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Activation of alternative NF-κB signaling during recovery of disuse-induced loss of muscle oxidative phenotype

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Remels AH, Pansters NA, Gosker HR, Schols AM, Langen RC. Activation of alternative NF-κB signaling during recovery of disuse-induced loss of muscle oxidative phenotype. *Am J Physiol Endocrinol Metab* 306: E615–E626, 2014. First published January 14, 2014; doi:10.1152/ajpendo.00452.2013.—Physical inactivity-induced loss of skeletal muscle oxidative phenotype (OXPHEN), often observed in chronic disease, adversely affects physical functioning and quality of life. Potential therapeutic targets remain to be identified, since the molecular mechanisms involved in reloading-induced recovery of muscle OXPHEN remain incompletely understood. We hypothesized a role for alternative NF-κB, as a recently identified positive regulator of muscle OXPHEN, in reloading-induced alterations in muscle OXPHEN. Markers and regulators (including alternative NF-κB signaling) of muscle OXPHEN were investigated in gastrocnemius muscle of mice subjected to a hindlimb suspension/reloading (HLS/RL) protocol. Expression levels of oxidative phosphorylation subunits and slow myosin heavy chain isoforms I and IIA increased rapidly upon RL. After an initial decrease upon HLS, mRNA levels of peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC) molecules PGC-1α and PGC-1β and mRNA levels of mitochondrial transcription factor A (Tfam) and estrogen-related receptor α increased upon RL. PPAR-δ, nuclear respiratory factor 1 (NRF-1), NRF-2α, and sirtuin 1 mRNA levels increased during RL although expression levels were unaltered upon HLS. In addition, both Tfam and NRF-1 protein levels increased significantly during the RL period. Moreover, upon RL, IKK-α mRNA and protein levels increased, and phosphorylation of P100 and subsequent processing to P52 were elevated, reflecting alternative NF-κB activation. We conclude that RL-induced recovery of muscle OXPHEN is associated with activation of alternative NF-κB signaling.

reloading; skeletal muscle; alternative nuclear factor-κB; peroxisome proliferator-activated receptor-γ coactivator

SKELETAL MUSCLE TISSUE DISPLAYS a remarkable degree of plasticity, in terms of size and metabolic activity, in response to different stimuli including physical (in)activity, inflammation, and hypoxia (3, 10, 15). In particular, decreased (neuro)muscular activity represents one of the most potent lifestyle- and disease-associated triggers detrimentally affecting skeletal muscle tissue quality and quantity. More insight in the molecular mechanisms orchestrating muscle functional recovery upon resumption of physical activity is needed for developing targeted intervention strategies in conditions in which increasing physical activity levels may be limited, e.g., in chronic disease.

In contrast to the adaptive response of molecular signaling pathways involved in muscle mass regulation to inactivity and subsequent resumed physical activity, which is well-described (36), significantly less is known about the muscle's metabolic response to these conditions. It has been convincingly demonstrated that physical inactivity or mechanical unloading, as frequently observed in clinical settings in response to immobilization or prolonged bed rest, leads to a coordinated downregulation of muscle oxidative phenotype (OXPHEN: muscle cell-intrinsic features determining the capacity for substrate oxidation) (23, 26, 30, 33, 39). Remarkably, the response of muscle OXPHEN, and specifically its molecular regulation, to resumed physical activity after a period of prolonged inactivity has been scarcely described.

Mitochondrial biogenesis, highly implicated in the determination of muscle OXPHEN, is a complex biological process that requires coordinated transcription of the nuclear as well as mitochondrial genome and import of nuclear-encoded proteins into the mitochondrial compartment (21). A multitude of regulatory proteins have been implicated herein. The peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1 (PGC-1) family of transcriptional coactivator molecules plays a key role in the regulation of mitochondrial biogenesis and determination of muscle OXPHEN as they interact with and coactivate numerous DNA-binding transcription factors to control the regulation of multiple genes encoding mitochondrial proteins. Transcription factors coactivated by PGC-1 include PPAR-α and PPAR-δ, estrogen-related receptor α (ERR-α), nuclear respiratory factor (NRF) 1, and NRF-2α (21, 38). Whereas PPAR molecules are involved in the transcription of genes coding for proteins involved in fatty acid uptake, transport, and oxidation (20), NRF-1 and NRF-2α transcribe constituents of the mitochondrial oxidative phosphorylation (OXPHOS) chain itself (2). Furthermore, PGC-1 and NRF molecules control transcription of the mitochondrial transcription factor A (Tfam), an essential mediator of mitochondrial biogenesis and replication of the mitochondrial genome (21, 22). In addition, upstream from PGC-1, sirtuin 1 (SIRT-1) regulates muscle OXPHEN by posttranslational modification (deacetylation) of PGC-1α thereby enhancing its function (48).

Mitochondria constantly fuse and divide, and an imbalance of these two processes alters mitochondrial morphology and function (28). In fact, several reports have demonstrated a direct correlation between the extent of mitochondrial fusion and the capacity for OXPHEN (16, 44). Mitochondrial fusion is mediated by so-called mitochondrial fusion (mfn) genes 1 and 2, whereas fission of mitochondria is controlled by fission genes (FIS1, MTP18). Mitochondrial fusion genes are under transcriptional control of PGC-1 molecules with involvement of ERR-α (40, 49).

Interestingly, the alternative NF-κB signaling pathway has recently been identified as a novel regulatory pathway impli-

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cated in the determination of skeletal muscle OXPHEN (5). Alternative NF-κB signaling is regulated by an IKK-α homodimer complex that phosphorylates the p100 precursor subunit of NF-κB. This, in turn, promotes partial p100 proteolysis to produce the p52 subunit that, together with RelB, forms a transcriptionally active complex (4). Bakkar et al. recently showed that IKK-α, through activation of the alternative NF-κB pathway, positively affects OXPHEN of mature skeletal muscle tissue in vivo (5). Because this represents a recently unknown function of this pathway, no data exist as to whether the alternative NF-κB pathway in muscle is modulated by exercise or physical (in)activity.

In the present study, we subjected wild-type mice to a hindlimb suspension/reloading protocol and systematically investigated skeletal muscle OXPHEN and its putative regulators (including alternative NF-κB signaling and PGC-1-coactivated signaling events). We hypothesized that reloading-induced increases in muscle mitochondrial markers and PGC-1-coactivated signaling events are associated with activation of alternative NF-κB signaling.

MATERIALS AND METHODS

Animals. The Institutional Animal Care Committee of Maastricht University approved the animal study (DEC-2009-074). C57/Bl6 mice were used, and, at the start of the experiment, mice were 13 wk old. Animals were housed in a temperature-controlled room (21–22°C) with a 12:12-h light-dark cycle with standard chow pellets and water ad libitum. Animals were randomly divided into different groups and followed the protocol duration as indicated. For baseline measurements, nine animals were used. Subsequently, eight animals were killed after 14 days of hindlimb suspension, and eight animals were killed 1, 2, 3, 5, or 8 days after reloading.

Hindlimb suspension was accomplished using a tail suspension device consisting of a plastic-coated iron wire taped around the mouse's tail and connected to a swivel hook to allow circular motility. The latter was attached to a Teflon-coated polyvinyl chloride ring that slid over an iron rod spanning the length of the cage to allow longitudinal motility. The tail harness was placed while mice were lightly anesthetized using isoflurane inhalation, and mice were raised so as to prevent the hindlimbs from touching the cage floor or sides. In this way, four hindlimb suspension mice could be housed in one standard cage. After 14 days of hindlimb suspension, mice were again lightly anesthetized, released from the tail harness, and allowed to resume normal cage activity. After death with pentobarbital sodium at the indicated time points, gastrocnemius muscles were excised using standardized dissection methods, cleaned of excess fat and connective tissue, pair weighed on an analytical balance, snap-frozen in liquid nitrogen, and stored at −80°C for RNA and protein extraction.

RNA isolation, cDNA synthesis, and quantitative PCR. Total RNA was extracted using an on-column RNA isolation kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA concentration was determined using a spectrophotometer. RNA (0.4 μg/sample) was reverse transcribed into cDNA using a Transcriptor cDNA synthesis kit (Roche, Almere, The Netherlands). cDNA was diluted 1/50 and quantitative PCR amplified with Sensimix SYBR green and Fluorescein mix (GC Biotech, Alphen a/d Rijn, The Netherlands) on a Bio-Rad PCR apparatus. Primers were designed to generate a PCR amplification product of 100–150 bp. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The expression of the genes of interest was normalized with a correction factor derived from geNorm, which is based on the geometric mean of the expression levels of multiple housekeeping genes (GAPDH, RPL13A, ARBP, calnexin, and β2M) to control for expression variation between mice.

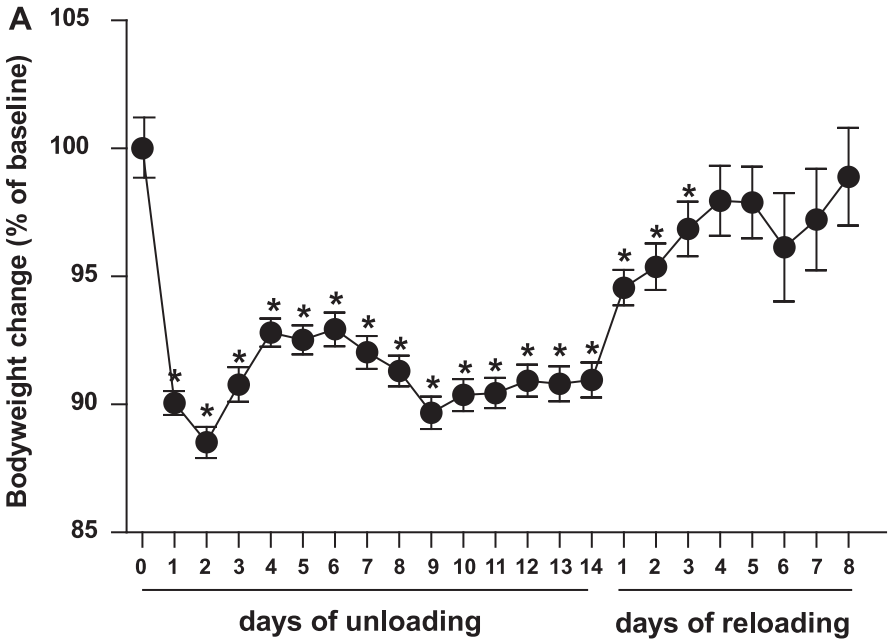
Preparation of whole cell lysates. Whole mouse muscle was powdered and lysed in a whole cell lysate buffer (20 mM Tris, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 1 mM DTT; 1 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml leupeptin; and 1% aprotenin). Next, crude lysates were incubated on ice for 30 min, followed by a 30-min centrifugation step at 16,000 *g* at 4°C. A portion of the supernatant was saved for protein determination, before the addition of 4× Laemmli sample buffer [0.25 M Tris·HCl, pH 6.8; 8% (wt/vol) SDS; 40% (vol/vol) glycerol; 0.4 M DTT; and 0.04% (wt/vol) bromphenol blue]. The samples were boiled for 5 min at 95°C and stored at −20°C. Total protein concentration was assessed with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Western blotting. For Western blotting, 0.4 μg (OXPHOS, GAPDH), 5 μg (phospho-p100, p100/p52, IKK-α, GAPDH), or 10 μg (Tfam and NRF-1) of protein were loaded per lane and separated on a 10% polyacrylamide gel (Mini Protean 3 system; Bio-Rad), followed by transfer to a 0.45-μm nitrocellulose membrane (Bio-Rad) by electroblotting. The membrane was blocked for 1 h at room temperature in 5% (wt/vol) nonfat dry milk followed by overnight incubation (4°C) with an antibody specific for OXPHOS proteins (Cell Signaling, Beverly, MA), GAPDH (Cell Signaling), phospho-p100 (Abcam, Cambridge, UK), P100/p52 (Bioke, Leiden, The Netherlands), IKK-α (Imgenex, San Diego, CA), NRF-1 (Abcam), or Tfam (Millipore). Antibodies were diluted 1/5,000 (OXPHOS, GAPDH) or 1/1,000 (phospho-p100, p100/p52, IKK-α, Tfam, and NRF-1) in (wt/vol) nonfat dry milk. After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Western blot films were imaged and quantified using the Quantity One analysis software from Bio-Rad. When 0.4 or 5 μg of protein were loaded, GAPDH was used as a loading control to correct for equal loading. When 10 μg of protein were loaded (Tfam and NRF-1), saturation of GAPDH signals prevented correct quantification and subsequent normalization. Therefore, these blots were subjected to a Ponceau S staining, which was used to calculate a correction factor, based on multiple bands of the Ponceau S staining (Quantity One analysis software from Bio-Rad). The whole hindlimb suspension group was included on every gel and was used to calculate an intergel correction factor. In general, all Western blot data are corrected by calculating the ratio with GAPDH (or Ponceau S when applicable). Phospho-p100, total p100, and p52 protein data were first corrected for GAPDH after which the ratio of interest was calculated and presented.

Statistics. Data were analyzed according to the guidelines of Altman et al. using SPSS (SPSS, Chicago, IL). In short, body weights and muscle weights from each group (hindlimb suspension and reloading time points) were analyzed nonparametrically and compared with the baseline group. All other data were analyzed by a one-way ANOVA (since each reloading time point was compared with both the baseline and the hindlimb suspension condition). A Levene's test was used to assess whether variances were equal or not. When equal variances were assumed, we used Tukey's post hoc correction. When unequal variances were assumed, the Games-Howell post hoc correction was used. Data are represented as the means ± SE as indicated. A P value of <0.05 was considered to be significant.

RESULTS

Expression of muscle structural and mitochondrial markers in response to hindlimb suspension/reloading. Body weights and gastrocnemius muscle weight decreased significantly during unloading and subsequently increased again upon reloading (Fig. 1, *A* and *B*). In the gastrocnemius muscle, myosin heavy chain (MyHC) I, MyHC IIA, and MyHC IIB isoform mRNA



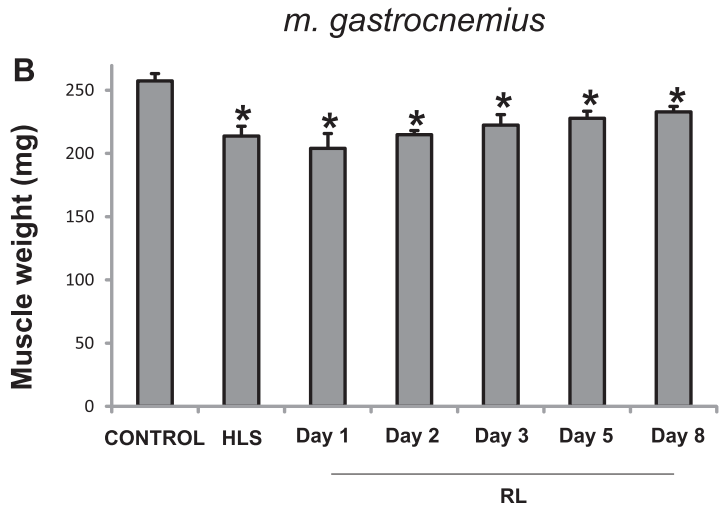


Fig. 1. Body weight and muscle weights during hindlimb suspension and reloading. Mice were subjected to a hindlimb suspension (14 days) and reloading (8 days) protocol (n = 8–9/time point). Body weight and weight of the gastrocnemius muscle (m. gastrocnemius) were assessed. *A*: body weights are presented as a percentage of baseline. *B*: paired gastrocnemius muscle weights (not corrected for body weight). Values are expressed as means ± SE. Significance compared with baseline: $*P \leq 0.05$.

levels decreased upon unloading and rapidly increased during the reloading phase. Increases in MyHC I and MyHC IIA mRNA during reloading, however, were more pronounced and more sustained in time compared with reloading-induced increases in MyHC IIB mRNA. mRNA levels of MyHC IIX increased slightly during the hindlimb suspension/reloading protocol (Fig. 2, *A–D*). mRNA expression levels of subunits of mitochondrial OXPHOS complexes II and III decreased significantly in response to unloading. In response to reloading, mRNA transcript levels of OXPHOS complexes I, II, and III increased and continued to increase throughout the recovery phase up to *day 3* of the reloading protocol (Fig. 3*A*). Correspondingly, after an unloading-induced decrease, OXPHOS complex III protein content gradually increased during the recovery phase compared with the unloaded condition (Fig. 3*B*). None of the investigated fusion and fission transcript levels were significantly altered by unloading. Upon reloading mfn-1 (fusion), FIS1 (fission), and mfn-2 (fusion), mRNA transcript levels were transiently increased at *day 3* of the reloading protocol (Fig. 3, *C–F*). MTP18 mRNA expression levels (fission) were largely unaffected by the unloading/reloading protocol.

In addition to markers of oxidative metabolism, transcript levels of the glycolytic enzymes phosphofructokinase (PFK) and hexokinase 2 (HKII) were investigated. PFK mRNA levels decreased slightly but significantly after hindlimb suspension and increased by twofold at the start of reloading after which levels gradually declined to approximate baseline levels. HKII transcript levels on the other hand were unaffected by unloading but did display a phasic increase during the recovery phase that persisted up to *day 3* of reloading (Fig. 4, *A* and *B*).

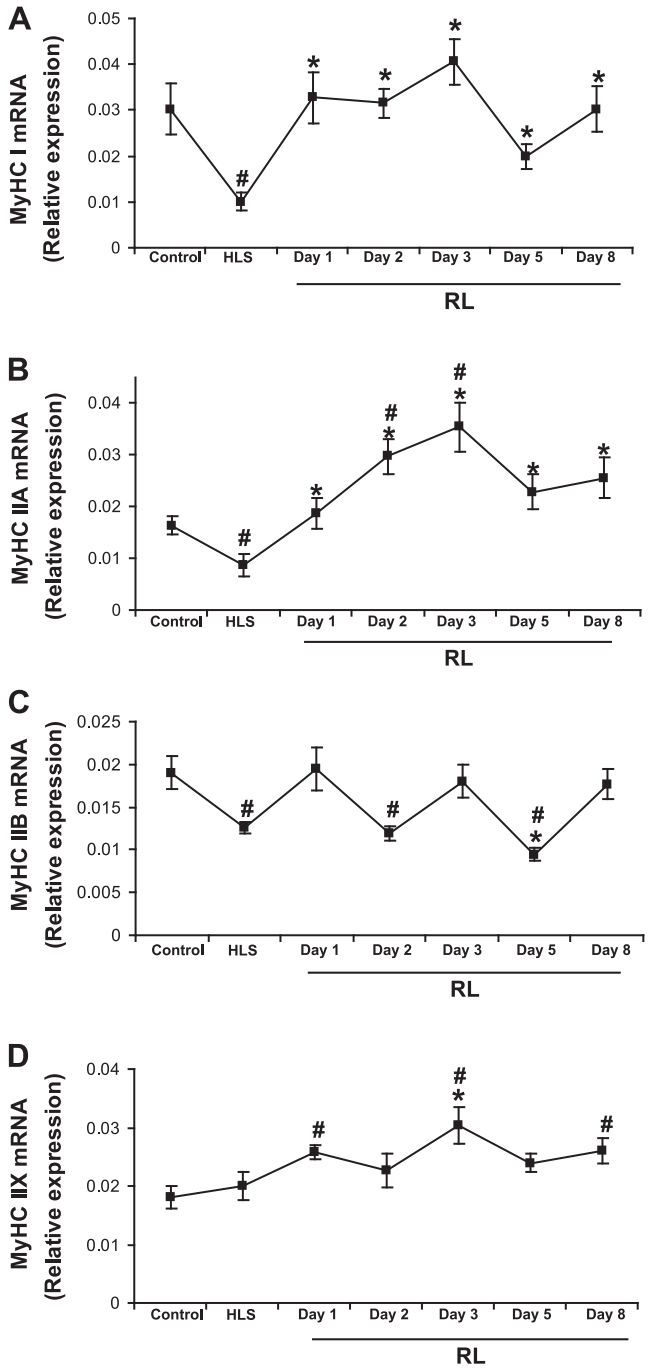


Fig. 2. Myosin heavy chain (MyHC) gene expression during hindlimb suspension (HLS) and reloading (RL). *A–D*: MyHC isoform mRNA expression was determined in gastrocnemius muscle of mice undergoing the HLS/RL protocol (n = 8–9/time point). Values are expressed as means ± SE. Significance compared with HLS: $*P \leq 0.05$. Significance compared with baseline (control): $\#P \leq 0.05$.

Regulation of muscle OXPHEN: PGC-1 signaling. PGC-1α, PGC-1β, and PPAR-α mRNA levels appeared to consistently decrease in response to unloading (Fig. 5, *A–C*). Decreases in PGC-1α and PPAR-α, however, failed to reach statistical significance. Furthermore, both PGC-1α and PGC-1β mRNA transcript levels displayed a biphasic increase in the first 3 days of the recovery phase (Fig. 5, *A* and *B*). PPAR-α mRNA levels were largely unaltered during recovery (Fig. 5*C*). PPAR-δ mRNA levels were unaffected by unloading but did, however, display a transient increase in the first 3 days of the reloading protocol (Fig. 5*D*). Tfam mRNA levels on the other hand were reduced after hindlimb suspension and increased upon reloading in a phasic manner similar to PGC-1α and PGC-1β (Fig. 5*E*). In addition, NRF-1α, NRF-2α, and SIRT-1 expression levels were unaltered in response to unloading but did increase during the reloading period (Fig. 5, *F–H*). Finally, ERR-α mRNA expression levels were reduced upon unloading, and increased ERR-α mRNA levels were observed from the start of the reloading protocol up to *day 3* of recovery (Fig. 5*I*).

In addition to assessment of mRNA expression levels, Tfam and NRF-1 protein levels were determined in response to hindlimb suspension and subsequent reloading. As depicted in Fig. 6, *A* and *B*, Tfam and NRF-1 protein levels were, respectively, unaltered or increased in response to unloading, whereas reloading resulted in a significant increase of both Tfam and NRF-1 protein (Fig. 6, *A* and *B*).

Regulation of muscle OXPHEN: Alternative NF-κB signaling. As described above, mechanical unloading decreased expression levels of multiple parameters and regulators of muscle OXPHEN. Importantly, subsequent reloading resulted in a potent induction of these constituents. This validated the hindlimb suspension/reloading model we used with regard to physical (in)activity-induced changes in muscle OXPHEN. We next investigated whether or not the induction of muscle OXPHEN upon reloading was paralleled by activation of the alternative NF-κB pathway. IKK-α mRNA levels were unaltered in response to unloading and rapidly increased by twofold upon reloading after which levels declined but remained elevated (compared with unloading levels) throughout the recovery phase (Fig. 7*A*). Protein levels of IKK-α responded correspondingly to the unloading/reloading protocol as they displayed a phasic increase during the first 3 days of recovery after which levels remained elevated throughout the recovery period (Fig. 7*B*). Consistent with increased IKK-α protein levels during recovery, phosphorylation of its downstream target p100 substantially increased during the reloading phase. Upon hindlimb suspension, however, levels of phosphorylated p100 were unaltered (Fig. 7*C*). In addition, processing of p100 to p52 was significantly increased during the recovery phase (Fig. 7*D*), further evidencing activation of the alternative NF-κB pathway.

DISCUSSION

In the present study, we show that reloading-induced upregulation of muscle OXPHEN is associated with a coordinated increase in mRNA and protein levels of multiple constituents of the PGC-1 signaling network evidencing activation of mitochondrial biogenesis and recovery toward a slow, oxidative muscle metabolic profile. Moreover, we show for the first time that reloading-induced increases in muscle OXPHEN and PGC-1-coactivated signaling events coincide with potent activation of the alternative NF-κB pathway, which was recently discovered as a novel positive regulator of muscle OXPHEN upstream of PGC-1 (5).

In both rodents and humans, the impact of unloading or disuse on skeletal muscle OXPHEN has been well-described (23, 26, 30, 33, 39). It has been convincingly demonstrated that

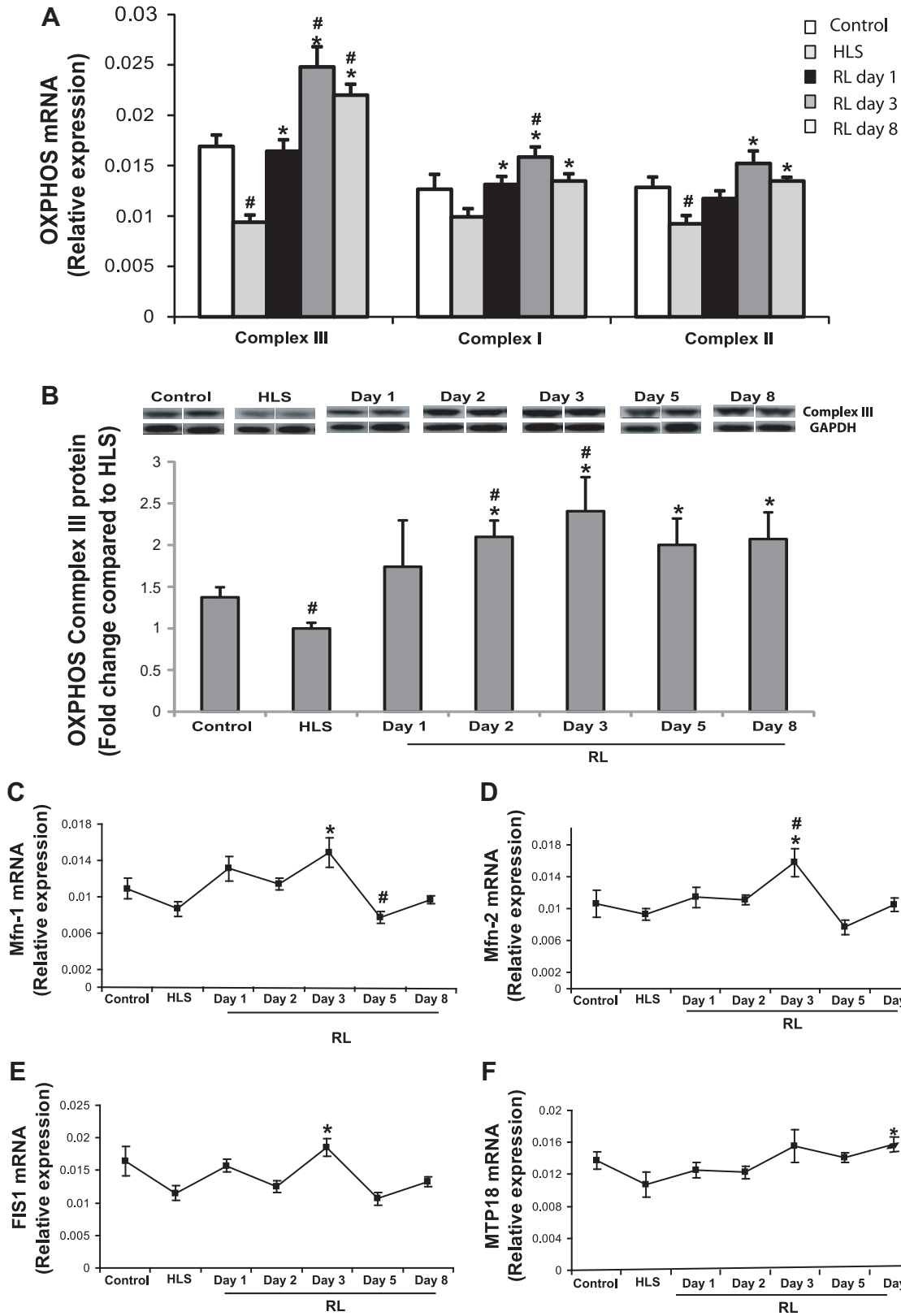


Fig. 3. Expression of oxidative phosphorylation (OXPHOS) and mitochondrial fusion and fission genes during HLS and RL. *A–F*: OXPHOS gene expression levels (*A*), OXPHOS complex III protein content (*B*), and mitochondrial fusion (*C* and *D*) and mitochondrial fission (*E* and *F*) gene expression levels were determined in gastrocnemius muscle of mice undergoing the HLS/RL protocol (n = 8–9/time point). Western blots were corrected for total protein content, and GAPDH was used as a loading control. Western blots and quantitative PCR (Q-PCR) data are expressed as means ± SE. Significance compared with HLS: *$P \leq 0.05$. Significance compared with baseline (control): #$P \leq 0.05$. Shown are representative Western blot images.

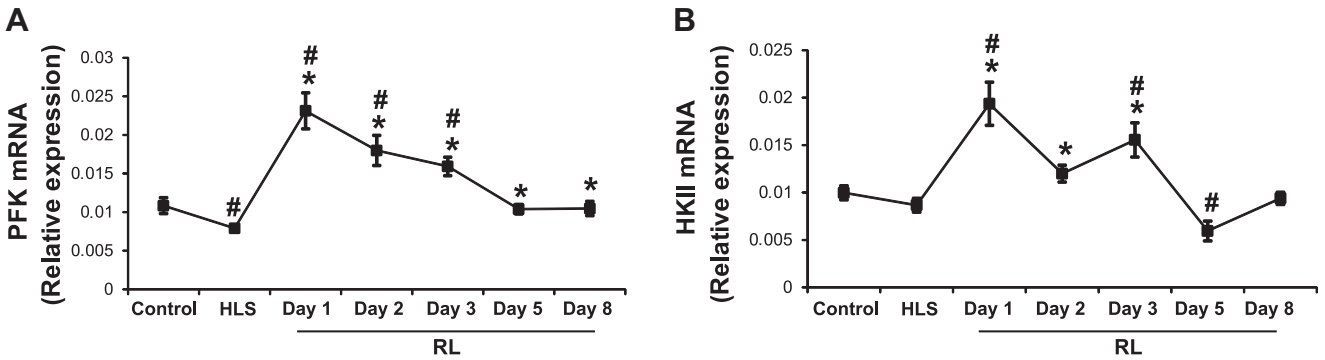


Fig. 4. Glycolytic gene expression levels during HLS and RL. Mice were subjected to a HLS (14 days) and RL (8 days) protocol (n = 8–9/time point). *A* and *B*: phosphofructokinase (PFK) and hexokinase 2 (HKII) mRNA levels were determined by Q-PCR. Values are expressed as means ± SE. Significance compared with HLS: *$P \leq 0.05$. Significance compared with baseline (control): #$P \leq 0.05$.

unloading of the musculature results in mitochondrial fragmentation (loss of tubular mitochondrial networks and appearance of swollen isolated mitochondria), loss of mitochondria, reduced mitochondrial oxygen consumption, and decreased mRNA and protein expression levels of components of the mitochondrial OXPHOS chain (23, 26, 30, 33, 39). This is in line with our finding that hindlimb suspension resulted in decreased mRNA and protein levels of several mitochondrial OXPHOS complex subunits.

In this study, we processed and analyzed whole gastrocnemius muscle, which can be considered as a mixed muscle because of the presence of both oxidative type I and IIA fibers and glycolytic type IIB and type IIX fibers (8). Although fiber-type analyses were not performed in the present study, it is known that unloading of mouse muscle results in a fiber-type shift toward a higher proportion of glycolytic fibers, whereas reloading induces a shift back toward a more oxidative fiber-type composition (25). In our study, we observed that unloading resulted in a robust decrease in MyHC I and MyHC IIA mRNA expression, whereas MyHC IIB and MyHC IIX levels were relatively less affected. This is in line with an unloading-induced fiber-type shift from slow to fast as described by several reports (25, 29, 42) but may also reflect a more pronounced atrophy of type I and IIA fibers, since atrophy of oxidative muscle fibers upon unloading has been described (9). In addition, decreases in MyHC IIB upon unloading are in contrast with a previous study reporting increased MyHC IIB mRNA levels upon unloading (14). The reason for this discrepancy is unclear, but loss of MyHC IIB upon hindlimb suspension in our study may reflect atrophy of type IIB fibers, in line with the observed loss of gastrocnemius muscle weight during hindlimb suspension. Increases in MyHC I and MyHC IIA mRNA during reloading were more pronounced and more sustained in time compared with reloading-induced increases in MyHC IIB mRNA, which is in line with previous studies reporting that reloading induces a fiber-type shift toward a more oxidative profile (25) but is also in coherence with our findings that progression toward a more OXPHEN is potently induced in the recovery phase.

A multitude of transcription factors, all coactivated by PGC-1 molecules, regulate muscle OXPHEN in response to altered physical activity levels (7). Several studies have investigated the impact of unloading on expression levels of these regulators. Decreased expression levels of PGC-1α and PGC-1β but also of the PGC-1-related cofactor upon unloading have been consistently reported (23, 27, 45). Although we did observe some variability in mRNA expression levels of several OXPHEN regulators in response to hindlimb suspension, our finding that hindlimb suspension induced significant decreases in PGC-1β mRNA expression levels is in line with previous reports. In addition, we observed that unloading significantly decreased mRNA expression levels of Tfam and ERR-α, which is in line with data from Wagatsuma et al. who reported decreased expression levels of these regulators in soleus muscle of hindlimb-suspended rats (45). Interestingly, decreased expression levels of PGC-1 molecules and ERR-α are also observed in other models of disuse-induced loss of muscle OXPHEN such as denervation and spaceflight-induced gravitational unloading, suggesting that disuse of the musculature in broad terms initiates a conserved transcriptional program that leads to a coordinated downregulation of muscle OXPHEN (1, 35, 46). Although the focus of this study was not to investigate the impact of disuse on muscle OXPHEN and its molecular regulation, we identified that only a subset of the regulators we investigated shows decreased expression levels upon unloading (PGC-1β, Tfam, and ERR-α), whereas the mRNA abundance of other regulatory molecules such as NRF-1, NRF-2α, PPAR-δ, and SIRT-1 remained unaltered. Also, whereas Tfam protein was unaltered, NRF-1 protein content increased upon hindlimb suspension, which may represent an adaptive, compensatory response aimed at regulating fuel utilization and maintaining metabolic flexibility upon unloading. Because Tfam and NRF-1 protein did not decrease upon unloading, other factors involved in the regulation of muscle OXPHEN must be responsible for loss of muscle OXPHEN upon unloading. In general, the unloading-induced alterations in mRNA expression levels of constituents of PGC-1 signaling are in line with studies from Cassano et al. and a report from Wagatsuma et al. that showed that, although ERR-α and Tfam mRNA levels were downregulated upon unloading, NRF-1 mRNA levels were unaltered in rat soleus muscle and mouse gastrocnemius muscle, respectively, after 14 or 7 days of unloading (12, 45). Qi et al., on the other hand, demonstrated that NRF-1 mRNA levels were potently decreased in rat skeletal muscle after 3 wk of hindlimb suspension (31). No studies so far have investigated the response of Tfam and NRF-1 protein levels upon changes in loading of the musculature. Importantly, data collected in previous studies indicate that the extent of the

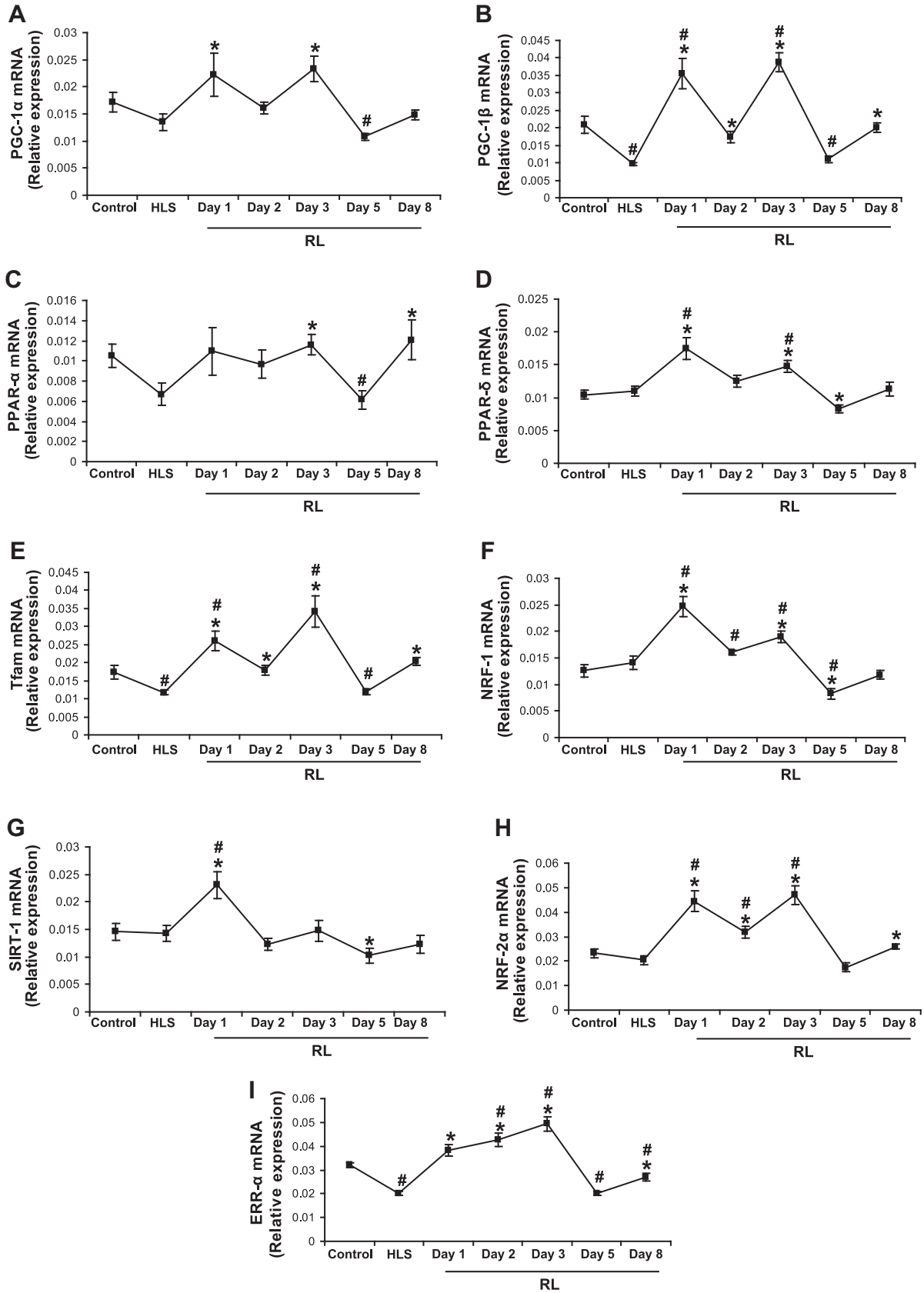


Fig. 5. Regulation of muscle oxidative phenotype during HLS and RL. *A–I*: gene expression levels of known regulators of muscle oxidative phenotype were determined in gastrocnemius muscle of mice undergoing the HLS/RL protocol (n = 8–9/time point). PPAR, peroxisome proliferator-activated receptor-γ; PGC, PPAR-γ coactivator; Tfam, mitochondrial transcription factor A; NRF, nuclear respiratory factor 1; SIRT-1, sirtuin 1; ERR-α, estrogen-related receptor α. Values are expressed as means ± SE. Significance compared with HLS: *$P \leq 0.05$. Significance compared with baseline (control): #$P \leq 0.05$.

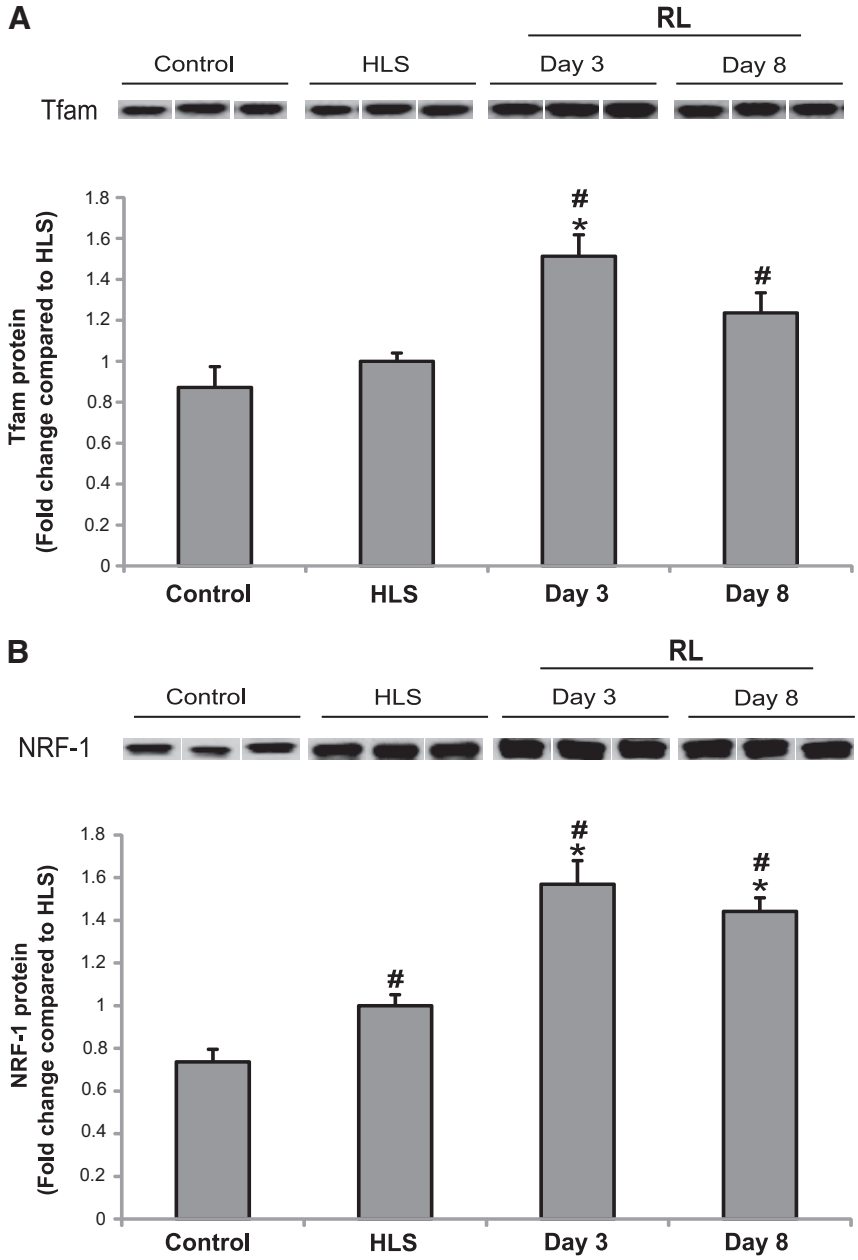


Fig. 6. Tfam and NRF-1 protein levels increase upon RL. *A* and *B*: protein levels of known regulators of muscle oxidative phenotype (Tfam and NRF-1) were determined in gastrocnemius muscle of mice undergoing the HLS/RL protocol (n = 8–9/time point). Western blots were corrected for Ponceau S staining to ensure equal loading. Values are expressed as means ± SE. Significance compared with HLS: *$P \leq 0.05$. Significance compared with baseline (control): #$P \leq 0.05$. Shown are representative Western blot images.

duration of the unloading period may account for differences between studies. In line with this notion, it was reported that 24 h of unloading increased PPAR-δ mRNA levels in rat soleus, whereas PPAR-δ transcript levels were unaltered after a 3-wk period of unloading (24). Consistent with this, PPAR-δ transcript levels remained unaltered after 14 days of unloading in our study.

Numerous studies have shown an increased reliance of muscle on glucose for energy production during hindlimb suspension. Indeed, enzymatic activity of the glycolytic enzymes HKII and PFK was shown to be increased upon unloading (13, 17, 41). In our study, however, hindlimb suspension failed to affect HKII mRNA levels, whereas PFK mRNA levels decreased. Noteworthy, the activity of these enzymes is not exclusively regulated at the transcriptional levels but is also subject to allosteric regulation by, e.g., metabolite levels, which may explain the apparent discrepancy with previous reports. In addition, reloading in our study enhanced glycolytic gene expression, which is in line with a previous report demonstrating increased transcript levels of genes involved in glucose uptake and glycolysis upon reloading (14). In concert with our data, this suggests that increases in glycolytic capacity are an integrated part of reloading-induced transcriptional reprogramming of skeletal muscle, which may serve to meet the metabolic requirements of the tissue during reloading-induced tissue remodeling.

In contrast to unloading-induced decreases in muscle OXPHEN, surprisingly little attention has been paid to the response of the muscle's metabolic profile to reloading after a prolonged period of unloading/inactivity. To the best of our knowledge, only a few studies previously investigated the response of skeletal muscle OXPHEN to reloading after a

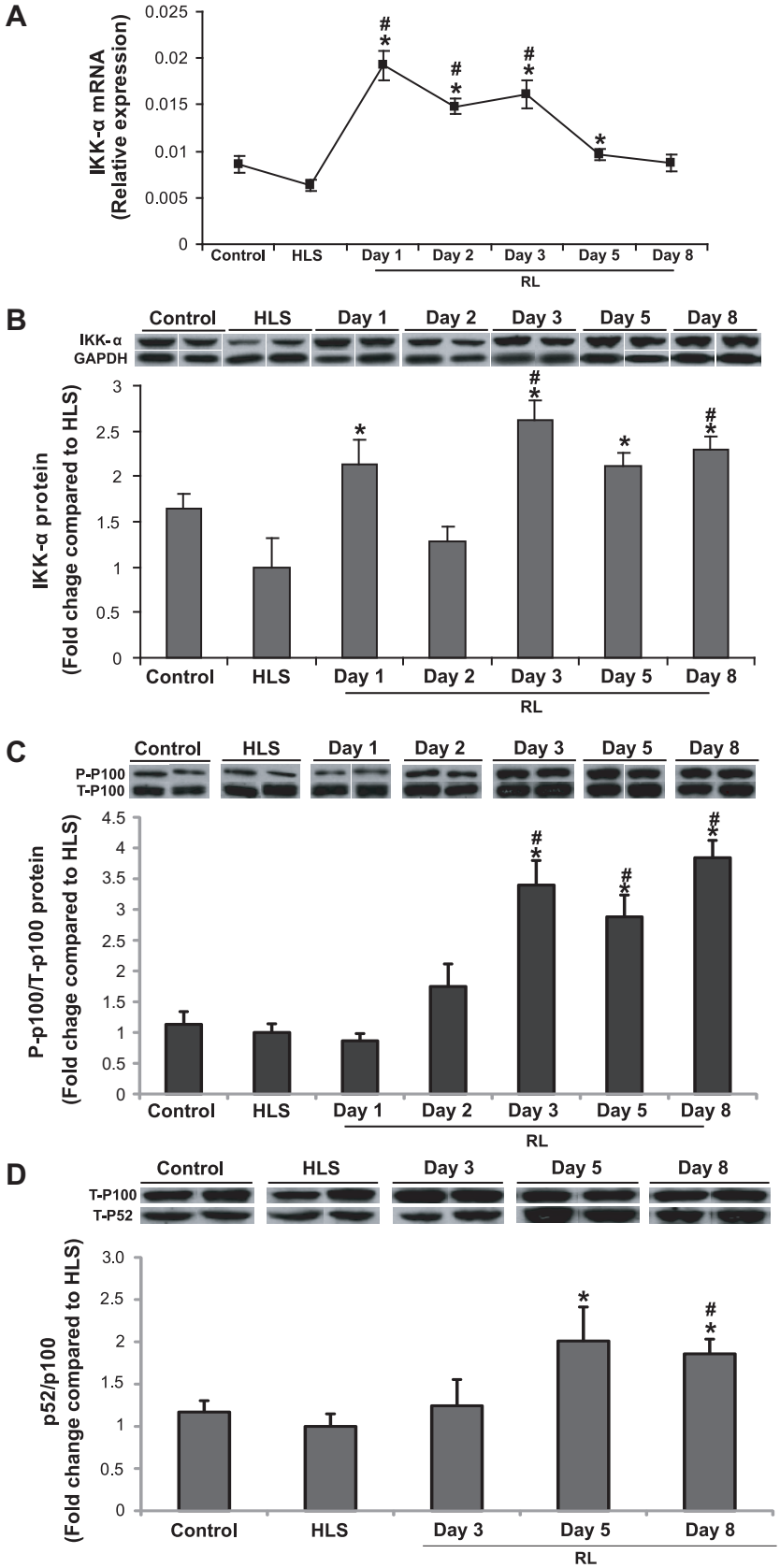


Fig. 7. Activation of the alternative NF-κB pathway during RL. *A*: IKK-α mRNA expression levels were determined in gastrocnemius muscle of mice undergoing the HLS/RL protocol (n = 8–9/time point). *B–D*: IKK-α, total P100, phosphorylated P100, and total P52 were determined in gastrocnemius muscle by Western blot (n = 4–9/time point). Western blots were corrected for total protein content, and GAPDH was used as a loading control. Western blot and Q-PCR data are expressed as means ± SE. Significance compared with HLS: $*P \leq 0.05$. Significance compared with baseline (control): $\#P \leq 0.05$. Shown are representative Western blot images.

period of hindlimb suspension (14, 27). In line with previous reports, these authors demonstrated that muscle OXPHEN was impaired during unloading evidenced by decreased protein levels of cytochrome *c* oxidase subunit IV (COXIV) and decreased mRNA expression levels of several other OXPHOS complexes. Upon reloading, protein levels of COXIV and OXPHOS mRNA expression levels gradually increased to approximate baseline levels. This is in line with our finding that OXPHOS mRNA and OXPHOS protein levels increased upon muscle reloading. Several reports have demonstrated a direct correlation between the extent of mitochondrial fusion and the capacity for OXPHOS (16, 44). In this context, we report, for the first time, that expression levels of mitochondrial fusion genes mfn-1 and mfn-2 are (transiently) induced upon reloading. Two previous reports showed that acute exercise in rodents and humans results in increased expression levels of both mfn-1 and mfn-2 fusion proteins and suggested that this was implicated in exercise-induced upregulation of muscle OXPHEN (11, 16). Because we also observed an increased FIS1 (mitochondrial fission) mRNA abundance in response to reloading, this suggests a role for altered mitochondrial dynamics in reloading-induced restoration of muscle OXPHEN. Furthermore, increased mitochondrial fusion gene expression is in line with our observation that reloading induced muscle PGC-1α mRNA levels, since PGC-1α has been shown to control transcription of mitochondrial fusion genes (40, 49). Besides confirming a reloading-induced increase in PGC-1α levels, as previously reported (27), we also observed that a multitude of molecular OXPHEN regulators, known to be coactivated by PGC-1, displayed a reloading-induced increase in their mRNA transcript abundance and protein level. Interestingly, reloading-induced expression profiles of known regulators of muscle OXPHEN occurred in a temporally distinct fashion. Indeed, whereas PPAR-δ, NRF-1, and SIRT-1 mRNA levels displayed a transient increase in expression, after which expression levels decreased to baseline levels or below, mRNA transcript abundance of other regulators, including PGC-1β, Tfam, and NRF-2α, displayed a more robust and biphasic increase during the reloading protocol. In line with mRNA data, Tfam and NRF-1 protein levels were significantly increased as from *day 3* of the reloading protocol. With regard to reloading-induced changes in mRNA levels of known regulators of muscle OXHEN, increases in PGC-1β expression upon reloading in particular were more pronounced (in terms of fold change) compared with the response of other regulators. Because alternative NF-κB signaling in muscle was shown to control PGC-1β expression (5), this is in line with the strong induction of alternative NF-κB activation that we observed in response to muscle reloading. Indeed, phosphorylation of P100 and subsequent processing of P100 to P52, as markers for alternative NF-κB activation, were significantly elevated in response to reloading, evidencing potent activation of alternative NF-κB signaling. Of note, GAPDH protein levels slightly increased during the reloading protocol, most likely because of its known involvement in the glycolytic pathway (which is induced by reloading in our study). Therefore, inductions of OXPHOS complex III protein content but also increases in IKK-α protein levels upon reloading likely represent a slight underestimation of the actual induction. As mentioned above, Bakkar et al. recently identified PGC-1β as a direct transcriptional target of the alternative NF-κB pathway (5). Consequently, these authors observed that knock down of IKK-α in mouse muscle potently impaired muscle OXPHEN and decreased PGC-1β expression levels. Conversely, overexpression of IKK-α increased muscle OXPHEN and PGC-1β mRNA levels (5).

The response of muscle alternative NF-κB signaling to altered physical activity levels thus far was unexplored. Hence, this is the first report demonstrating activation of alternative NF-κB signaling in skeletal muscle in response to resumed physical activity after a period of unloading, which coincided with reloading-induced restoration of muscle OXPHEN. Reloading-induced upregulation of muscle OXPHEN may well involve myogenesis-induced development of muscle OXPHEN. Indeed, muscle reloading has been shown to be associated with muscle regeneration, which involves myogenesis (19, 37, 43). Moreover, we and others have convincingly shown, not only that myogenesis and muscle regeneration are associated with a potent induction of mitochondrial biogenesis and activation of PGC-1-coactivated signaling events (32, 47), but also that myogenesis and myogenesis-induced activation of oxidative metabolism are highly interdependent processes (18, 34). Although a causal involvement of activated alternative NF-κB signaling in reloading-induced activation of muscle OXPHEN cannot be concluded from our experiment, our data and the recently identified role of the alternative NF-κB pathway in the control of muscle OXPHEN (5) and the observation that alternative NF-κB activation is potently induced during myogenesis (6) in concert with myogenesis-induced OXPHEN development strongly suggest a role for the alternative NF-κB signaling pathway in reloading-induced activation of muscle OXPHEN. In contrast to reloading-induced activation of alternative NF-κB signaling, muscle unloading did not affect activity of the alternative NF-κB pathway. This suggests that, although the reloading-induced increases in muscle OXPHEN and PGC-1 signaling may well be mediated by activation of the alternative NF-κB pathway, unloading-induced decreases in PGC-1β and OXPHEN appear not to be the consequence of reduced alternative NF-κB signaling, which suggests involvement of signaling mechanisms independent of the alternative NF-κB pathway.

Of interest, we observed that increases in OXPHOS expression levels and in expression levels of PGC-1 signaling constituents as well as inductions in alternative NF-κB signaling induced by reloading displayed an "overshoot" compared with the baseline condition. This is in line with data from Däpp et al. (14) and suggests that increasing expression levels and activity of pathways involved in muscle OXPHEN regulation above baseline levels is part of an integrated response of muscle signaling pathways to ensure adequate restoration of muscle OXPHEN during recovery from unloading.

In conclusion, reloading-induced upregulation of muscle OXPHEN is clearly associated with potent activation of the alternative NF-κB signaling pathway. Because IKK-α has been convincingly shown to control muscle OXPHEN (5), this suggests an important role for IKK-α and alternative NF-κB signaling in the recovery of muscle OXPHEN following disuse. Whether or not the alternative NF-κB pathway in muscle can be modulated pharmacologically and whether this can be exploited to improve OXPHEN restoration in conditions in

which increasing physical activity is impaired (e.g., in chronic disease) remains to be determined.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: A.H.R. and N.A.M.P. conception and design of research; A.H.R. and N.A.M.P. performed experiments; A.H.R. and N.A.M.P. analyzed data; A.H.R. interpreted results of experiments; A.H.R. prepared figures; A.H.R. drafted manuscript; A.H.R., H.R.G., A.M.S., and R.C.L. edited and revised manuscript; A.H.R., H.R.G., A.M.S., and R.C.L. approved final version of manuscript.

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