Muscle immobilization and remobilization downregulates PGC-1α signaling and the mitochondrial biogenesis pathway

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Muscle immobilization and remobilization downregulates PGC-1α signaling and the mitochondrial biogenesis pathway. J Appl Physiol 115: 1618–1625, 2013. First published August 22, 2013; doi:10.1152/japplphysiol.01354.2012.—Prolonged immobilization (IM) results in skeletal muscle atrophy accompanied by increased reactive oxygen species (ROS) generation, inflammation, and protein degradation. However, the biological consequence of remodeling such muscle has been studied only sparsely. In this study, we examined the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α)-controlled mitochondrial biogenesis pathway and inflammatory response in mice subjected to 2 wk of hindlimb IM followed by 5 days of remobilization (RM). We hypothesized that ROS generation and activation of redox-sensitive signaling pathways play important roles in the etiology of muscle injury. FVB/N mice (age 2 mo) were randomly assigned to either 14 days of IM by casting one of the hindlimbs (n = 7), IM followed by 5 days of RM with casting removed (n = 7), or to a control group (Con; n = 7). Muscle to body weight ratios of three major leg muscles were significantly decreased as a result of IM. Two ubiquitin-proteasome pathway enzymes, muscle atrophy F-box (MAFbx or atrogin-1) and muscle ring finger-1 (MuRF-1), were upregulated with IM and maintained at high levels during RM. Protein contents of PGC-1α and nuclear respiratory factors 1 and 2 in tibialis anterior (TA) muscle were reduced by 50% (P < 0.01) in IM vs. Con, with no recovery observed during RM. IM suppressed mitochondrial transcription factor A and cytochrome-c content by 57% and 63% (P < 0.01), respectively, and cytochrome-c oxidase activity by 58% (P < 0.05). Furthermore, mitochondrial DNA content was reduced by 71% (P < 0.01) with IM. None of these changes were reversed after RM. With RM, TA muscle showed a 2.3-fold (P < 0.05) higher H2O2 content and a 4-fold (P < 0.01) higher 8-isoprostane content compared with Con, indicating oxidative stress. Tumor necrosis factor-α and interleukin-6 levels in TA muscle were 4- and 3-fold higher (P < 0.05), respectively, in IM and RM vs. Con. The nuclear factor-xB (NF-κB) pathway activation was observed only after RM, but not after IM alone. These data indicate an increase in ROS generation during the initial phase of muscle RM that could activate the NF-κB pathway, and elicit inflammation and oxidative stress. These events may hinder muscle recovery from IM-induced mitochondrial deterioration and protein loss.

Skeletal muscle inactivity due to denervation, bed rest, microgravity, and immobilization (IM) can result in significant muscle atrophy that not only reduces mobility and quality of life, but also elicits disease and affects overall health (36, 37). IM is characterized by decreased protein synthesis, increased protein degradation, muscle fiber type shift, oxidative stress, and suppression of mitochondrial biogenesis (12, 13, 15). Proteosome-dependent proteolysis has been shown to be the primary reason for IM-induced muscle atrophy (20, 22). A common etiological mechanism for increased protein degradation appears to be the activation of the ubiquitin-proteasome system, in which two muscle-specific E3 ubiquitin ligases, muscle atrophy F-box (MAFbx; also known as atrogin-1) and muscle ring finger-1 (MuRF-1), play a critical role (4, 7, 23). Both atrogin-1 and MuRF-1 genes are highly overexpressed in an animal model of acute muscle atrophy, and control the ubiquitination and degradation of both regulatory (e.g., calcineurin and MyoD) and structural (e.g., myosin and troponin I) proteins (20, 34).

Skeletal muscle recovery from disuse atrophy has been an interesting topic bearing both scientific and clinical importance. During the early phase of muscle remobilization (RM) endurance capacity and the resistance to fatigue remain to be low (5). Lipid peroxidation and glutathione oxidation are enhanced in the remobilized muscle suggesting involvement reactive oxygen species (ROS) generation and oxidative stress (18, 29). There is evidence that mitochondria are a major source of ROS in mechanically ventilated diaphragm muscle atrophy (17), but xanthine oxidase (XO) and NADPH oxidase (NOX) may also contribute to inactivity-induced ROS production in muscle (26, 40). Increased ROS can activate the redox-sensitive nuclear factor-kappa B (NF-κB) pathway that controls the gene expression of atrogin-1 and MuRF-1, as well as several proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and interferon-α (IFN-α) (16, 38). By binding to their respective receptors, these cytokines are known to activate the NF-κB pathway and further promote their expression through autocrine signaling, thus escalating the vicious cycle within muscle cells. However, the response of these prooxidant and proinflammatory pathways to the early phase of muscle RM is largely unclear.

Recent research suggests that the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) plays an essential role in regulating mitochondrial biogenesis and antioxidant defense in skeletal muscle, thus directly affecting muscle functional performance, inflammation, and disease state (8, 9, 19). As the master transcription regulator, PGC-1α stimulates the transcription of nuclear respiratory factors (NRFs) 1 and 2, and mitochondrial transcription factor A (Tfam), leading to increased synthesis of nuclear-encoded proteins and DNA replication (32). PGC-1α also regulates the gene expression of superoxide dismutase 2 (SOD2), catalase, glutathione peroxidase 1 (GPx1), and uncoupling protein (UCP), thus protecting intracellular oxidant-antioxidant homeostasis and preventing oxidative stress (34). A downregulation of PGC-1α was observed in muscle atrophy of different models accompanied by...
enhanced FoxO dephosphorylation and transactivation of atrogin-1 (31). Furthermore, PGC-1α-knockout mice displayed higher basal expression of TNF-α than wild-type mice (8). Conversely, transgenic mice with PGC-1α overexpression have shown decreased inflammatory cytokine production and protein degradation caused by denervation and aging (26, 39). However, few studies have elucidated the dynamics of the PGC-1α signaling pathway during the transition of muscle IM to RM.

The goal of the present study was to understand the responses of PGC-1α-controlled signaling pathways and their target gene products in a 2-wk IM regimen followed by a short period of RM in mouse skeletal muscle. Specifically, we investigated the role of the PGC-1α mitochondrial biogenesis pathway, ROS generation, and proinflammatory cytokine production using a mouse hindlimb IM model. Our hypotheses were 1) that IM can result in a downregulation of PGC-1α expression and mitochondrial function, and can activate protein degradation pathway in mouse hindlimb muscle; and 2) that short-term RM can increase mitochondrial ROS generation and NF-κB activation, inflammatory response, and oxidative stress.

**MATERIALS AND METHODS**

**Animals and experimental design.** Female FVB/N mice (age 8–10 wk; body wt 21–23 g) were housed individually in the animal facilities at the University of Wisconsin-Madison in a temperature-controlled room (22°C) on a reverse 12-h light/dark cycle. All animals were fed a chow diet and tap water ad libitum. The animal use protocol was approved by the University of Wisconsin-Madison research Animal Resource Center. After a 1-wk acclimation, mice were randomly assigned to one of three groups: 2-wk immobilization (IM; n = 7), 2-wk IM followed by 5 days RM (RM; n = 7), and sham controls (Con; n = 7). To verify that 14 days elicited a sufficient level of muscle atrophy, a fourth group of mice (n = 7) were subjected to IM for 19 days and their results were compared with those from the 14-day IM group. We found no significant difference in terms of muscle mass loss between 19 and 14 days of IM. Furthermore, PGC-1α protein content showed a further modest reduction, but it was not significantly different from 14 days of IM. Therefore, 14 days was chosen as our final protocol for the IM group.

**Mouse hindlimb IM.** We used a mouse hindlimb IM procedure adapted from that described by Caron et al. (5). Briefly, mice were anesthetized with 100 mg/kg ketamine plus 10 mg/kg xylazine via intraperitoneal injection. IM and IM-RM mice in this study were immobilized wherein one of the hindlimbs was randomly selected to be fixed in knee extension and ankle plantarflexion. This was accomplished by cutting a 1.5-ml microtube longitudinally and placing the limb between two halves. The two pipette tip halves were secured around the limb via medical adhesive bandage. The animals were free to move and to eat and drink ad libitum. The IM procedure prevented movement of the immobilized leg alone.

**Tissue collection and mitochondria isolation.** Following each treatment session described above, each mouse was killed, and the gastrocnemius (Gast) and tibialis anterior (TA) muscles from one hindlimb were dissected and weighed. Mitochondria were isolated from a portion of the fresh TA muscle with differential centrifugation using a mitochondrial isolation kit (Thermo Scientific). Briefly, tissues were washed with PBS and homogenized on ice. Mitochondrial isolation reagent C was added and the homogenate was centrifuged at 700 g for 10 min at 4°C. The debris was discarded and the supernatant was transferred to a new tube. The solution was centrifuged at 3,000 g for 15 min at 4°C and the supernatant was removed from the mitochondrial pellets, which were suspended in 0.25 M sucrose (pH 7.6) and placed on ice.

**Mitochondrial ROS production.** ROS production by isolated mitochondria was measured using the Amplex Red method in combination with horseradish peroxidase (Invitrogen). Briefly, succinate was used as a respiratory substrate and SOD was added to convert all superoxide anion produced by mitochondria into H2O2. Resorufin formation at an excitation wave length of 551 nm and an emission wave length of 581 nm was determined using a fluorescence spectrophotometer (F-2000; Hitachi). The rate of H2O2 emission was calculated using the slope coefficient of the fluorescence traces per amount of mitochondrial protein present in the cuvette, expressed in pmol/min × mg protein (33).

**Western blotting analysis.** A portion of the TA muscle was immediately frozen in liquid nitrogen and then homogenized with a tissue grinder (Tissumizer; Tekmar) for 15 s in ice-cold buffer. An NE-PER kit (Thermo Scientific) was used according to the manufacturer’s description to separate nuclear and cytosolic extracts in the presence of protease inhibitor cocktails (Roche) during nuclear and cytoplasmic extraction. Proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore) and blocked with either 5% BSA or skim milk prior to primary antibody incubation. The following antibodies were used for primary antibody incubation: anti-NRF-1 (sc-23624), NRF-2 (sc-13032), and NF-κB p65 (3034), phospho-IκB kinase (p-IKK; Ser176, 2078), p-IκBα (Ser12 and Ser36, 9246), and IkBα (9242) (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA); anti-NF-κB p65 (3034), phospho-IκB kinase (p-IKK; Ser176, 2078), p-IκBα (Ser12 and Ser36, 9246), and IkBα (9242) (all purchased from Cell Signaling Technology, Danvers, MA); anti-atrogin-1 (AP2041) and MuRF-1 (MP3401) (purchased from ECM Bioscience, Versailles, KY); anti-α-tubulin (loading control, ab18251), VDAC1/porin (mitochondrial loading control, ab15895), histone H2B (nuclear loading control, ab1790), SOD2 (ab13533), cytochrome-c (ab53056), Traf (ab131607), and COX IV (ab16056) (purchased from Abcam, Cambridge, MA); and anti-PGC-1α (ST1202) (purchased from Calbiochem, Merck Chemicals, UK).

Following secondary antibody incubation, the membranes were developed using peroxide and ECL Plus Western Blotting Detection Reagents (GE Healthcare) to expose Hyperfilm ECL (GE Healthcare) and analyzed using ImageJ software (version 1.42q, National Institutes of Health, Bethesda, MD).

**Quantitative analysis of mitochondrial DNA (mtDNA).** Mitochondria were lysed in the presence of 0.5% SDS and 0.2 mg/ml proteinase K in 10 mM Tris-HCl, 0.15 M NaCl, and 0.005 M EDTA. Mitochondrial DNA was then purified with a DNA purification kit (Qiagen). Total DNA was isolated using standard protocols. To quantify the amount of mtDNA present per nuclear genome, we used the following primers (6): mtDNA (NADH dehydrogenase 1; ND1) forward, CCTACACCCCTGGGCCATATC; mtDNA reverse, GAGGCTGTTGGTGTGAC; nuclear DNA (Pecame gene on chromosome 6) forward, ATGGAAAGCGCGCCATG; nuclear DNA reverse, TCTTGGTTGTCGACATC. Quantification of relative copy number differences was carried out using analysis of the difference in threshold amplification between mtDNA and nuclear DNA (ΔΔC(t) method). The quantitative RT-PCR thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

**Mitochondrial ATP production rate.** Mitochondrial ATP production rate (MAPR) was measured using three additional groups of mice subjected to the same treatment protocols of IM (n = 7), RM (n = 7), and Con (n = 7) as described previously. After the mitochondria were isolated from TA muscle, the pellet was resuspended in buffer (180 mM succrose, 35 mM KH2PO4, 10 mg acetate, 5 mM EDTA) and kept on ice. The reaction mixture included a luciferin-luciferase ATP monitoring reagent (ENLITEN ATP Assay System; Promega, Madison, WI), 35 μg ADP, and substrates. Pyruvate (1 mM) and 1 mM malate were added to the reaction mixture as a substrate for oxidation phosphorylation. When ATP was the limiting component in the luciferase reaction, the intensity of the emitted light was proportional to the ATP concentration. Measurement of the light intensity using a luminometer.
permitted direct quantitation of ATP. ATP production reactions were monitored at 25°C for 20–25 min with a luminometer (41).

**Enzyme activity.** Another portion of TA muscle was homogenized with a tissue grinder (Tekmar) with a 1:10 (wt:vol) dilution in ice-cold lysis buffer containing 50 mM Tris (pH 7.4 at 4°C), 150 mM NaCl, 1% Triton X-100, 1% IGEPAL (NP-40), and 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. An aliquot of the homogenate without PMSF was retained for cytokine assay (see below). Thereafter, the homogenate was incubated on ice for 10 min, and centrifuged at 1,000 g for 5 min at 4°C. The supernatant was separated into aliquots and stored at −80°C. Cytochrome-c oxidase (COX) activity was measured in mitochondria as recommended by the manufacturer’s protocol (Sigma, St. Louis, MO). Protein concentrations of homogenates, extracts, and mitochondria were determined using the Bradford method.

**TNF-α and IL-6 measurements.** TNF-α and IL-6 protein concentrations were measured in TA muscle homogenate (Tris-HCl buffer pH 7.4) using an ELISA assay kit (BD Bioscience) following the manufacturer’s instructions. 8-Isoprostane was measured in TA homogenate using a chemiluminescence competitive ELISA assay kit following the manufacturer’s instructions (Cayman Chemical).

**Statistical analysis.** Experimental data were expressed as means ± SE and group comparisons were made by one-way ANOVA. The Tukey-Kramer method was used as a post hoc test when ANOVA reached significance (P < 0.05).

### RESULTS

**Muscle mass and ubiquitin-proteasome pathway.** There was no body weight change as a result of IM or RM (Table 1). However, the ratio of muscle to body weight was decreased by 20% in TA and 18% in Gast after 2 wk of IM, compared with the Con group (P < 0.05). After 5 days of RM, the muscle/body weight ratio of both muscles recovered to 89% of Con values and were no longer significantly different from those of the Con group.

Atrophy in striated muscle results from enhanced protein breakdown. Therefore, activation of the ubiquitin-proteasome pathway was determined, including expression of the muscle-specific ubiquitin protein ligases atrogen-1 and MuRF1. After IM, atrogen-1 protein content was significantly increased by 3.9- and 3.4-fold (P < 0.01) in TA and Gast muscle, respectively. After RM, atrogen-1 content remained 2.9- and 3.5-fold higher than Con in TA and Gast muscle, respectively (P < 0.01) (Fig. 1A). MuRF1 content was increased by 2.7- and 3.6-fold (P < 0.05) with IM in TA and Gast, respectively (P < 0.01) (Fig. 1B). Its content decreased significantly after RM compared with that of IM (P < 0.01), but remained 70% higher than Con in TA muscle (P < 0.05).

**Downregulation of the mitochondrial biogenesis pathway.** To assess IM induced PGC-1α downregulation and its potential regulation of mitochondrial biogenesis and function, protein content of PGC-1α and PGC-1α-controlled mitochondrial biogenesis markers NRF-1, NRF-2, cytochrome-c, COX IV, and Tfam were determined in TA muscle by Western blot.

**Table 1. Body weight and muscle/body weight ratio in mice**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>IM</th>
<th>RM</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>22.07 ± 3.01</td>
<td>23.5 ± 2.25</td>
<td>23.6 ± 2.34</td>
</tr>
<tr>
<td>TA/body weight, mg/g</td>
<td>1.71 ± 0.025</td>
<td>1.38 ± 0.016*</td>
<td>1.52 ± 0.027</td>
</tr>
<tr>
<td>Gast/body weight, mg/g</td>
<td>6.02 ± 0.122</td>
<td>4.97 ± 0.107*</td>
<td>5.34 ± 0.271</td>
</tr>
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IM, immobilization; RM, remobilization; TA, tibialis anterior; Gast, gastrocnemius. All values are means ± SE; *P < 0.05, IM vs. control.

IM decreased PGC-1α protein content in TA by 50% (P < 0.01) (Fig. 2). Five days of RM did not restore the PGC-1α level. Therefore, IM-induced PGC-1α downregulation in skeletal muscle could not be restored with a short period of RM.

Nuclear NRF-1 and 2 contents were decreased by 47% and 44% in IM vs. Con, respectively (P < 0.05), and remained suppressed after RM with no significant change shown between RM and IM (Fig. 3). Cytochrome-c, a mitochondrial protein marker, was 63% lower in IM vs. Con (P < 0.01), and showed an additional 11% drop after RM (Fig. 4B). Interestingly, the content of COX IV, a nuclear-encoded mitochondrial protein, was not affected by either IM or RM (Fig. 4C). In contrast, Tfam content was reduced by 57% in IM (P < 0.01) and remained suppressed in RM (P < 0.05) (Fig. 4D).
To further examine the effect of IM and RM on mitochondrial proliferation, we measured the mtDNA level using nuclear (n) DNA as a reference. The mtDNA/nDNA ratio decreased by 71% after IM (P < 0.01) and showed no recovery after RM (Fig. 5A). To assess the effect of IM and RM on mitochondrial metabolic potential, COX activity was measured under optimal assay conditions. COX activity was decreased by 53% (P < 0.05) and recovered markedly after RM to 70% of Con values (Fig. 5B). As a result, there was no significant difference between RM and Con. To assess the effect of IM-led decrease in mitochondrial biogenesis and protein degradation on mitochondrial energy metabolism, we measured the ATP production rate in two separate groups of mice undergoing the same IM-RM regimen and compared them with the Con group (Fig. 5C). There was a 29% (P < 0.05) decrease in MAPR with both IM and RM, and no improvement was observed when RM and IM were compared.

Effect of IM and RM on muscle oxidative stress. Mitochondrial H2O2 production in TA muscle was not affected by IM (Fig. 6A); however, H2O2 content increased 2.3-fold (P < 0.05) in RM vs. Con. As a marker of lipid peroxidation, 8-isoprostane showed a 4-fold increase in RM vs. Con and a 2-fold increase between RM and IM (P < 0.01) (Fig. 6B). The difference between IM and Con was not significant. Thus IM alone had little effect on ROS generation and lipid peroxida-
associated with activation of the NF-κB pathway.

**DISCUSSION**

The most significant finding of the current investigation is that during the early stage of a short-term remobilization from previously immobilized mouse hindlimb muscle, ROS generation is increased, causing oxidative stress, NF-κB activation, and proinflammatory cytokine overexpression, which probably contribute to a sustained activation of proteolysis and mitochondria dysfunction.

During prolonged skeletal muscle IM, atrophy is associated with activation of several proteolytic systems including the ubiquitin-proteasome pathway controlled by NF-κB, wherein

![Figure 5](https://jap.physiology.org/doi/10.1152/japplphysiol.01354.2012)

**Fig. 5.** Effect of IM and RM on mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratio (A), cytochrome-c oxidase activity (COX) (C), and mitochondrial ATP production rate (MAPR) (D) in mouse TA muscle. Each bar represents means ± SE of Con (n = 7), IM (n = 7), and RM (n = 7) groups. *P < 0.05, **P < 0.01; IM or RM vs. Con.

![Figure 6](https://jap.physiology.org/doi/10.1152/japplphysiol.01354.2012)

**Fig. 6.** Effect of IM and RM on mitochondrial hydrogen peroxide production rate (A), 8-isoprostane content (B), tumor necrosis factor (TNF)-α (C), and interleukin (IL)-6 (D) levels in mouse TA muscle. Each bar represents means ± SE of Con (n = 7), IM (n = 7), and RM (n = 7) groups. *P < 0.05, **P < 0.01; IM or RM vs. Con. +P < 0.05, RM vs. IM.
two muscle-specific ubiquitin ligases, atrogin-1 and MuRF-1, play a key role (4, 7). In the current study, atrogin-1 and MuRF-1 expressions were increased 3- to 4-fold in two hindlimb muscles after 14 days of IM (Fig. 1). Atrogin-1 levels showed a more dramatic increase in response to IM and remained elevated after 5 days of RM, whereas MuRF-1, although having declined after RM, was still above control levels in TA muscle. Our data are in agreement with previous studies using casting, hindlimb suspension, and bed rest in humans as experimental models. For example, Caron et al. showed that 3.5 days after hindlimb immobilization in mice the expression of atrogin-1 and MuRF-1 mRNA was increased 5.9- and 1.9-fold, respectively (5). It is well known that muscle IM can downregulate the PI3K-Akt pathway that not only diminishes the signals required to maintain protein synthesis via the mTOR transcription factor, but also dephosphorylates FoxO, a condition that activates atrogin-1 transcription (16). Furthermore, recent research has revealed that the autophagy-lysosome systems may be activated during muscle IM and contribute to the loss of muscle mass (30). Activation of the FoxO pathway may stimulate several pathways such as parkin, PINK1, Bnip3, and Bnip3L, eventually leading to mitochondrial degradation (mitophagy). In the current study, IM resulted in an approximately 18–20% decline in muscle/body weight ratio, whereas TA and Gast muscles regained only 42% and 35%, respectively, of their lost mass after RM. The sustained activation of two major ubiquitin ligases, atrogin-1 and MuRF1, could at least in part explain the patterns of muscle weight changes in response to IM and RM. However, given more extended time for recovery, the muscle would probably regain its lost mass; therefore, our atrogin-1 and MuRF-1 data should not be interpreted as the cause of sustained muscle mass loss during IM-RM. It is also noteworthy that the TA was immobilized in the stretched position, in which muscle might not be completely at rest but subjected to hormonal, nerve, and other input that could mitigate IM-induced atrophy. Caution should be exercised in comparing our findings to muscles immobilized at conditions closer to its natural length.

Previous research showed that mitochondrial dysfunction could be an important etiological contributor to muscle disuse atrophy (1, 17). Our data showed that after 14 days of IM, PGC-1α protein content was less than half that of Con, whereas 5 days of RM did little to restore PGC-1α levels (Fig. 2). Furthermore, both NRF-1 and 2 and Tfam were downregulated to 50% of Con and remained at this level after RM. As a result, the mitochondrial inner membrane protein marker cytochrome-c and COX enzyme activity were severely depressed with IM, and only COX showed a marked recovery after RM. The reduction in Tfam apparently affected mitochondrial proliferation because the mtDNA:nDNA ratio was dramatically decreased with IM and RM (Fig. 5). The combined effects of mitochondrial volume and protein loss with IM and RM caused a ~30% decrease in mitochondrial ATP production rate, which could severely reduce muscle energy production and functional performance observed in many muscle disuse atrophy models (1, 15, 29).

One surprising exception that escaped the IM-induced mitochondrial degeneration was COX IV (Fig. 4C), a nuclear encoded inner membrane enzyme considered a classic marker of mitochondrial biogenesis. The resistance of this enzyme to muscle IM indicates that pathways other than that of PGC-1α may regulate mitochondrial biosynthesis. Furthermore, the
mitophagic pathway that controls mitochondrial degradation may also contribute to overall mitochondrial protein levels in IM muscle (30).

It is widely believed that in the absence of chronic disease, inflammation is not a major pathway leading to disuse muscle atrophy. However, NF-κB activation, overproduction of proinflammatory cytokines, and disturbance of redox status have been found in some previous studies (3, 11, 16, 28). Although there is clear evidence that the mitochondrial electron transport chain can be detrimentally affected by IM, which increases superoxide production in some IM models (27), we did not observe a significant change in ROS production or 8-isoprostane level with IM, indicating that IM alone does not impose an oxidative stress under our experimental condition. Activation of NADPH oxidase and xanthine oxidase has been reported in inactivated respiratory muscles (25, 40), but their involvement in ROS production in hindlimb muscle IM is not certain (12).

However, our IM protocol resulted in a large increase in TNF-α and IL-6 levels (Fig. 6, C and D). Although previous literature denied a major role for TNF-α in muscle disuse atrophy except under cachexic condition, our observation was consistent with some recently reported studies using denervation (26) or hindlimb suspension (10) as an experimental model. TNF-α is known to stimulate mitochondrial ROS production, whereas both TNF-α and IL-6 activate membrane-borne cyclooxygenase and lipoxygenase, thereby increasing superoxide production (15). TNF-α and IL-6 also increase the expression of adhesion molecules that promote blood-borne polymorphonuclear (PMN) infiltration into muscle cells and cause respiratory burst, which also increases the generation of superoxide and other reactive oxidants (29). Thus, the sustained elevation of these two proinflammatory cytokines during IM and RM may play a critical role in escalating ROS production and oxidative stress, and facilitating protein degradation in the mouse muscle.

It is well established that DNA binding of NF-κB subunit p65 is a required step in the transcriptional activation of a variety of genes involved in protein degradation, inflammation, and antioxidant defense (38). It is thus not surprising that muscle IM induces a cellular milieu in favor of NF-κB activation (12, 24). From our data revealing no significant NF-κB activation in mouse muscle after 14 days of IM (Fig. 7), it seems that increases in proinflammatory cytokines alone due to short-term IM were not sufficient to activate this redox-sensitive pathway in the IM muscle. This point of view is supported by the observation that neither H₂O₂ production nor lipid peroxidation was enhanced by IM alone (Fig. 6, A and B). However, after 5 days of RM there was a dramatic, twofold increase in ROS production, a more than fourfold increase in 8-isoprostane, along with a significant increase in IKK activation, IκB phosphorylation, and p65 nuclear translocation. These data could explain several biochemical and physiological changes observed in the IM and RM muscle. First, NF-κB activation could be a main stimulus for the sustained high levels of TNF-α and IL-6 expression when muscle movement restriction was removed. The upregulation of the proinflammatory cytokines might be accomplished via a paracrine/autocrine mechanism controlled by NF-κB and other redox-sensitive signaling pathways such as members of mitogen-activated protein kinase (14). Second, NF-κB activation could promote increased expression of cytokine-induced adhesion molecules that enhance PMN infiltration and NADPH oxidase activation, and hence ROS production, forming a vicious cycle and sustained oxidative stress. This mechanism may be especially relevant when blood flow is restored to RM muscle. Third, NF-κB has been proposed to negatively regulate PGC-1α activity in muscle cells via direct binding to PGC-1α protein (2). Thus, the increased nuclear concentration of p65 observed in our study may interact with PGC-1α and prevent its binding to key transcriptional factors (e.g., NRF-1) and reduce mitochondrial biogenesis. Finally, NF-κB activation could be a main reason for the continued activation of atrogin-1 and MuRF-1 observed after RM (21). Although different signaling pathways may be involved in triggering muscle atrophy, NF-κB was identified as the most important pathway to activate the two muscle-specific E3 ubiquitin ligases atrogin-1 and MuRF-1 (22). Ultimately, the sustained activation of this pathway may explain the slow recovery of muscle mass and metabolic function during the initial stage of muscle remobilization.

In summary, our data clearly demonstrated that ROS generation and NF-κB activation may play a critical role in the continuous suppression of mitochondrial protein expression and metabolic function during the early stage of RM after a prolonged period of IM in skeletal muscle. The high levels of proinflammatory cytokines and activation of ubiquitin-proteolysis may be one of the etiological mechanisms that keeps a muscle in catabolic mode and under oxidative stress, hindering its quick recovery.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.K. and L.L.J. conception and design of research; C.K. performed experiments; C.K. and L.L.J. analyzed data; C.K. and L.L.J. wrote the first draft of the manuscript; C.K. and L.L.J. edited and revised manuscript; L.L.J. approved final version of manuscript.

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