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Lack of caspase-3 attenuates immobilization-induced muscle atrophy and loss of tension generation along with mitigation of apoptosis and inflammation

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Abstract

Introduction—Immobilization by casting induces disuse muscle atrophy (DMA).

Methods—Using wild type (WT) and caspase-3 knockout (KO) mice, we evaluated the effect of caspase-3 on muscle mass, apoptosis and inflammation during DMA.

Results—Caspase-3 deficiency significantly attenuated muscle mass decrease [gastrocnemius: $28 \pm 1\%$ in KO vs. $41 \pm 3\%$ in WT; soleus: $47 \pm 2\%$ in KO vs. $56 \pm 2\%$ in WT; ($P < 0.05$)] and gastrocnemius twitch tension decrease ($23 \pm 4\%$ in KO vs. $36 \pm 3\%$ in WT, $P < 0.05$) at day 14 in immobilized versus contralateral hindlimb. Lack of caspase-3 decreased immobilization-induced increased apoptotic myonuclei (3.2-fold) and macrophage infiltration (2.2-fold) in soleus muscle and attenuated increased monocyte chemoattractant protein-1 mRNA expression (2-fold in KO vs. 18-fold in WT) in gastrocnemius.

Conclusion—Caspase-3 plays a key role in DMA and associated decreased tension, presumably by acting on the apoptosis and inflammation pathways.

Keywords

skeletal muscle; inflammation; caspase-3; immobilization; apoptosis

INTRODUCTION

Immobilization is an accompanying feature of critical illness, as exemplified by sepsis or burn injury. Immobilization, *per se*, can lead to muscle atrophy even in the absence of critical illness. There is decreased fiber size and muscle mass, resulting in decreased tension generation capacity.^{1–4} Pathways leading to disuse muscle atrophy (DMA) may be different or similar to those involved in critical illness myopathy, which is associated with systemic inflammation. Several mechanisms, including activation of caspases, ubiquitin–proteasome systems, apoptosis, and inflammation, have been implicated in the skeletal muscle atrophy induced by various conditions, including immobilization.^{5–13}

Caspases are a family of cysteine proteases that play a key role in apoptosis in various tissues.¹⁴ Caspase-3 is an effector caspase involved in the execution of apoptosis. Immobilization or denervation is associated with activation of caspase-3, apoptosis, and muscle loss.^{13,15–18} In addition to its role in apoptosis, caspase-3 has been shown to be

involved in proteolysis of muscle via the ubiquitin–proteasome pathway induced by diabetes and uremia in rats.¹⁹ The ubiquitin–proteasome pathway plays an important role in skeletal muscle atrophy in many muscle wasting conditions including immobilization through pinning of joints or plaster casting.^{1,20,22} Atrogin-1/Muscle Atrophy F-box (MAFbx) and muscle-specific ring finger protein 1 (MuRF1) are 2 muscle-specific E3 ligases that are upregulated in muscle disuse.²⁰

Activation of caspase-3 and inflammation occur concomitantly with immobilization.¹³ Inflammation is associated with the muscle atrophy observed in aging or disorders such as chronic heart failure and chronic obstructive pulmonary disease.^{23,24} Inflammation in most tissues, including muscle, is accompanied by macrophage infiltration and increased expression of monocyte chemoattractant protein-1 (MCP-1).^{13,25,26} MCP-1, which is secreted by monocytes/macrophages and other cell types, plays a key role in the migration of monocytes/macrophages to sites of inflammation.^{25,27,28}

Although evidence from several investigations indicates that immobilization is associated with induction of caspase-3, apoptosis, the ubiquitin–proteasome pathway, and inflammation,^{13,17,18,20} the relationships between them in DMA produced by casting remain unclear. In this study, using wild type (WT) and caspase-3 knockout (KO) mice, we tested the hypothesis that caspase-3 is essential for apoptosis and inflammation during immobilization-induced muscle wasting and reduced tension generation.

MATERIALS AND METHODS

Animals

WT C57BL/6 mice and caspase-3 KO (KO) mice on a C57BL/6 background were used. The KO mice were kindly provided by Dr. T. Mak from the Department of Immunology and Medical Biophysics, University of Toronto (Toronto, Ontario, Canada) and have been previously described.²⁹ WT mice (C57BL/6) were purchased from Harlan laboratory (Indianapolis, IN). Male mice aged 6–8 weeks, weighing 20–26 g were used. This study was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Mice were housed under a 12-hour light/dark cycle with food and water available *ad libitum*.

Immobilization model

The procedure of immobilization is a modification of the plaster casting method. Briefly, the mice were anesthetized with pentobarbital sodium (60 mg/kg) by intraperitoneal injection (i.p.). The right hindlimb was immobilized by external casting using a plastic casing tube with the knee in extension and the ankle in plantar flexion for 3, 7 or 14 days to induce maximal atrophy of the gastrocnemius and soleus muscles.^{30,31} The external plastic casing was made from a 3 ml Fisherband transfer pipette (Fisher scientific, Pittsburg, PA), by cutting off the top half of the pipette bulb and the bottom half of the stem. The length of the plastic tube was adjusted to the length of the mouse hindlimb. The casing, after sliding it over the limb, was stabilized by attaching the skin of the hindlimb to the casing using histoacryl clear skin adhesive (Aesculap, Center Valley, PA). Two bore holes ($r = 2.5$ mm) were made in the case to ensure free air flow within the tube. The contralateral limbs of immobilized mice or sham-immobilized animals were used for controls. Sham-immobilized animals received anesthesia and limb manipulation, but no immobilization.

Muscle preparation

After 3, 7 or 14 days of immobilization, the mice were euthanized. Then, gastrocnemius and soleus muscles were harvested. For biochemical analysis, the muscles were rapidly dissected and weighed. The gastrocnemius muscles were snap frozen in liquid nitrogen, while the

soleus muscles were snap frozen in isopentane pre-cooled in liquid nitrogen or fixed in formalin. Frozen gastrocnemius muscles were used for western blotting analysis or RNA isolation. Frozen soleus muscles were used for TdT-mediated dUTP nick end label (TUNEL) assay and subsequent immunofluorescence of dystrophin for identification of muscle tissue, while formalin-fixed soleus muscles were used for immunohistochemistry (cleaved caspase-3 and macrophage).

Western blotting

50µg gastrocnemius muscle protein was subject to SDS-PAGE under reduced conditions. Immunoblotting was performed with monoclonal anti-GAPDH (Calbiochem, MA) and polyclonal anti-caspase-3 antibodies (Santa Cruz biotechnology, CA). Immunoreactive proteins were visualized by using a chemiluminescent detection kit (Denville Scientific Inc., Metuchen, NJ) according to the manufacturer's instructions.

Real-time RT-PCR for atrogin-1, MuRF1, MCP-1 and F4/80

Total RNA was isolated from gastrocnemius muscles of the immobilized and contralateral sides at day 14 of immobilization using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After cDNA was synthesized using 1 µg RNA, real-time quantitative PCR was performed with a Stratagene Mx3005P system (La Jolla, CA) by using a Superscript III Platimun Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One single product was ensured by examining the resulting dissociation curve. Primers used in this study are shown in Table 1. Levels of mRNA encoding each gene of interest were normalized to GAPDH mRNA in the same samples.

Immunohistochemistry, TUNEL assay and Morphometrics

Soleus muscles were fixed in 10% buffered formalin phosphate for 24 hours at room temperature, embedded in paraffin, and cross sectioned (4-µm thick). The sections were rehydrated in a series of xylene and ethanol, followed by hydrogen peroxide treatment for quenching endogenous peroxidases. To retrieve antigen, the sections were microwaved in 10 mM sodium citrate (pH 6.0) until 97°C and incubated for 10–15 minutes. Sections were rinsed and incubated with primary antibodies [anti-cleaved-caspase-3 (Cell Signaling Technology, Danvers, MA) or anti-CD68 (Abcam, Cambridge, MA)] to detect cleaved (activated) caspase-3 or macrophages, respectively. Then the slides were incubated with biotinylated secondary antibody and followed by streptavidin-HRP/diaminobenzidine (DAB) (Vectastain ABC peroxidase kit, Vector Laboratories, Burlingame, CA). The primary antibody omitted during staining was used as the negative control. Hematoxylin was used as a counterstain.

For quantitation of macrophages, photomicrographs of sections were taken using a Nikon Eclipse E800 microscope and spot advanced imaging software (Diagnostic Instrument Inc., Sterling Heights, MI). The number of macrophages and myofibers were counted and averaged for at least 4 microscope fields at 400× magnification. Data are presented as the number of positive cells per 100 myofibers in cross-section.³²

For TUNEL assay, soleus muscles were snap frozen in isopentane pre-cooled in liquid nitrogen and cross sectioned (10-µm thick). *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostic, Indianapolis, IN) was used for TUNEL staining. Subsequent immunofluorescence was performed with anti-dystrophin (1:100; Sigma-Aldrich Corp., St. Louis, MO).³³ Nuclei were counterstained with DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA).

For quantitation of TUNEL-positive myonuclei, photomicrographs of sections were taken as described above. The number of TUNEL-positive myonuclei and total nuclei were counted and averaged for at least 4 microscope fields at 400 $\times$ magnification. Data are presented as the percentage of TUNEL-positive myonuclei in total nuclei per cross-section.¹⁵

For morphometric assessment, the formalin-fixed soleus cross sections, prepared as described above were used to measure the cross-sectional area via ImageJ software. A minimum of 100 fibers were measured per section.

Muscle function study

Neuromuscular transmission tests were performed as previously described.² Mice were anesthetized with pentobarbital (60 mg/kg, i.p.) 14 days following immobilization, and tracheostomy was performed for mechanical ventilation with room air at 120 breaths/minute with a tidal volume of 10 ml/kg (MiniVent Type 845, Harvard Apparatus, Holliston, MA). Body temperature was monitored with a rectal probe and maintained at 36 $^{\circ}$ –38 $^{\circ}$ C by a heat lamp. Adequate depth of anesthesia was confirmed by the absence of the withdrawal response to tail clamping. Anesthesia was maintained with supplemental intermittent doses of pentobarbital 10–20 mg/kg i.p., empirically administered every 20–30 minutes. Neuromuscular transmission and function were monitored by evoked mechanomyography using a peripheral nerve stimulator (NS252, Fisher & Paykel Health Care, Irvine, CA) along with a Grass force transducer and software (Grass Technologies, West Warwick, RI).

For the neuromuscular function study, mice were placed in the dorsal recumbent position. The tendon of the insertion of the gastrocnemius muscle was surgically exposed on each side and individually attached to separate Grass FT03 force displacement transducers (Grass Technologies, West Warwick, RI). Both sciatic nerves were exposed at the thigh and tied with ligatures. Distal to the ligatures, stimulation electrodes were attached for nerve-mediated stimulation of the gastrocnemius muscle. The knees were stabilized rigidly with a clamp. A baseline tension of approximately 10 g was applied to the tendon of each gastrocnemius muscle, which yielded optimal evoked tensions. The nerve-evoked tension of each gastrocnemius muscle was recorded via a Grass P122 amplifier and displayed using the Grass Polyview Software (Grass Technologies, West Warwick, RI). Initially, during supramaximal stimuli, tetanic stimulation (50 Hz, 3 seconds) was applied to recruit muscle, and then the baseline mechanomyographic responses were stabilized over a period of 15 minutes using the train-of-four (TOF) stimulation pattern every 20 seconds. At the end of this period, the evoked muscle peak twitch tension (g force) developed during TOF stimulation was recorded.

Statistical analysis

All values are expressed as mean $\pm$ SEM. An unpaired student *t*-test was used to investigate the difference between 2 groups (muscle mass reduction and fold change of mRNA levels in WT mice versus KO mice). All other data were analyzed by two-way ANOVA with the student Newman-Keuls *post-hoc* test. Level of significance was set at *P* < 0.05.

RESULTS

Time course of muscle mass change and caspase-3 activation after immobilization in WT mice

First, we tested the effect of immobilization on gastrocnemius and soleus muscle mass change in WT mice at 3, 7 and 14 days after the perturbation. At 3 and 7 days of immobilization, no difference was noted in gastrocnemius muscle mass between the immobilized and contralateral legs. No difference was detected in soleus muscle mass

between the immobilized and contralateral legs at day 3 of immobilization. Immobilization for 7 and 14 days, however, significantly decreased soleus muscle mass to 68% and 44% of the contralateral leg, respectively, while 14 days of immobilization significantly reduced gastrocnemius muscle mass to 59% of the contralateral leg (Fig. 1A & B). Then, we examined caspase-3 activation by western blotting analysis. Western blotting analysis showed that cleaved caspase-3, the active form of caspase-3, was significantly increased in the gastrocnemius muscle of the immobilized hindlimbs at day 14 of immobilization compared with the contralateral hindlimbs in WT mice (Fig. 1C & D). At days 3 and 7, cleaved caspase-3 appeared to be increased in the immobilized hindlimbs of WT mice, but no statistical difference was found. On the other hand, there is little, if any, detectable cleaved caspase-3 in the gastrocnemius muscle of the contralateral hindlimbs and that of the immobilized hindlimbs at day 0 in WT and KO mice (Fig. 1C).

Caspase-3 KO mice are partially protected from immobilization-induced muscle mass loss, fiber cross-sectional area decrease, and tension generation reduction

Without immobilization, no differences in muscle mass between the right and left sides of sham-immobilized WT and KO mice at day 14 were observed (data not shown). Although soleus muscle mass of KO was significantly smaller compared to that of WT, the mass of gastrocnemius and soleus muscles also did not differ between the sham-immobilized and contralateral unimmobilized sides of immobilized mice in both WT and KO mice (Table 2). Likewise, the fiber cross-sectional area in soleus muscle was not different between sham-immobilized mice and contralateral unimmobilized sides of immobilized mice in both WT and KO mice (Table 2). Thus, all results are compared to the contralateral side of the same animal, unless otherwise stated.

We next examined muscle mass loss in KO mice at day 14 of immobilization. No significant difference was observed in body weights between WT and KO mice (24.0 ± 0.7 g in WT vs. 23.7 ± 0.6 g in KO, $P > 0.10$). There was significant muscle mass loss in the gastrocnemius ($28 \pm 1\%$, $P < 0.05$) and soleus ($47 \pm 2\%$, $P < 0.05$) muscles on the immobilized compared with the contralateral side in KO mice. However, the muscle mass loss on the immobilized sides of WT mice was significantly greater ($41 \pm 3\%$, $P < 0.05$ in gastrocnemius, and $56 \pm 2\%$, $P < 0.05$ in soleus muscle, respectively) compared with that of KO mice (Table 3). The fiber cross-sectional area on the immobilized side was also significantly smaller compared with the contralateral side in both WT and KO mice after immobilization. Consistent with the muscle mass changes, the decrease in fiber cross-sectional area was more pronounced in WT ($55 \pm 3\%$, $P < 0.05$) compared with KO mice ($32 \pm 8\%$, $P < 0.05$) (Table 3).

The tension generation after immobilization was next examined by nerve-evoked muscle contraction simultaneously on the immobilized and contralateral unimmobilized gastrocnemius muscles. The nerve evoked peak twitch tension (g force) was lower on the immobilized compared with the contralateral unimmobilized side in both WT and KO mice. However, the decrease in tension generation was significantly more profound in WT ($36 \pm 3\%$, $P < 0.05$) compared with KO mice ($23 \pm 4\%$, $P < 0.05$) (Table 3). The peak twitch tension normalized to muscle mass (g force/mg muscle), was also significantly reduced in WT mice ($14 \pm 5\%$, $P < 0.05$). In KO mice, however, the peak twitch tension normalized to muscle mass was not different between the immobilized and contralateral unimmobilized side (reduction: $-2 \pm 8\%$, $P > 0.10$). Therefore, there was a significant decrease in peak twitch tension normalized to muscle mass in WT compared with KO mice after immobilization ($P < 0.05$) (Table 3).

Caspase-3 deficiency inhibits immobilization-induced apoptosis in soleus muscles

The following experiments examined whether lack of caspase-3 could block increased apoptosis in muscle cells induced by immobilization, which was evaluated by TUNEL assay. To distinguish TUNEL-positive muscle cell nuclei versus infiltrating cell nuclei, immunofluorescence for dystrophin, a muscle fiber marker, was performed subsequently on the same slides. Increased numbers of apoptotic myonuclei were detected on the immobilized compared with the contralateral side in WT mice [$2.9 \pm 0.6\%$ in immobilized vs. $0.9 \pm 0.03\%$ in contralateral side, $P < 0.05$, (3.2-fold)]. Similar to the myonuclei distribution in normal muscle, the apoptotic myonuclei were also located at the periphery of the muscle cell membrane, while infiltrating cells were randomly distributed. The percentage of apoptotic myonuclei on the immobilized and contralateral side was not different in KO mice ($0.9 \pm 0.07\%$ in immobilized vs. $0.8 \pm 0.11\%$ in contralateral side, $P > 0.10$) (Fig. 2A & B). To confirm the activation of caspase-3 in soleus muscles after immobilization, immunohistochemistry was performed to examine the expression of cleaved caspase-3. Cleaved activated caspase-3 was detected on the immobilized, but not on the contralateral side of WT mice; cleaved caspase-3 was not detected on either the immobilized or contralateral side of KO mice (Fig. 3).

KO and WT mice demonstrate equivalent upregulation of atrogin-1 and MuRF1 in the immobilized gastrocnemius muscles

The mRNA levels of atrogin-1 and MuRF1 were examined in WT and KO mice by RT-PCR. There was significant upregulation of atrogin-1 and MuRF1 mRNA on the immobilized compared with the contralateral side (4.7-fold $\pm$ 1.3 and 2.2-fold $\pm$ 1.2, $P < 0.05$, respectively, in WT; 4.5-fold $\pm$ 1.5 and 3.0-fold $\pm$ 1.5, $P < 0.05$, respectively, in KO mice). The immobilization-induced upregulation of atrogin-1 and MuRF1 mRNA was similar in WT and KO mice (Fig. 4).

Absence of caspase-3 results in decreased inflammatory response in the gastrocnemius and soleus muscles after immobilization

Inflammation and activation of caspase-3 occurs concomitantly with immobilization.¹³ Inflammation in tissues is associated with infiltration of macrophages which express CD68.³⁴ We therefore examined whether caspase-3 had any effect on the inflammatory response seen with immobilization. Immunohistochemistry using anti-CD68 antibody indicated that immobilization led to a significant increase of macrophage infiltration in soleus muscles of WT mice [$2.8 \pm 0.4\%$ in immobilized vs. $1.3 \pm 0.2\%$ in contralateral side, $P < 0.05$, (2.2-fold)], but not in KO mice ($1.66 \pm 0.1\%$ in immobilized vs. $1.70 \pm 0.1\%$ in contralateral side, $P > 0.10$) (Fig. 5A & B). Consistently, RT-PCR exhibited significant upregulation of F4/80 (a specific marker of mature macrophages)³⁵ in gastrocnemius muscles of WT mice (5.4-fold $\pm$ 2.7, $P < 0.05$), but not in KO mice (0.5-fold $\pm$ 0.6, $P > 0.10$) (Fig. 5C). Real-time RT-PCR also showed significant upregulation of MCP-1 mRNA on the immobilized compared with the contralateral side in gastrocnemius muscles of both WT (18-fold $\pm$ 8.2, $P < 0.05$) and KO (2-fold $\pm$ 0.3, $P < 0.05$) mice. However, absence of caspase-3 (KO mice) resulted in smaller upregulation of MCP-1 mRNA compared with WT mice ($P < 0.05$) (Fig. 5C).

DISCUSSION

This study shows that: 1) caspase-3 deficiency significantly ameliorated the reductions in muscle mass, muscle fiber cross-sectional area, and peak single twitch tension that occurs during immobilization; 2) deficiency of caspase-3 blocked immobilization-induced increased apoptosis in skeletal muscle; 3) caspase-3 deficiency did not affect the upregulation of atrogin-1 and MuRF1 mRNA levels after immobilization; 4)

immobilization-induced inflammation was attenuated by the absence of caspase-3, as evidenced by decreased expression of MCP-1 and F4/80 mRNA and macrophage infiltration in KO compared with WT mice.

Immobilization produced by application of external plastic casting induced significant muscle atrophy in soleus muscles at days 7 (32% reduction) and 14 (56% reduction) in 6–8 week old mice, while significant muscle atrophy in gastrocnemius muscles was only observed at day 14 (59% reduction) of immobilization. These results are in complete agreement with previous studies, which show that soleus muscles (slow-twitch muscle) have greater muscle mass reduction compared with gastrocnemius muscles (mixed fast-twitch and slow-twitch muscle) after immobilization.³⁶

Other studies have shown that caspase-3 activation and apoptosis occur together in DMA.^{13,18} It has been shown that increased caspase-3 activity was detected in gastrocnemius muscles at day 8 of immobilization in adult rats with an Orfit-soft plaque cast.¹⁸ In our study, we detected significant caspase-3 activation in gastrocnemius and soleus muscles as well as apoptosis in soleus muscles at day 14 of immobilization. In addition, we further demonstrate that caspase-3 deficiency blocked the increased apoptosis in soleus muscle and attenuated muscle atrophy, as evidenced by less gastrocnemius and soleus muscle mass loss and less soleus muscle fiber cross-sectional area decrease. Furthermore, we demonstrate that DMA led to tension generation reduction in both WT and KO mice, as shown by peak twitch tension reduction. However, caspase-3 deficiency mitigated the decrease in gastrocnemius muscle peak twitch tension generation seen during immobilization; immobilization caused around a 36% decrease in peak twitch tension generation in WT mice, while there was only around a 23% decrease in KO mice. Taken together, these findings suggest that activation of caspase-3 is a definitive component of immobilization-induced muscle wasting and decreased tension generation. Caspase-3 inhibitors have been used to block activation of caspase-3 *in vivo* to prevent apoptosis in several tissues.^{37–40} It is tempting to speculate that targeted therapeutic inactivation of caspase-3 may reduce muscle wasting and tension generation reduction after immobilization.

Muscle atrophy induced by hindlimb suspension is not associated with inflammation.⁹ However, it has been shown that immobilization with a plaster case for 7 days induces inflammation and necrosis but not apoptosis in adult mice.⁴¹ Plaster casting is different from hindlimb suspension; there is loss of weight bearing in both models, but there is indeed limb movement in the latter, and not in the former. Therefore, pathways of muscle atrophy induced by these 2 different methods may not be the same. In addition, it has been recently reported that immobilization is related to inflammation, apoptosis, and caspase-3 activation in old rats using an Orfit-soft plaque cast for 8 days.¹³ Furthermore, experimental inflammation (e.g., induced by endotoxin and anti-Fas antibody administration) is also associated with caspase-3 activation, and inhibition of caspase-3 blocks the inflammatory response.^{42,43} While increased expression of MCP-1 protein has been detected after immobilization,¹³ it has been shown that MCP-1 activates caspase-3 via MCP-1-induced protein in ischemic heart disease.⁴⁴ Our findings complement these observations in muscle where immobilization-induced inflammation, particularly upregulation of MCP-1 mRNA and increased macrophage infiltration, was almost completely abolished by the lack of caspase-3. In addition, our previous study demonstrated that systemic inflammation induced by heat-inactivated *Corynebacterium parvum* leads to more profound muscle wasting and tension generation reduction.⁷ Therefore, caspase-3 is essential for immobilization-induced inflammation, which may contribute to muscle wasting and tension generation reduction.

Another aspect of analysis of tension generation reduction after perturbation is to examine the change in twitch tension normalized to muscle mass (g force/mg muscle), which can obviate any reduction in tension due to prejunctional or junctional factors.^{2,7} Even after it was normalized to muscle mass, the peak twitch tension in gastrocnemius muscle was significantly lower on the immobilized compared with the contralateral side in WT mice. In contrast, no difference was observed in the peak twitch tension normalized to muscle mass between the immobilized and contralateral control side in KO mice. These findings lead us to posit that in addition to loss of muscle mass, other factors decrease the ability of tension generation capacity in WT mice. We demonstrate that caspase-3 deficiency blocked inflammation induced by immobilization in KO mice. It is possible, therefore that the presence and absence of inflammation in WT and KO mice, respectively, may account for this difference. In addition, caspase-3 deficiency may block heretofore unknown factors involved in the reduction of twitch tension normalized to muscle mass after immobilization.

The ubiquitin–proteasome system plays important roles in muscle atrophy indicated by upregulation of atrogin-1 and MuRF1 after immobilization.²⁰ Caspase-3 activation has been shown to be upstream of the ubiquitin–proteasome system during muscle wasting induced by diabetes and uremia, and the ubiquitin–proteasome system accelerates muscle proteolysis by breaking down proteins.¹⁹ Recently, caspase-3 has been shown to promote muscle protein breakdown *in vitro* by enhancing proteasome activity.⁴⁵ RT-PCR analysis in our study, however, demonstrated that upregulation of atrogin-1 and MuRF1 on the immobilized side was similar in KO and WT mice. This suggests that the upregulation of the muscle-specific E3 ligases during immobilization may not require activation of caspase-3. Taken together, these results, including the partial inhibition of immobilization-induced muscle atrophy by caspase-3 deficiency, argue that muscle wasting of immobilization is mediated by caspase-3-dependent and -independent pathways.

The soleus muscle mass at baseline (on the contralateral unimmobilized side of immobilized animals) was significantly lower in the KO compared to WT mice. The cross-sectional area of soleus muscle on the contralateral unimmobilized side, however, was not significantly different between KO and WT mice. This observation suggests that caspase-3 deficiency might lead to less soleus muscle fiber numbers, which could be related to changes in muscle tissue during development. Muscle fibers are formed from fusion of developmental myoblasts, and the number of muscle fibers will not change after birth in normal condition. In addition, fiber specification occurs during early development, and the specification of vertebrate slow-twitch muscle fiber fate is related to several molecules, such as Sonic hedgehog and Blimp1.^{46,47} The genetic modification in caspase-3 knockout mice might have some negative effects on these 2 or as yet undetermined molecules, which play roles in directing slow-twitch fiber-specific development by naive myoblasts during development, resulting in less soleus muscle fibers in KO compared to WT mice. Further studies are required to clarify this point.

In summary, these studies characterized the role of caspase-3 activation in immobilization-induced muscle atrophy and tension generation reduction. Lack of caspase-3 attenuated immobilization-induced muscle wasting and tension generation reduction by blocking apoptosis and inflammation. Caspase-3 is, however, dispensable for the induction of muscle-specific ubiquitin ligases, atrogin-1 and MuRF1. Our findings suggest that caspase-3 could be a potential therapeutic target to mitigate muscle atrophy and tension generation reduction resulting from immobilization.

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Abbreviations

CONT	contralateral unimmobilized side of immobilized animals
CSA	fiber cross-sectional area
DMA	disuse muscle atrophy
GC	gastrocnemius muscle
IMM	immobilized side
KO	caspase-3 knockout
MCP-1	monocyte chemoattractant protein-1
MuRF1	muscle-specific ring finger protein 1
P_t	peak twitch tension
SOL	soleus muscle
WT	wild type

References

1. Fang CH, Tiao G, James JH, Ogle C, Fischer JE, Hasselgren PO. Burn injury stimulates multiple proteolytic pathways in skeletal muscle, including the ubiquitin-energy-dependent pathway. *J Am Coll Surg*. 1995; 180:161–170. [PubMed: 7850049]
2. Ibeunjo C, Martyn JA. Fiber atrophy, but not changes in acetylcholine receptor expression, contributes to the muscle dysfunction after immobilization. *Crit Care Med*. 1999; 27:275–285. [PubMed: 10075050]
3. Ingemann-Hansen T, Halkjaer-Kristensen J. Computerized tomographic determination of human thigh components. The effects of immobilization in plaster and subsequent physical training. *Scand J Rehabil Med*. 1980; 12:27–31. [PubMed: 7384763]
4. Nicks DK, Beneke WM, Key RM, Timson BF. Muscle fibre size and number following immobilisation atrophy. *J Anat*. 1989; 163:1–5. [PubMed: 2558097]
5. Franch HA, Price SR. Molecular signaling pathways regulating muscle proteolysis during atrophy. *Curr Opin Clin Nutr Metab Care*. 2005; 8:271–275. [PubMed: 15809529]
6. Frick CG, Fink H, Gordan ML, Eckel B, Martyn JA, Blobner M. Chronic *Escherichia coli* infection induces muscle wasting without changing acetylcholine receptor numbers. *Intensive Care Med*. 2008; 34:561–567. [PubMed: 17952407]
7. Fink H, Helming M, Unterbuchner C, Lenz A, Neff F, Martyn JA, Blobner M. Systemic inflammatory response syndrome increases immobility-induced neuromuscular weakness. *Crit Care Med*. 2008; 36:910–916. [PubMed: 18431280]
8. Hirose M, Yasuhara S, Martyn JAJ. Immobilization depresses insulin signaling in skeletal muscle. *Am J Physiol Endocrinol Metab*. 2000; 279:E1235–1241. [PubMed: 11093909]
9. Kandarian SC, Jackman RW. Intracellular signaling during skeletal muscle atrophy. *Muscle Nerve*. 2006; 33:155–165. [PubMed: 16228971]
10. Murton AJ, Constantin D, Greenhaff PL. The involvement of the ubiquitin proteasome system in human skeletal muscle remodelling and atrophy. *Biochim Biophys Acta*. 2008; 1782:730–743. [PubMed: 18992328]

11. Smith HK, Maxwell L, Martyn JA, Bass JJ. Nuclear DNA fragmentation and morphological alterations in adult rabbit skeletal muscle after short-term immobilization. *Cell Tissue Res.* 2000; 302:35–41.
12. Supinski GS, Callahan LA. Caspase activation contributes to endotoxin-induced diaphragm weakness. *J Appl Physiol.* 2006; 100:1770–1777. [PubMed: 16484358]
13. Magne H, Savary-Auzeloux I, Vazeille E, Claustre A, Attaix D, Anne L, Véronique SL, Philippe G, Dardevet D, Combaret L. Lack of muscle recovery after immobilization in old rats does not result from a defect in normalization of the ubiquitin-proteasome and the caspase-dependent apoptotic pathways. *J Physiol.* 2011; 589:511–524. [PubMed: 21115641]
14. Nunñez G, Benedict MA, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. *Oncogene.* 1998; 17:3237–3245. [PubMed: 9916986]
15. Plant PJ, Bain JR, Correa JE, Woo M, Batt J. Absence of caspase-3 protects against denervation-induced skeletal muscle atrophy. *J Appl Physiol.* 2009; 107:224–234. [PubMed: 19390003]
16. Siu PM, Alway SE. Mitochondria-associated apoptotic signalling in denervated rat skeletal muscle. *J Physiol.* 2005; 565:309–323. [PubMed: 15774533]
17. Smith HK, Maxwell L, Martyn JA, Bass JJ. Nuclear DNA fragmentation and morphological alterations in adult rabbit skeletal muscle after short-term immobilization. *Cell Tissue Res.* 2000; 302:35–41.
18. Vazeille E, Codran A, Claustre A, Averous J, Listrat A, Béchet D, Taillandier D, Dardevet D, Attaix D, Combaret L. The ubiquitin-proteasome and the mitochondria-associated apoptotic pathways are sequentially downregulated during recovery after immobilization-induced muscle atrophy. *Am J Physiol Endocrinol Metab.* 2008; 295:E1181–1190. [PubMed: 18812460]
19. Du J, Wang XN, Miereles C, Bailey JL, Debigare R, Zheng B, Price SR, Mitch WE. Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J Clin Invest.* 2004; 113:115–123. [PubMed: 14702115]
20. Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science.* 2001; 294:1704–1708. [PubMed: 11679633]
21. Vermaelen M, Sirvent P, Raynaud F, Astier C, Mercier J, Lacampagne A, Cazorla O. Differential localization of autolyzed calpains 1 and 2 in slow and fast skeletal muscles in the early phase of atrophy. *Am J Physiol Cell Physiol.* 2007; 292:C1723–1731. [PubMed: 17182728]
22. Williams A, Sun X, Fischer JE, Hasselgren PO. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery.* 1999; 126:744–749. [PubMed: 10520924]
23. Degens H, Alway SE. Control of muscle size during disuse, disease, and aging. *Int J Sports Med.* 2006; 27:94–99. [PubMed: 16475053]
24. Remels AH, Gosker HR, van der Velden J, Langen RC, Schols AM. Systemic inflammation and skeletal muscle dysfunction in chronic obstructive pulmonary disease: state of the art and novel insights in regulation of muscle plasticity. *Clin Chest Med.* 2007; 28:537–552. [PubMed: 17720042]
25. Melgarejo E, Medina MA, Sánchez-Jiménez F, Urdiales JL. Monocyte chemoattractant protein-1: A key mediator in inflammatory processes. *Int J Biochem Cell Biol.* 2009; 41:998–1001. [PubMed: 18761421]
26. Smith C, Kruger MJ, Smith RM, Myburgh KH. The inflammatory response to skeletal muscle injury: illuminating complexities. *Sports Med.* 2008; 38:947–969. [PubMed: 18937524]
27. Matsushima K, Larsen CG, DuBois GC, Oppenheim JJ. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J Exp Med.* 1989; 169:1485–1490. [PubMed: 2926331]
28. Yoshimura T, Robinson EA, Tanaka S, Appella E, Kuratsu J, Leonard EJ. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J Exp Med.* 1989; 169:1449–1459. [PubMed: 2926329]
29. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. Essential contribution of

- caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev.* 1998; 12:806–819. [PubMed: 9512515]
30. Booth FW. Effect of limb immobilization on skeletal muscle. *J Appl Physiol.* 1982; 52:1113–1118. [PubMed: 7047468]
 31. Goldspink DF. The influence of immobilization and stretch on protein turnover of rat skeletal muscle. *J Physiol.* 1977; 264:267–282. [PubMed: 839454]
 32. Przybyla B, Gurley C, Harvey JF, Bearden E, Kortebein P, Evans WJ, Sullivan DH, Peterson CA, Dennis RA. Aging alters macrophage properties in human skeletal muscle both at rest and in response to acute resistance exercise. *Exp Gerontol.* 2006; 41:320–327. [PubMed: 16457979]
 33. Evans M, Morine K, Kulkarni C, Barton ER. Expression profiling reveals heightened apoptosis and supports fiber size economy in the murine muscles of mastication. *Physiol Genomics.* 2008; 35:86–95. [PubMed: 18593863]
 34. Holness CL, Da Silva RP, Fawcett J, Gordon S, Simmons DL. Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem.* 1993; 268:9661–9666. [PubMed: 8486654]
 35. Lee SH, Starkey PM, Gordon S. Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. *J Exp Med.* 1985; 161:475–489. [PubMed: 3973536]
 36. Witzmann FA, Kim DH, Fitts RH. Hindlimb immobilization: length-tension and contractile properties of skeletal muscle. *J Appl Physiol.* 1982; 53:335–345. [PubMed: 7118655]
 37. Clark RS, Kochanek PM, Watkins SC, Chen M, Dixon CE, Seidberg NA, Melick J, Loeffert JE, Nathaniel PD, Jin KL, Graham SH. Caspase-3 mediated neuronal death after traumatic brain injury in rats. *J Neurochem.* 2000; 747:40–53.
 38. Endres M, Namura S, Shimizu-Sasamata M, Waeber C, Zhang L, Gómez-Isla T, Hyman BT, Moskowitz MA. Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J Cereb Blood Flow Metab.* 1998; 18:238–247. [PubMed: 9498840]
 39. Jaeschke H, Fisher MA, Lawson JA, Simmons CA, Farhood A, Jones DA. Activation of caspase 3 (CPP32)-like proteases is essential for TNF-alpha-induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. *J Immunol.* 1998; 160:3480–3486. [PubMed: 9531309]
 40. Zhou C, Yamaguchi M, Kusaka G, Schonholz C, Nanda A, Zhang JH. Caspase inhibitors prevent endothelial apoptosis and cerebral vasospasm in dog model of experimental subarachnoid hemorrhage. *J Cereb Blood Flow Metab.* 2004; 24:419–431. [PubMed: 15087711]
 41. Appell HJ, Ascensão A, Natsis K, Michael J, Duarte JA. Signs of Necrosis and Inflammation Do Not Support the Concept of Apoptosis as the Predominant Mechanism During Early Atrophy in Immobilized Muscle. *Basic Appl Myol.* 2004; 14:191–196.
 42. Faouzi S, Burckhardt BE, Hanson JC, Campe CB, Schrum LW, Rippe RA, Maher JJ. Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-kappa B-independent, caspase-3-dependent pathway. *J Biol Chem.* 2001; 276:49077–49082. [PubMed: 11602613]
 43. Supinski GS, Callahan LA. Caspase activation contributes to endotoxin-induced diaphragm weakness. *J Appl Physiol.* 2006; 100:1770–1777. [PubMed: 16484358]
 44. Zhou L, Azfer A, Niu J, Graham S, Choudhury M, Adamski FM, Younce C, Binkley PF, Kolattukudy PE. Monocyte chemoattractant protein-1 induces a novel transcription factor that causes cardiac myocyte apoptosis and ventricular dysfunction. *Circ Res.* 2006; 98:1177–1185. [PubMed: 16574901]
 45. Wang XH, Zhang L, Mitch WE, LeDoux JM, Hu J, Du J. Caspase-3 cleaves specific 19 S proteasome subunits in skeletal muscle stimulating proteasome activity. *J Biol Chem.* 2010; 285:21249–21257. [PubMed: 20424172]
 46. Liew HP, Choksi SP, Wong KN, Roy S. Specification of vertebrate slow-twitch muscle fiber fate by the transcriptional regulator Blimp1. *Dev Biol.* 2008; 324:226–235. [PubMed: 18948093]
 47. Te KG, Reggiani C. Skeletal muscle fibre type specification during embryonic development. *J Muscle Res Cell Motil.* 2002; 23:65–69. Review. [PubMed: 12363287]

Fig. 1. Muscle weights in gastrocnemius & soleus muscles and caspase-3 activation in gastrocnemius muscles after immobilization

A. Gastrocnemius muscle mass change. Gastrocnemius (GC) muscle mass did not differ between the immobilized (IMM) and contralateral sides (CONT) at days 3 and 7 of immobilization. At day 14, however, there was a significant reduction in GC muscle mass. **B. Soleus muscle mass change.** The soleus (SOL) muscle mass did not differ between the CONT and IMM sides at day 3 of immobilization, but there was significant muscle mass reduction at days 7 and 14 of immobilization. **C. Western blotting analysis for caspase-3 activation in gastrocnemius muscles.** Cleaved caspase-3 was detected at days 3, 7 and 14 of immobilization in gastrocnemius muscles of WT, but not at day 0 in WT and day 14 in KO mice. Representative blots from 5 animals are shown. **D. Significant caspase-3 activation was only present at day 14 of immobilization in WT mice.** Values are mean $\pm$ SEM for 4 (days 3 and 7) or 7 (day 14) animals. N.S.: no significant difference between IMM and CONT; $P < 0.05$: significant difference between IMM and CONT.

Fig. 2. Absence of caspase-3 protects mice from increase of apoptotic myonuclei after immobilization

A. *TdT-mediated TUNEL staining for apoptotic nuclei and immunofluorescence staining for dystrophin were performed on frozen transverse sections of soleus muscles after 14 days of immobilization.* TUNEL-positive apoptotic myonuclei are stained green and indicated by arrows. Muscle fibers were stained red using anti-dystrophin antibody and nuclei were stained blue using DAPI. The presence of the apoptotic nuclei in the periphery along the cell membrane of the muscle cells, as opposed to a random location, suggests that those nuclei are myogenic in origin. Representative sections of 4 animals are shown. **B.** *Percentage of apoptotic myonuclei in soleus muscles after 14 days of immobilization based on TUNEL assay and immunofluorescence staining for dystrophin.* Immobilization induced a significant increase in percentage of apoptotic myonuclei on the immobilized (IMM) compared with the contralateral sides (CONT) in soleus muscles of WT mice. A total of 300 nuclei in 4 fields of view (400× magnification) were counted for each section. Values are mean $\pm$ SEM for 4 animals. N.S.: no significant difference between IMM and CONT; $P < 0.05$: significant difference between IMM and CONT.

Fig. 3. Immobilization induces caspase-3 activation in soleus muscles of WT mice, but not KO mice

Immunohistochemistry analysis for cleaved-caspase-3 at day 14 of immobilization in soleus muscles. Cleaved caspase-3 was detected in WT mice but not in KO mice after immobilization. Representative sections of 4 animals are shown. Arrows point to positive staining for cleaved caspase-3.

Fig. 4. KO and WT mice demonstrate equivalent upregulation of atrogin-1 and MuRF1 in immobilized gastrocnemius muscles

RT-PCR analysis for atrogin-1 and MuRF1 in gastrocnemius muscles at day 14 of immobilization. There was significant upregulation of atrogin-1 and MuRF1 mRNA on the immobilized sides (IMM) compared with the contralateral unimmobilized sides (CONT) in both WT and KO mice. However, the increase levels of atrogin-1 and MuRF1 mRNA between WT and KO mice were not different. The mRNA expression of these genes was normalized to that of GAPDH in the same sample. Values are expressed as the fold change of mRNA levels in the IMM versus that in the CONT sides. Data are the mean $\pm$ SEM for 4 (WT) or 5 (KO) animals. N.S.: no significant difference between IMM and CONT.

Fig. 5. Absence of caspase-3 attenuates immobilization-induced inflammatory response

A. Immunohistochemistry analysis for macrophage marker CD68 at day 14 of immobilization in soleus muscles. Representative sections of 4 animals are shown. Arrows point to positive staining for CD68 in the interstitial space. **B.** Immobilization induced a significant increase of macrophage infiltration on the immobilized (IMM) compared with the contralateral sides (CONT) in soleus muscles of WT mice, but not in KO mice. A total of 300 muscle fibers in 4 fields of view (400 $\times$ magnification) were counted for each section. **C.** Real-time RT-PCR analysis for mRNA of F4/80 and MCP-1 in gastrocnemius muscles at day 14 of immobilization. Expression of the genes was normalized to that of GAPDH in the same sample. Values shown are fold change of mRNA levels in the IMM relative to those of the CONT sides. Data are the mean $\pm$ S.E.M for 4 (WT) or 5 (KO) animals. N.S.: no significant difference between IMM and CONT (macrophage infiltration) or between WT and KO (mRNA level of F4/80 and MCP-1); $P<0.05$: significant difference between IMM and CONT (macrophage infiltration) or between WT and KO (mRNA level of F4/80 and MCP-1).

Table 1

Primer sequences used for real-time RT-PCR

Target	Primer sequences (5'-3')	Product size (bp)
MCP-1	Forward 5'-TTGTCACCAAGCTCAAGAGAGA-3' Reverse 5'-GAGGTGGTTGTGAAAAGGTAG-3'	211
MuRF1	Forward 5'-AGG CAG CCA CCC GAT GT -3' Reverse 5'-CAC CTC ACA CGT GAG ACA GT -3'	70
atrogin-1	Forward 5'-GGG AAC TTC TCC AGA CTC TCT AC -3' Reverse 5'-GCA CAG ACT TGC CGA CTC T -3'	44
F4/80	Forward 5'-TTTCCTCGCCTGCTTCTTC-3' Reverse 5'-CCCCGTCTCTGTATTCAACC-3'	223
GAPDH	Forward 5'-CCC CCA ATG TAT CCG TTG T-3' Reverse 5'-CAC TCC TTG GAG GCC ATG TA-3'	296

Table 2

Absolute gastrocnemius & soleus muscle mass and soleus muscle fiber cross-sectional area in sham-immobilized and contralateral unimmobilized side of immobilized WT and KO mice

Group	GC (mg)*	SOL (mg)*	CSA (μm^2) [†]
WT-Sham	139 $\pm$ 4.6	8.6 $\pm$ 0.5	2848 $\pm$ 243
WT-CONT	123 $\pm$ 6.2	8.7 $\pm$ 0.5	2677 $\pm$ 176
KO-Sham	135 $\pm$ 8.5	6.7 $\pm$ 0.4 [‡]	2456 $\pm$ 220
KO-CONT	121 $\pm$ 3.7	6.4 $\pm$ 0.2 [‡]	2598 $\pm$ 150

* n = 7 (CONT) or 4 (sham);

[†] n = 4;

[‡] $P < 0.05$: significant difference from WT mice

CONT, contralateral unimmobilized side of immobilized animals; CSA, fiber cross-sectional area; KO, caspase-3 knockout; GC, gastrocnemius muscle; SOL, soleus muscle; WT, wild type.

Absolute gastrocnemius & soleus muscle mass, soleus muscle fiber cross-sectional area, gastrocnemius muscle evoked peak twitch tension and peak twitch tension normalized to muscle mass, together with percent reduction after immobilization in WT and KO mice

Table 3

Group	GC (mg) [*]	SOL (mg) [*]	CSA (μm ²) [†]	P _t (g force)	P _t /mass (g force/mg muscle) [‡]
WT-CONT	123 ± 6.2	8.7 ± 0.5	2677 ± 176	85 ± 7.7	0.71 ± 0.06
WT-IMM	73 ± 5.6 [§]	3.8 ± 0.3 [§]	1194 ± 30 [§]	54 ± 5.2 [§]	0.60 ± 0.05 [§]
KO-CONT	121 ± 3.7	6.4 ± 0.2 ^{//}	2598 ± 150	74 ± 3.5	0.67 ± 0.04
KO-IMM	87 ± 2.0 ^{§//}	3.4 ± 0.2 [§]	1718 ± 129 ^{§//}	60 ± 3.7 [§]	0.67 ± 0.04 ^{//}
WT-Reduction	41 ± 3%	56 ± 2%	55 ± 3%	36 ± 3%	14 ± 5%
KO-Reduction	28 ± 1% ^{//}	47 ± 2% ^{//}	32 ± 8% ^{//}	23 ± 4% ^{//}	-2 ± 8% ^{//}

^{*} n = 7;
[†] n = 4;
[‡] n = 5;

[§] *p* < 0.05: significant difference from CONT;

^{//} *p* < 0.05: significant difference from WT mice

CONT, contralateral unimmobilized side of immobilized animal; CSA, fiber cross-sectional area; KO, caspase-3 knockout; IMM, immobilized side; GC, gastrocnemius muscle; P_t, peak twitch tension; SOL, soleus muscle; WT, wild type; %, percent reduction relative to contralateral side.