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Differential gene expression of muscle-specific ubiquitin ligase MAFbx/Atrogin-1 and MuRF1 in response to immobilization-induced atrophy of slow-twitch and fast-twitch muscles

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Abstract We examined muscle-specific ubiquitin ligases MAFbx/Atrogin-1 and MuRF1 gene expression resulting from immobilization-induced skeletal muscle atrophy of slow-twitch soleus and fast-twitch plantaris muscles. Male C57BL/6 mice were subjected to hindlimb immobilization, which induced similar percentage decreases in muscle mass in the soleus and plantaris muscles. Expression of MAFbx/Atrogin-1 and MuRF1 was significantly greater in the plantaris muscle than in the soleus muscle during the early stage of atrophy. After a 3-day period of atrophy, total FOXO3a protein level had increased in both muscles, while phosphorylated FOXO3a protein had decreased in the plantaris muscle, but not in the soleus muscle. PGC-1α protein expression did not change following immobilization in both muscles, but basal PGC-1α protein in the soleus was markedly higher than that in plantaris muscles. These data suggest that although soleus and plantaris muscles atrophied to a similar extent and that muscle-specific ubiquitin protein ligases (E3) may contribute more to the atrophy of fast-twitch muscle than to that of slow-twitch muscle during immobilization.

Keywords Fiber type · Immobilization · Muscle-specific ubiquitin ligase · Skeletal muscle atrophy

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Introduction

Skeletal muscle atrophy occurs in many catabolic conditions, including fasting, cancer, sepsis, diabetes, and reduced mechanical loading [1–6]. However, muscle atrophy does not occur similarly in all types of muscle fibers. Skeletal muscles of different fiber type composition have different contractile and metabolic properties. Oxidative muscles (predominantly slow-twitch fibers) are generally rich in mitochondria and capillaries, while glycolytic muscles (predominantly fast-twitch fibers) are not. Oxidative muscles are more resistant to atrophy than glycolytic muscles; however, much remains unknown regarding the molecular mechanism(s) of fiber type-specific muscle atrophy. During fasting or exposure to glucocorticoids, sepsis, and cancer cachexia, glycolytic muscle (fast-twitch) fibers show greater atrophy than oxidative (slow-twitch) fibers [7–12]. Conversely, during hindlimb suspension, which is a commonly used animal model of reduced mechanical loading to mimic prolonged spaceflight, slow-twitch muscles often show more pronounced atrophy than fast-twitch muscles [13, 14]. Some exceptions do exist, however; hindlimb immobilization induces similar percentage decreases in muscle mass in both slow and fast muscles [15, 16]. Data from human bed rest studies have also demonstrated that fast-twitch fiber cross-sectional area markedly declines in vastus lateralis [17].

Muscle atrophy results from an imbalance between protein synthesis and breakdown rates. An increased rate of proteolysis is a major step in muscle atrophy induced by reduced mechanical loading, whereas the rate of protein synthesis is not always reduced [18]. Three intracellular proteolytic systems, the lysosomal (cathepsins), the Ca^{2+}-dependent proteinases (calpains), and the ubiquitin–proteasome system, play major roles in muscle protein

degradation. Although the lysosomal and Ca^{2+}-dependent proteolytic pathways may contribute to muscle protein degradation, the ubiquitin–proteasome pathway is most important in the breakdown of intracellular proteins, including myofibrillar proteins, during reduced mechanical loading [19, 20]. Proteins destined for degradation by the ubiquitin–proteasome pathway are covalently linked to a chain of ubiquitin (Ub) molecules, which marks them for rapid breakdown to short peptides by the 26S proteasome [21]. The key enzyme responsible for attaching ubiquitin to protein substrates is a ubiquitin-protein ligase (E3) that catalyzes the transfer of an activated form of ubiquitin from a specific ubiquitin-carrier protein (E2) to a lysine residue on the substrate.

Two muscle-specific ubiquitin ligases (E3s), MAFbx (muscle atrophy F-box)/Atrogin-1 and muscle ring finger 1 (MuRF1), are upregulated in different models of muscle atrophy and are responsible for the increased protein degradation through the ubiquitin–proteasome system [22, 23]. The expression of these genes now serves as an early marker of skeletal muscle atrophy, and accordingly, these genes are considered to be master regulators of muscle atrophy. The expression of MAFbx/Atrogin-1 and MuRF1 is regulated by the FOXO family of transcription factors [24, 25]. FOXOs comprise three isoforms, denoted FOXO1, FOXO3a, and FOXO4. FOXO phosphorylation promotes the export of FOXOs from the nucleus to the cytoplasm. The translocation and activity of FOXO is required for the upregulation of MAFbx/Atrogin-1 and MuRF1. FOXO3 promotes MAFbx/Atrogin-1 expression and muscle atrophy when transfected in skeletal muscles in vivo [24]. Sandri et al. [26] showed that PGC-1α, the master regulatory gene for mitochondria biogenesis and slow-twitch fiber formation, protects against atrophy in response to denervation and fasting by inhibiting FOXO-dependent transcription. PGC-1α expression is downregulated in different models of muscle atrophy, including diabetes, uremia, cancer cachexia, and chronic heart failure [26, 27]. Taken together, these findings indicate that muscle atrophy is an active process controlled by a specific signaling pathway and transcriptional program.

Patterns of MAFbx/Atrogin-1 and MuRF1 gene expression have been studied using predominantly fast-twitch muscle in atrophy models, including fasting and cancer models [23, 28]. In muscle atrophy resulting from sepsis or chronic heart failure, MAFbx/Atrogin-1 and MuRF1 are expressed to a greater extent in the predominantly fast-twitch white vastus muscle than in the predominantly slow-twitch soleus muscle [27, 29]. Conversely, with fasting and cancer, increased levels of FOXOs are found predominantly in fast-twitch muscle [28, 30, 31]. These findings suggest that muscle-specific ubiquitin ligases and FOXOs may contribute more to

skeletal muscle atrophy in fast-twitch muscle fibers than in slow-twitch muscle fibers in various muscle atrophy models. Authors of studies using models of inactivity-induced atrophy have reported that both muscle-specific ubiquitin ligase and FOXO expression are upregulated in the slow-twitch fiber soleus and/or the mixed fiber-type gastrocnemius [32, 33]. However, to the best of our knowledge, there are as yet no available data on whether the gene expression kinetics of MAFbx/Atrogin-1 and MuRF1 is muscle fiber specific in response to immobilization-induced muscle atrophy.

The aim of this study was, therefore, to examine changes in the expression of muscle-specific ubiquitin ligase genes and FOXO3a and PGC-1α proteins controlling the expression of muscle-specific E3s in both slow and fast muscles following immobilization. We hypothesized that following hindlimb immobilization, muscle mass would be similar, but MAFbx/Atrogin-1 and MuRF1 mRNA expression would be markedly increased in fast muscle compared with slow muscle.

Methods

Animals

Fifty-eight 10-week old male C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). The animals were allowed to acclimatize to their new surrounding for 1 week before the study. All animals were maintained under a 12/12-h light/dark photocycle during the hindlimb immobilization experiment. Mouse chow and water were provided ad libitum. All animal experiments were conducted in compliance with the ethical requirements of the Animal Committee at Tokai University.

Hindlimb immobilization

Hindlimb immobilization of the mice was performed according to previously described procedures [2, 16]. Briefly, the mice were lightly anaesthetized with diethyl ether for attachment of the casting material. Both the right and left hindlimbs of the animals were fixed in a shortened position with casting tape (Scotchcast Plus-J; 3M Health Care, St. Paul, MN). After casting, the mice were housed two animals per cage and provided free access to standard mouse chow and water. The animals were checked daily for damage to casting material, which was subsequently repaired as necessary. Immobilization was imposed for 0 (as the control), 3, 7, 14, and 21 days. At the end of the immobilization, casts were removed under pentobarbital sodium anesthesia (50 mg/kg), and hindlimb skeletal muscles (soleus and plantaris) from both legs were

carefully dissected, weighed, and frozen in liquid nitrogen for storage at −80°C. Both right and left hindlimb muscles from individual animals were pooled for preparation of the homogenate and for RNA isolation and protein extraction.

RNA isolation and real-time reverse transcription-PCR

Total RNA was isolated from soleus and plantaris muscles tissues using TRIzol Reagent (Invitrogen Japan, Tokyo, Japan) following the manufacturer's protocol. Each pair of muscles was homogenized in 1 mL TRIzol reagent with a handheld homogenizer (PRO200 Homogenizer; PRO Scientific, Oxford, CT) and then allowed to stand for 5 min at room temperature. Next, 2 mL of chloroform was added to the homogenate, which was shaken vigorously for 15 s and then kept at room temperature for 2–3 min. The mixture was then centrifuged at 14,000 g for 15 min at 4°C and the aqueous phase (about 0.5 mL) transferred to a fresh tube, following which 2 μL glycogen (Nacalai Tesque, Kyoto, Japan) was added to improve RNA precipitation. Isopropanol (0.5 mL) was added to this aqueous solution to precipitate the RNA. After standing at room temperature for 10 min, the tube was centrifuged at 20,000 g for 30 min at 4°C. The RNA pellet recovered was washed with 1 mL of 75% ethanol and subsequently centrifuged at 20,000 g for 5 min at 4°C. The RNA pellet was then air-dried for 5 min and the RNA dissolved in RNase-free water. RNA extracts were treated with DNase (Invitrogen Japan) to remove any residual genomic DNA. The purity of the RNA was determined by calculating the absorbance ratio at 260 and 280 nm. RNA concentrations were determined by measuring absorbance at 260 nm. For each sample, cDNA was synthesized from 1 μg of total RNA using components from a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Reverse transcription (RT) was performed first at 25°C for 10 min, then at 37°C for 120 min and finally at 85°C for 5 s.

Real-time PCR was performed to analyze the expression of mRNAs for MAFbx/Atrogin-1 and MuRF1 using the Applied Biosystems 7500 Fast Real-time PCR System. The PCR reactions were performed in a total volume of 10 μL that included 5 μL TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.5 μL of the primer and probe mix, and 4.5 μL cDNA. PCR analyses were carried out using the following cycle parameters: one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, with a final cycle of 63°C for 1 min. The results were expressed using the comparative cycle threshold (Ct) method as described in User Bulletin No. 2 (Applied Biosystems). The Ct or threshold cycle represents the polymerase PCR cycle at which an increase in reporter fluorescence above a baseline signal can initially be detected. The ΔCt values were calculated for each gene of interest as follows: Ct (gene of interest) − Ct (internal control gene). The housekeeping gene 18 s rRNA was used as the internal control gene. Relative changes in the expression levels of one specific gene (ΔΔCt) were calculated by subtracting the ΔCt of the soleus control group (used as the calibrator) from the corresponding immobilization groups (7, 14, and 21 days). The values shown in Figs. 2a and b and 3a and b were determined as follows: $2^{-\Delta\Delta Ct}$, and the soleus control was arbitrarily set to 1. The slopes of amplification curves were not different between groups in mRNA analysis, and differences in amplification efficiency were not observed. Primer and probe sets for MAFbx/Atrogin-1 (Mm00499518) and MuRF1 (Mm01188690) were purchased from Applied Biosystems.

Protein isolation and Western blots

Protein isolation and western blots were performed according to previously described procedures [34, 35]. In brief, each pair of muscles was homogenized on ice with buffer [50 mM HEPES pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$–H_2O, 100 mM beta-glycerophosphate, 25 mM NaF, protease inhibitor (Roche Diagnostics, Tokyo, Japan) and phosphatase inhibitor (Roche Diagnostics]. Following homogenization, the protein concentrations of the samples were measured using a BCA Protein Assay kit (PIERCE, Pittsburgh, PA). Protein samples were solubilized at a concentration of 1.75 mg/mL in loading buffer [62.5 mM Tris–HCl pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 0.025% bromphenol blue] and boiled for 5 min. Total protein was then loaded (20 μg/lane) onto 8% SDS–polyacrylamide gel electrophoresis gels. Separated proteins were then transferred onto nitrocellulose membranes. To verify the transfer of proteins and equal loading of lanes, the membranes were stained with Ponceau S (Sigma-Aldrich Japan, Tokyo, Japan), which allows for both the qualitative visualization and quantitation of the amount or protein in a given lane [36].

The primary antibody PGC-1α (AB3242) was purchased from Chemicon International (Temecula, CA). The primary antibodies [total-FOXO3a (#2497S) and phospho-Ser^{253}-FOXO3a (#9466S)] were purchased from Cell Signaling Technology Japan (Tokyo, Japan). All membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBST). The membranes were then probed overnight with the appropriate antibody. Antibodies were used at the following concentrations: PGC-1α, 1:1,000 in 5% milk–TBST; total-FOXO3a, 1:1,000 in 5% bovine serum albumin (BSA)–TBST; phospho-FOXO3a, 1:1,000 in 5% BSA–TBST. After a second serial wash with TBST, the membranes were incubated with both a horseradish peroxidase (HRP)-

conjugated secondary antibody [anti-rabbit immunoglobulin (IgG; 1:2,000; GE Healthcare Japan, Tokyo, Japan)] and a HRP-linked anti-biotin antibody (1:10,000, Cell Signaling Technology, Beverly, MA) in blocking buffer for 1 h, followed by another serial wash with TBST. Immunocomplexes were visualized using the enhanced chemiluminescence reagent (ECL Plus Western blotting detection reagents: GE Healthcare Japan). Finally, enhanced signals were detected by Light-Capture II (AE-6981; ATTO, Tokyo, Japan). The signal was analyzed using ATTO densitograph software library (CS Analyzer ver 3.0).

Statistics

The data are shown as the mean ± standard error (SE). The data were analyzed for the homogeneity of variance using Levene's test, and based on the results, we determined the appropriate statistical test. Differences in muscle mass and total protein content in soleus and plantaris muscles at 0, 3, 7, 14, and 21 days of hindlimb immobilization were assessed by two-way analysis of variance (ANOVA) with the Bonferroni's post hoc test. Differences in mRNA and protein expression at 0, 3, 7, 14, and 21 days of hindlimb immobilization were assessed by one-way ANOVA with the Dunnett's post hoc test. If homogeneity of variance was not observed, effects of immobilization on mRNA and protein expressions were analyzed by the Kruskal–Wallis non-parametric test. When the P values for the overall comparisons were <0.05 in the Kruskal–Wallis test, we performed post hoc pairwise comparisons by using Bonferroni's adjusted Mann–Whitney U test. Differences in mRNA expression and protein expressions between control and 3 days of immobilization were assessed suing the Mann–Whitney U test. All statistical significance is defined at P < 0.05, except for Bonferroni's adjusted Mann–Whitney U test. Statistical calculations were performed using SPSS ver. 2 (SPSS, Chicago, IL).

Results

Muscle mass and total protein content

Changes in muscle mass and total protein content after 3, 7, 14, and 21 days of hindlimb immobilization are shown in Fig. 1a and b. Soleus and plantaris muscle mass decreased significantly after 3, 7, 14, and 21 days of immobilization (Fig. 1a): in comparison with the control group, soleus muscle mass decreased by 11.4, 20.3, 31.2, and 35.9%, respectively, at these four time points and plantaris muscle mass decreased by 11.4, 17.3, 28.3, and 31.0%, respectively. There was no statistically significant difference in the change of muscle mass between soleus and plantaris muscles throughout the entire immobilization period. There was a significant difference in mass in both muscles between 7 and 14 days of immobilization, but not between 14 and 21 days. These data indicate that the immobilization procedure induced the greatest atrophy in both muscles by day 14, and thereafter induced mild atrophy.
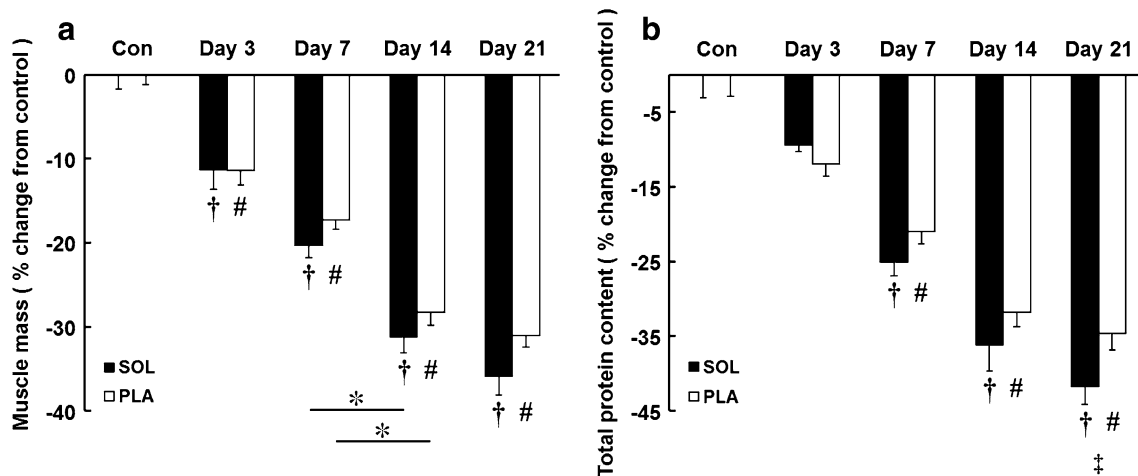

Fig. 1 Effects of 3, 7, 14, and 21 days of hindlimb immobilization on muscle mass (a) and total protein content (b) in soleus and plantaris muscles. SOL Soleus muscle, PLA plantaris muscle, Con control, Day 3, 7, 14, 21 period of hindlimb immobilization. The data are shown as the percentage change from the control group for each muscle. Each bar represents the mean ± standard error (SE) (a n = 9–17/group, b n = 5–12/group). Differences in muscle mass and total protein content were assessed by two-way analysis of variance (ANOVA) with the Bonferroni's post hoc test. Significant differences from those of the control group for each muscle: SOL †P < 0.05; PLA #P < 0.05. Asterisk (*) indicates significant differences (P < 0.05) between day 7 and 14 of hindlimb immobilization in both soleus and plantaris muscles. Double plus sign (‡) indicates significant differences between soleus and plantaris muscles at each time point after immobilization (P < 0.05)

Total protein content in soleus and plantaris muscles during immobilization followed a similar pattern of change as muscle mass (Fig. 1b). At 3, 7, 14, and 21 days of immobilization, total protein in the soleus muscle had decreased by 9.4, 25.1, 36.2, and 41.7%, respectively, compared with the control group. Similarly, at the same time points plantaris muscle showed a decrease of 11.9, 21.0, 31.8, and 34.6%, respectively compared with the control group. There was no statistically significant difference in change of total protein content between soleus and plantaris muscles throughout the entire immobilization period, except for day 21 of immobilization. The changes in total protein content to atrophy induced by inactivity were qualitatively similar to changes in myofibrillar protein content in both soleus and plantaris muscles [37–39].

Expression of muscle-specific E3 genes throughout the entire immobilization period

Hindlimb immobilization increased mRNA expression of MAFbx/Atrogin-1 and MuRF1 in the soleus and plantaris muscles. MAFbx/Atrogin-1 mRNA in the soleus muscle peaked after 3 days (6.3-fold), then declined at 7 days (4.7-fold) and 14 days (2.0-fold), and returned to control levels at 21 days (Fig. 2a). In comparison, the expression of MAFbx/Atrogin-1 mRNA in plantaris muscle also peaked after 3 days (13.5-fold), then declined progressively although it remained above control levels after 14 days (5.1-fold) and 21 days (2.1-fold) (Fig. 2a). These results indicate that although both muscles atrophied by a similar percentage, immobilization induced a greater expression of MAFbx/Atrogin-1 mRNA in the predominantly fast-twitch plantaris muscle compared with the predominantly slow-twitch soleus muscle during the entire treatment. MuRF1 mRNA expression during immobilization followed a similar pattern of changes as MAFbx/Atrogin-1 mRNA (Fig. 2b). While the increases in muscle-specific E3 mRNAs were not significant at 3, 7, 14, and 21 days (based on the Kruskal–Wallis test), the expression levels of these E3s are the same or higher than statistically significant increases reported in other studies in which inactivity was imposed at one point [33, 40]. When we compared muscle-specific E3 mRNAs between the soleus and plantaris muscles after 3 days of immobilization, immobilization was found to have induced significantly a higher expression (2.2- and 1.7-fold) of MAFbx/Atrogin-1 and MuRF1 mRNA in the predominantly fast-twitch plantaris muscle compared with the predominantly slow-twitch soleus muscle (Fig. 3a, b). There were no significant differences in MAFbx/Atrogin-1 and MuRF1 mRNA between the soleus and plantaris muscles in the control groups (data not shown).
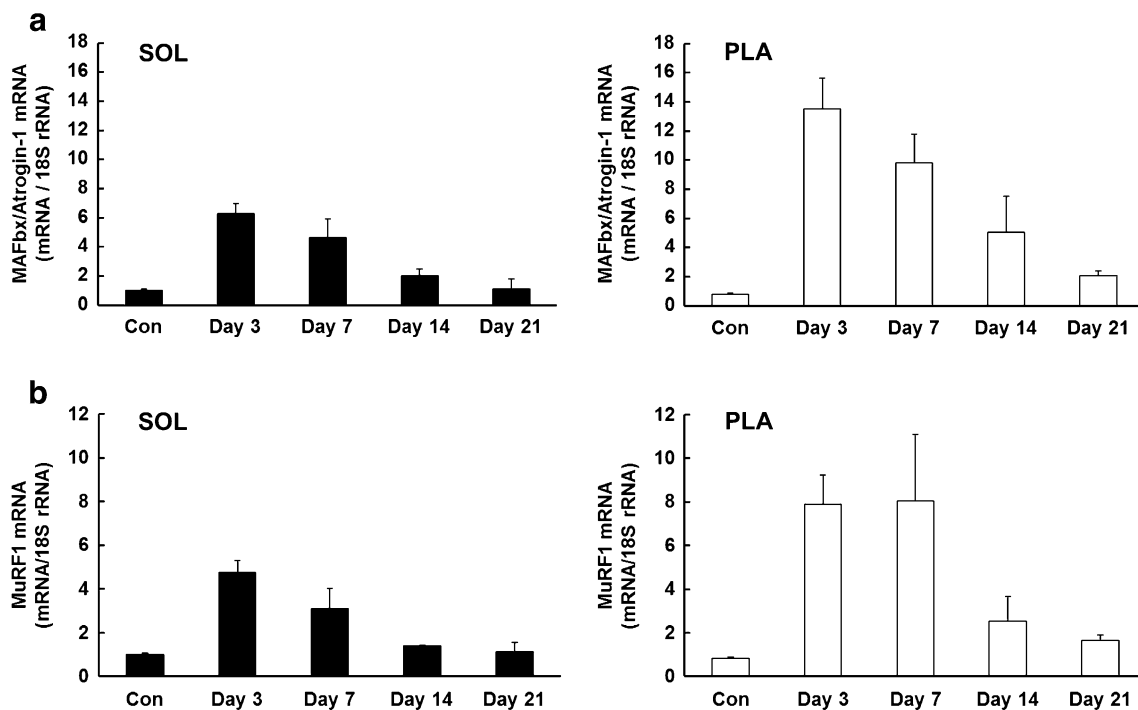

Fig. 2 Effects of 3, 7, 14, and 21 days of hindlimb immobilization on MAFbx/Atrogin-1 (a) and MuRF1 (b) mRNA expression in soleus (SOL) and plantaris (PLA) muscles. The data are shown as the relative values [to those of SOL muscle in the control group (Con)]. Each bar represents the mean ± SE ($n = 4$/group). Differences in mRNA expression were assessed by the Kruskal–Wallis non-parametric test with Bonferroni's adjusted Mann–Whitney U test because homogeneity of variance was not observed
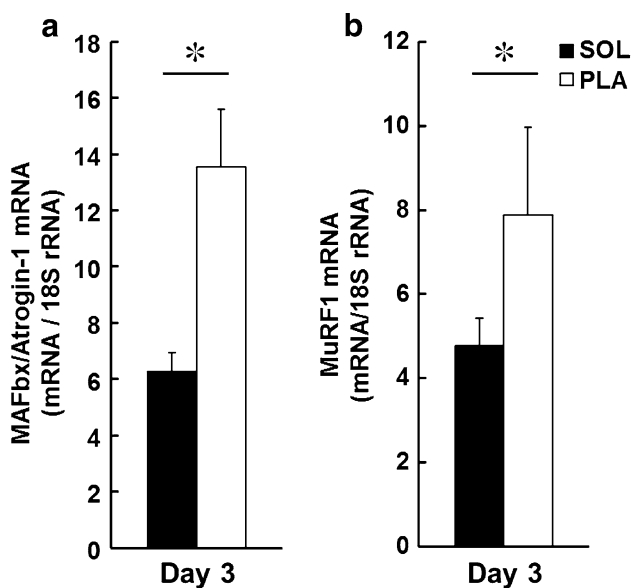
Fig. 3 Muscle atrophy F-box (*MAFbx*)/Atrogin-1 (**a**) and muscle ring finger 1 (*MuRF1*) (**b**) mRNA expression in soleus (*SOL*) and plantaris (*PLA*) muscles after 3 days of hindlimb immobilization. Differences in mRNA expression between SOL and PLA muscles after 3 days of immobilization were assessed by the Mann–Whitney *U* test. The data are shown as the relative values [to those of the SOL muscle in the control group (*Con*)]. Each *bar* represents the mean ± SE (*n* = 4/group). *Asterisk* (*) indicates significant differences (*P* < 0.05) between soleus and plantaris muscle after 3 days of hindlimb immobilization

Expression of factors (FOXO3a and PGC-1α) controlling muscle-specific E3 expression

The basal expression of FOXO3a protein in the soleus muscle was markedly greater than that in the plantaris muscle, whereas there was no significant difference in basal expression of phosphorylated FOXO3a between the soleus and plantaris muscles. On day 3 of hindlimb immobilization, total FOXO3a protein was significantly increased in the soleus (+161%) and plantaris (+168%) muscles compared to the control (Fig. 4a). In contrast, FOXO3a phosphorylation was significantly decreased in the plantaris muscle (−46%), but not in the soleus muscle (−16%) in the early stages of immobilization (Fig. 4b). On day 3 of hindlimb immobilization, the ratio of phosphorylated to total FOXO3a protein levels was significantly decreased in the soleus (−68%) and plantaris (−80%) muscles (Fig. 4c). Our comparison of the percentage change in the ratio of phosphorylated to total FOXO3a protein in the soleus and plantaris muscles after 3 days of immobilization in comparison to the control showed that immobilization induced a significant decrease of this ratio in the predominantly fast-twitch plantaris muscle compared with the predominantly slow-twitch soleus muscle (Fig. 4d). After 7 days of immobilization, total FOXO3a

protein and phosphorylation were elevated; these remained above control levels in the soleus and plantaris muscles for the duration of the experiment (data not shown).

Basal PGC-1α protein expression in the soleus muscle was markedly greater than that in plantaris muscle (Fig. 5). However, PGC-1α protein expression generally remained unchanged in both muscles after 3 days of immobilization (Fig. 5) and throughout the period of immobilization, with the exception of an increase in plantaris muscle 21 days after immobilization (data not shown).

Discussion

The muscle-specific ubiquitin ligases (E3s) MAFbx/Atrogin-1 and MuRF1 are considered to be master genes that regulate skeletal muscle atrophy. However, it is still unknown whether changes in muscle-specific E3s differ between muscle fiber types following immobilization-induced muscle atrophy. Here, we have presented data indicating the differential gene expression of MAFbx/Atrogin-1 and MuRF1 in response to immobilization-induced atrophy of slow-twitch and fast-twitch muscles. We have shown that immobilization produced similar percentage decreases in muscle mass in the slow-twitch soleus and the fast-twitch plantaris muscles, whereas MAFbx/Atrogin-1 and MuRF1 were markedly upregulated in plantaris muscle relative with soleus muscle during immobilization.

Expression of muscle-specific ubiquitin ligase mRNAs in immobilization-induced atrophied muscles

Muscle-specific E3s are induced early during the atrophy process, and the increase in muscle-specific expression of E3s precedes the loss of muscle mass [23]. Following denervation, MAFbx/Atrogin-1 and MuRF1 mRNA expression peaks at 3 days, even before muscle atrophy occurs [41, 42]. In a previous study using a cast-immobilized model, the expression of muscle-specific E3 mRNAs peaked at 3 days in the mixed fiber-type gastrocnemius [18]. In our study, MAFbx/Atrogin-1 and MuRF1 mRNA expression peaked in both the soleus and plantris muscles after 3 days of immobilization, suggesting that proteolysis under the actions of muscle-specific E3s may be involved in the early stage of immobilization-induced atrophy in both muscle types. We found that plantaris muscle expressed more MAFbx/Atrogin-1 and MuRF1 mRNA than soleus muscle (Fig. 3a, b). Fast-twitch muscles are more sensitive to atrophy than slow-twitch muscles in many catabolic conditions, including fasting, cancer, sepsis, and chronic heart failure, all of which induce MAFbx/Atrogin-1 and MuRF1 expression [23, 27–29]. Although
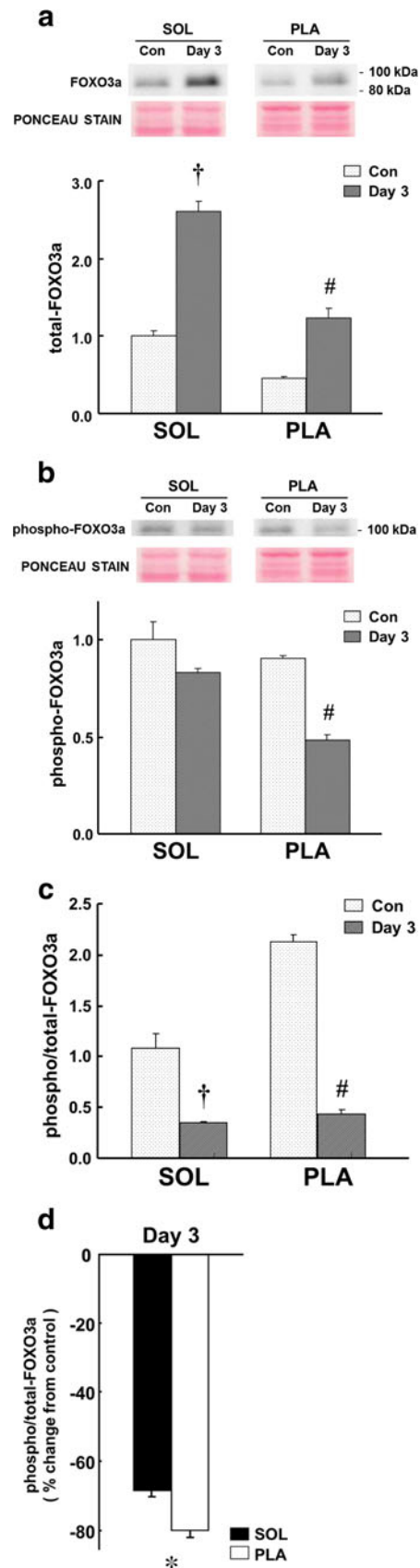
Fig. 4 Effects of 3 days of hindlimb immobilization on total ▶ FOXO3a (*total-FOXO3a*; **a**) and phosphorylated FOXO3a (*phospho-FOXO3a*; **b**) protein expression, on the ratio of phospho-FOXO3a to total-FOXO3a protein levels (**c**), and on the percentage differences in this ratio from the control group (**d**) in soleus (*SOL*) and plantaris (*PLA*) muscles. The data are shown as relative values [to those of SOL muscle in the control (*Con*) group]. Each *bar* represents the mean ± SE ($n = 3$/group). Differences in protein expression between control muscles and those subjected to 3 days of immobilization were assessed by the Mann–Whitney U test. Significant differences from those of the control group for each muscle: SOL [†]$P < 0.05$; PLA [#]$P < 0.05$. Differences in percentage change of the ratio of phospho-FOXO3a to total-FOXO3a protein levels between soleus and plantaris after 3 days of immobilization were assessed by the Mann–Whitney U test. *Asterisk* (*) indicates significant difference between ($P < 0.05$) soleus and plantaris muscle after 3 days of hindlimb immobilization

the molecular mechanisms for fiber type-specific muscle atrophy remain largely unknown, slow-twitch muscles do show markedly greater atrophy than fast-twitch muscles in the hindlimb suspension model. The hindlimb suspension model is a commonly used model to assess the effects of inactivity on skeletal muscle [13, 14]. We observed that immobilization in this study produced similar percentage decreases in muscle mass in the slow-twitch soleus muscle and the fast-twitch plantaris muscle, but that gene expression of MAFbx/Atrogin-1 and MuRF1 in the plantaris muscle was higher than that in soleus muscle during immobilization. MAFbx/Atrogin-1 and MuRF1 may therefore contribute more to skeletal muscle atrophy in fast-twitch muscle fibers than in slow-twitch muscle fibers in immobilization-induced atrophy models.

A limitation to the study was that we did not measure changes or differences in muscle development between 11 and 14 weeks of age in fast versus slow muscles. However, we found that the soleus muscle showed significant atrophy, with a low expression of muscle-specific E3 mRNAs compared with the plantasis muscle. These findings suggest that other proteolytic pathways, such as the lysosomal or calcium-dependent systems, are responsible for the remaining loss in muscle mass in soleus muscle. In support of this concept, Vermaelen et al. [43] reported that calpain autolysis was only seen in the slow-twitch soleus muscle, while there was no change in the fast-twitch plantaris muscle after 5 days of hindlimb immobilization. The relative contribution of the intracellular proteolytic systems that regulate muscle protein degradation after immobilization need to be addressed in future studies.

Expression of FOXO3a and PGC-1α proteins controlling muscle-specific E3 expression

FOXO proteins are transcription factors for muscle-specific E3s [24, 25]. FOXOs are induced under many catabolic
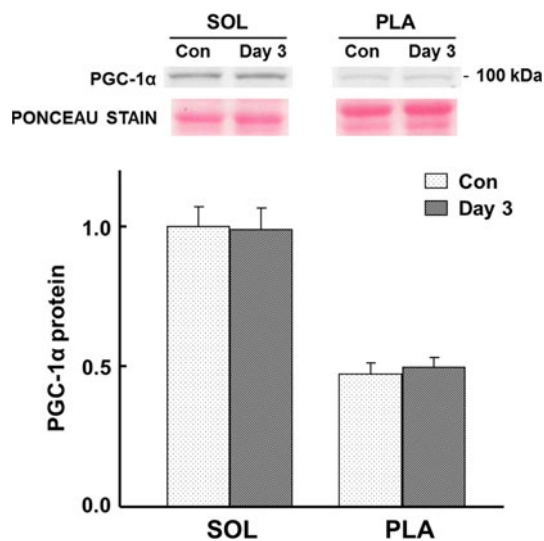
Fig. 5 Effects of 3 days of hindlimb immobilization on PGC-1α protein expression in soleus (*SOL*) and plantaris (*PLA*) muscles. The data are shown as the relative values to those of soleus muscle in the control (*Con*) group. Each *bar* represents the mean ± SE ($n = 4$–5/group). Differences in protein expression between control and 3 days of immobilization were assessed by Mann–Whitney U test

conditions, including fasting and cancer [28, 30, 31]. Studies using inactivity-induced atrophy models also report that FOXO expression in the slow-twitch soleus muscle and/or the mixed fiber-type gastrocnemius muscle is upregulated at both the mRNA and protein levels [32, 33]. However, much is unknown about the regulation of FOXO proteins in the fast-twitch muscle during immobilization-induced muscle atrophy. In our study, we found that FOXO3a protein was significantly elevated to a similar extent in both soleus and plantaris muscles in the early stages of immobilization (Fig. 4a). Based on expression patterns of muscle-specific E3 mRNAs, we initially hypothesized that the expression of FOXO3a protein would be higher in immobilized fast-twitch plantaris muscle than in immobilized slow-twitch soleus muscle. On the contrary, FOXO3a protein was higher in immobilized soleus muscle than in immobilized plantaris muscle throughout the time course of our immobilization study.

In their unphosphorylated form, FOXO transcription factors are predominantly located in the nuclear compartment where they are active and bound to DNA [44]. Phosphorylation of FOXO leads to nuclear exclusion, thereby inhibiting their transcriptional activity [24, 25]. An important observation made in our study was that only the expression of phosphorylated FOXO3a protein was markedly reduced in the plantaris muscle after 3 days of immobilization (Fig. 4b). This observation is consistent with the time point that the expression of MAFbx/Atrogin-1 mRNA was markedly upregulated in the plantaris muscle compared with the soleus muscle (Fig. 3a). As noted

above, phosphorylation of FOXO results in the inactivation and retention of FOXO in the cytoplasm. Although we did not measure nuclear FOXO3a protein, these results suggest that the translocation of FOXO to the nucleus by dephosphorylation may be preferentially induced in the plantaris muscle in the early stage of immobilization, thereby causing preferential expression of MAFbx/Atrogin-1 mRNA in the plantaris muscle compared with the soleus muscle.

Sandri et al. [26] demonstrated that PGC-1α, the master regulatory protein for mitochondria biogenesis and slow-twitch fiber formation, could protect against atrophy in response to denervation and fasting by inhibiting FOXO-dependent transcription. When the expression of PGC-1α was maintained, either by the use of transgenic mice or by transfecting adult muscle fibers, muscles were protected from the atrophy induced by denervation, fasting, or expression of FOXO3a. PGC-1α expression is downregulated in different models of muscle atrophy, including diabetes, uremia, cancer cachexia, and chronic heart failure [26, 27]. In contrast, PGC-1α expression increases in both slow-twitch and fast-twitch muscles following acute exercise and chronic training [45, 46]. Therefore, we hypothesized that immobilization would reduce PGC-1α expression in both slow-twitch and fast-twitch muscles. In our study, however, PGC-1α protein expression did not change in either muscle throughout the entire immobilization period. Basal PGC-1α protein expression in the soleus muscle was markedly greater than in the plantaris muscle, and this difference in PGC-1α protein level persisted after 21 days of immobilization. One possible explanation is that this remarkable expression of PGC-1α in slow-twitch muscle may help to suppress the transcriptional activity of FOXO3a in the nucleus during conditions of immobilization. Interestingly, immunohistochemical analysis demonstrated that 1 and 3 weeks of immobilization did not induce any shift in the myosin heavy chain (MyHC) isoform (data not shown). The lack of any change in PGC-1α protein expression could explain why the MyHC isoform did not change.

Conclusion

To the best of our knowledge, this is the first report of the muscle-specific ubiquitin ligases (E3s) MAFbx/Atrogin-1 and MuRF1 being expressed at higher levels in the predominantly fast-twitch plantaris muscle compared with the predominantly slow-twitch soleus muscle following hindlimb immobilization—despite a similar degree of muscle atrophy. Mammalian skeletal muscles have a wide distribution of muscle fiber types. If different muscle types react differently to muscle atrophy, muscle-specific treatments

should be design to suppress muscle atrophy. Future investigations should aim at identifying predominant targets of E3s in slow-twitch and fast-twitch muscles, as this information will assist in developing new treatments for muscle atrophy.

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