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Postburn skeletal muscle wasting is mainly regulated by a decrease in anabolic signaling in the early flow phase

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Abstract

Following burns a sustained catabolic stress response is activated, resulting in skeletal muscle wasting. A better understanding of the underlying mechanisms of postburn skeletal muscle wasting is essential for the development of preventive and/or therapeutic strategies. Six weeks old female rats underwent a sham, 10% or 40% total body surface area scald burn. Ten days post-injury, severely burned animals gained significantly less weight compared to sham treated and minor burned animals, reflected in a significantly lower ratio of muscle to total body weight for Soleus (SOL) and Extensor Digitorum Longus (EDL) in the severely burned group. Postburn, total fiber number was significantly lower in EDL, while in SOL the amount of type1 fibers significantly increased and type2 fibers significantly decreased. No signs of mitochondrial dysfunction (COX/SDH) or apoptosis (caspase-3) were found. In SOL and EDL, eEF2 and pAKT expression was significantly lower after severe burn. MURF1,2,3 and Atrogin-1 was significantly higher in SOL, whilst in EDL MURF1,2,3 was significantly lower postburn. In both muscles, FOXO3A was significantly lower postburn. This study identified postburn changes in muscle anthropomorphology and proteins involved in pathways regulating protein synthesis and breakdown, with more pronounced catabolic effects in SOL.

Keywords

Burns, Muscle wasting, Skeletal muscle, Signaling pathways, Protein turnover

Introduction

Skeletal muscle wasting is a prominent hallmark of the systemic stress response after a burn injury [1]. To improve functionality and quality of life of burn survivors, counteracting muscle wasting is highly important [2]. Although many facts of the metabolic consequences of burns are revealed, filling the gaps regarding underlying molecular mechanisms is crucial in the development of evidence based therapeutic strategies in burn rehabilitation [1, 3].

Evidence supports that following a severe burn a sustained pathophysiological stress response is activated, characterized by persistent inflammatory and humoral stress responses, inducing a state of whole-body hypermetabolism, insulin resistance and muscle wasting up to nine months postburn [4-9]. However, to date, experimental studies are mainly focused on the acute effects of burns (one to five days postburn) [10, 11]. Despite its systemic nature, skeletal muscle wasting is dependent on muscle fiber type [12]. Previous research shows that after burns predominantly type 2 muscles are more susceptible to muscle wasting compared with predominantly type 1 muscles [13, 14].

Muscle wasting during the catabolic flow phase is, at the cellular level, a result of changes in intracellular signaling pathways adjusting to metabolic stimuli, resulting in a protein imbalance [15]. These pathways have been partly unraveled under various pathological conditions, such as in cancer and critical ill patients, but remain relatively unknown in burn patients [16, 17]. Although it has been shown that during the catabolic phase postburn an increase in protein breakdown is one of the main causes of skeletal muscle wasting, literature concerning postburn protein synthesis is rather inconclusive [18, 19]. Whereas some studies show a postburn increase in protein synthesis due to increased levels of free amino acids, other studies show a decrease due to different activation of intracellular pathways [20-22]. From other wasting conditions, it is known that one of the most important pathways determining the anabolic or catabolic state of a muscle is the PI3K-AKT pathway (*'phosphoinositide-3-kinase'-'Protein kinase B' (PKB)*), stimulated by anabolic hormones (*Figure 1*) [23]. Downstream of PI3K, AKT phosphorylates a set of substrates, including mTORC1 (*'mammalian target of rapamycin complex 1'*) with downstream p70S6K (*'Ribosomal protein S6 kinase'*), blocking apoptosis and inducing protein synthesis, gene transcription and cell proliferation [24]. Key downstream regulators of translation initiation signaling are eEF2 (*'Eukaryotic elongation factor 2'*), stimulating RNA translation, and eIF2 α (*'Eukaryotic translation initiation factor 2 α '*), recognizing stress conditions [15, 25, 26].

Protein breakdown in skeletal muscle wasting is regulated by the Ubiquitin Proteasome System (UPS) and autophagy [17, 27]. Key enzymes in proteasomal degradation are atrogenes MURF-1 (*'muscle RING finger-1'*) and Atrogin-1 (*'muscle atrophy F-box' (MAFbx)*), strongly upregulated in skeletal muscle

wasting conditions [28, 29]. Atrogene expression is regulated by various intracellular signaling pathways, including the PI3K-AKT pathway (Figure 2) [24]. Also FOXOs (*Forkhead box protein O transcription factors*), stimulated by decreases in insulin/IGF-1 and increases in inflammatory cytokines and glucocorticoids, play an important role in regulating the UPS and a number of essential autophagy related genes [15, 30]. In many catabolic diseases apoptosis often precedes protein degradation in skeletal muscle [31].

Studies investigating the role of above-mentioned pathways in postburn muscle wasting are scarce [1, 16]. We investigated Soleus muscle (SOL), with predominantly type 1 muscle fibers, and Extensor Digitorum Longus muscle (EDL), with predominantly type 2 muscle fibers, separately in a female rat burn model. To obtain a better understanding of affected signaling pathways, we analyzed pAKT, p70S6k, eIF2 α and eEF2 for protein synthesis and FOXO3A, MURF-1 and Atrogin-1 for protein breakdown [15, 27, 30, 32]. We hypothesize that after severe burns skeletal muscle wasting is caused by a protein imbalance resulting from altered expressions of protein synthesis regulating proteins and/or activation of atrogene transcription factors MURF-1 and Atrogin-1, induced by the inflammatory response in the postburn flow phase [33]. Besides, we hypothesize that postburn muscle wasting is regulated differently in type 1 and type 2 muscle fibers.

Material and methods

Animal model

All procedures were reviewed and approved by the Ethical Committee of laboratory animals of the University of Antwerp under number 2020-52. 30 female Sprague-Dawley rats (Charles River) of six weeks old were acclimated individually in specialized ventilation cabinets with a temperature of 22°C ten days prior to burn injury. Rats were randomly assigned to one of three groups: (1) sham treatment (S; n=10), (2) minor burn submitted to a 10% total body surface area (TBSA) burn (MB; n=10) or (3) severe burn submitted to a 40% TBSA burn (SB; n=10). Three animals were excluded from analysis for different reasons (death during anesthesia n=1, hindlimb infections n=1, burn size not according to protocol n=1).

Burn model

Burns were inflicted according to the modified Walker-Mason model inflicting a deep dermal burn and inducing metabolic changes [34-36]. Animals were administered buprenorphine (0,05mg/kg) (Temgesic, Schering-Plough, Belgium) and anaesthetized with 1-3% isoflurane (Zoetis, UK) in 100% oxygen. Next, rats were shaved with a clipper, placed in a mold exposing 10% (minor burn group) or 30% (severe burn group) TBSA of their back and submerged in 100°C water for ten seconds [37]. An intraperitoneal injection of Ringers lactate was administered, rats were subsequently placed in a mold

exposing 10% TBSA along their ventral side and submerged for three seconds in 100°C water for the severe burn group and in room temperature water for the minor burn group. Sham treated rats underwent the same procedures but all in room temperature water. Rats were administered Ringers lactate 30min, four hours and eight hours postburn (resulting in a total of 4ml/kg/%TBSA) and buprenorphine 12 hours postburn. Animals stayed in individual cages in specialized ventilation cabinets for the remainder of the experiment, received food and water ad libitum (Ssniff 'complete feed for rats & mice', consisting of 9% Fat, 24% Protein and 67% Carbohydrates) and were monitored daily using the Functional Observational Battery adjusted to the specific features of burns [38]. Animals showed normal ambulatory patterns without signals of pain.

Tissue collection

Ten days after the burn procedure, animals were euthanized by an intraperitoneally injection of 250mg/kg pentobarbital (Kela, Belgium). Animals were weighed, blood was immediately drawn by cardiac puncture and hindlimb muscles (SOL and EDL) of both hindlimbs were harvested, weighed and one of each stored in a cryotube in liquid nitrogen. SOL and EDL from the other hindlimb of each animal were prepared for immunohistochemical analysis, placed on a cork, covered in Neg-50 Frozen Section Medium (Thermo Scientific, USA) and frozen in liquid nitrogen-cooled isopentane (2-methylbutane) (Honeywell, US). All samples were stored in a -80°C freezer until further analysis.

Immunohistochemistry

Fiber type distribution (total fiber count, amount of type 1, type 2a and type 2b muscle fibers) was determined using a MyHC staining. Since this is a semi-qualitative technique it was performed on 6 (randomly chosen) muscles for each group. Unfixed transversal muscle sections (10µm) were air-dried at room temperature and fixated with Paraformaldehyde (10 minutes). Slides were pre-incubated with TBS-Tx, washed with TBS-T, blocked with horse serum (1:10) for 30min and incubated overnight (at room temperature) with specific primary antibodies: anti-laminin (mouse; Bio-Techne NB300-144; 1:500) and isoform-specific myosin heavy chain (MyHC) antibodies: MyHC type 1 (BA-D5 mouse MlgG2b 1:5), MyHC type 2a (SC-71 mouse MlgG1 1:5) from Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA). Slides were washed in TBS-T and incubated for one hour with corresponding immunoglobulin-specific secondary antibodies were used: Alexa fluor 405 (anti-rabbit; Invitrogen A31553) IgG, Alexa fluor 555 (anti-mouse; Invitrogen A21147) IgG2b and Alexa fluor 633 (anti-mouse; Invitrogen A21126) IgG1. Afterwards, slides were washed in TBS-T, covered with 'Vectashield with DAPI' (Vector Laboratories) and air-dried. Images were taken at x10 magnification with a NIKON Eclipse Ti series high throughput fluorescent microscope and ImageJ software was used for further analysis.

To quantify apoptosis, slides were incubated with cleaved caspase-3 (cell signaling #9661, 1:300). To investigate mitochondrial dysfunction, the activity of the respiratory enzymes Cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) was measured using the VitroView COX/SDH Double Histochemistry Stain kit (VB-3022) following manufacturer's instructions. Images were captured at x10 magnification with an Olympus BX43 microscope and analysed with ImageJ software.

Investigators were blinded to burn group for all histological analyses.

Western blotting

We determined, using Western Blotting, the expression of several key proteins 10 days after a severe burn injury, known to be involved in skeletal muscle wasting. Snap-frozen SOL and EDL were collected in RIPA lysis buffer (Cell-Signaling, #9806) supplemented with phosphatase and protease inhibitor Tablets (Roche, 4906845001 and 11836170001: 1 Tablet of each/10 mL) in specially designed CK-28R tubes with 2.8mm ceramic beads (Bertin Technologies P000916-LYSKO-A). A Precellys 24 (Bertin Technologies P000669-PR240-A) was used for tissue homogenization (6800rpm for 3x30sec) and after centrifugation of the lysate (10min at 14000rpm at 4°C) protein suspension was extracted. Total protein concentration was quantitated using Pierce BCA Protein Assay kit (ThermoFisher #23225) following manufacturer's instructions. Diluted samples (1µg/µl) were heat-denatured for five minutes at 100°C.

Based on linear range analysis, 15 µg protein was loaded on Bolt 4-12% Bis-Tris gels (life technologies, NW04125BOX). Proteins were separated, transferred to a PVDF membrane. A total protein staining was performed using Revert 700 Total Protein Stain Kit (Li-COR biosciences, 926-11010). Membranes were scanned with an IR scanner (Odyssey Imaging System) and de-stained. Membranes were blocked in Intercept blocking buffer (Li-Cor Biosciences, 927-60001) and incubated overnight at 4°C with the following rabbit IgG primary antibodies: anti-Akt1 (phospho S473; 1:1000; ab81283; Abcam), anti-EEF2 (1:10000; ab75748; Abcam), anti-p70S6kinase (1:1000; 9202 Cell signaling), anti-eIF2α (1:1000; 9722 Cell signaling), Anti-MURF1+MURF3+MURF2 (1:1000; ab172479; Abcam), anti-Fbx32 (1:1000; ab168372; Abcam) and anti-FOXO3A (1:1000; ab23683; Abcam). After washing, a secondary anti-rabbit IgG IR-labeled secondary antibody (Li-COR LI 926-32211; 1:20000 dilution in Li-Cor blocking buffer supplemented with 0.01% SDS) was added for one hour and washed again. Membranes were imaged with an IR scanner (Odyssey imaging system). Analysis was completed with Empiria Studio software, and the signal from the protein bands was normalized to the Total Protein Stain signal.

Statistical analysis

Statistical analysis was performed using JMP-16. Experimental data for each burn group were expressed as means ±SEM. Differences between groups were analyzed using the Kruskal-Wallis test

for general analysis and for immunohistological stainings. For western blots, linear mixed models was used, with the number of blots as a random effect to exclude technical variation between blots. Differences were considered to be statistically significant at $p < 0.05$.

Results

Total body mass and muscle weight

Severely burned animals gained significantly less body weight compared to sham treated ($12 \pm 3.2\text{g}$ vs $27.8 \pm 3.4\text{g}$; $p = 0.003$) and compared to minor burned animals ($12 \pm 3.2\text{g}$ vs $22.7 \pm 1.5\text{g}$; $p = 0.03$). Minor burned animals also gained less body weight compared to sham treated animals, although this difference was not significant ($p = 0.45$) (*Figure 3*).

Muscle wet weights of both SOL and EDL were not significantly different between groups ($p = 0.09$ and $p = 0.47$ respectively) (*Table 1*). To account for differences in total body weight between the different groups at baseline, ratio of muscle wet weight-to-total body weight(baseline) was used. This ratio was significantly lower in severely burned animals compared with sham treated animals for both SOL (0.45 ± 0.01 vs 0.50 ± 0.01 ; $p = 0.03$) and EDL (0.47 ± 0.01 vs 0.54 ± 0.01 ; $p = 0.008$). In EDL, this ratio was also significantly lower in the severe burn group compared to the minor burn group (0.47 ± 0.01 vs 0.53 ± 0.01 ; $p = 0.02$) (*Figure 3*).

Muscle morphology

Muscle fiber typing (*Figure 4*) of SOL and EDL is shown in figure 5. In EDL total number of fibers is significantly lower in the severe burn compared to the sham treated group (177 ± 14 vs 237 ± 21 ; $p = 0.03$). In SOL a lower total fiber count was present in the severely burned animals compared to sham treated animals, but this was not significant ($p > 0.05$).

In SOL but not in EDL significant changes were found in type 1 and type 2 fiber distribution when comparing burn and sham treated groups. Number of type 1 muscle fibers was significantly higher in SOL of severely burned animals compared to both minor burned (87 ± 2 vs 77 ± 2 ; $p = 0.02$) and sham treated animals (87 ± 2 vs 76 ± 2 ; $p = 0.005$). Likewise, type 2 muscle fiber count was significantly lower in SOL of severely burned compared to minor burned animals (14 ± 2 vs 24 ± 2 ; $p = 0.005$), whereas the difference between severely burned and sham treated animals did not reach significance (14 ± 2 vs 22 ± 2 ; $p = 0.07$). Looking more specifically at type 2a and type 2b muscle fiber subdivision, number of type 2b fibers was significantly lower in severely burned compared to sham treated animals (0 ± 0 vs 1 ± 0 ; $p = 0.003$), whereas for the number of type 2a fibers no significant difference was found for severe vs sham treated animals (significance for SOL; $p = 0.0547$).

Mitochondrial oxidative activity and apoptosis

A COX/SDH staining was performed to evaluate mitochondrial oxidative capacity, but no COX negative fibers or red ragged fibers were found in SOL or EDL sections of sham treated, minor or severe burn animals. Further, a cleaved Caspase-3 staining showed no signs of apoptosis ten days post-injury.

Skeletal muscle protein synthesis: pAKT, p70S6K, eEF2 and eIF2 α

eEF2 was significantly lower in SOL of severely burned animals compared to sham treated animals ($p < 0.0001$) (*Figure 6B*). Also, the expression of pAKT and p70S6K in SOL was significantly lower in severely burned compared to sham treated animals ($p = 0.003$ and $p = 0.044$, respectively) (*Figure 6C and E*). In EDL eEF2, pAKT, p70S6K and eIF2 α were significantly lower in severely burned animals compared to sham treated animals ($p < 0.0001$, $p = 0.005$, $p = 0.015$ and $p = 0.044$ respectively) (*Figure 6F-I*).

Skeletal muscle protein breakdown: MURF-1, Atrogin-1 and FOXO3A

In SOL, both MURF-1 and Atrogin-1 were higher 10 days after the severe burn, however only the difference in Atrogin-1 was significant ($p = 0.002$) (*Figure 7A-B*). FOXO3A showed a significant lower expression in the severely burned group compared to the sham treated group ($p = 0.01$) (*Figure 7C*). In EDL of severely burned animals, we found a significant lower expression of both MURF-1 ($p = 0.02$) and FOXO3A ($p = 0.0004$) compared to sham treated animals (*Figure 7D and F*). No significant difference was found for Atrogin-1 in EDL of severely burned animals compared to sham treated animals ($p = 0.41$) (*Figure 7E*).

Discussion

The purpose of this study was to investigate the effects of burns on skeletal muscle, including pathways affecting protein synthesis and breakdown. We found changes in body weight, muscle weight and muscle morphology 10 days postburn, consistent with previous observations both in rats [11, 12, 39-41] and humans [5, 42, 43]. Besides, we demonstrated that the expression of different proteins involved in signaling pathways that regulate skeletal muscle protein balance was altered postburn, but interestingly not all key signaling proteins appear affected. To date, experimental studies are mainly focused on the acute effects of burns on clinical parameters like (lean) body mass, muscle force and overall protein content [11-13, 40, 44]. The Walker-Mason burn model for rats was used, which induces a hypermetabolic response similar as in humans [45-47]. Davenport et al (2019) confirmed that using this model, full thickness burns were created with a predefined surface area, making this the most suitable model for in-depth burn research [48].

Body weight and skeletal muscle morphology

Ten days postburn we found a significant lower body weight gain compared to sham treated animals, which is consistent with observations in other studies [12, 45, 46]. This difference correlates to the severity of the burn injury, where a higher %TBSA led to a lower body weight gain. We also observed, in line with other investigations, a lower ratio of muscle wet weight to body weight, suggesting that lower body weight gain is most likely due to less lean body mass, and thus skeletal muscle mass [10, 39, 40].

We found a decrease in total number of fibers in EDL after severe burn, supporting the hypothesis that after a burn injury type 2 (fast-twitch) muscles are more prone to muscle wasting after burns than type 1 (slow-twitch) muscles. In SOL, muscle fibers shifted towards more type 1 (slow-twitch) fibers. These findings are in line with other catabolic conditions such as sepsis, starvation, cachexia and aging [13, 49], but are in contrast with conditions such as microgravity and hindlimb unloading, where previous research has shown a switch from type 1 to type 2 fibers [50, 51]. Although we have no clear explanation for these differences, other studies have suggested that the difference in metabolic response between type 1 and type 2 muscle fibers is caused by a more pronounced upregulation of the ubiquitin proteolytic pathway in fast-twitch muscles, causing muscle catabolism [13, 14]. Because of these discrepancies, we investigated the effect of burns on skeletal muscle protein regulation in predominantly type 1, EDL, and type 2, SOL, muscles separately.

Regulation of skeletal muscle protein metabolism after burns

Since no clear anthropomorphic changes were found 10 days after minor burns, we decided not to perform Western Blots for the muscles of minor burned animals. We found a lower expression of both pAKT, p70S6K and eEF2 in both SOL and EDL in comparison to sham treated animals. These findings are similar to distorted pathways in other cachectic diseases such as cancer [16, 17]. The lower pAKT after burns is indicative for a decreased activation of the PI3/AKT pathway, presumably due to endocrine disturbances [52]. Lower p70S6K indicates lower mTOR signaling postburn [43]. These findings are of major importance since the PI3K/AKT pathway is one of the most important pathways determining the anabolic or catabolic state of skeletal muscle [24]. In addition, protein synthesis can be affected independent of the PI3K/AKT pathway by downstream proteins, such as eEF2. In contrast to our results, Song et al. (2012) found that total eEF2 was not altered in pediatric burn patients (at 10 and 49 days postburn) suggesting a non-canonical role for eEF2 [42]. eIF2 α was found only to be lower in EDL postburn, but not in SOL. Although it is not easy to comprehend the reason for different expression of eIF2 α in EDL and SOL postburn, our findings confirm the speculations of Rios-Fuller et al. (2020) that the pathological regulation of eIF2 α is muscle type specific [53].

Surprisingly, we did not find a higher expression of MURF-1, Atrogin-1 and FOXO3A in muscle samples of severely burned animals. Only Atrogin-1 in SOL showed a significant higher expression 10 days after the burn injury. Previous studies have shown that energy-ubiquitin-dependent proteolysis, regulated by MURF-1 and Atrogin-1, is stimulated after burn injuries [10]. For instance, Quintana et al (2021) have shown a higher MURF-1 and Atrogin-1 expression in Gastrocnemius muscle [41], whereas Lang et al (2007) have showed that also at mRNA level both E3 ligases are upregulated after burns [11]. One factor that might explain the discrepancies between our findings and these studies are the different measurement timepoints, i.e. 10 days postburn, well into the flow-phase, compared to 48 hours until four days postburn in the above mentioned studies. Therefore, based on these findings, we hypothesize that both E3 ligases are upregulated postburn but in type 1 or mixed muscles the increases are sustained over a longer period of time.

Mitochondrial oxidative activity and apoptosis

There is no agreement as to the role of apoptosis in postburn skeletal muscle wasting. Previous studies reported an increase in apoptosis in skeletal muscle postburn, while our results showed no increase in caspase-3 activity either in SOL or EDL [40, 54]. However, our results are in agreement with the studies by Duan et al (2016), reporting that caspase-3 levels decreased to basal levels at day 10 postburn, and by Merrit et al (2012), reporting no significant increases in activated caspases during the postburn flow phase [40, 43]. We assume that the contribution of apoptosis to muscle wasting after burns is important in the first few days after the burn injury, as Duan et al. (2016) found marked increases in caspase-3 levels on one and four days postburn [40]. It should be noted that apoptosis has been shown to be upregulated in immobilization and sedentary behavior, which also contributes to muscle wasting after burns, but was not taken into account in the present study [55, 56].

On muscle sections of the burned animals, COX/SDH staining revealed no positive fibers. Positive complexes II (SDH) and IV (COX) would indicate mitochondrial impairments encoded by both nuclear and mitochondrial DNA (mtDNA)[57]. Our results indicate that at 10 days postburn the nuclear DNA, encoding SDH, is not impaired and that there are no mutations of the mitochondrial DNA, encoding subunits of COX. This is in contrast to previous studies in cancer cachexia, where loss of oxidative phenotype and mitochondrial content was found concomitant with muscle wasting [58]. Even after sepsis impaired mitochondrial complex enzymes have been observed long after the sepsis has resolved, shown by lower complex II and complex IV activity [59].

Conclusion

In conclusion, we have shown that at 10 days postburn changes in muscle anthropometry are present and that proteins related to signaling pathways regulating protein synthesis and protein breakdown

are altered, with more pronounced catabolic effects in SOL. In both SOL and EDL anabolic signaling was clearly reduced postburn. Overall, our findings support the idea that severe burns lead to a protein imbalance, induced by metabolic changes altering intracellular signaling pathways. This understanding may help to guide future research to elucidate the relative contribution of pathways and molecules leading to postburn muscle wasting in the postburn flow phase.

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References

1. Dombrecht D, Van Daele U, Van Asbroeck B, Schieffelaers D, Guns PJ, Gebruers N, et al. Molecular mechanisms of post-burn muscle wasting and the therapeutic potential of physical exercise. *J Cachexia Sarcopenia Muscle*. 2023.
2. Porter C, Hardee JP, Herndon DN, Suman OE. The Role of Exercise in the Rehabilitation of Patients with Severe Burns. *Exercise and Sport Sciences Reviews*. 2015;43(1):34-40.
3. Porter C, Tompkins RG, Finnerty CC, Sidossis LS, Suman OE, Herndon DN. The metabolic stress response to burn trauma: current understanding and therapies. *The Lancet*. 2016;388(10052):1417-26.
4. Jeschke MG, Gauglitz GG, Kulp GA, Finnerty CC, Williams FN, Kraft R, et al. Long-term persistence of the pathophysiologic response to severe burn injury. *PLoS One*. 2011;6(7):e21245.
5. Hart DW, Wolf SE, Mlcak R, Chinkes DL, Ramzy PI, Obeng MK, et al. Persistence of muscle catabolism after severe burn. *Surgery*. 2000;128(2):312-9.
6. Gauglitz GG, Herndon DN, Kulp GA, Meyer WJ, III, Jeschke MG. Abnormal Insulin Sensitivity Persists up to Three Years in Pediatric Patients Post-Burn. *The Journal of Clinical Endocrinology & Metabolism*. 2009;94(5):1656-64.
7. Izamis M-L, Uygun K, Uygun B, Yarmush ML, Berthiaume F. Effects of Burn Injury on Markers of Hypermetabolism in Rats. *Journal of Burn Care & Research*. 2009;30(6):993-1001.
8. Chao T, Herndon DN, Porter C, Chondronikola M, Chaidemenou A, Abdelrahman DR, et al. Skeletal Muscle Protein Breakdown Remains Elevated in Pediatric Burn Survivors up to One-Year Post-Injury. *Shock*. 2015;44(5):397-401.
9. Stanojic M, Abdullahi A, Rehou S, Parousis A, Jeschke MG. Pathophysiological Response to Burn Injury in Adults. *Annals of Surgery*. 2018;267(3).
10. Fang CH, Tiao G, James H, Ogle C, Fischer JE, Hasselgren PO. Burn injury stimulates multiple proteolytic pathways in skeletal muscle, including the ubiquitin-energy-dependent pathway. *J Am Coll Surg*. 1995;180(2):161-70.
11. Lang CH, Huber D, Frost RA. Burn-induced increase in atrogin-1 and MuRF-1 in skeletal muscle is glucocorticoid independent but downregulated by IGF-I. *Am J Physiol Regul Integr Comp Physiol*. 2007;292(1):R328-36.
12. Wu X, Baer LA, Wolf SE, Wade CE, Walters TJ. The impact of muscle disuse on muscle atrophy in severely burned rats. *J Surg Res*. 2010;164(2):e243-51.
13. Fang CH, Li BG, Tiao G, Wang JJ, Fischer JE, Hasselgren PO. The molecular regulation of protein breakdown following burn injury is different in fast- and slow-twitch skeletal muscle. *Int J Mol Med*. 1998;1(1):163-9.
14. Wu X, Wolf SE, Walters TJ. Muscle contractile properties in severely burned rats. *Burns*. 2010;36(6):905-11.

15. Glass DJ. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat Cell Biol.* 2003;5(2):87-90.
16. Bowen TS, Schuler G, Adams V. Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. *J Cachexia Sarcopenia Muscle.* 2015;6(3):197-207.
17. Cohen S, Nathan JA, Goldberg AL. Muscle wasting in disease: molecular mechanisms and promising therapies. *Nat Rev Drug Discov.* 2015;14(1):58-74.
18. Gordon BS, Kelleher AR, Kimball SR. Regulation of muscle protein synthesis and the effects of catabolic states. *Int J Biochem Cell Biol.* 2013;45(10):2147-57.
19. Pereira C, Murphy K, Jeschke M, Herndon DN. Post burn muscle wasting and the effects of treatments. *Int J Biochem Cell Biol.* 2005;37(10):1948-61.
20. Sommerhalder C, Blears E, Murton AJ, Porter C, Finnerty C, Herndon DN. Current problems in burn hypermetabolism. *Curr Probl Surg.* 2020;57(1):100709.
21. Biolo G, Fleming RY, Maggi SP, Nguyen TT, Herndon DN, Wolfe RR. Inverse regulation of protein turnover and amino acid transport in skeletal muscle of hypercatabolic patients. *J Clin Endocrinol Metab.* 2002;87(7):3378-84.
22. Fong YM, Minei JP, Marano MA, Moldawer LL, Wei H, Shires GT, 3rd, et al. Skeletal muscle amino acid and myofibrillar protein mRNA response to thermal injury and infection. *Am J Physiol.* 1991;261(3 Pt 2):R536-42.
23. Sartorelli V, Fulco M. Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy. *Sci STKE.* 2004;2004(244):re11.
24. Glass DJ. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. *Curr Top Microbiol Immunol.* 2010;346:267-78.
25. Anthony TG. Mechanisms of protein balance in skeletal muscle. *Domest Anim Endocrinol.* 2016;56 Suppl(Suppl):S23-32.
26. Miyake M, Nomura A, Ogura A, Takehana K, Kitahara Y, Takahara K, et al. Skeletal muscle-specific eukaryotic translation initiation factor 2 α phosphorylation controls amino acid metabolism and fibroblast growth factor 21-mediated non-cell-autonomous energy metabolism. *Faseb j.* 2016;30(2):798-812.
27. Bonaldo P, Sandri M. Cellular and molecular mechanisms of muscle atrophy. *Dis Model Mech.* 2013;6(1):25-39.
28. Goll DE, Neti G, Mares SW, Thompson VF. Myofibrillar protein turnover: the proteasome and the calpains. *J Anim Sci.* 2008;86(14 Suppl):E19-35.
29. Cao PR, Kim HJ, Lecker SH. Ubiquitin-protein ligases in muscle wasting. *Int J Biochem Cell Biol.* 2005;37(10):2088-97.
30. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyeva Y, Kline WO, et al. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell.* 2004;14(3):395-403.
31. Argilés JM, López-Soriano FJ, Busquets S. Apoptosis signalling is essential and precedes protein degradation in wasting skeletal muscle during catabolic conditions. *Int J Biochem Cell Biol.* 2008;40(9):1674-8.
32. Fanzani A, Conraads VM, Penna F, Martinet W. Molecular and cellular mechanisms of skeletal muscle atrophy: an update. *J Cachexia Sarcopenia Muscle.* 2012;3(3):163-79.
33. Jeschke MG, Chinkes DL, Finnerty CC, Kulp G, Suman OE, Norbury WB, et al. Pathophysiologic response to severe burn injury. *Ann Surg.* 2008;248(3):387-401.
34. Walker HL, Mason AD, Jr. A standard animal burn. *J Trauma.* 1968;8(6):1049-51.
35. Venter NG, Monte-Alto-Costa A, Marques RG. A new model for the standardization of experimental burn wounds. *Burns.* 2015;41(3):542-7.
36. Joshi AP, Saad MK, Mohan M, editors. A review on burn and burn models in animals 2017.
37. Gouma E, Simos Y, Verginadis I, Lykoudis E, Evangelou A, Karkabounas S. A simple procedure for estimation of total body surface area and determination of a new value of Meeh's constant in rats. *Lab Anim.* 2012;46(1):40-5.

38. Mathiasen JR, Moser VC. The Irwin Test and Functional Observational Battery (FOB) for Assessing the Effects of Compounds on Behavior, Physiology, and Safety Pharmacology in Rodents. *Curr Protoc Pharmacol*. 2018;83(1):e43.
39. Chai J, Wu Y, Sheng Z. The relationship between skeletal muscle proteolysis and ubiquitin-proteasome proteolytic pathway in burned rats. *Burns*. 2002;28(6):527-33.
40. Duan H, Chai J, Sheng Z, Yao Y, Yin H, Liang L, et al. Effect of burn injury on apoptosis and expression of apoptosis-related genes/proteins in skeletal muscles of rats. *Apoptosis*. 2009;14(1):52-65.
41. Quintana HT, Baptista VIA, Lazzarin MC, Antunes HKM, Le Sueur-Maluf L, de Oliveira CAM, et al. Insulin Modulates Myogenesis and Muscle Atrophy Resulting From Skin Scald Burn in Young Male Rats. *J Surg Res*. 2021;257:56-68.
42. Song J, Finnerty CC, Herndon DN, Kraft R, Boehning D, Brooks NC, et al. Thermal injury activates the eEF2K-dependent eEF2 pathway in pediatric patients. *JPEN J Parenter Enteral Nutr*. 2012;36(5):596-602.
43. Merritt EK, Cross JM, Bamman MM. Inflammatory and protein metabolism signaling responses in human skeletal muscle after burn injury. *J Burn Care Res*. 2012;33(2):291-7.
44. Gore DC, Chinkes DL, Wolf SE, Sanford AP, Herndon DN, Wolfe RR. Quantification of protein metabolism in vivo for skin, wound, and muscle in severe burn patients. *JPEN J Parenter Enteral Nutr*. 2006;30(4):331-8.
45. Herndon DN, Wilmore DW, Mason AD, Jr. Development and analysis of a small animal model simulating the human postburn hypermetabolic response. *J Surg Res*. 1978;25(5):394-403.
46. Barrow RE, Meyer NA, Jeschke MG. Effect of varying burn sizes and ambient temperature on the hypermetabolic rate in thermally injured rats. *J Surg Res*. 2001;99(2):253-7.
47. Wolfe RR. Review: acute versus chronic response to burn injury. *Circ Shock*. 1981;8(1):105-15.
48. Davenport L, Dobson G, Letson H. A new model for standardising and treating thermal injury in the rat. *MethodsX*. 2019;6:2021-7.
49. Wang Y, Pessin JE. Mechanisms for fiber-type specificity of skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care*. 2013;16(3):243-50.
50. Caiozzo VJ, Haddad F, Baker MJ, Herrick RE, Prietto N, Baldwin KM. Microgravity-induced transformations of myosin isoforms and contractile properties of skeletal muscle. *J Appl Physiol* (1985). 1996;81(1):123-32.
51. Desplanches D, Mayet MH, Sempore B, Flandrois R. Structural and functional responses to prolonged hindlimb suspension in rat muscle. *J Appl Physiol* (1985). 1987;63(2):558-63.
52. Sugita H, Kaneki M, Sugita M, Yasukawa T, Yasuhara S, Martyn JA. Burn injury impairs insulin-stimulated Akt/PKB activation in skeletal muscle. *Am J Physiol Endocrinol Metab*. 2005;288(3):E585-91.
53. Rios-Fuller TJ, Mahe M, Walters B, Abbadi D, Pérez-Baos S, Gadi A, et al. Translation Regulation by eIF2 α Phosphorylation and mTORC1 Signaling Pathways in Non-Communicable Diseases (NCDs). *Int J Mol Sci*. 2020;21(15).
54. Yasuhara S, Perez ME, Kanakubo E, Yasuhara Y, Shin YS, Kaneki M, et al. Skeletal muscle apoptosis after burns is associated with activation of proapoptotic signals. *Am J Physiol Endocrinol Metab*. 2000;279(5):E1114-21.
55. Allen DL, Linderman JK, Roy RR, Bigbee AJ, Grindeland RE, Mukku V, et al. Apoptosis: a mechanism contributing to remodeling of skeletal muscle in response to hindlimb unweighting. *Am J Physiol*. 1997;273(2 Pt 1):C579-87.
56. Zhu S, Nagashima M, Khan MA, Yasuhara S, Kaneki M, Martyn JA. Lack of caspase-3 attenuates immobilization-induced muscle atrophy and loss of tension generation along with mitigation of apoptosis and inflammation. *Muscle Nerve*. 2013;47(5):711-21.
57. Ross JM. Visualization of mitochondrial respiratory function using cytochrome c oxidase/succinate dehydrogenase (COX/SDH) double-labeling histochemistry. *J Vis Exp*. 2011(57):e3266.

58. Brown JL, Rosa-Caldwell ME, Lee DE, Blackwell TA, Brown LA, Perry RA, et al. Mitochondrial degeneration precedes the development of muscle atrophy in progression of cancer cachexia in tumour-bearing mice. *Journal of Cachexia, Sarcopenia and Muscle*. 2017;8(6):926-38.
59. Owen AM, Patel SP, Smith JD, Balasuriya BK, Mori SF, Hawk GS, et al. Chronic muscle weakness and mitochondrial dysfunction in the absence of sustained atrophy in a preclinical sepsis model. *Elife*. 2019;8.

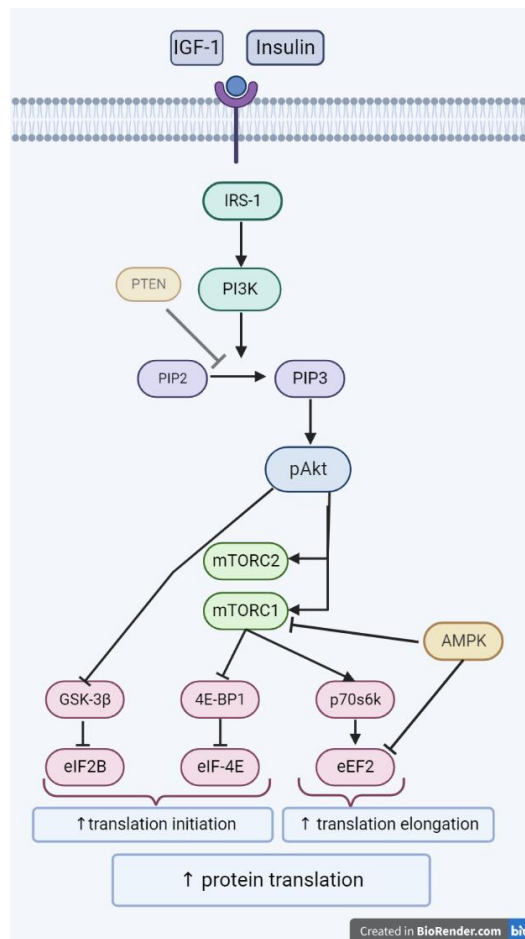


Figure 1: PI3K-AKT ('phosphoinositide-3-kinase'-'AKT') signaling pathway of protein synthesis in skeletal muscle. Upon Insulin or IGF-1 ('Insulin like growth factor-1') binding to its receptor, IRS-1 ('insulin receptor substrate 1') activates PI3K, generates PIP3 ('phosphatidylinositol (3,4,5)-trisphosphate') at the plasma membrane, facilitating phosphorylation of AKT. Subsequently, downstream GSK-3 β is inhibited (consequently inhibiting eIF2B) and parallel downstream mTORC1/2 ('mammalian target of rapamycin complex 1/2') are activated, of which mTORC1 inhibits 4EBP1 ('eukaryotic initiation factor 4E binding protein') (consequently inhibiting eIF-4E ('Eukaryotic translation initiation factor 4E')), and stimulates p70s6k ('70kDa ribosomal protein S6 kinase') (consequently inhibiting eEF2k which inhibits eEF2 ('translation initiation factor 2B')), resulting in the initiation of protein translation.

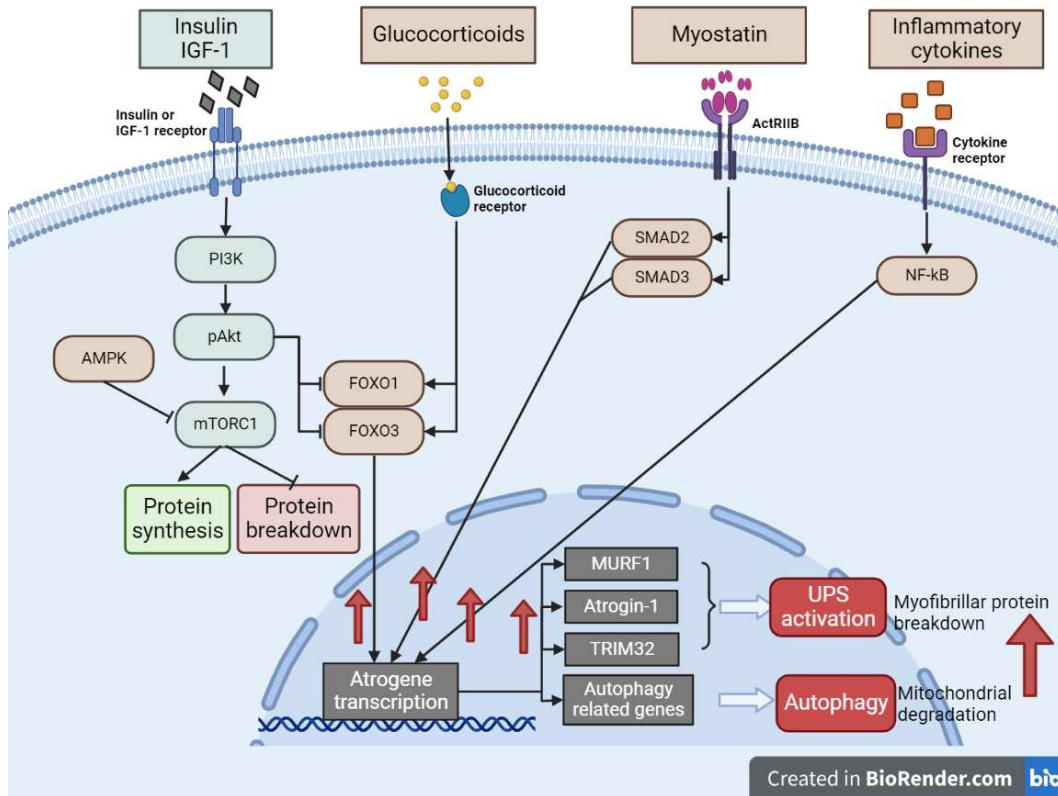


Figure 2: Main intracellular signaling pathways of skeletal muscle activating the UPS and Autophagy as found during other atrophy conditions. The atrogene transcription program is activated by 1) decreases in insulin or IGF-1 signaling, decreasing the activity of the PI3K-Akt-mTOR pathway, increasing expression of transcription factors FOXO1 and FOXO3; 2) increases in glucocorticoid release resulting in increases of FOXO1/3; 3) increased amounts of inflammatory cytokines leading to higher NF-κB expression; 4) transcription factors SMAD2 and SMAD3, stimulated by myostatin. The atrogene transcription program consequently leads to proteolysis via increased activity of MURF-1, atrogin-1, TRIM32 and autophagy related genes, stimulating the Ubiquitin Proteasome System (UPS) and Autophagy. IGF-1, insulin like growth factor 1; PI3K-Akt-mTOR, phosphoinositide 3-kinase-Akt-mammalian target of rapamycin; FOXO, forkhead box protein O; NF-κB, nuclear factor-κB; MURF-1, muscle-specific RING-finger 1; TRIM32, Tripartite Motif Containing 32.

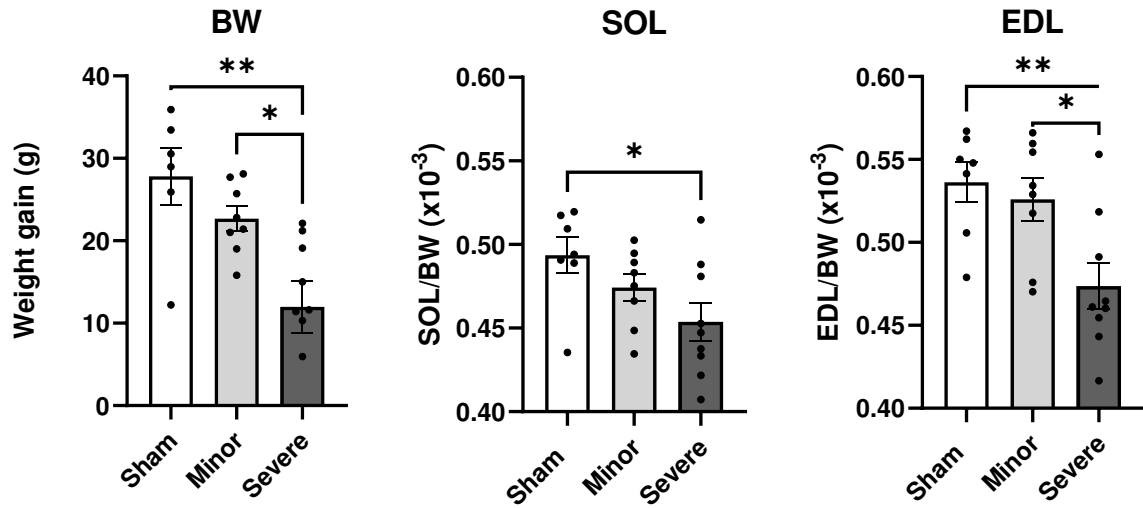


Figure 3: Difference in total body weight (BW) gain (weight day 10 minus weight day 0 postburn), ratio of *m. Soleus* (SOL) and *m. Extensor Digitorum Longus* (EDL) wet weight (mg) at 10 days postburn to total body weight at baseline (BW) (g) for sham, minor and severe burned animals. N=9 per group, error bars represent SEM, significant difference * $p < 0.05$, ** $p < 0.01$

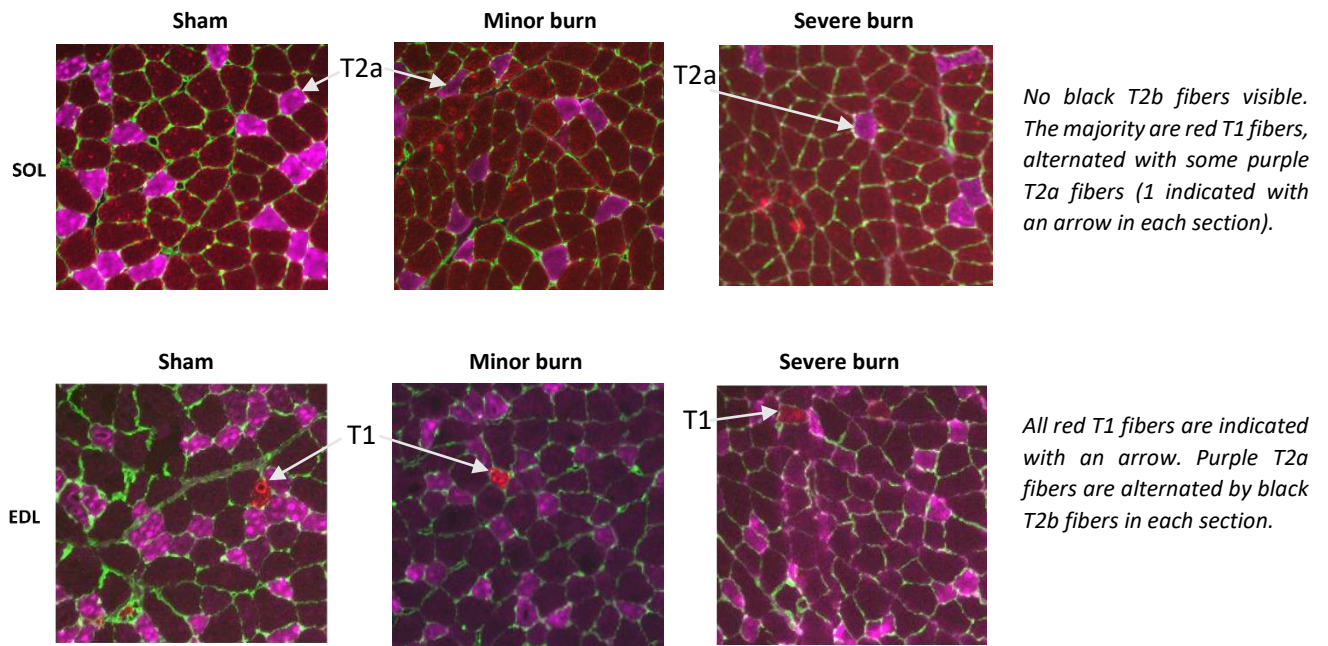


Figure 4: Example of the used immunohistology MyHC staining on transversal muscle sections (10 μ m) of sham treated, minor and severely burned Soleus (SOL) muscle samples and of sham treated, minor and severely burned Extensor Digitorum (EDL) muscle samples captured at 10x magnification. Green indicates the basal membrane, red type 1 fibers, purple type 2a fibers and black type 2b fibers

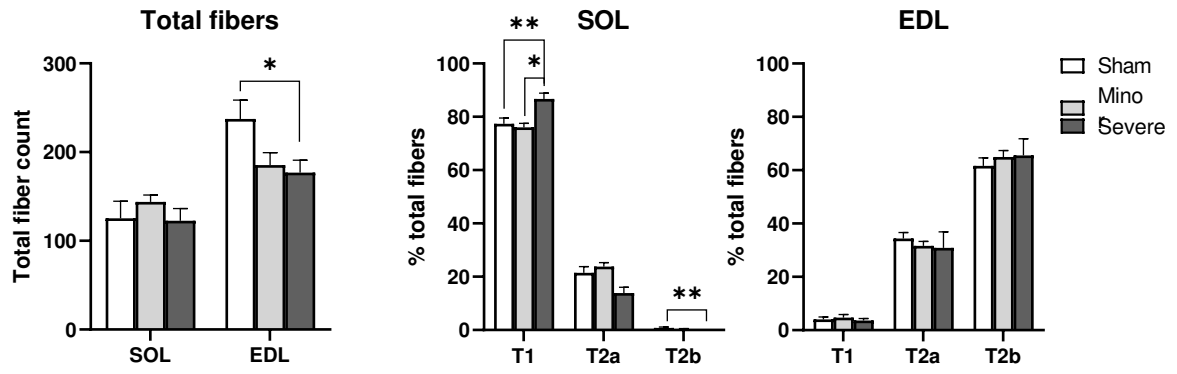


Figure 5: Muscle fiber type distribution: total fiber count, % of type 1 (T1), type 2a (T2a) and type 2b (T2b) fibers (specific fiber type count/total fiber count) for Soleus (SOL) and Extensor Digitorum Longus (EDL) transversal muscle sections (10 μ m) muscles from sham treated, minor and severe burned animals after 10 days. N=6 per group, error bars represent SEM, Significant difference * $p < 0.05$, ** $p < 0.01$

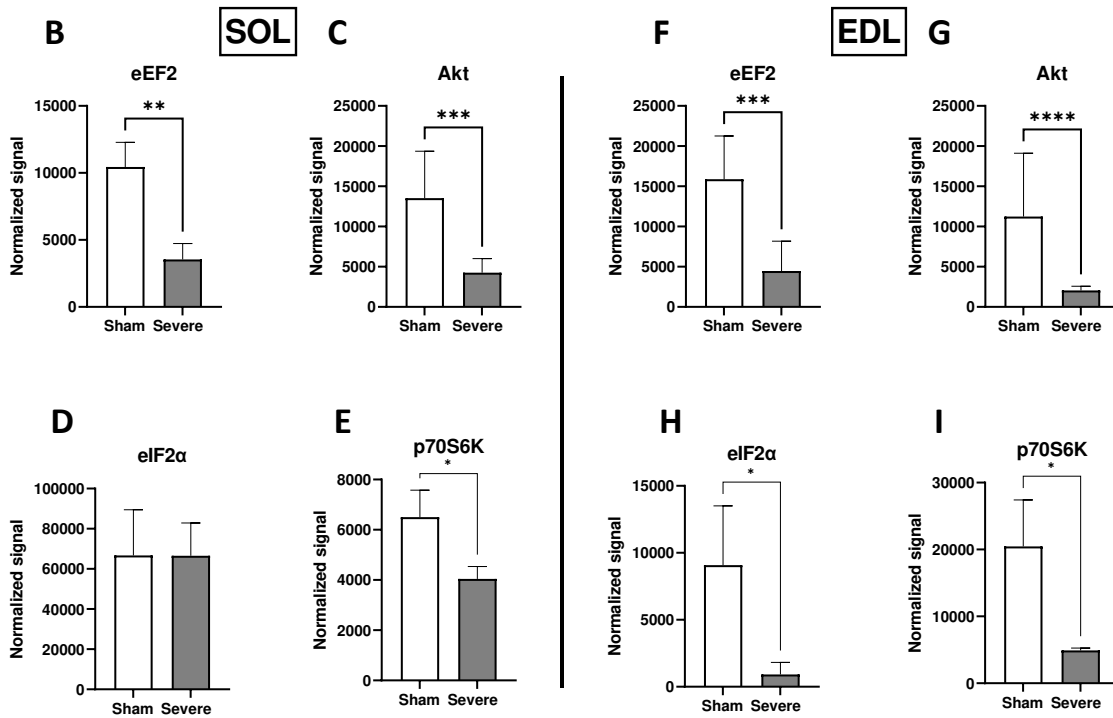
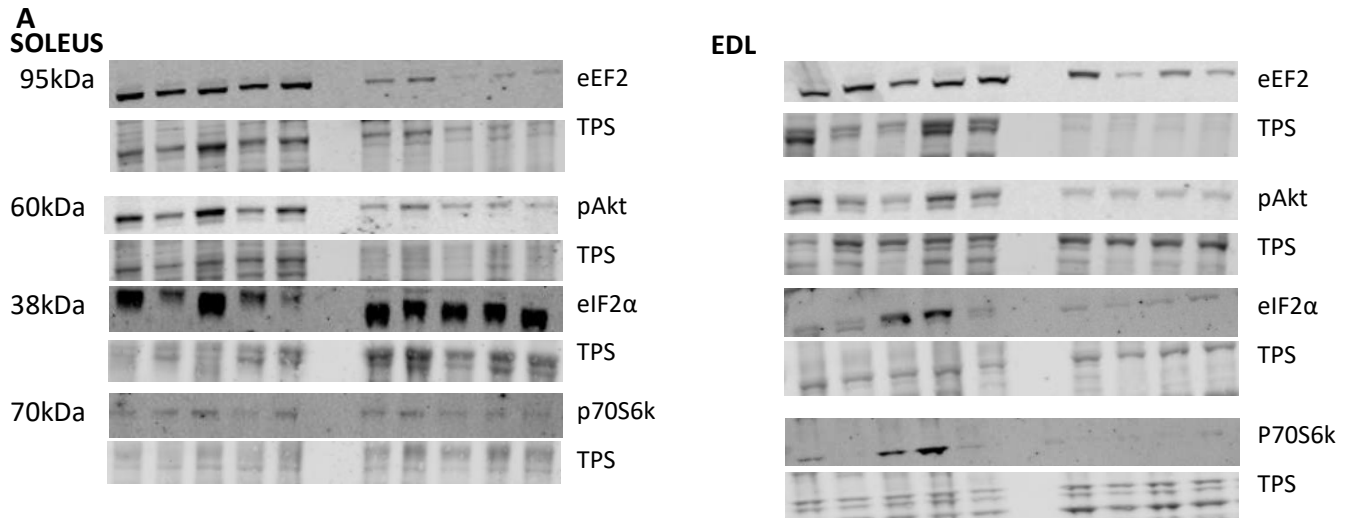


Figure 6: **A:** Western blots for eEF2, Akt, eIF2 α and p70S6K of Soleus (SOL) and Extensor Digitorum Longus (EDL) muscle. The immunoblots depicted are representative of samples of sham treated and severely burned animals 10 days post injury. For every protein, the Total Protein Stain (TPS) at the corresponding molecular height is depicted. **B-I:** Protein signals of Western blots normalized to Total protein stains in sham treated versus severe burn groups 10 days postburn. Bars represent SEM. **A-E:** Normalized protein signals in SOL **F-I:** Normalized protein signals in EDL. N=9 per group, error bars represent SEM, significant difference ** $p < 0.01$, **** $p < 0.0001$

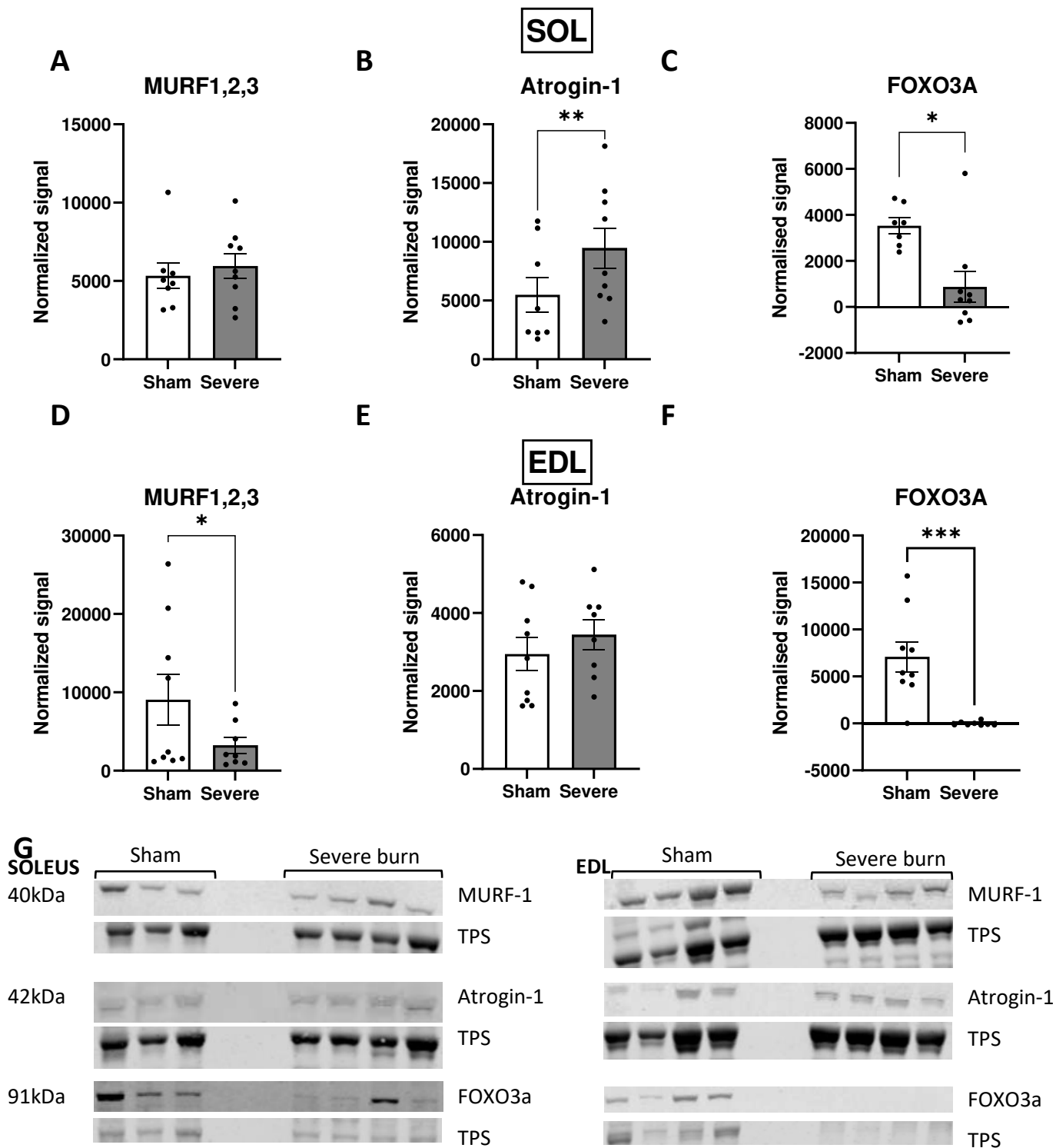


Figure 7: Protein signals of Western blots normalized to Total protein stains in sham versus severe burn groups 10 days postburn. Bars represent SEM. **A.** Normalized MURF1,2,3 protein signal in Soleus muscle. **B.** Normalized Atrogin-1 protein signal in Soleus muscle. **C.** Normalized FOXO3A protein signal in Soleus muscle **D.** Normalized MURF1,2,3 protein signal in EDL muscle. **E.** Normalized Atrogin-1 protein signal in EDL muscle. **F.** Normalized FOXO3A protein signal in EDL muscle. **G.** : Western blots for MURF1,2,3, Atrogin-1 and FOXO3a of Soleus and Extensor Digitorum Longus muscle. The immunoblots depicted are representative of samples of sham and severely burned animals 10 days post injury. For every protein, the Total Protein Stain (TPS) at the corresponding molecular height is depicted. N=9 per group, error bars represent SEM, significant difference * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$

	Body weight baseline (g)	Body weight end (g)	Body weight gain (g)	SOL (mg)	EDL (mg)
Sham	200.7±8.7	228.5±6.4	27.8±3.4	96.1±4.3	104±4.7
Minor burn	194.5±2.6	217.2±2.2	22.7±1.5	89.5±3.3	99.4±4
Severe burn	227±7.2**	239±7.5#	12±3.2**	103.4±5.3	107.8±5.5

*Table 1: Total body weight(g) at baseline and 10 days postburn (end) and wet weights (mg) of m. Soleus (SOL) and m. Extensor Digitorum Longus (EDL) 10 days postburn. Values are expressed as mean ± SEM. N=9 per group. *significant difference vs sham; #significant difference vs minor burn group; Significance if $p < 0.05$*