Oxidative stress, apoptosis, and proteolysis in skeletal muscle repair after unloading

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Andrianjafinony T, Dupré-Au Couturier S, Letexier D, Couchox H, Desplanches D. Oxidative stress, apoptosis, and proteolysis in skeletal muscle repair after unloading. Am J Physiol Cell Physiol 299: C307–C315, 2010. First published May 26, 2010; doi:10.1152/ajpcell.00069.2010.—Although several lines of evidence link muscle-derived oxidants and inflammation to skeletal muscle wasting via regulation of apoptosis and proteolysis, little information is currently available on muscle repair. The present work was designed to study oxidative stress response, inflammatory cytokines, apoptotic, or proteolytic pathways during the early (1 and 5 days) and later (14 days) stages of the regrowth process subsequent to 14 days of hindlimb unloading. During the early stages of reloading, muscle mass recovery (day 5) was facilitated by transcriptional downregulation (day 1) of pathways involved in muscle proteolysis [β-calpain, atrogin-1/muscle atrophy F-box (MAFbx), and muscle RING finger-1 (MurF1) mRNA] and upregulation of an autophagy-related protein Beclin-1 (day 5). At the same time, oxidative stress (glutathione vs. glutathione disulfide ratio, superoxide dismutase, catalase activities) remained still enhanced, whereas the increased uncoupling protein 3 gene expression recovered. Increased caspase-9 (mitochondrial-driven apoptosis) and decreased caspase-12 (sarcoplasmic reticulum-mediated apoptosis) activation was also normalized at early stages (day 5). Conversely, the receptor-mediated apoptotic pathway initiated by ligand-induced (tumor necrosis factor-α, TNF-α) binding and promoting the activation of caspase-8 remained elevated until 14 days. Our data suggest that at early stages, muscle repair is mediated via the modulation of mitochondrial-driven apoptosis and muscle proteolysis. Despite full muscle mass recovery, oxidative stress and TNF-α-mediated apoptotic pathway are still activated till later stages of muscle remodeling.

muscle recovery; oxidative response; cytokines; protein degradation; caspases

MUSCLE ATROPHY is commonly associated with prolonged bed-rest, spaceflight, and many diseases such as cancer cachexia, sepsis, diabetes, obstructive pulmonary disease, and chronic heart failure. During the last several years, extensive research has been done on the potential triggers and molecular signaling events that underlie skeletal muscle atrophy (8, 25, 36, 47). Studies using rodent models, including hindlimb unloading, showed that muscle wasting results from a rapid decrease in protein synthesis rate followed by a slower transient increase in muscle protein synthesis and breakdown. The purpose of this study was to unravel some of the signaling pathways associated with muscle wasting. Thus far, limited attention has been paid to muscle unloading-induced proteolysis, but the primary regulator of muscle wasting is the ATP-ubiquitin-dependent proteasome pathway including two critical muscle-specific ubiquitine ligases muscle RING finger 1 (MurF1) and muscle atrogin1/muscle atrophy F-box (MAFbx) (8, 22, 51). Moreover, a unique group of cysteine-dependent proteases termed caspases is activated by hindlimb unloading (2, 6, 29, 49). These caspases are endoproteases that degrade proteins but also cause programmed cell death (13, 15).

Several lines of evidence link muscle-derived oxidants and inflammation to skeletal muscle wasting via regulation of apoptosis and proteolysis (39, 45). Reactive oxygen species (ROS) and elevated proinflammatory cytokines, in particular tumor necrosis factor (TNF-α), mediate muscle atrophy via the redox-sensitive transcription factor nuclear factor-κB (NF-κB). ROS can also initiate the mitochondrial-driven apoptotic pathway, promoting the activation of caspase-9 and -3, a key cell death protease via the release of cytochrome c into the cytoplasm (29, 45, 46). Moreover, TNF-α initiates the receptor-mediated apoptotic pathway leading to the activation of caspase-8 but also upregulates ubiquitin ligases E3 such as MurF1 and MAFbx (40).

Although extensive research has been done on proteolytic and apoptotic signaling pathways involved in muscle unloading-induced atrophy, less attention has been paid to muscle recovery (10, 20, 27, 50, 54). Muscle protein synthesis and breakdown are elevated at 18 h of reloading, suggesting that both are necessary to recover from hindlimb unloading-induced soleus muscle atrophy (50). During the early stage of muscle reloading, the unusual increase in mechanical stress promotes muscle fiber injury with invasion of macrophages and other phagocytic cells, essential to allow effective regeneration (14, 19, 54). Evidence for an important remodeling of soleus muscle fibers is also provided by structural observations demonstrating the appearance of regenerating, i.e., centrally nucleated fibers and alterations toward the reestablishment of normal slow fiber type distribution (19).

Abnormal cellular redox status and chronic inflammation may induce apoptosis and interfere with the imbalance in protein synthesis and breakdown. The purpose of this study was to unravel some of the signaling pathways associated with muscle recovery. We specifically investigated the contribution of muscle-derived oxidants and inflammatory cytokines (TNF-α, IL-1β, IL-6 proteins) to skeletal muscle repair via regulation of caspase-dependent apoptosis (death-receptor-, endoplasmic reticulum stress-, and mitochondrial-mediated pathways) and proteolysis (β-calpain, MurF1, MAFbx) during the early (1 and 5 days) and later (14 days) stages of the regrowth process subsequent to 14 days of hindlimb unloading.

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EXPERIMENTAL PROCEDURES

Animal care and protocol. Pathogen-free female Wistar rats, weighing 200 g (3 mo) from Charles River, were housed in a temperature-controlled room (24 ± 2°C) with a light-dark cycle (12:12 h). After 1 wk of acclimatization, four groups of eight rats were hindlimb unloaded for a period of 14 days in individual cages using Morey’s tail-suspension model (20). Adhesive tape was wrapped around the tail and connected to a pulley by a plastic bar. Rats were able to move in 360° of arc with their forelimbs, thus allowing exercise and access to food and water, although the hindlimbs were nonload bearing. They were suspended at about 20° head-down tilt angle to minimize lordosis. After 14 days of suspension, one group (H) was euthanized and three groups were allowed to perform normal cage activity for 1, 5, or 14 days, therefore permitting reloading of the soleus muscle (H+R1, H+R5, and H+R14). Animals of the same age as the rats before (C0), after 14 days of hindlimb suspension (C14), and after 14 days of recovery (C28) served as experimental controls. At the end of the different protocols, rats were anesthetized with halothane. Soleus muscles were excised, weighed, frozen in isopentane chilled with liquid nitrogen, and stored at −80°C until analysis. The animal experiments were approved by the Institutional Animal Care and Use Committee from the University Lyon 1, following the recommendations provided by the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe number 123, Strasbourg, 1985).

Histological analysis. Serial transverse sections (10 μm) from the midsbella region of each muscle were cut on a microtome at −25°C and stained for the myosin ATPase as previously described (20). Muscle fibers were classified into two major types (I, IIa) and hybrid type I/IIa fibers. The fiber cross-sectional areas were calculated using a computerized planimetry system coupled to a digitizer. Mean fiber area is expressed as the amount of at least 100 fiber areas divided by the number of fibers.

Immunohistochemistry. Briefly, muscle sections were fixed in cold acetone for 10 min, air dried for 10 min, and washed in 50 mM phosphate-buffered saline (PBS, pH 7.4, 10 min). After incubation for 60 min with the primary mouse anti-rat CD 68 antibody (1:100, AbD Serotec), sections were washed three times for 5 min in PBS. They were incubated for 30 min with a biotinylated horse anti-mouse secondary antibody IgG (Vector) again washed with PBS. Sections were incubated with the Vectastain Elite ABC reagent for 30 min, washed in PBS, and reacted with 3,3′-diaminobenzidine for 1 min. Muscle slides were rinsed in water, counterstained with hematoxylin, dehydrated with increasing ethanols, and mounted. The primary antibody was omitted for control slides.

Glutathione levels. The ratio of reduced to oxidized glutathione is a widely used indicator of cell oxidative stress. Reduced (GSH) and oxidized glutathione (GSSG) were measured according to the method of Anderson (3) by monitoring the reduction by GSH of 5,5'-dithiobis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoate at 412 nm (25°C). Proteins were precipitated with ice-cold 5% metaphosphoric acid and centrifuged at 5,000 g for 5 min. For GSH measurements, samples were derivatized with 2-vinyl-pyridin. The assay was initiated by the addition of 10 μl of 50 U/ml glutathione reductase. GSH was used as a standard and was assayed in parallel under the same condition as the tissue samples.

Antioxidant enzymes activities. A portion of soleus muscle was homogenized with a potter Elvejhem at 4°C, in buffer containing KH2PO4 (100 mM), dithiothreitol (1 mM), and EDTA (2 mM), pH 7.4. After centrifugation (3,000 g/min, for 5 min), the supernatant was used for enzymatic assays. Superoxide dismutase (SOD) activity was assayed by monitoring the rate of acetylated cytochrome c reduction by superoxide radicals generated by the xanthine-xanthine oxidase system (18). One activity unit of SOD was defined as the quantity of SOD required to cause a 50% inhibition of the rate of acetylated cytochrome c reduction. To distinguish manganese SOD (MnSOD), exclusively located in mitochondrial matrix, from cuprozinc SOD (CuZnSOD), which is primarily located in the cytosol, SOD activity was determined after incubation with NaCN (1 mM). At this concentration, cyanide inhibits the CuZn isofrom of the enzyme but does not affect the Mn isofrom (18). The total activity of glutathione peroxidase (GPx) was assayed with cumene hydroperoxide as a substrate according to Tappel (52). Catalase (CAT) activity was measured by the method of Aebi (1). This technique used the first-order rate constant of the decomposition of H2O2 by tissue CAT at 20°C. One unit of CAT activity was calculated by using k = (2.3/dt)(log A1/A2), where k is CAT activity, dt is change in time, A1 is initial absorbance, and A2 is final absorbance. All enzyme activities were expressed in units per milligram of proteins.

Western blot analysis. Frozen tissue samples were disrupted in ice-cold buffer [50 mM Tris-HCl, 2.5% sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 5% glycerol and protease inhibitor cocktail (Roche Applied Science)] and incubated at 100°C for 5 min. After centrifugation (10,000 g, 10 min, 4°C), the supernatants were collected and stored at −80°C. Protein content was determined according to Lowry using the Bio-Rad protein assay. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Blots were incubated overnight at 4°C with antibodies against caspase-3, -8, -9 (1.750, Cell Signaling Technology), caspase-12, and TNF-α (1.000, Cell Signaling Technology), IL-1β (1.333, R&D Systems), IL-6 (1.333, BioVision), Beclin-1 (1.500, Cell Signaling TechnologOM), and β tubulin (1.750, Sigma Aldrich). Incubation with corresponding IgG-peroxidase-conjugated goat anti-rabbit (1.800, Sigma Aldrich) and goat anti-mouse (1.500, Jackson ImmunoResearch Laboratories) were performed for chemiluminescence detection (ECL, Amersham). Specific bands were analyzed by using a Kodak EDAS 120 system including digital camera and images software (Eastman Kodak, Rochester, NY). To evaluate successful equal protein loading and transfer, membranes were incubated in Ponceau S solution. For quantification, a housekeeping protein such as β-tubulin was used as an internal loading control to normalize protein loading.

mRNA concentration in soleus muscle. Total RNA were extracted from muscle samples (50 mg) of rats using TRIzol (Invitrogen Life Technologies, Bethesda, MD). mRNA relative abundance was measured by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) using cyclopsin as a reference (48). Primer sequences are shown in Table 1. For each sample, an RT was performed from 1 μg of total RNA with 100 units of M-MLV reverse transcriptase. Primer sequences were designed using the Primer3 program (16). Table 1 shows the primer sequences used for RT-PCR. The concentrations of the primers were measured by calculating the ratio of the primer to 100 μg of total RNA.
transcriptase (Promega, Madison, WI), 5 μL of M-MLV RT 5× buffer, 20 units of RNAsin ribonuclease inhibitor, 15 nmoles of deoxyribonucleotide triphosphate, and 1 μg of oligo dT in a final volume of 25 μL. The reaction consisted of 5 min at 70°C (RNA and oligo dT), then 90 min at 42°C (all mix), followed by 10 min at 70°C. After being chilled, 2.5 μL was used for PCR. The 2.5 μL of RT medium was added to 47.5 μL of PCR mix containing 5 μL of 10× Taq PCR buffer (Eurobio, Les Ulis, France), 75 nmoles of MgCl₂, 15 nmoles of deoxyribonucleotide triphosphate, 2.5 units of Taq DNA polymerase (Eurobio), and 22.5 pmoles of corresponding antisense and sense primers. The PCR conditions were 2 min at 94°C followed by n cycles of PCR (1 cycle = 1 min at 94°C, 1 min at 60°C, 1 min at 72°C). The number of cycles that represents the linear portion of the amplification plot was determined for each target and is indicated in Table 1. PCR was ended by 10 min at 72°C. Products were analyzed on 1.5% agarose gel prestained with ethidium bromide. For quantification of relative bands intensities, pictures were taken with a camera DC120 (Kodak), and the ratio of each target to cyclophilin was determined for each sample with image analysis system (Eastman).

Statistical analysis. All data reported are means ± SE. A multifactorial analysis of variance was used for intergroup comparisons. The Fisher paired least significant difference was used to identify specific means differences. Values were considered statistically different when P < 0.05.

RESULTS

Muscle mass, fiber type, and size. In the present experiment, three groups of controls (C0, C14, and C28) were designed to verify whether muscle weight gain during reloading was due to postnatal growth and/or to muscle regrowth after atrophy. Soleus muscle-to-body mass ratio was 45.2 ± 1.6, 44.9 ± 1.1, and 46.5 ± 2.0 mg/100 g for C0, C14, and C28 rats, respectively. No postnatal growth was obvious in soleus muscle mass between the beginning (C0) and the end (C28) of the study. So C14 group termed C was kept as a control group.

Soleus muscle-to-body mass ratio and cross-sectional areas of type I, I/IIa, and IIa fibers are shown in Table 2. Soleus muscle atrophy (~41%) induced by 14 days of hindlimb unloading had already returned to control values after 5 days of reloading. The percent distribution of type I fibers (88.2 ± 2.6%) was reduced by 13% (76.4 ± 4.0%) after 14 days of unloading. The percentage of hybrid fibers I/IIa increased from 1.7% in unloaded rats). The percent distribution of all fibers returned to the normal range within 5 days. Furthermore, 14 days of recovery led to a 92.5 ± 1.1 type I and 7 ± 1.1% type I/IIa percent distribution. Type I, I/IIa, and IIa fiber size were all decreased after hindlimb unloading. Recovery was complete after 5 days. The small number of type Ila fibers did not allow us to determine fiber areas after 14 days of reloading.

GSH/GSSG and antioxidant defense systems. In the soleus muscle, the ratio of reduced versus oxidized glutathione was decreased by 44% after unloading and returned to control values within 14 days (Fig. 1A). Hindlimb unloading increased CuZnSOD (+70%) and CAT (+34%) activities in H rats. CuZnSOD and CAT activities regained baseline values within 14 days (Fig. 2, A and D). MnSOD and GPx activities remained statistically unchanged through all experimental procedures (Fig. 2, B and C). To further examine another potent antioxidant defense system (uncoupling protein), we performed measurements of uncoupling protein 3 (UCP3) gene expression. Unloading induced a large increase (5-fold) in UCP3 mRNA with respect to baseline controls, and recovery was complete at day 5 (Fig. 1B).
Macrophages, TNF-α, IL-1β, and IL-6 proteins. Infiltrating cells immunoreactive for CD68 (macrophages expressing the ED1 antigen) and central nuclei were observed during the unloading period (Fig. 3). TNF-α protein content was significantly upregulated (2-fold) after unloading and returned to control values within 14 days (Fig. 4A). Additionally, unloaded rats showed a 1.5-fold increase in both IL-1β and IL-6 protein contents, and a full recovery was observed by day 5 (Fig. 4, B and C).

Caspases-8, -9, -12, -3 proteins. The active cleaved unit (43 kDa) of caspase-8 was increased twofold after unloading and remained elevated even after 14 days of reloading (Fig. 5A), whereas the increased cleavage of procaspase-9 (38 kDa) and

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**Fig. 2.** Oxidative stress response in soleus muscle after un- and reloading. Activity of cytosolic (CuZnSOD) (A) and manganese superoxide dismutase (Mn SOD) (B), glutathione peroxidase (GPx) (C), and catalase (D). Values are means ± SE for 8 animals; see Fig. 1 for additional abbreviations. *P < 0.05, significantly different from control rats. #P < 0.05, significantly different from hindlimb unloaded rats.

**Fig. 3.** Cross sections of the belly portion of soleus muscle stained with anti-ED1. A: 14-day control rats; B: 14-day hindlimb unloaded rat, rats; C: reloaded 5 days; D: reload 14 days. Positive immunostaining is brown and nuclei appear in blue. Central nuclei and ED1+ cells were indicated respectively by arrowheads and arrows. Bar, 50 μm.
procaspase-3 (17 kDa) was normalized at day 5 (Fig. 5, B and D). Conversely, the cleaved unit (42 kDa) of procaspase-12 was decreased by unloading but returned to baseline values within 5 days of reloading (Fig. 5C).

Beclin-1 protein, μ-calpain, and E3 ubiquitine ligases mRNA. No change occurred in basal Beclin-1 protein level in H rats. However, a significant increase (+230%) in Beclin-1 was observed after 5 days of reloading (Fig. 6). The upregulation of μ-calpain (+73%, Fig. 7A), MAFbx (+73%, Fig. 7B), and MuRF1 (+57%, Fig. 7C) occurring in unloaded rats was reversed at early stage after 1 day of reloading.

DISCUSSION

Among potential triggers and signaling pathways underlying muscle atrophy, oxidative stress, and chronic inflammation might contribute to muscle wasting via apoptosis ad proteolysis activation. In the present study, evidence for an important remodelling of soleus muscle was provided by the appearance of centrally nucleated fibers, infiltrated macrophages, and alterations toward a reestablishement of fiber-type distribution. Recovery of muscle mass and fiber size was very rapid and already complete after only 5 days.

It is still debated whether cellular redox unbalance would be a key “trigger” or a by-product for soleus muscle atrophy and repair (9, 26, 39, 45). We report here that to oxidized glutathione ratio returned to baseline values at the late stages of reloading (day 14). A complex cytoprotective system that includes enzymes scavenging ROS is recruited against free radical damage. The unloading-induced rise in CuZnSOD activity indicates a cytosolic oxidative stress, whereas the lack of adaptation of MnSOD (localized in the mitochondrial matrix) activity suggests that muscle atrophy was not associated with a mitochondrial stress. However, a mild uncoupling of oxidative phosphorylation might also reduce the mitochondrial production of ROS by lowering mitochondrial membrane potential (43). ROS or by-products of lipid peroxidation leads to a mild uncoupling via the activation of an UCP3-mediated proton leak (16). Bezaire et al. (7) postulate that UCP3 would have an antioxidant function selectively at times when proton motrice force is high, i.e., in a resting muscle with a low ATP demand. Recent results from our laboratory (12, 48) and others (34) showed a marked upregulation of UCP3 in unloaded soleus muscle. We hypothesize that this rise in UCP3 expression might contribute to protect less active skeletal myocytes from oxidative damage by stimulating oxygen consumption, depleting the local concentration of oxygen, and decreasing the generation of ROS (12, 48). Therefore, a concomitant increase in MnSOD activity would be less necessary. Alternatively, Argiles et al. (4) suggested that a rise in UCP3 gene expression could occur before apoptosis to moderate excessive apoptosis during muscle wasting. However, we observed a coordinate downregulation of caspase-3 (apoptosis effector) and UCP3 during reloading (day 5). We may not disregard that UCP3 expression recovery might also imply the increased demand in ATP synthesis during the rebuilding of damaged muscle fibers. The small increase in catalase activity associated with no change in GPx activity suggests an increased production of hydroxyl radicals by Fenton reaction. Redox active metal ions such as Fe²⁺ can react with hydrogen peroxide to generate the hydroxyl radical. Interestingly, increased iron level has been
previously demonstrated in skeletal muscle atrophied by immobility (26). Similar to GSH/GSSG changes, CuZnSOD and CAT activities returned to normal range at the late stage of reloading (14 days) despite an earlier muscle mass recovery. ROS and elevated proinflammatory cytokines such as IL-6, TNF-α, and IL-1β (44) mediate muscle atrophy via the redox-sensitive transcription factor nuclear NF-κB. However, after a short period of muscle unloading (7 days), Hunter et al. (24) reported the activation of an alternative NF-κB pathway and a moderate downregulation of TNF-α protein levels. The upregulation of TNF-α, IL-1β, and IL-6 protein levels observed in the present study might be due to the longer duration of the unloading period (14 days). Muscle repair was associated with a downregulation of IL-6 and IL-1β protein levels at day 5, whereas TNF-α and oxidative stress were normalized later (14 days). A synergistic costimulation of other catabolic cytokines (IL-1α, IL-6) occurred at the early stages of reloading, increasing muscle effects of TNF-α. In a recent study, Li et al. (30) reported that proinflammatory cytokines IL-1 and TNF-α promote muscle protein loss through common pathways. TNF-α is mainly secreted from activated monocytes and macrophages. Several studies showed that muscle reloading induced an invasion of inflammatory cells (14, 27, 54). Specifically, Tidball and Wehling-Henricks (54) reported that macrophages are mostly concentrated near damaged fibers at early stages of reloading (2 days), whereas at later stages (4 days) they are more distributed within the perimysium. Moreover, using anti-F4/80 injections (macrophage depletion), they showed that the late-invading macrophages between 2 and 4 days of reloading contribute to muscle repair, growth, and regeneration by promoting muscle membrane repair and satellite cell differentiation.
tion. We also observed infiltrating cells immunoreactive for CD68 (macrophages) and the appearance of regenerating fibers; i.e., centrally nucleated fibers during the reloading period. Our previous experience (19) has shown that centrally nucleated fibers are equally present in 14-day unloaded and 1-day reloaded soleus muscles and increase in 5- and 14-day reloaded soleus muscles. The current findings suggest that release of substantial amount of ROS from macrophages might contribute to ROS generation at the early and late stages of reloading, but further investigations are needed to better delineate ROS production pathways during reloading.

TNF-α stimulates both inflammatory and apoptotic pathways. Caspases play a central role in the implementation of apoptosis and are divided into two groups: initiator caspases (caspase-8, -9, -12) and effector caspases (caspase-3, -6, -7). TNF-α initiates the death-receptor-mediated apoptotic pathway leading to the activation of caspase-8 (15). A second endoplasmic reticulum (ER) stress pathway may play a critical role in apoptosis induction (41). Caspase-12, highly expressed in muscle, is an ER (localized on the sarcoplasmic reticulum in skeletal muscle) stress-specific caspase, functioning as the initiator caspase in response to ER stress (41). A third intrinsic mitochondrial pathway of apoptosis is partly initiated by ROS (15). The release of cytochrome c from mitochondria facilitates the formation of an apoptosome complex with Apaf-1 and pro-caspase-9, leading to the activation of the major effector caspase-3. After unloading was completed, cleaved-caspase-8, -9 and -3 protein levels were all increased according to numerous studies showing that caspases contribute to muscle atrophy (2, 6, 17, 29, 49). Surprisingly, a decrease in cleaved caspase-12 was always observed after unloading and at the early stage (1 day) of recovery. Caspase-12 mediates an ER-specific apoptosis pathway through a calcium-caspase-12-caspase-3 cascade. Given the role played by calcium homeostasis, one could presumably explain the decrease in cleaved caspase-12 by the reduced resting intracellular calcium previously reported in unloaded rat soleus muscle by Fraysse et al. (21). To our knowledge, this is the first report to demonstrate a full recovery of cleaved caspase-12, -9, and caspase-3 after 5 days of reloading. Conversely, the receptor-mediated apoptotic pathway initiated by ligand-induced (TNF-α) binding and promoting the activation of caspase-8 remained elevated until 14 days. Some delayed response might be explained by the cascade of events involved in the extrinsic apoptotic pathway: binding of TNF-α to its death receptor, formation of spatially and temporally distinct receptor composed of adaptor complexes such as Fas-associated protein with death domain (FADD), TNF receptor-associated protein with death domain (TRADD), and finally activation of the initiator caspase-8 (23). These data highlight the importance of selecting the appropriate time point when analyzing signaling pathways during muscle regrowth. We cannot rule out that cleaved caspase-8 would be normalized with some more days of reloading.

The remodeling of skeletal muscle during atrophy and repair implies removal of myonuclei by apoptosis followed by addition of new nuclei (38). During the recovery process subsequent to 14 days of unloading, Mitchell and Pavlath (38) showed that early soleus muscle growth (first week) occurs independently of an increase in myonuclear number, whereas later growth (second week) requires proliferating muscle precursor cells leading to myonuclear accretion. However, the current paradigm of myonuclei apoptosis and “constant nuclear domains” has been challenged by recent studies using in vivo time lapse microscopy. Brusgaard and Gundersen (10) reported that denervation led to high levels of apoptotic nuclei, but apoptosis likely occurred in stroma cells and satellite cells. There is no doubt that the origin of
nuclei undergoing apoptosis need to be evaluated in our present
disse model (hindlimb unloading).

Apoptosis and autophagy are closely interconnected types of
programmed cell death (37). Interestingly, Luo and Rubinsztein
(31) recently reported a cleavage of Beclin-1 (mammalian Atg6),
an autophagy-related protein, by caspase-3, this event inhibiting
autophagy and decreasing its antia apoptotic effects. Our observa-
tions of a rise in Beclin-1 only at day 5 of muscle repair when
muscle mass and cleaved caspase 3 were totally recovered, differ
from data reported by O’Leary and Hood (42) who found an
increase in Beclin-1 during denervation-induced muscle atrophy.
It will be essential to gather more information on the possible
autophagic role of Atg proteins in muscle mass atrophy and
regrowth. Autophagy may be either lethal or cytoprotective de-
pending on the cellular context (37). In support, studies from
Masiero et al. (33) recently showed that muscle-specific deletion
of a crucial autophagy gene Atg7 resulted in a large muscle
atrophy with abnormal mitochondria and inclusions, implying that
the autophagic pathway may protect muscle mass.

The autophagic/lysosomal and proteasomal pathways are coor-
dinately regulated by FoxO3 in atrophying mouse muscles (55).
Expression of other proteases were then investigated during the
early and late stages of skeletal muscle recovery after unloading.
In muscle wasting, the nonlysosomal Ca2+– regulated cystein pro-
tases called calpains are the initiators of myofibrillar degradation
(5). Consistent with our previous observations (48), μ-calpain
mRNA was upregulated during muscle unloading, and 1 day of
recovery was sufficient to regain baseline values. The in vivo
calpain regulation is currently unclear. In vitro, the ubiquitous
μ-calpain has a calcium sensitivity in the range of 3–50 μM.
Given that resting intracellular calcium was previously estimated
to be 52 and 30 nM in control and unloaded (14 days) rat soleus
muscle, this finding indicates that calcium transients are not
sufficiently large to activate calpains directly (21). However,
McClung et al. (35) clearly demonstrated that calpain-1 (i.e.,
μ-calpain) in contrast to caspase-3 was absolutely required for
hydrogen peroxide-induced myotube atrophy in vitro. Neverthe-
less, caspase-3 is also essential for the activation of proteolysis as
it cleaves actomyosin, producing a characteristic 14-KDa actin
fragment that is degraded by the proteasome system (13). The
ubiquitin-proteasome pathway is mainly responsible for the un-
loaded soleus muscle wasting and two muscle-specific ubiquitin
ligases (E3): MAFbx (atrogin-1) and MuRF1 (muscle RING
finger 1) are critical regulators in the enhanced proteolysis leading
to muscle atrophy (8, 21). In addition to the calpain-dependent
system, MuRF1 might also participate in the disruption of the Z
disk by binding the giant sarcomeric titin (11). Similar to μ-cal
pain, the upregulation of Murf1 was complete after 1 day of
recovery, while MAFbx mRNA was downregulated at 1 day and
reached baseline values only within 14 days. These transcriptional
reprogramming at the early stage of reloading suggest that all
these genes (μ-calpain, MAFbx, MuRF1) might be early
markers of reversed muscle wasting.

Rebuilding of atrophic muscle is a complex process and
involve initial damage and regeneration before recovery of
muscle mass. Taken together, our data show that at early
stages, muscle regrowth is mediated via the modulation of
muscle proteolysis and mitochondrial-driven apoptosis. How-
ever, oxidative stress and TNF-α-mediated apoptotic pathway
remained activated until later stages of muscle remodelling.
These findings suggest that caspase-dependent apoptosis and
proteolysis-related pathways due to oxidative stress and in-
flammation work in a sequential pattern during the muscle
regrowth process after unloading. Understanding in vivo the
crosstalk between all these intricate pathways will be important
to define new pharmacological strategies for optimizing the
recovery of muscle mass subsequent to catabolic diseases such as
bedrest, spaceflight, ageing, diabetes, and cancer cachexia.

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DISCLOSURES

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