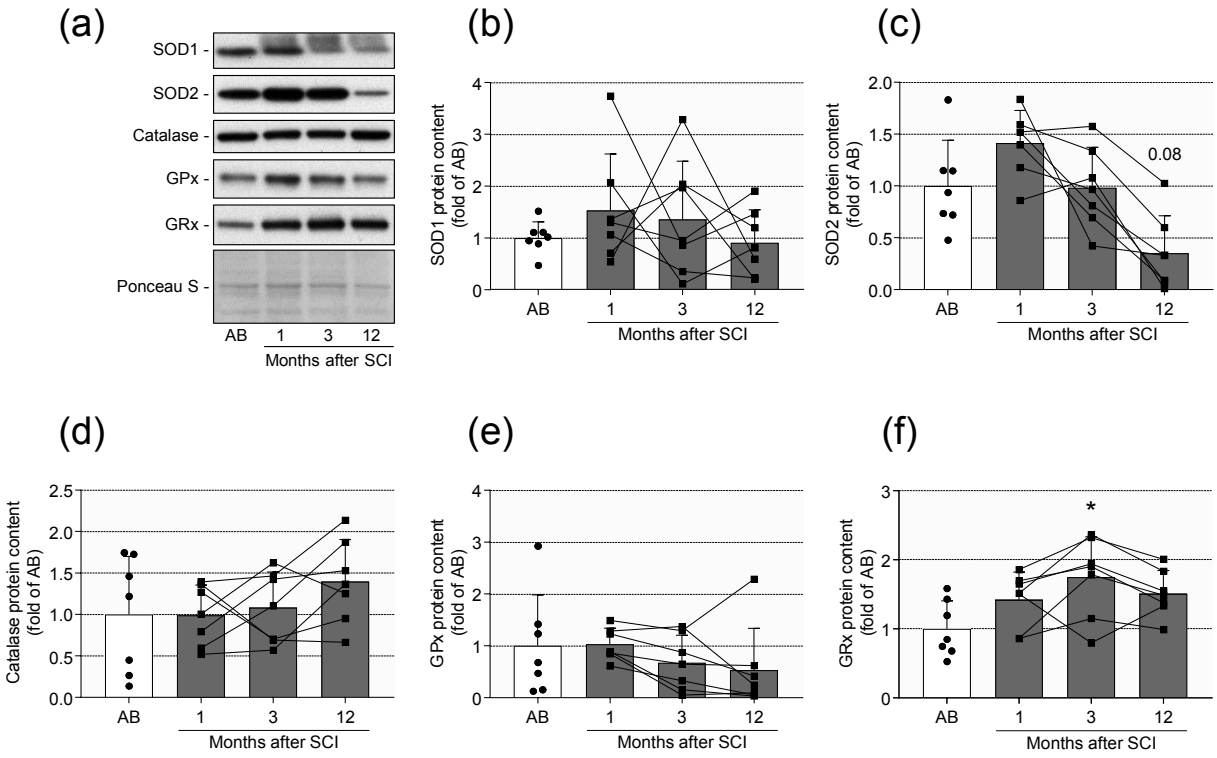

Figure 4. Antioxidant enzymes in skeletal muscle following spinal cord injury. Representative western blot images (a) and quantifications for SOD1 (b), SOD2 (c), catalase (d), GPx (e) and GRx (f) measured in skeletal muscle from able-bodied (AB, white bars) and spinal cord-injured individuals (SCI, grey bars) at one, three and 12 months post-injury. Units are scaled to the mean of the able-bodied controls. Data are presented as mean and standard deviation, and individual data points are overlaid. * - $p<0.05$ vs able-bodied controls, Kruskal-Wallis followed by Dunn's multiple comparisons test.

Antioxidant enzymes in skeletal muscle following spinal cord injury.

In order to determine the enzymatic antioxidant defence capacity in skeletal muscle after spinal cord injury, we measured protein levels of several enzymes responsible for ROS detoxification (Fig. 4a). Protein abundance of [Cu-Zn]-superoxide dismutase (SOD1) was similar between spinal cord-injured and able-bodied individuals (Fig. 4b). Conversely, protein content of [Mn]-superoxide dismutase (SOD2) tended ($p=0.08$) to be decreased at 12, but not at one and three months after spinal cord injury (Fig. 4c). Skeletal muscle catalase and glutathione peroxidase (GPx) protein content was unaltered in spinal cord-injured versus able-bodied individuals (Fig. 4d and e). Glutathione reductase (GRx) was increased at three ($p<0.05$), but not at one or 12 months after spinal cord injury (Fig. 4f). Overall, our results indicate moderate adaptations in the antioxidant defence capacity in skeletal muscle during the first year following spinal cord injury.

Apoptotic signalling in skeletal muscle following spinal cord injury.

As oxidative stress can lead to an activation of apoptosis, we determined the content of several proteins involved in apoptotic signalling (Fig. 5a). Protein levels of dually-phosphorylated (Thr^{183}/Tyr^{185}) c-Jun N-terminal kinase (JNK) were decreased at 12 months ($p<0.05$), but unaltered one or three months after spinal

cord injury in comparison to able-bodied controls (Fig. 5b). Conversely, total protein content of JNK was higher ($p<0.05$) at one and three, but not at 12 months after spinal cord injury (Fig. 5c). Protein abundance of cleaved caspase-9, cleaved calpain-1 and total calpain-1 did not differ between spinal cord-injured and able-bodied individuals (Fig. 5d, e and f). We detected higher pro-caspase-3 protein content at one and three months ($p<0.05$) and a similar trend for cleaved caspase-3 at one month ($p=0.06$) after spinal cord injury (Fig. 5g and h). Overall, our data provide evidence to suggest that apoptotic signalling is increased in skeletal muscle at one and three months after spinal cord injury.

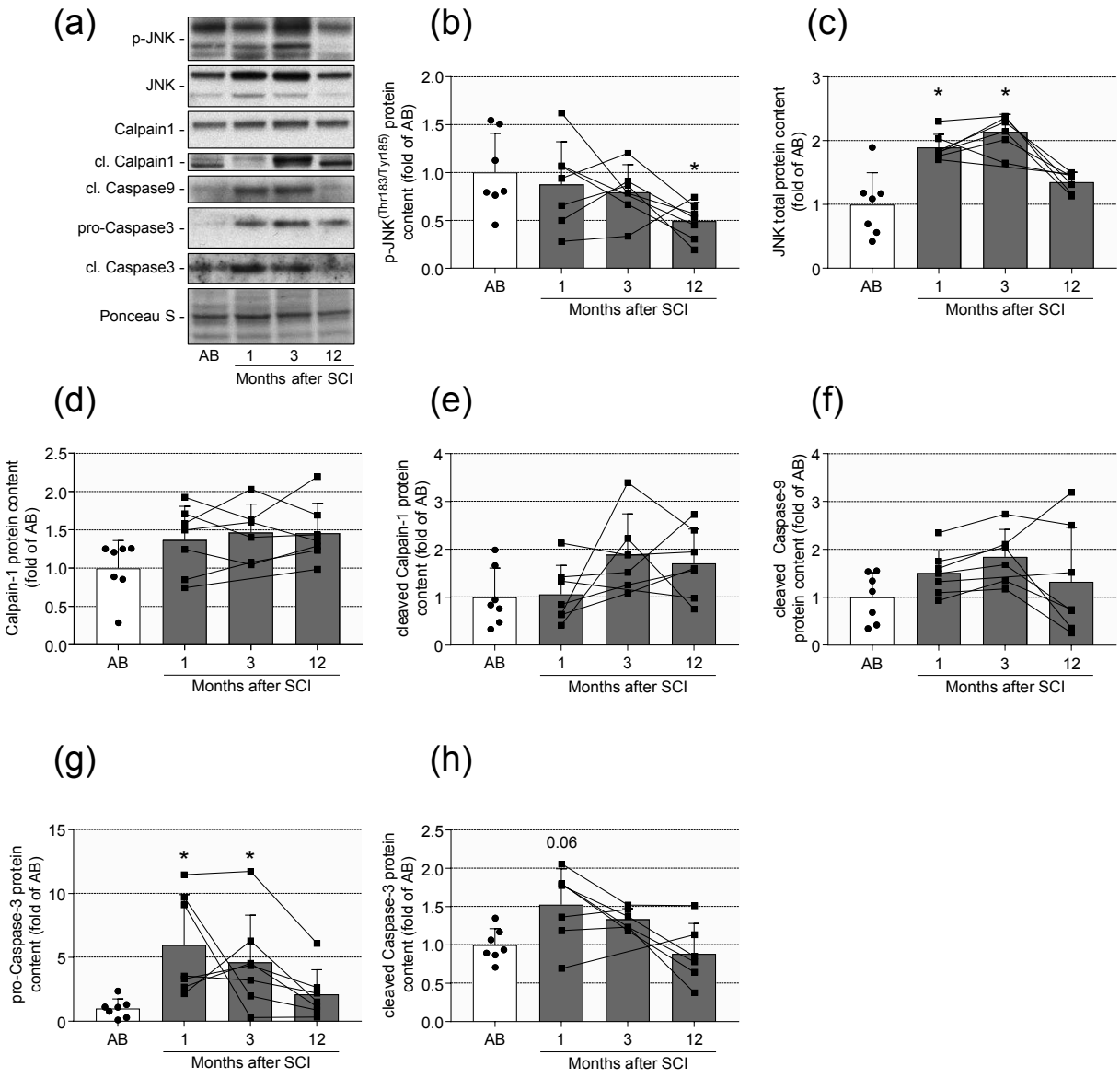

Figure 5. Apoptotic signalling in skeletal muscle following spinal cord injury. Representative western blot images (a) and quantifications for p-JNK (Thr183/Tyr185) (b), total JNK (c), calpain-1 (d), cleaved calpain-1 (e), cleaved caspase-9 (f), pro-caspase-3 (g) and cleaved caspase-3 (h) measured in skeletal muscle from able-bodied (AB, white bars) and spinal cord-injured individuals (SCI, grey bars) at one, three and 12 months post-injury. Units are scaled to the mean of the able-bodied controls. Data are presented as mean and standard deviation, and individual data points are overlaid. * - $p<0.05$ vs able-bodied controls, Kruskal-Wallis followed by Dunn's multiple comparisons test.

Discussion

We determined whether spinal cord injury disturbs ROS homeostasis and induces oxidative stress in human skeletal muscle. We detected increased protein abundance of NADPH oxidases and xanthine oxidase at one and three months after injury, concurrent with increased total and reduced glutathione content and GRx protein levels. Furthermore, our data provide evidence to suggest that caspase-3 is activated at one and three months after spinal cord injury. At 12 months following spinal cord injury we observed increased 4HNE protein adducts and decreased mitochondrial content and SOD2 protein levels. Collectively, our study reveals increases in apoptosis and non-mitochondrial ROS production at one and three months after spinal cord injury, concomitant with an increase in antioxidant defences.

Mitochondria are a major source of ROS in skeletal muscle undergoing disuse-induced atrophy.[10] We detected decreased mitochondrial protein content (complexes I, III, IV and V) at 12 months after spinal cord injury as compared with able-bodied controls, consistent with our earlier findings.[22] Given that mitochondrial enzymatic activity is decreased,[23] mitochondrial ROS production is likely lower in the late post-injury phase. Conversely, mitochondrial protein content was unchanged at one and three months post-injury. Mitochondrial enzymatic activity increases during the first six months post-injury,[2] suggesting that ROS production might be higher, independent of unchanged protein content.

Several non-mitochondrial enzymes have been implicated in excessive ROS production in skeletal muscle undergoing atrophy.[8, 9, 13] We observed increased skeletal muscle protein content of NOX4 at one month and NOX2 at one and three months following spinal cord injury, but unchanged $p47^{phox}$ phosphorylation. While NOX2 ROS production requires phosphorylation of $p47^{phox}$ regulatory subunit, NOX4 is considered to be constitutively active and transcriptionally regulated.[24] Thus, increased NOX4 protein content may constitute an important source of ROS in early post-injury phase. Conversely, unchanged $p47^{phox}$ phosphorylation indicates NOX2 activity is unaltered between skeletal muscle from spinal cord-injured and able-bodied individuals. However, $p47^{phox}$-dependent activation of NOX2 occurs during muscle contraction.[25] Thus, increased total NOX2 protein content indicates increased functional capacity, and could implicate NOX2 as an important source of ROS during muscle spasms.

Skeletal muscle protein abundance of xanthine oxidase was increased at one and three months after spinal cord injury. ROS producing xanthine oxidase activity has been noted in the acute phase of murine disuse atrophy.[9] Additionally, in murine skeletal muscle, ROS production by NOX promotes Ca^{2+} leak from the endoplasmic reticulum through ryanodine receptor 1 modification.[26] Xanthine oxidase is proteolytically cleaved into its ROS producing form by a Ca^{2+}-dependent protease other than calpain. [27, 28] Thus, NOX and xanthine oxidase could form a

feed-forward loop to increase non-mitochondrial ROS production during the first months post-injury.

Superoxide is converted into hydrogen peroxide by action of SOD1 localized in the cytoplasm and SOD2 localized in the mitochondrial matrix.[29] Although SOD1 protein content was unaltered by spinal cord injury, a trend for decreased SOD2 protein content was observed at 12 months after spinal cord injury. As most posttranslational modifications of SOD enzymes lead to decreased activity,[30] the unaltered SOD1 and SOD2 protein content at one and three months after spinal cord injury indicates that the capacity to decompose superoxide radicals is either conserved or decreased. Conversely, the decreased abundance of mitochondrial SOD2 at 12 months after injury reflects lower antioxidant protection and mirrors the decreased mitochondrial content. Hence, our data indicate that superoxide scavenging capacity is conserved in the first months following spinal cord injury.

Hydrogen peroxide is converted into water and molecular oxygen by the action of catalase or GPx.[31] GSH is used as a substrate by GPx and oxidized into glutathione disulphide (GSSG), which can be reduced to GSH by GRx.[32] We found that protein content of GPx and catalase was unaltered between spinal cord-injured and able-bodied individuals. Conversely, GSH and total glutathione content was increased at one and three months after spinal cord injury. Glutathione biosynthesis is promoted through the Akt/mTOR signalling,[33] which is transiently increased early after spinal cord injury.[34] Thus, a compensatory increase in glutathione biosynthesis could account for increased total glutathione content. Additionally, GRx activity increases with GSSG content during a single bout of exercise in murine myocardium, via a NOX-dependent mechanism.[32] Thus, long exposure to increased NOX activity could lead to adaptive changes in GRx protein content in skeletal muscle after spinal cord injury. Our results provide evidence suggesting compensatory increases of antioxidant systems in skeletal muscle at one and three months following spinal cord injury.

Protein carbonylation in skeletal muscle of spinal cord-injured individuals was unchanged compared to able-bodied controls, while 4HNE protein adducts were increased at 12 months post-injury. Potentially, the compensatory increases in antioxidants are sufficient to protect proteins from oxidative damage following spinal cord injury. However, proteins damaged by oxidation are preferentially degraded by the ubiquitin-proteasome system.[35] We have reported that skeletal muscle atrophy following spinal cord injury is accompanied by increased proteolysis and transient increase in protein synthesis one month after spinal cord injury.[34] Thus, a high protein turnover state, with preferential degradation of oxidized proteins, may lead to underestimation of protein carbonylation in early post-injury phase. Conversely, at 12 months post-injury skeletal muscle enters a new "steady-state" of low protein turnover,[34, 36] therby allowing for accumulation of 4HNE protein adducts.

Excessive ROS exposure and oxidative stress induce apoptosis.[37] The increase in total JNK protein at one and three months post-injury suggests transcriptional and translational mechanisms promoting apoptosis. However, JNK (Thr^{183}/Tyr^{185}) phosphorylation was unaltered at these time points, reflecting that signalling through the upstream extrinsic apoptotic pathway is retained.[38] Conversely, the reduction in JNK (Thr^{183}/Tyr^{185}) phosphorylation and unchanged total protein content at 12 months post-injury, may suggest decreased apoptotic signalling. Surprisingly, we do not observe increased cleaved-caspase-9 protein content, indicative of stable mitochondrial apoptotic pathways. However, as caspase-9 is regulated through the interaction with cytoplasmic factors,[39] the mitochondrial apoptotic pathway may be activated despite unchanged caspase-9 protein content. Interestingly, we detected that pro-caspase-3 protein content was increased at one and three months after injury, whereas cleaved caspase-3 was increased at one month following spinal cord injury. Caspase-3 is an effector caspase,[40] and an essential protease for inducing protein catabolism in skeletal muscle undergoing atrophy.[41] Thus, increased protein content of both pro- and cleaved forms of caspase-3 suggests apoptosis and proteolysis are increased in the early stages after spinal cord injury. Even though we were unable to discern the specific pathways involved, our data provides indirect evidence of increased apoptosis at one and three months post-injury.

The one- and three-month time points were chosen based on the rapid skeletal muscle atrophy occurring in the initial six weeks post-injury.[2] Conceivably, more profound changes could precede the first one-month biopsy. However, it was not feasible to obtain biopsies at earlier time-points due to the severe nature of the acute phase of spinal cord injury. Furthermore, due to a relatively low number of participants we might not be able to detect more subtle alterations. Nevertheless, our study provides valuable insight into the more profound changes in ROS homeostasis in skeletal muscle during the first year following spinal cord injury.

Non-mitochondrial ROS production and an increased antioxidant capacity has potential clinical relevance in spinal cord injury. NADPH oxidase and xanthine oxidase ROS production is upregulated during skeletal muscle contraction,[25, 42] which may contribute to developing oxidative stress during muscular spasms or during electrically-stimulated rehabilitative exercise. Investigations into the ROS-response to such rehabilitative efforts, especially in the initial rapid stage of skeletal muscle atrophy after spinal cord injury, are warranted. The development of antioxidant adjuvant therapy in the early phase could improve the beneficial responses to rehabilitative interventions and mitigate skeletal muscle atrophy following spinal cord injury.

In conclusion, we provide evidence of increased ROS production from non-mitochondrial sources and apoptotic signalling in the initial rapid phase of

skeletal muscle atrophy, concomitant with compensatory adaptations in antioxidant systems. Conversely, 12 months after injury, apoptotic signalling and non-mitochondrial ROS production are restored to able-bodied levels, while mitochondrial ROS production is decreased. Since oxidative stress markers were unaltered, these adaptations may partly protect skeletal muscle against oxidative stress, indicating retained redox plasticity following spinal cord injury.

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Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Table 1. Clinical characteristics

		Able-Bodied (n=7)	Spinal Cord-Injured (n=7)		
Age, yr (mean ± SD)		49 ± 6	43 ± 15		
			Months after injury		
			1	3	12
BMI, kg/m^2 (mean ± SD)		26 ± 2	25 ± 4	25 ± 4	27 ± 3
Injury level			AIS motor score (0-100)		
Cervical, n=2	C4	n/a	8	8	16
	C6	n/a	27	27	28
Thoracic, n=5	Th3-12	n/a	50	50	50

AIS - American Spinal Cord Injury Association Impairment Scale; BMI – Body Mass Index; n/a – not applicable

Materials and methods

Study participants.

Seven individuals with a complete spinal cord injury according to international standards for neurological classification of spinal cord injury[43] (injury range C4-Th12, four men and three women) and seven able-bodied controls (men) matched for age and body mass index participated in the study. Clinical characteristics of the participants are presented in Table 1, have been described before.[34] None of the participants had any known systemic diseases. Able-bodied participants were not receiving any medications, while participants with spinal cord injury received low molecular weight heparin therapy during the first three months after injury, as well as spasmolytic therapy (baclofen or equivalent) when indicated. Participants with spinal cord injury received standard rehabilitative care at Sunnaas Rehabilitation Hospital, Nesoddtangen, Norway. All participants gave their written informed consent and the study was conducted according to the

Declaration of Helsinki. The study was approved by the Regional Committee for Medical and Health Research Ethics at Helse Sør-Øst Trust, Norway.

Preparations of skeletal muscle biopsies.

Skeletal muscle biopsies were obtained using a Bergström needle from the *vastus lateralis* of the *quadriceps femoris* muscle under local anaesthesia (Lidocaine 5 mg ml^{-1}), cleaned from visible fat and blood, and rapidly frozen in liquid nitrogen. Biopsies were lysed in ice cold lysis buffer (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 1 mmol l^{-1} MgCl, 20 mmol l^{-1} Tris pH 7.8, 10 mmol l^{-1} NaF, 1 mmol l^{-1} EDTA, 0.5 mmol l^{-1} $NaVO_3$, 1 mmol l^{-1} PMSF, 10% glycerol (w/v), 1% Triton X-100 (w/v) and protease inhibitor cocktail Set 1 (Calbiochem, EMD Biosciences, San Diego, CA, US)). Extracellular debris was removed by centrifugation at 12000 *g* for 10 min at 4°C, and the supernatant containing soluble material was collected. Protein concentration was determined using a commercially available Pierce bicinchoninic acid (BCA) protein assay (#23225, Thermo Scientific, Waltham, MA, US) according to manufacturer's instructions.

Protein carbonylation assay.

Analysis of protein carbonylation was performed as described,[44] using a commercially available kit (OxyBlot, #S7150, Merck Millipore, Burlington, MA, US). Equal amounts of protein were loaded, denatured by addition of 6% (v/v) sodium dodecyl sulfate (SDS) and carbonyl groups on protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH). After 15 minutes at room temperature the derivatization reaction was stopped by adding the neutralizing solution and 5% (v/v) of 2-mercaptoethanol.

SDS-PAGE and Western blot.

Western blotting was performed as described.[34] Equal amounts of protein were diluted in Laemmli buffer and loaded for an SDS-PAGE (#3450124, Criterion XT Precast gels, BioRad, Hercules, CA, US). Protein was transferred to a polyvinyl fluoride (PVDF) membrane (IPVH00010, Immobilon-P, Merck Millipore), Ponceau S staining (#P7170, Sigma-Aldrich, St. Louis, MO, US) was performed and results were normalized to total protein per lane. Membranes were blocked using 7.5% (w/v) non-fat dried milk in tris-buffered saline (TBS) with Tween 20. Primary antibodies were diluted 1:1000 (v/v) in TBS containing 0.1% (w/v) bovine serum albumin and NaN_3. Membranes were incubated in primary antibody dilutions overnight (~16 hours) at 4°C under gentle agitation. The list of primary antibodies used is presented in Table 2. Species appropriate horseradish peroxidase-conjugated secondary antibodies, diluted 1:25000 (v/v) in 5% non-fat dried milk in TBS-Tween were used, and protein amounts were visualized using chemiluminescence (#RPN2106 ECL and #RPN2235 ECL select, GE Healthcare, Chicago, IL, US). Optical density of detected bands was quantified using Image Lab v.5.2.1 (BioRad). The optical density of detected bands for protein carbonylation

(molecular weights 250, 225, 100, 80, 50, 40 and 35 kDa) and 4HNE adducts (molecular weights 150, 100, 60 and 35kDa) was determined and the sum of the bands is presented as total.

Table 2. Primary antibodies list

Antigen	Molecular weight (kDa)	Manufacturer (Cat #)
4-Hydroxynonenal	n/a	Abcam (ab46545)
OXPHOS Cocktail	n/a	Abcam (ab110411)
Complex I	18	Abcam (ab110242)
Complex II	29	Abcam (ab14714)
Complex III	48	Abcam (ab14745)
Complex IV	22	Abcam (ab110258)
Complex V	54	Abcam (ab14748)
p-p47$^{(Ser328)}$	47	Abcam (ab111855)
NOX2	65	Abcam (ab80508)
NOX4	67	Abcam (ab133303)
XO	85	Abcam (ab109235)
SOD1	17	Abcam (ab16831)
SOD2	25	Abcam (ab13534)
Catalase	64	Santa Cruz, Dallas, TX, US (sc-50508)
GPx	22	Abcam (ab22604)
GRx	58	Abcam (ab16801)
p-JNK	46/54	Cell Signalling, Danvers, MA, US (9251)
JNK	46/54	Cell Signalling (9252)
Calpain-1	68/82	Abcam (ab28258)
Caspase-9	37	Abcam (ab2324)
Caspase-3	19/35	Cell Signalling (9662)

Reduced and total glutathione detection

Glutathione content was determined using a commercially available assay kit (ab138881, Abcam, Cambridge, UK). Aliquots, normalized for protein concentration were loaded for the reaction. To avoid assay enzymatic interference, deproteinization was performed by 1:5 (v/v) addition of 100% (w/v) trichloroacetic acid (TCA) and precipitated protein and TCA were removed by centrifugation (12000 *g*, 5 min at 4°C). The remaining TCA in the supernatant was neutralized by addition of 1M $NaHCO_3$, the samples centrifuged (12000 *g*, 15 min at 4°C) and the supernatant collected for further analysis. The amount of reduced (GSH) and total glutathione was determined fluorometrically, and the amount of oxidised (GSSG) glutathione was calculated according to manufacturer's instructions.

Statistical analyses.

The arbitrary units of optical density were normalized to the mean of the able-bodied control group. Data are presented as mean and standard deviation (SD) with the individual data points overlaid. Statistical comparisons were made using the non-parametric Mann-Whitney (Table 1) or Kruskal-Wallis tests, followed by Dunn's multiple comparison where appropriate. P values below 0.05 were considered as significant, while $0.1 \geq p \geq 0.05$ indicated trends. Statistical analyses were performed using Prism v.7.01 (GraphPad, San Diego, CA, US).

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Paper III

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ORIGINAL RESEARCH

Retained differentiation capacity of human skeletal muscle satellite cells from spinal cord-injured individuals

Mladen Savikj[1,2,3], Maxwell A. Ruby[4,5], Emil Kostovski[2,6,7], Per O. Iversen[7,8], Juleen R. Zierath[3,4], Anna Krook[3,4] & Ulrika Widegren[4]

1 Faculty of Medicine, University of Oslo, Oslo, Norway
2 Science Department, Sunnaas Rehabilitation Hospital, Nesoddtangen, Norway
3 Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden
4 Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden
5 Department of Nutrition and Preventive Medicine, Norwich Medical School, University of East Anglia, Norwich, Norfolk, United Kingdom
6 Institute of Clinical Medicine, University of Oslo, Oslo, Norway
7 Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway
8 Department of Haematology, Oslo University Hospital, Oslo, Norway

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Correspondence
Ulrika Widegren, Section for Integrative Physiology, Department of Molecular Medicine and Surgery, Karolinska Institutet, SE-171 77 Stockholm, Sweden.
Tel: +46 70 298 67 47
Fax: +46 8 33 88 33
E-mail: ulrika.widegren@ki.se

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Abstract

Despite the well-known role of satellite cells in skeletal muscle plasticity, the effect of spinal cord injury on their function in humans remains unknown. We determined whether spinal cord injury affects the intrinsic ability of satellite cells to differentiate and produce metabolically healthy myotubes. We obtained *vastus lateralis* biopsies from eight spinal cord-injured and six able-bodied individuals. Satellite cells were isolated, grown and differentiated in vitro. Gene expression was measured by quantitative PCR. Abundance of differentiation markers and regulatory proteins was determined by Western blotting. Protein synthesis and fatty acid oxidation were measured by radioactive tracer-based assays. Activated satellite cells (myoblasts) and differentiated myotubes derived from skeletal muscle of able-bodied and spinal cord-injured individuals expressed similar ($P > 0.05$) mRNA levels of myogenic regulatory factors. Myogenic differentiation factor 1 expression was higher in myoblasts from spinal cord-injured individuals. Desmin and myogenin protein content was increased upon differentiation in both groups, while myotubes from spinal cord-injured individuals contained more type I and II myosin heavy chain. Phosphorylated and total protein levels of Akt-mechanistic target of rapamycin and forkhead box protein O signalling axes and protein synthesis rate in myotubes were similar ($P > 0.05$) between groups. Additionally, fatty acid oxidation of myotubes from spinal cord-injured individuals was unchanged ($P > 0.05$) compared to able-bodied controls. Our results indicate that the intrinsic differentiation capacity of satellite cells and metabolic characteristics of myotubes are preserved following spinal cord injury. This may inform potential interventions targeting satellite cell activation to alleviate skeletal muscle atrophy.

Introduction

Traumatic spinal cord injury has a prevalence of between 250 and 906 cases per million in developed countries (Singh et al. 2014), and leads to severe physical and psychosocial consequences. It is characterized by varying degrees of motor, sensory and autonomic neurological deficits below the level of injury, affecting most bodily

systems (Binder 2013). Decentralization of skeletal muscle from the nervous system causes inactivity and marked atrophy, with a decrease of both single fiber and whole muscle cross sectional area (Castro et al. 1999; Gorgey and Dudley 2007). The decrease in muscle mass is attributed to an imbalance between protein synthesis and degradation, with associated changes in the protein kinase B (Akt) – mechanistic target of rapamycin (mTOR) signalling axis, and activity of the forkhead box protein O (FoxO) transcription factors and their targets, respectively (Jackman and Kandarian 2004; Dreyer et al. 2008; Leger et al. 2009). Atrophy reduces the size of all fibers and shifts the fiber type composition from type I oxidative fibers to predominantly type IIx glycolytic fibers (Lotta et al. 1991; Aksnes et al. 1996; Castro et al. 1999; Kostovski et al. 2013). Along with morphological changes, spinal cord injury reduces the fatty acid oxidation capacity of skeletal muscle (Wang et al. 1999; Kjaer et al. 2001; Long et al. 2011; McCully et al. 2011). Moreover, the reduced muscle mass diminishes peripheral glucose disposal (Aksnes et al. 1996). Impairments in both lipid and glucose metabolism ultimately increase the risk of noncommunicable diseases such as type 2 diabetes and cardiovascular disease (Cragg et al. 2013a,b).

Satellite cells are located between the sarcolemma and the basement membrane of the muscle fibers (Mauro 1961). These cells play an integral role in muscle plasticity and regeneration through self-renewal and fusion into the existing fibers (Schiaffino et al. 1976; Collins et al. 2005; Bruusgaard et al. 2010; Lepper et al. 2011). Paired box protein 7 (Pax7) positive satellite cells express myogenic regulatory transcription factors, which show commitment to the myogenic lineage (e.g., myogenic factor 5 – Myf5 and myogenic differentiation factor 1 – Myod1) and passage into terminal differentiation (e.g. myogenin) (Almeida et al. 2016).

Individuals with spinal cord injury have a reduced number of satellite cells per skeletal muscle fiber (Verdijk et al. 2012). It is yet unclear whether spinal cord injury affects the differentiation capacity of human skeletal muscle satellite cells. Altered function of satellite cells has been indicated by animal models. Spinal cord transection and contusion in rats lead to satellite cell activation (Dupont-Versteegden et al. 1999; Jayaraman et al. 2013). However, the differentiation of satellite cells may be lacking, as the myonuclear number continues to decrease in these animals in spite of satellite cell activation (Dupont-Versteegden et al. 1999). Several types of skeletal muscle atrophy, with an underlying neurological mechanism, are accompanied with abnormal satellite cell differentiation. Denervation of rat skeletal muscle leads to formation of new myotubes with defective contractile machinery (Carraro et al. 2015). Skeletal muscle satellite cells from individuals with amyotrophic lateral sclerosis have reduced differentiation capacity and form myotubes with abnormal morphology (Pradat et al. 2011; Scaramozza et al. 2014). However, whether skeletal muscle satellite cell differentiation is affected by spinal cord injury in humans remains unknown. As satellite cells play a role in regulating skeletal muscle mass and are responsive to exercise stimuli (Bruusgaard et al. 2010), their ability to differentiate is of importance in efforts to maintain skeletal muscle mass following spinal cord injury.

Here, we determined the effect of spinal cord injury on the intrinsic ability of satellite cells to differentiate. Additionally, we assessed the metabolic properties of cultured myotubes to determine whether changes seen in vivo in skeletal muscle after spinal cord injury are reflected in satellite cell-derived myotubes in vitro. Myotubes differentiated from satellite cells in vitro retain characteristics of the donor skeletal muscle in several metabolic and neurological conditions (Bouzakri et al. 2004; Pradat et al. 2011; Boyle et al. 2012; Green et al. 2011, 2013; Jiang et al. 2013; Scaramozza et al. 2014). Thus, primary human skeletal muscle cultures have provided meaningful insight into satellite cell function in muscle disorders.

Materials and Methods

Study participants

Fourteen individuals were studied, including eight men with a longstanding (more than 1 year) spinal cord injury currently undergoing routine follow-ups at Sunnaas rehabilitation hospital (Oslo, Norway), and six age-matched able-bodied controls. Participants with spinal cord injury received anticoagulant (clexane or equivalent) therapy during the first 3–6 months after injury as well as spasmolytic therapy (baclofen or equivalent). They did not receive any corticosteroid treatment. Six men with no history of smoking, nonathletes, with no current use of medications were recruited as able-bodied controls. Neither spinal cord-injured nor able-bodied participants had any known malignant, systemic or musculoskeletal disease, nor an intercurrent infection. Participant characteristics are shown in Table 1. The study was conducted according to the ethical principles expressed in the declaration of Helsinki (World Medical, A, 2013). All participants gave their written informed consent and the study was approved by the Regional Committee for Medical and Health Research Ethics at Helse Sør-Øst Trust, Norway.

Skeletal muscle biopsy procedure

Participants were instructed to abstain from physical activity the day before the biopsy. Skeletal muscle samples

Table 1. Participant characteristics.

	Age years (range)	BMI (kg m^{-2}; mean ± SEM)
Able-bodied participants (n = 6)	42 (33; 54)	22 ± 1
Spinal cord-injured participants (n = 8)	52 (26; 71)	26 ± 2[1]
Level of injury (n)	Motor deficiency	Time since injury (years)
C7 (2)	Complete	11; 4
C7	Incomplete	8
Th4	Complete	34
Th5	Complete	20
Th12 (3)	Incomplete	6; 5; 4

[1]P = 0.06 for BMI (able-bodied vs. spinal cord-injured).

were obtained by either a semi-open or by an open biopsy procedure (Henriksson 1979; Berman et al. 1985) from the mid portion of *vastus lateralis* of the *quadriceps femoris* muscle under local anesthesia (Lidocaine 5 mg mL^{-1}). A single piece of skeletal muscle (50–100 mg) was obtained by either procedure and immediately transferred to ice cold phosphate buffered saline (PBS) supplemented with 1% PenStrep (100 UI mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) and kept on ice.

Satellite cell isolation and culture of primary skeletal muscle cells

Satellite cells were isolated from skeletal muscle samples and cultured as previously described (Mudry et al. 2017). Satellite cells were extracted using a collagenase solution; attachment of nonsatellite cells was allowed for 1 h, after which the supernatant containing satellite cells was collected. Cells proliferated at 37°C and 7.5% CO_2 in growth medium (20% fetal bovine serum – FBS). Differentiation was induced at ~80% confluence, using fusion medium (100 μg mL^{-1} apo-transferrin and 0.286 IU mL^{-1} insulin) for 4 days. Experiments were performed on differentiated myotubes after an additional 4 days in post-fusion medium (2% FBS). For assessment of differentiation cells were harvested prior to (myoblasts), and 8 days after (myotubes) the induction of differentiation.

Immunohistochemistry of primary skeletal muscle cell cultures

Immunohistochemistry was performed as previously described (Sjogren et al. 2015). Myoblasts and myotubes were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Desmin and Ki67 were detected by indirect immunofluorescence using primary antibodies against desmin (#15200 Abcam, Cambridge, U.K.) at a concentration of 1:500 and against Ki67 (#9449 Cell Signaling, Danvers, MA) at a concentration of 1:800. Secondary antibodies used (5 μg mL^{-1}) were Alexa Fluor 488 (#A-11008, Invitrogen, Carlsbad, CA) and Alexa Fluor 594 (#A-11005, Invitrogen). Nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI) (#D1306, Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

RNA isolation, cDNA synthesis and qPCR

Cells were harvested in TRIzol (#15596-018, Life Technologies, Carlsbad, CA) and RNA was isolated according to the manufacturer's instructions. RNA concentration was determined by Nanodrop ND-1000 (Thermo Fischer Scientific) and 1000 ng was loaded as template for cDNA synthesis, which was performed using the high-capacity cDNA reverse transcriptase kit (#4368814, Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was performed using Fast SYBR Green Master Mix (#4385612, Applied Biosystems) and Ct values were determined using StepOne software v2.1 (Applied Biosystems). The results were normalized to the geometric mean of two controls genes, TATA-binding protein (TBP) and 60S acidic ribosomal protein P0 (RPLP0), and the expression calculated by the ΔCt method. Primers used for the reactions were designed to exon to exon junctions of the targeted genes. Sequences of used primers are listed in Table 2. Each assay was accompanied by a melt curve step, which have shown a single clear peak for each set of primers.

SDS-PAGE and Western blot

Cells were harvested in ice cold lysis buffer (20 mmol L^{-1} Tris-HCl pH 7.8) containing protease and phosphatase inhibitors (#539131, Protease Inhibitor Coctail Set I – Calbiochem, Merck Millipore, Billerica, MA; 1 mmol L^{-1} PMSF; 0.5 mmol L^{-1} Na_3VO_4). Protein concentration was determined using Pierce BCA protein assay kit (#23225, Thermo Fischer Scientific). Equal amounts of protein were diluted in Laemmli buffer. Sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Sjogren et al. 2015), using Criterion XT Bis-Tris 4–12% precast gels (#3450124, BioRad, Hercules, CA). Protein was transferred to an Immobilion-P polyvinylidene fluoride (PVDF) membrane (#IPVH00010, Merck Millipore). Ponceau staining was performed and the results are normalized to the total amount of protein per lane. Western blotting was performed using primary antibodies overnight, at a 1:1000 concentration in tris-buffered saline

Table 2. Sequences of primers used for qPCR.

Target gene	Forward primer	Reverse primer
TBP	AGTTCTGGGATTGTACCGCA	TATATTCGGCGTTTCGGGCA
RPLP0	TGGAGAAACTGCTGCCTCAT	GATTTCAATGGTGCCCCTGG
Pax7	GAGGACCAAGCTGACAGAGG	CTGGCAGAAGGTGGTTGAA
Myf5	CCACCTCCAACTGCTCTGAT	GCAATCCAAGCTGGATAAGG
Myod1	AGCACTACAGCGGCGACT	GCGCCTCGTTGTAGTAGGC
Myf6	GGATCAGCAGGAGAAGATGC	CCTGGAATGATCGGAAACAC
Myogenin	GCTCAGCTCCCTCAACCA	GCTGTGAGAGCTGCATTCG
Desmin	CTGGAGCGCAGAATTGAATC	GGCAGTGAGGTCTGGCTTAG

(TBS) containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3. The antibodies used are listed in Table 3. Species-appropriate horseradish peroxidase conjugated secondary antibodies were used at a concentration of 1:25,000 in 5% milk in TBS-Tween. Proteins were visualized by chemiluminescence (#RPN2232 ECL and #RPN2235 ECL select Western blotting detection reagent – GE Healthcare, Little Chalfont, U.K.) and the quantification was performed by ImageLab software v. 5.2.1 (BioRad).

[^{14}C]Phenylalanine incorporation into protein

Fully differentiated myotubes were incubated in Dulbecco's Modified Eagle Medium (DMEM) (1 g L^{-1} glucose) with 425 μmol L^{-1} phenylalanine and 0.4 μCi mL^{-1} of [^{14}C] phenylalanine (#NEC284E050UC, Perkin Elmer, Waltham, MA) for 6 h at 37°C and 7.5% CO_2. The cells were then washed in PBS and lysed in 0.03% SDS for 1 h at room temperature. The total amount of protein per well was determined by the Pierce BCA protein assay kit (#23225, Thermo Fischer Scientific). Protein from the cell lysates was precipitated in 50% Trichloracetic acid with 1% BSA, overnight at −20°C, followed by centrifugation. The protein pellet was then washed in acetone, dissolved in 0.5 mol L^{-1} NaOH and the amount of [^{14}C] determined by scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac, Turku, Finland). Counts per minute were normalized to the total amount of protein per well, and the amount of phenylalanine incorporated into protein is presented as pmol mg^{-1} h^{-1}.

[^{3}H] Palmitic acid oxidation assay

[^{3}H] Palmitic acid oxidation was measured by production of [^{3}H] labeled water as previously described (Rune et al. 2009). Differentiated myotubes were incubated for 6 h in DMEM (1 g L^{-1} glucose) with 1 mCi mL^{-1} [^{3}H] palmitic acid (#NET043005MC, Perkin Elmer), 25 μmol L^{-1} palmitic acid and 0.02% fatty acid free BSA. The collected medium was incubated in a charcoal slurry (0.1 g activated charcoal per 1 mL 0.02 mol L^{-1} Tris-HCl at pH 7.5), subjected to centrifugation, and the supernatant was collected for scintillation counting (WinSpectral 1414 Liquid Scintillation Counter; Wallac). Cells were lysed in 0.03% SDS and the protein concentration was determined using a commercially available Bradford protein assay kit (#5000006, BioRad). Results were normalized to the total protein amount per well and the oxidation rate of palmitic acid is presented as pmol mg^{-1} h^{-1}.

Statistical analysis

Statistical significance of differences in gene expression and protein content in myoblasts and myotubes from spinal cord-injured and able-bodied individuals were determined by a two-way ANOVA, followed by the Sidak's corrected multiple comparison test (Figs. 1, 2). Differences between myotubes of the two groups were determined by the Mann–Whitney test (Figs. 3, 4, 5). *P* values below 0.05 were considered as statistically significant, while values below 0.1 are reported as trends. Statistical analyses were performed, using the GraphPad Prism v. 7.01 (GraphPad, La Jolla, CA).

Results

Differentiation of skeletal muscle satellite cells

To determine the differentiation capacity of skeletal muscle satellite cells, activated satellite cells (myoblasts) were differentiated into myotubes. We assessed myotube formation by immunohistochemistry for differentiation (desmin) and proliferation (Ki67) markers and nuclear staining with DAPI. Qualitative assessment of

Table 3. Primary antibodies used for Western blot.

Antigen	Molecular weight (kDa)	Product number and supplier
Desmin	55	#15200 Abcam, Cambridge, U.K.
Myogenin	34	#12732 Santa Cruz, Dallas, TX
MHC II (Myh1/2)	225	#53088 Santa Cruz
MHC I (Myh7)	225	#A4.840 DSHB, Iowa City, IA
pAkt$^{(Ser473)}$	60	#4060 Cell Signaling Danvers, MA
pAkt$^{(Thr308)}$	60	#4056 Cell Signaling
Akt	60	#9272 Cell Signaling
pmTOR$^{(Ser2448)}$	289	#600-401-422 Rockland, Pottstown, PA
mTOR	289	#2983 Cell Signaling
Raptor	150	#2280 Cell Signaling
p4EBP1$^{(Thr37/46)}$	20	#2855 Cell Signaling
4EBP1	20	#9452 Cell Signaling
pS6$^{(Ser235/236)}$	32	#2211 Cell Signaling
S6	32	#2317 Cell Signaling
pFoxO1$^{(Ser256)}$	78	#9461 Cell Signaling
FoxO1	78	#12161 Abcam
pFoxO3a$^{(Ser253)}$	97	#13129 Cell Signaling
FoxO3a	97	#47409 Abcam
LC3	16/18	#L8918 Sigma Aldrich, St. Louis, MO
p62	62	# P0067 Sigma Aldrich
MAFbx	35	#166806 Santa Cruz
Pan 20Sα	30–32	#22674 Abcam
pACC$^{(Ser222)}$	280	#3661 Cell Signaling
ACC	280	#3676 Cell Signaling

MHC, myosin heavy chain; mTOR, mechanistic target of rapamycin; 4EBP1, 4E binding protein 1; FoxO, forkhead box protein O; LC, light chain; MAFbx, muscle atrophy F-box; ACC, acetyl-CoA carboxylase.

immunohistochemistry images has shown that, upon differentiation, myoblasts from both spinal cord-injured and able-bodied individuals fused and formed multinucleated myotubes with an abundance of desmin and depletion of Ki67 positive nuclei (Fig. 1A).

Western blot was used to assess protein levels of differentiation markers of myoblasts and myotubes. Differentiation increased ($P < 0.05$) cellular desmin, myogenin and both type I and type II myosin heavy chain (MHC I and II; Fig. 1B and C, respectively). Myotubes from spinal cord-injured and able-bodied individuals had similar ($P > 0.05$) levels of desmin and myogenin. Myotubes from spinal cord-injured individuals displayed higher ($P < 0.05$) protein abundance of MHC II, whereas MHC I tended ($P = 0.06$) to be increased (Fig. 1B and C).

We next determined whether spinal cord injury affects the transcriptional regulation of differentiation by assessing the expression of myogenic regulatory factors in myoblasts and myotubes from spinal cord-injured and able-bodied individuals. The expression of Pax7, Myf5, Myod1 and myogenic factor 6 (Myf6) decreased ($P < 0.05$) throughout differentiation (Fig. 2A–D). Conversely, the expression of myogenin and desmin was increased ($P < 0.05$) in myotubes compared to myoblasts (Fig. 2E and F). Expression of Pax7, Myf5, Myf6, myogenin and desmin was similar ($P > 0.05$) in myoblasts, as well as in myotubes from the spinal cord-injured and able-bodied individuals (Fig. 2A, B and D–F; $P > 0.05$). Expression of Myod1 was higher ($P < 0.05$) in myoblasts from spinal cord-injured individuals compared to able-bodied controls (Fig. 2C).

Protein synthesis in differentiated myotubes

To determine whether myotubes from spinal cord-injured individuals have an altered capacity for protein synthesis we measured incorporation of [^{14}C]-labeled phenylalanine into protein. The incorporation of radiolabeled tracer into protein was similar ($P > 0.05$) between myotubes from spinal cord-injured and able-bodied individuals (Fig. 3A). We found similar ($P > 0.05$) levels of phosphorylated Akt$^{(Ser473)}$ and $^{(Thr308)}$, mTOR$^{(Ser2448)}$, 4E binding protein 1 (4EBP1)$^{(Thr37/46)}$, ribosomal protein S6 (S6)$^{(Ser235/236)}$, and the corresponding total protein content in spinal cord-injured and able-bodied individuals (Fig. 3B and C). Similarly, content of regulatory-associated protein of mTOR (raptor) was not different ($P > 0.05$) between myotubes from spinal cord-injured and able-bodied individuals (Fig. 3B and C).

Protein degradation signalling in differentiated myotubes

We used Western blot analysis to investigate pathways regulating protein degradation. Phosphorylated FoxO1$^{(Ser256)}$ and FoxO3a$^{(Ser253)}$, as well as the total abundance of FoxO3a were similar (Fig. 4A and B; $P > 0.05$) between spinal cord-injured and able-bodied individuals. Total protein content of FoxO1 tended to be decreased (Fig. 4A and B; $P = 0.06$) in myotubes from spinal cord-injured compared to able-bodied individuals. Protein levels of the FoxO transcriptional targets including ubiquitin-binding protein p62 (p62), microtubule-associated protein 1 light chain 3 (LC3) I and II, and muscle atrophy F-box (MAFbx) were unchanged (Fig. 4A and B; $P > 0.05$). Similarly, protein abundance of the proteasomal 20Sα catalytic subunit was comparable between myotubes from spinal cord-injured and able-bodied individuals (Fig. 4A and B; $P > 0.05$).

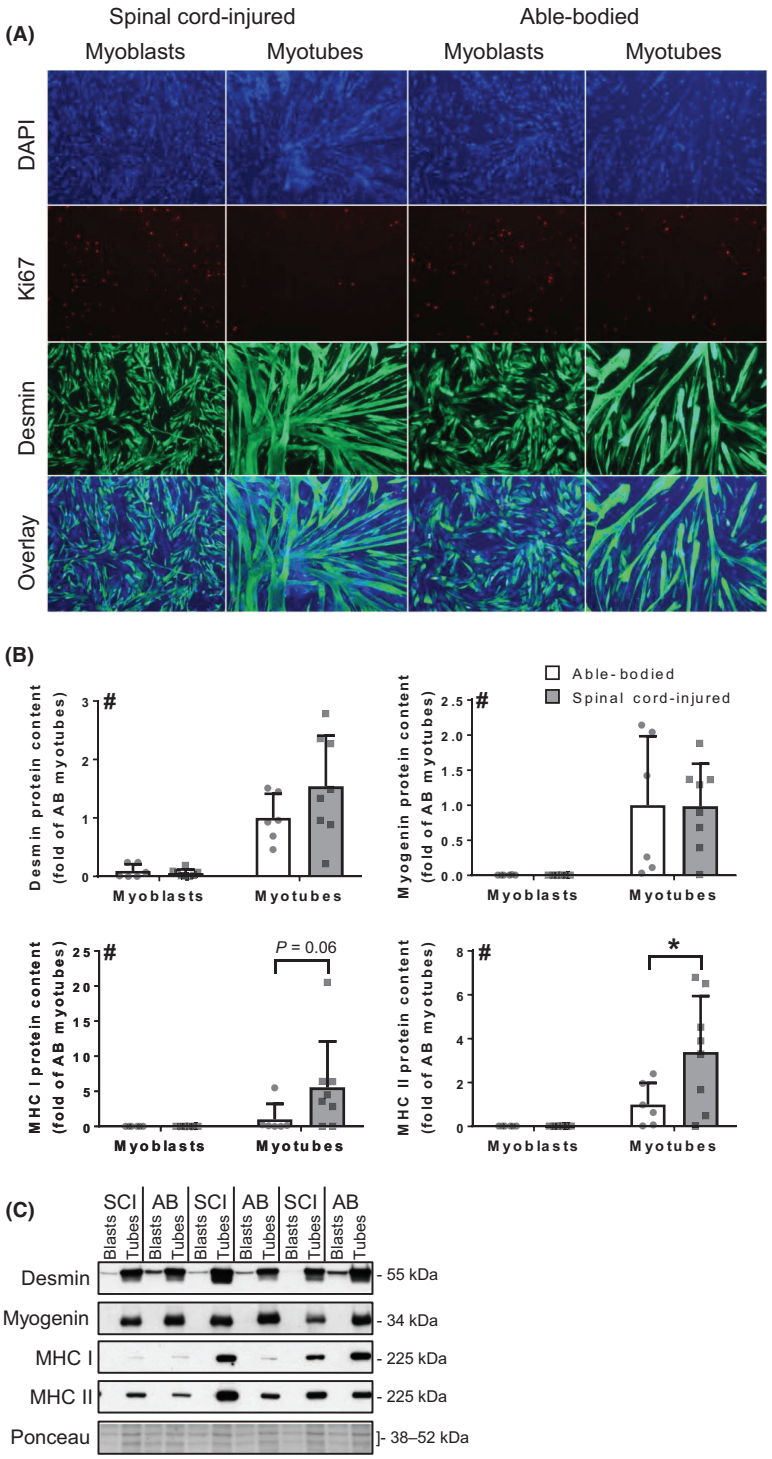

Figure 1. Differentiation of skeletal muscle satellite cells. (A) Representative images of immunohistochemistry for Ki67 and desmin, as well as DAPI staining of the nuclei and an overlay of the three signals. (B) Protein content of muscle-specific differentiation markers (desmin, myogenin, MHC I and MHC II) in myoblasts and myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. n = 6–8; Two-way ANOVA: # – overall effect of differentiation ($P < 0.05$); * – Sidak's post hoc comparison ($P < 0.05$). (C) Representative Western blot images. DAPI, 4,6-diamidino-2-phenylindole; MHC, myosin heavy chain.

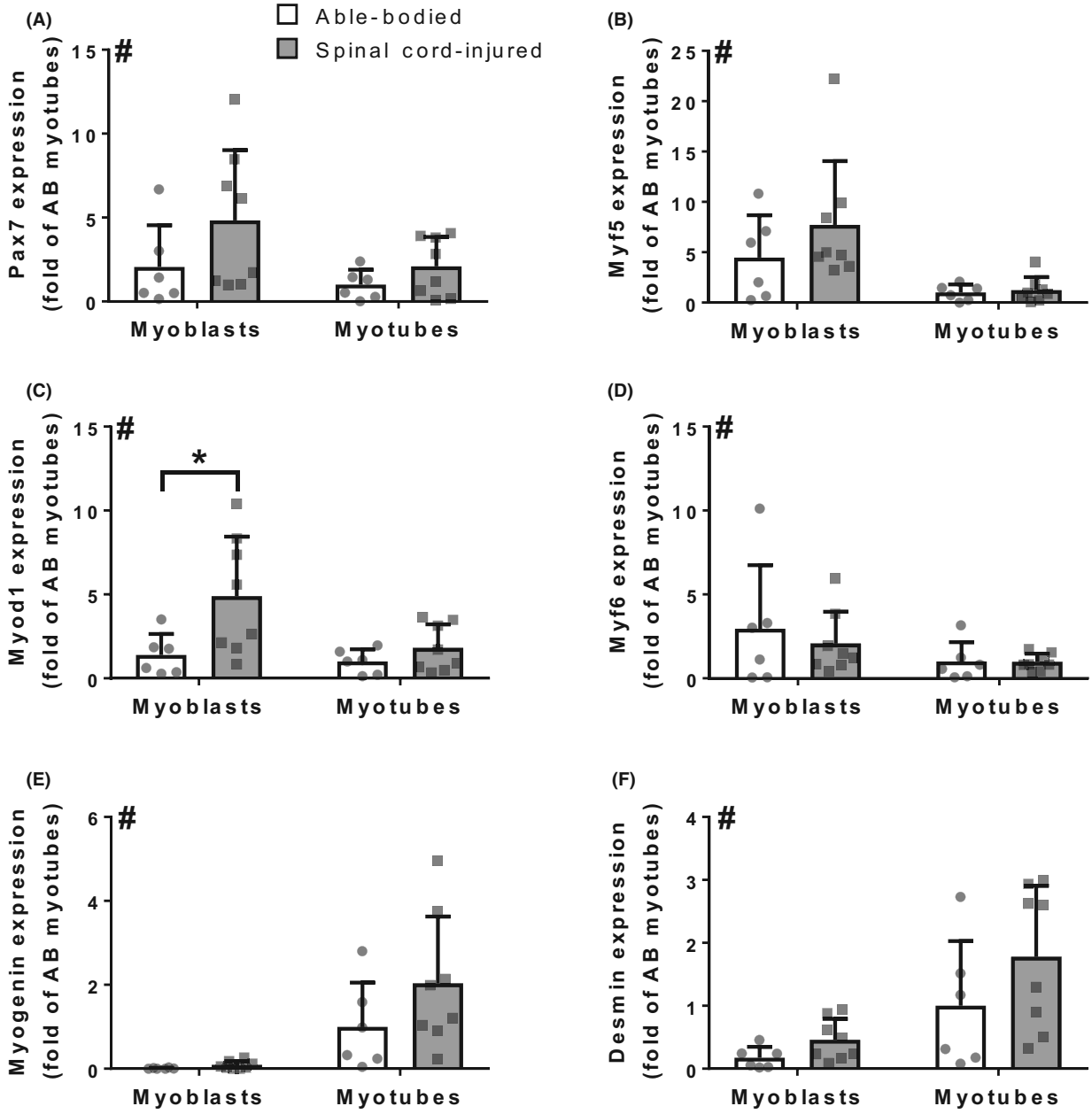

Figure 2. Regulation of differentiation of skeletal muscle satellite cells. Expression of myogenic regulatory factors (A) Pax7, (B) Myf5, (C) Myod1, (D) Myf6, (E) myogenin, and (F) desmin, in myoblasts and myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. n = 6–8; Two-way ANOVA: # – overall effect of differentiation ($P < 0.05$); * – Sidak's post hoc comparison ($P < 0.05$).

Fatty acid oxidation in differentiated myotubes

To determine the oxidative capacity of myotubes derived from satellite cells of spinal cord-injured individuals, we measured palmitic acid oxidation by production of [^{3}H]-labeled water from [^{3}H]-labeled palmitic acid. The oxidation of palmitic acid was similar between satellite cells of spinal cord-injured and able-bodied individuals ($P > 0.05$; Fig. 5A). Moreover, total and phosphorylated protein content of acetyl-CoA carboxylase (ACC)$^{(Ser222)}$ was similar ($P > 0.05$) between skeletal muscle satellite

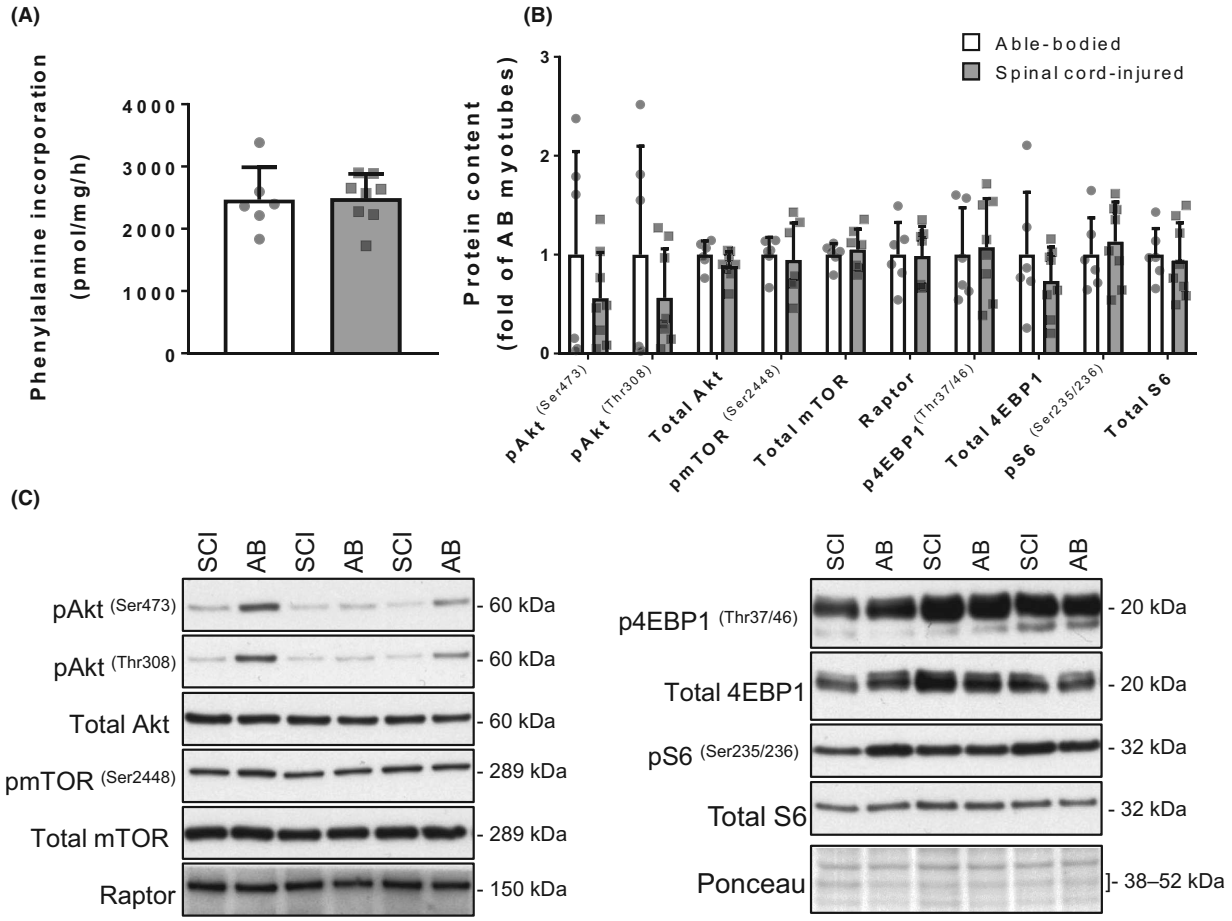

Figure 3. Protein synthesis in differentiated myotubes. (A) Phenylanine incorporation into protein in myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. (B) Phosphorylated and total protein content of molecules in the Akt-mTOR signalling axis (pAkt$^{(Ser473)}$, pAkt$^{(Thr308)}$, total Akt, mTOR$^{(Ser2448)}$, total mTOR, Raptor, p4EBP1$^{(Ser37/46)}$, total 4EBP, pS6$^{(Ser235/236)}$, total S6) in myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. n = 6–8; Mann–Whitney test (significance $P < 0.05$). (C) Representative Western blot images. mTOR, mechanistic target of rapamycin; 4EBP, 4E binding protein.

cells of spinal cord-injured and able-bodied individuals (Fig. 5B and C).

Discussion

Satellite cells play an important role in skeletal muscle regeneration and plasticity (Schiaffino et al. 1976; Collins et al. 2005; Bruusgaard et al. 2010; Lepper et al. 2011). However, little is known regarding their differentiation capacity in human skeletal muscle following a spinal cord injury. We assessed the myogenic programming of satellite cells obtained from skeletal muscle of spinal cord-injured individuals. We provide evidence that skeletal muscle satellite cells retain their capacity to differentiate after spinal cord injury. Moreover, once differentiated the myotubes show comparable metabolic characteristics to those from able-bodied individuals. Collectively, our data indicates that the intrinsic myogenic programming of skeletal muscle satellite cells is retained in individuals with spinal cord injury.

During satellite cell isolation, we attempted to filter out nonsatellite cells by allowing their attachment, although a formal assessment of purity was not performed. Our study included spinal cord-injured participants after a longstanding (>1 year) injury, a period when skeletal muscle has undergone dramatic skeletal muscle atrophy (Moore et al. 2015). We show that satellite cells do not mirror such prominent changes when grown in vitro. However, due to the relatively low number of participants (n = 6–8), it is possible that more subtle changes exist

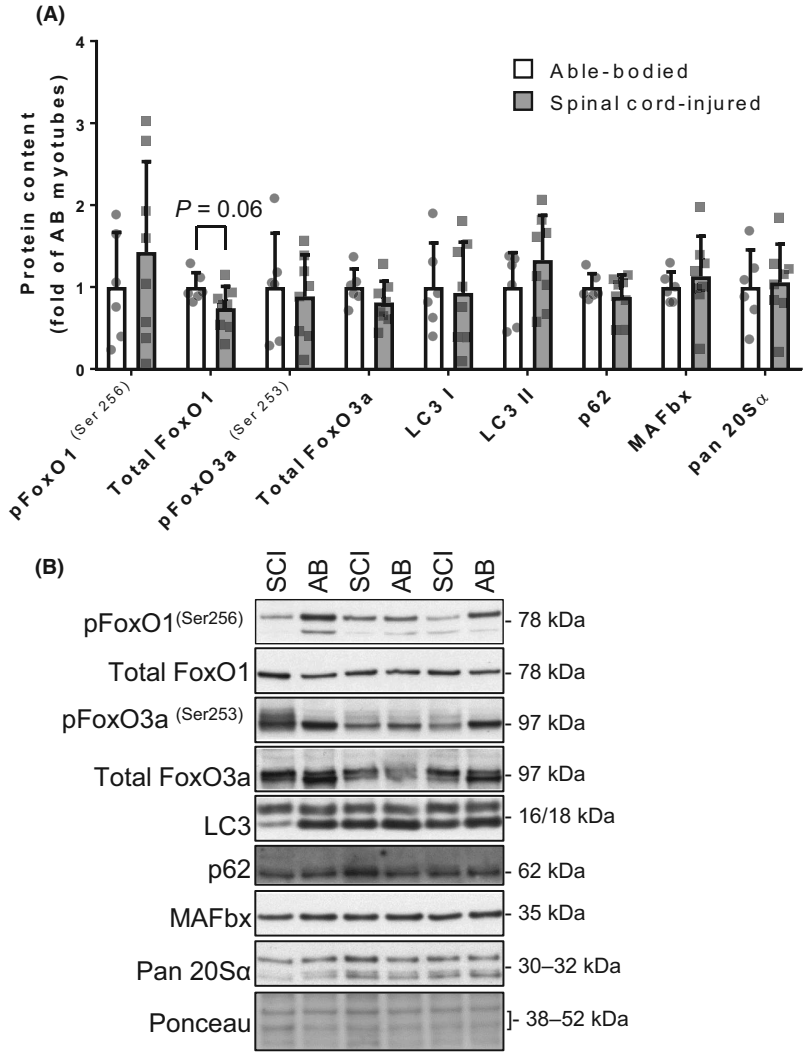

Figure 4. Protein degradation signalling in differentiated myotubes. (A) Phosphorylated and total protein content of FoxO1$^{(Ser256)}$ and FoxO3$^{(Ser253)}$ transcription factors, and their targets (LC3I, LC3II, p62, MAFbx), as well as the total protein levels of 20Sα proteosomal subunit in myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. n = 6–8; Mann–Whitney test (significance $P < 0.05$). (B) Representative Western blot images. FoxO, forkhead box protein O; LC, light chain; MAFbx, muscle atrophy F-box.

which we are underpowered to detect. Finally, the spinal-cord injury group consisted of participants with both incomplete and complete spinal-cord injury, but none were freely ambulating. However, we cannot rule out that remaining motor activity or muscular spasms may influence satellite cell function.

We have shown that during differentiation, myoblasts from spinal cord-injured individuals retain the capacity to exit the cell cycle, as evidenced by the decrease of Ki67 nuclear localization through differentiation (Scholzen and Gerdes 2000), and form multinucleated myotubes, with levels of desmin similar to myotubes from able-bodied controls. Additionally, protein abundance of muscle specific structural proteins (desmin and MHC) was increased during differentiation in both cells from able-bodied and spinal cord-injured individuals. Surprisingly, we found the abundance of MHC I and MHC II was increased in myotubes from spinal cord-injured versus able-bodied individuals. Spinal cord injury is associated with a decrease of MHC I and an increase of MHC II in skeletal muscle in vivo, reflecting the change in fiber type-composition from type I to predominantly type II fibers (Lotta et al. 1991; Castro et al. 1999; Kostovski et al. 2013). Our findings of increased MHC II in differentiated myotubes

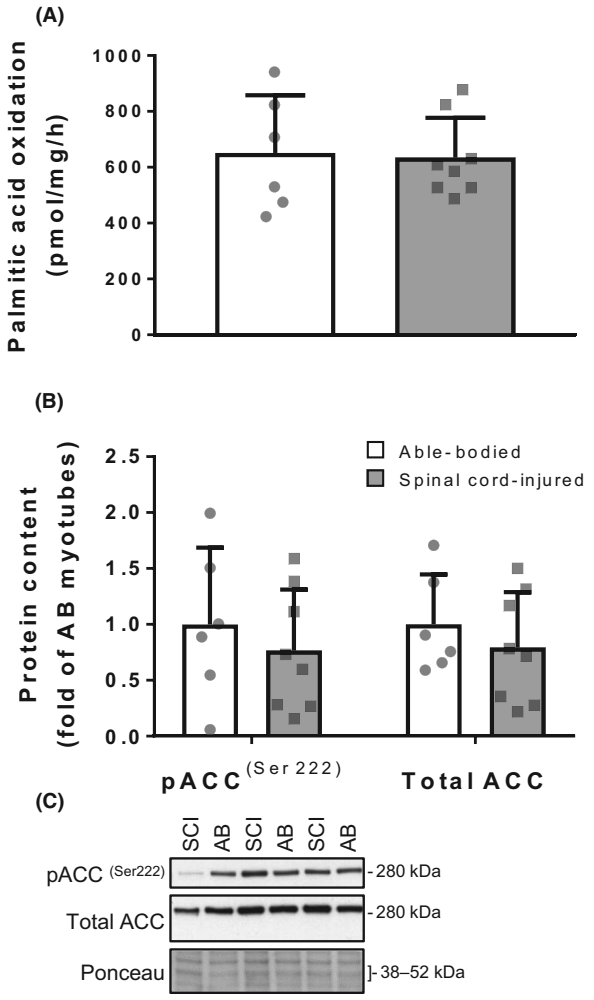

Figure 5. Oxidative capacity of differentiated myotubes. (A) Palmitic acid oxidation in myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. (B) Protein content of total and phosphorylated ACC$^{(Ser222)}$ in myotubes from spinal cord-injured (gray bars) and able-bodied (white bars) individuals. Bars represent mean ± SD and individual data points are overlaid. n = 6–8; Mann–Whitney test: (significance $P < 0.05$). (C) Representative Western blot images. ACC, acetyl-CoA carboxylase.

from spinal cord-injured individuals may indicate that satellite cells retain a fiber-type memory. However, we found a trend ($P = 0.06$) for increased MHC I in myotubes from spinal cord-injured subjects, suggesting that the alteration in the MHC content in myotubes may be caused by different mechanisms. Nevertheless, the capacity to increase skeletal muscle-specific structural proteins during differentiation indicates that the differentiation capacity in satellite cells from spinal cord-injured individuals is preserved.

Pax7 and myogenic regulatory factors direct differentiation (Almeida et al. 2016). In the early stages of differentiation, commitment to the myogenic lineage is evident by high expression of Myf5 and Myod1 (Almeida et al. 2016). Distinct populations of myoblasts express either Myf5 or Myod1 upon activation, and these factors show an overlapping role in differentiation (Rudnicki et al. 1993; Cooper et al. 1999). As myoblasts from spinal cord-injured individuals had higher Myod1 expression compared to those from able-bodied controls, a larger portion of myoblasts from spinal cord-injured individuals may belong to the Myod1 positive group. As differentiation progresses, the terminal stages are characterized by increased myogenin expression (Almeida et al. 2016). In cells from both able-bodied and spinal cord-injured individuals, the expression of Pax7, Myf5 and Myod1 decreased through differentiation, while the expression of myogenin increased through differentiation. These data suggest that satellite cells from spinal cord-injured individuals are committed to the myogenic lineage, evidenced by high Myf5 and Myod1 expression, and are able to undergo terminal differentiation and increase myogenin expression in myotubes. Thus, in addition to their differentiation capacity, the regulation of differentiation in satellite cells from spinal cord-injured individuals is preserved. In the current study, we have not determined the proliferation capacity of satellite cells. As spinal cord injury leads to their activation (Dupont-Versteegden et al. 1999; Jayaraman et al. 2013), this is a parameter of interest, especially if inherent satellite cells are to be used to combat muscle atrophy.

Loss of skeletal muscle mass is mainly attributed to an imbalance between anabolic and catabolic protein metabolism (Jackman and Kandarian 2004). Akt-mTOR signalling undergoes a coordinated decrease in skeletal muscle of spinal cord-injured in vivo, indicating decreased protein synthesis (Dreyer et al. 2008). Our findings of unchanged protein abundance of the Akt-mTOR signalling axis members and amino acid incorporation into protein in myotubes directly contrast the in vivo observations. We conclude that skeletal muscle satellite cells after spinal cord injury retain their ability to produce myotubes with a normal capacity for, and regulation of protein synthesis.

With chronic spinal cord injury, FoxO transcriptional activity and the expression of their downstream targets involved in protein degradation are reduced (Leger et al. 2009; Milan et al. 2015). We reported a tendency ($P = 0.06$) for reduced total FoxO1 protein in myotubes from spinal cord-injured individuals, with no changes in phosphorylated (inactive) levels of both FoxO1$^{(Ser256)}$ and FoxO3a$^{(Ser253)}$, as well as the total protein content of FoxO3a. The tendency of reduced FoxO1 total protein

content could indicate lower transcriptional activity. However, as there were no changes in protein abundance of FoxO transcriptional targets (p62, LC3I, LC3II and MAFbx) (Milan et al. 2015) we conclude that in contrast to the observations in skeletal muscle in vivo following spinal cord injury, the FoxO transcriptional regulation of protein degradation is unchanged in satellite cell-derived myotubes in vitro.

Additionally, the comparable levels of p62, the ubiquitin-binding autophagy cargo adapter, and LC3I and II, the autophagosome-forming proteins, indicate similar levels of macroautophagy between myotubes from spinal cord-injured and able-bodied individuals (Mizushima and Yoshimori 2007). Unchanged abundance of MAFbx, an ubiquitin E3 ligase and pan20Sα proteasomal proteolytic subunit in myotubes from spinal cord-injured individuals, indicate stable levels of ubiquitination and proteosomal degradation, respectively. Together, this could indicate stable levels of protein degradation in myotubes from spinal cord-injured individuals.

Skeletal muscle following spinal cord injury in vivo has decreased β-oxidation, mirrored by reductions in free fatty acid uptake, mitochondrial content and levels of oxidative enzymes (Wang et al. 1999; Kjaer et al. 2001; Long et al. 2011; McCully et al. 2011). Conversely, myotubes from spinal cord-injured and able-bodied individuals were able to oxidize palmitic acid at a comparable level. Total and phosphorylated protein content of $ACC^{(Ser222)}$ were similar between the two groups, indicating stable regulation of the fatty acid metabolism. Thus, in contrast to the reduced β-oxidation capacity in vivo, skeletal muscle satellite cells from spinal cord-injured individuals are able to produce myotubes with oxidative capacity comparable to those from able-bodied controls. Collectively, our data shows that the metabolic memory of satellite cells is retained and they are able to produce myotubes with normal protein and fatty acid metabolism, in spite of the changes occurring in skeletal muscle in vivo.

Previous animal studies indicate that spinal cord injury leads to activation of satellite cells in the affected skeletal muscle in vivo (Dupont-Versteegden et al. 1999; Jayaraman et al. 2013). However, the terminal differentiation of the cells may be lacking as the myonuclear number continues to decrease (Dupont-Versteegden et al. 1999). Other rat models of skeletal muscle atrophy, such as lower motor neuron injury and denervation, also lead to activation of satellite cells followed by inefficient differentiation and underdeveloped myotubes, with deficient or absent contractile machinery (Carraro et al. 2015). Similar mechanisms, through activation and inefficient differentiation, may be responsible for the reduction of the satellite cell pool in the skeletal muscle of spinal cord-injured individuals (Verdijk et al. 2012). However, our data demonstrates that the intrinsic myogenic differentiation capacity and the metabolic memory of satellite cells from spinal cord-injured individuals are preserved following spinal cord injury. Once extracted from the skeletal muscle and grown in vitro, they differentiate and produce myotubes that retain metabolic characteristics. Thus, defects in satellite cell differentiation in skeletal muscle of spinal cord-injured individuals may be connected to the decentralized and atrophying skeletal muscle "environment", rather than a dysfunction in their programming.

As satellite cells play a role in regulating skeletal muscle mass (Bruusgaard et al. 2010), specific rehabilitative interventions targeting their activation could be efficient in reducing skeletal muscle atrophy after spinal cord injury. Electrical stimulation coupled with exercise improves the metabolic characteristics of skeletal muscle in spinal cord-injured individuals (Hjeltnes et al. 1998; Gorgey et al. 2017) and may be used as a potential activator of skeletal muscle regenerative machinery (Kern and Carraro 2014). Different protocols of functional electrical stimulation mimic different types of exercise (Fornusek and Davis 2008; Bickel et al. 2015). Both endurance and resistance training lead to an increase in satellite cell number, while resistance training also leads to an increase in myonuclear number (Bruusgaard et al. 2010; Kurosaka et al. 2012). In light of this, studies of satellite cell response to different modalities and intensities of functional electrical stimulation could help design specialized interventions for satellite cell activation. Further studies analysing the response to electrical pulse stimulation in vitro could reveal the full extent of functionality of myotubes and inform these efforts in regard to any underlying electrical stimulus intensity, frequency and duration preference. Our findings may encourage these future efforts, and highlight the prospect of inherent satellite cell activation in attempts to prevent skeletal muscle atrophy following spinal cord injury.

Conflict of Interest

No conflicts of interest are declared by the authors.

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