Absence of caspase-3 protects against denervation-induced skeletal muscle atrophy

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Plant PJ, Bain JR, Correa JE, Woo M, Batt J. Absence of caspase-3 protects against denervation-induced skeletal muscle atrophy. J Appl Physiol 107: 224–234, 2009. First published April 23, 2009; doi:10.1152/japplphysiol.90932.2008.—The ubiquitin-proteasome system is a key proteolytic pathway activated during skeletal muscle atrophy. The proteasome, however, cannot degrade intact myofibrils or actinomyosin complexes. In rodent models of diabetes mellitus and uremia, caspase-3 is involved in actinomyosin cleavage, generating fragments that subsequently undergo ubiquitin-proteasome-mediated degradation. Here, we demonstrate that caspase-3 also mediates denervation-induced muscle atrophy. At 2 wk after tibial nerve transection, the denervated gastrocnemius of caspase-3-knockout mice weighed more and demonstrated larger fiber-type-specific cross-sectional area than the denervated gastrocnemius of wild-type mice. However, there was no difference between caspase-3-knockout and wild-type denervated muscles in the magnitude or pattern of actinomyosin degradation, as determined by Western blotting for actin and the 14-kDa actin fragment. Similarly, there was no difference between caspase-3-knockout and wild-type denervated muscles in the magnitude of increase in proteasome activity, total protein ubiquitination, or atrogin-1 and muscle-specific ring finger protein 1 transcript levels. In contrast, there was an increase in TdT-mediated dUTP nick end label-positive nuclei in the denervated muscle of wild-type compared with caspase-3-knockout mice. Apoptotic signaling upstream of caspase-3 remained intact, with equivalent mitochondrial release and caspase-9 activation in the denervated gastrocnemius muscle of wild-type and caspase-3-knockout mice. However, there was no difference between caspase-3-knockout and wild-type denervated muscles in the degree of muscle proteolysis in dialysis patients and in other catabolic conditions (51).

The bulk of myocyte structural proteins is contained in actinomyosin complexes and myofibrils, but the proteasome is unable to degrade these intact complexes (11, 42). The proapoptotic protease caspase-3 has been shown to cleave actinomyosin complexes in vitro, generating monomeric actin and actin fragments, including a characteristic 14-kDa fragment, which are subsequently degraded by the 26S proteasome (11). In rodent models of systemic catabolic conditions such as chronic uremia and diabetes, caspase-3 activation and subsequent actin cleavage have been shown to be critical steps in the loss of muscle mass (11, 25, 43, 47), and ex vivo suppression of caspase-3 diminishes actin degradation and ubiquitin-proteasome-mediated muscle proteolysis. In fact, the 14-kDa actin fragment has been proposed as a biomarker of increased muscle degradation, because it correlates well with the degree of muscle proteolysis in dialysis patients and in other catabolic conditions (51).

Denervation of skeletal muscle also results in caspase-3 activation (40, 41), and ubiquitin-proteasome-mediated protein degradation is the predominant proteolytic pathway of denervation-induced muscle atrophy. In this study, we sought to determine whether the absence of caspase-3 would protect against skeletal muscle atrophy induced by denervation, as it does in catabolic conditions. We hypothesized that the lack of caspase-3 would limit the substrate pool of fragmented actin available, diminishing ubiquitin-proteasome-mediated proteolysis. We found that caspase-3-knockout mice were indeed partially protected from denervation-induced skeletal muscle atrophy. However, this protection did not arise from inhibition of actin cleavage or inhibition of ubiquitin-mediated protein degradation in the caspase-3-deficient muscle. Rather, we found that proapoptotic signaling was upregulated in the denervated muscle of wild-type compared with caspase-3-knockout mice. These findings suggest that the absence of caspase-3 protects against denervation-induced skeletal muscle atrophy by suppressing apoptotic signaling in the denervated muscle, rather than regulating actin substrate provision in ubiquitin-proteasome-mediated proteolysis.
MATERIALS AND METHODS

Caspase-3-knockout mice. The generation of caspase-3-knockout mice has been previously described (49). 129J/C57Bl/6 chimeric mice were backcrossed to C57Bl/6 for at least four generations. Knockout mice are not born at the expected Mendelian ratio, but at least half of those surviving the postnatal stage survive to be healthy adults. Mice used in this study were 3–4 mo of age. Wild-type sibling control animals were derived from the breeding of animals that were heterozygous for the caspase-3-knockout cassette.

Experimental denervation model and muscle preparation. The gastrocnemius muscle denervation model (4) was approved by the Research Ethics Board of Hamilton Health Sciences at McMaster University. Briefly, the right tibial nerve was transected under inhalational isoflurane anesthesia, completely denervating the gastrocnemius muscle in 16 caspase-3-knockout and 16 wild-type sibling control mice. The proximal portion of the tibial nerve was sutured to the superficial surface of the biceps femoris muscle to prevent erratic reinnervation of the gastrocnemius muscle. The gastrocnemius muscle of the left limb served as an internal control in each animal. Mice were maintained under conditions of routine care for 2 wk. This time point was selected, because caspase-3 activation has been previously demonstrated in 2-wk-denervated muscle (40, 41). Subsequently, the mice were killed, and the gastrocnemius muscles were harvested from the denervated limb and the contralateral control limb. After rapid, atraumatic dissection and weighing, the muscle was divided and snap frozen in liquid nitrogen or fixed in formalin. Frozen muscle was used for total RNA isolation using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and for extraction of cellular proteins.

The method used for protein extraction depended on the experiment that was to be performed. For determination of actin fragments, total protein was extracted by three 30-s cycles of homogenization (Polytron PT 1200E, Kinematica, Lucerne, Switzerland) of the muscle in ice-cold muscle lysis buffer A [5 mM Tris·HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol, 1% glycerol, and PMSF (1 mM), leupeptin, and aprotinin at 10 μg/ml each], and homogenates were centrifuged at 1,600 g for 10 min at 4°C. The supernatant (soluble cytoplasmic fraction) was centrifuged further for 10 min at 4°C to 10,000 g and used for ubiquitination assays and Western blotting (see below). The pellet was washed with PBS, resuspended, dissolved by sonication in 200 μl of muscle lysis buffer A containing 0.5% SDS, and further centrifuged at 10,000 g at 4°C for 10 min. This supernatant was designated the insoluble fraction containing myofibrillar proteins, actinomyosin, and actin fragments, as described elsewhere (11).

For enzymatic assays, muscles were homogenized on ice in muscle lysis buffer B [10 mM NaCl, 1.5 mM MgCl2, 20 mM HEPES (pH 7.4), 20% glycerol, 1 mM DTT, and 0.1% Triton X-100] for 1 h at 4°C to lyse the nuclei. Lysates were then centrifuged at 21,900 g for 15 min at 4°C, and supernatants were collected and stored as cytosol-free nuclear fractions. For generation of mitochondria-free cytosolic fractions, the cytosolic supernatant was centrifuged three times at 16,000 g for 20 min at 4°C to pellet mitochondria, and the final supernatant was collected as nuclei-free, mitochondria-free cytosolic protein fractions. Mitochondrial pellets were solubilized in 600 mM NaCl, 1.5 mM MgCl2, 20 mM HEPES (pH 7.4), 20% glycerol, 1 mM DTT, and 0.1% Triton X-100 with protease inhibitors. All protein lysates and fractions were quantified using the Pierce (Rockford, IL) bichromatic acid protein assay kit and normalized for equal loading for SDS-PAGE and Western blot analysis.

Western blotting. Muscle lysate (25–50 μg; fractions isolated as described above) was separated by SDS-PAGE, transferred to nitrocellulose, exposed to Ponceau stain, and immunoblotted. The primary antibodies used for Western blotting included monoclonal Apoptack Apoptosis Fraction Analysis MAb cocktail [which contains anti-cytochrome c, anti-GAPDH, and anti-F, F6-F-ATP synthase (complex V) subunit antibodies; Mitosciences, Eugene, OR; all at 1:500 dilution], polyclonal anti-actin (catalog no. A2066, Sigma, St. Louis, MO: 1:200 dilution), polyclonal anti-Bax (Cell Signaling Technology, Beverly, MA: 1:1,000 dilution), monoclonal anti-ubiquitin (Covance, Berkeley, CA: 1:1,000 dilution), monoclonal anti-GAPDH antibody (Abcam, Cambridge, MA: 1:100,000 dilution), polyclonal anti-histone H2B (Upstate, Lake Placid, NY: 1:1,000 dilution), and monoclonal anti-poly(ADP-ribose) polymerase (PARP; Sigma; 1:2,000 dilution). Protein bands were detected with horseradish peroxidase (HRP)-linked goat anti-rabbit or mouse secondary antibody (Cell Signaling Technology; 1:10,000 dilution). The chemiluminescent signal was acquired using a charge-coupled device camera (Fluor-S Max, Bio-Rad Laboratories, Hercules, CA), and the total signal was quantified using Quantity One software (Bio-Rad Laboratories) with adjusted volume analysis.

Real-time RT-PCR. Real-time RT-PCR was used to assess expression levels of transcripts for the ubiquitin ligases atrogin-1 and MuRF1. Transcript, rather than protein, levels were assessed, because good commercial antibodies were not available. These genes do not produce proteins that require posttranslational modification for activity; therefore, determination of the level of expression of the transcripts was deemed to be adequate.

RNA quality was assessed with a bioanalyzer (model 2100, Agilent Technologies, Palo Alto, CA) and quantified by absorption spectroscopy at 260 and 280 nm. For removal of any contaminating genomic DNA, the muscle total RNA samples were treated with DNA-free (Ambion, Austin, TX) according to the manufacturer’s instructions. cDNA was generated from the total RNA for each muscle in a first-strand synthesis reaction using Superscript II reverse transcriptase (Invitrogen) at 42°C for 2 h according to the manufacturer’s instructions. Relative quantitative real-time RT-PCR was subsequently performed with an Applied Biosystems 7900 system and SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Primers were designed using Primer Express software (Applied Biosystems): atrogin-1 [5′ gag aaa tact ctc cca gct gc 3′ (forward) and 5′ gat cag aag cgt cca ctc 3′ (reverse)] and MuRF1 [5′ agg cag cca ccc gat gc 3′ (forward) and 5′ cac cca cag gag gc 3′ (reverse)]. For each gene, real-time PCR was performed in triplicate wells on cDNA generated from the reverse transcription of 10 ng of total RNA. Negative controls for each gene included no template (water) and no reverse transcriptase (10 ng of RNA). The PCR amplification consisted of 10 min of denaturation followed by 40 cycles of amplification (15 s at 95°C, 60 s at 60°C). After amplification, amplicons were melted, and the resulting dissociation curve was assessed to ensure a single product. Ten microliters of all products were run on DNA polyacrylamide gels to ensure the presence of a single amplicon of the expected size. Relative quantitation of atrogin-1 and MuRF1 was determined using the cycle threshold (ΔΔCt) method according to instructions from Applied Bio-
systems. Hydroxymethylbilane synthetase, a gene we previously identified to be stably expressed during the development of denervation atrophy in gastrocnemius muscle (5), was used as a housekeeping gene [5’ tcc aag agg agc cca get a 3’ (forward) and 5’ att aag ctc ggc tgc aac a 3’ (reverse)]. Primer amplification efficiencies were equal for all genes tested and the housekeeping gene.

**Ubiquitination assays.** Soluble protein (500 μg) was combined with an ATP-regenerating system (1.5 mM ATP, 40 mM phosphocreatine, and 80 μg/ml phosphocreatine kinase) and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and incubated at 37°C for 1 h (32). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and subjected to Western blotting with an anti-ubiquitin antibody (Covance). The chemiluminescent signal was acquired using a charge-coupled device camera (Fluor-S Max), and the total signal was quantified using Quantity One software (Bio-Rad Laboratories) with adjusted volume analysis. Blots

**Fig. 1.** Caspase-3-knockout (caspase-3/−/−) mice are partially protected from denervation-induced muscle atrophy. Caspase-3-knockout mice and wild-type (caspase-3+/+/+) sibling control mice were subjected to right hindlimb tibial nerve transection to completely denervate the gastrocnemius muscle for 2 wk. The left limb served as an internal control within each animal. A: weight of denervated gastrocnemius muscle expressed as a percentage of weight of contralateral control muscle. Denervated gastrocnemius muscle of caspase-3-knockout mice demonstrated an attenuated atrophic response and weighed significantly more than denervated gastrocnemius muscle of wild-type mice (62.0 ± 2.2% vs. 51.9 ± 1.6%, n = 9, P < 0.05). B: type II fibers were identified on histological cross sections by immunostaining with an anti-skeletal muscle myosin, fast isoform, antibody (brown-staining fibers). Type I slow-twitch fibers appear white. Representative sections are shown. C: fiber cross-sectional area. Type II fibers were significantly smaller in wild-type than caspase-3-knockout mice (640.7 ± 13.1 vs. 836.6 ± 19.1 μm², n = 4, P < 0.05). At least 100 fibers were measured per muscle. NS, not significant (P > 0.05).
were stripped and reprobed with anti-GAPDH antibodies (Abcam) to demonstrate equivalent loading of lysates.

Caspase-9 and proteasome enzymatic assays. Caspase-9 activation was determined using a fluorometric assay, whereby caspase-9 cleavage of the fluorogenic 7-amino-4-trifluoromethylcoumarin (AFC)-conjugated substrate Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC; Alexis Biochemicals, San Diego, CA) is measured, as described elsewhere (40, 41). Briefly, 100 μg of total cytosolic protein, without protease inhibitors, was incubated in 100 μl of assay buffer [50 mM PIPES, 0.1 mM EDTA, 10% glycerol, and 10 mM DTT (pH 7.2)] with 100 μM Ac-LEHD-AFC at 37°C for 2 h. The caspase-specific inhibitor carbobenzoxy-Val-Ala-Asp fluoromethylketone (Z-VAD-FMK; Alexis Biochemicals) was used as a control to validate specificity of caspase, and a standard curve was generated using free AFC (Alexis Biochemicals). Fluorescence was measured in a SpectraMax Gemini EM 96-well microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) with a 400-nm excitation filter and a 505-nm emission filter at time 0 and at 2 h. Caspase-9 activity was determined as the change in fluorescence at 2 h and expressed as a ratio of denervated to control muscle.

To measure proteasome activity, we monitored the release of the fluorophore 7-amino-4-methylcoumarin (AMC) from the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVL-AMC; Calbiochem, La Jolla, CA), as described in the Calbiochem 20S proteasome assay kit. Briefly, 100 μg of total cytosolic proteins were incubated at 37°C in 100 μl of assay buffer [25 mM HEPES, 0.5 mM EDTA (pH 7.6), and 0.03% SDS] with 50 μM Suc-LLVL-AMC in the presence or the absence of the proteasome-specific inhibitor MG132 (100 μM final concentration). Purified 20S proteasome (Alexis Biochemicals) served as a positive control, and a standard curve was generated using free AMC (Alexis Biochemicals). Fluorescence was measured in the spectrofluorometer over time with a 380-nm excitation filter and a 460-nm emission filter. Proteasome activity was derived by subtraction of the fluorescence obtained in the presence of MG132 from the fluorescence obtained in its absence at time 0 and 1 h. Proteasome activity in the denervated muscle is expressed as a fold change relative to the contralateral control muscle (denervated/control).

Immunohistochemistry, morphometrics, and TUNEL assays. Muscles were fixed in 1% buffered formalin phosphate for 24 h at room temperature, rinsed in ethanol, embedded in paraffin, and sectioned (10 μm thick) on cross section. The sections were rehydrated in a series of xylene and ethanol washes, and endogenous peroxidases were quenched with a 30-min incubation in 0.3% H2O2. For antigen retrieval, the sections were microwaved in 10 mM sodium citrate (pH 6.0) three times for 5 min each. Sections were rinsed, and activated caspase-3 was detected using a cleaved caspase-3 (Asp175) antibody (catalog no. 9661, Cell Signaling; specific for the cleaved large fragment of activated caspase-3; 1:200 dilution) followed by biotinylated secondary antibody and streptavidin-HRP/diaminobenzidine (DAB; Vectastain ABC Elite peroxidase kit, Vector Laboratories, Burlingame, CA). For a negative control, the primary antibody was omitted during staining. Hematoxylin was used as a counterstain.

For morphometric assessment, the gastrocnemius cross sections, prepared as described above, were immunostained with an anti-skeletal muscle myosin, fast isoform (catalog no. MY-32, Sigma; 1:500 dilution), followed by biotinylated secondary antibody and
strepavidin-HRP/DAB, as described above, to identify type II fibers. Type II myofiber cross-sectional area was measured using Sigma Scan Pro software. A minimum of 100 fibers was measured per section.

For terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays, the gastrocnemius cross sections, prepared as described above, were permeabilized with One-Phor-All buffer (Pharmacia Biotech, Piscataway, NJ) with 0.1% Triton X-100 for 10 min, incubated with 10 U of TdT enzyme (Pharmacia), 3 µM biotin-16-dUTP (Roche), 1 µM dATP (Pharmacia), and 0.01% Triton X-100 in One-Phor-All buffer for 2 h at 37°C followed by streptavidin-HRP/DAB, and counterstained with hematoxylin.

Statistical analyses. Continuous data are reported as means ± SE and were compared using t-tests. If comparison of multiple groups was performed, overall significance was established with one-way ANOVA before Tukey’s post hoc analysis to determine differences between specific groups. Statistical significance was assumed in all cases if P < 0.05.

RESULTS

Caspase-3-knockout mice are partially protected from denervation-induced muscle atrophy. To assess the effect of the lack of caspase-3 on the development of denervation-induced muscle atrophy, we employed the tibial nerve transection model to denervate the gastrocnemius muscle in caspase-3-knockout and wild-type mice for 2 wk and then determined muscle weight and fiber-type-specific cross-sectional area. Caspase-3-knockout and wild-type mice developed gastrocnemius muscle atrophy in response to denervation injury, but the loss of muscle mass was diminished in the absence of caspase-3. Gastrocnemius muscle weighed significantly more in the caspase-3-knockout than wild-type mice 2 wk after denervation (Fig. 1A).

The mouse gastrocnemius muscle is composed primarily of fast-twitch type II muscle fibers (7, 27), which are markedly sensitive to denervation-induced atrophy. We identified these fibers using immunostaining for skeletal muscle myosin, fast isoform, and then measured fiber area on gastrocnemius muscle cross sections. Denervation resulted in a greater decrease in cross-sectional area of type II fibers in wild-type than caspase-3-knockout mice (Fig. 1, B and C).

Caspase-3 is activated in gastrocnemius muscle after denervation. To confirm the activation of caspase-3 in muscle by denervation, we immunostained gastrocnemius muscle transverse sections for activated caspase-3 (Fig. 2). Expression of activated caspase-3 was evident in the denervated, but not the control, gastrocnemius muscle of caspase-3 wild-type mice. As expected, activated caspase-3 immunoreactivity was absent in the denervated muscle and control muscle of caspase-3-knockout mice.

Caspase-3-knockout mice generate the 14-kDa actin fragment. Since caspase-3 is activated by denervation and caspase-3 degrades actinomysin, we determined whether denervation generated gastrocnemius muscle expression of the 14-kDa actin degradation fragment. SDS-PAGE and Western blotting for actin in insoluble portion of gastrocnemius muscle protein lysates revealed basal levels of expression of the 14-kDa actin fragment in control muscle (Fig. 3A). Muscle denervation significantly increased expression of the 14-kDa actin fragment compared with contralateral control muscle in caspase-3-knockout and wild-type mice, but there was no difference in the pattern of actin degradation fragments generated by denervation or the magnitude of the increase in expression of the 14-kDa actin fragment between the two cohorts of mice (Fig. 3B).

Caspase-3-knockout and wild-type mice demonstrate equivalent increases in ubiquitination and proteasome activation in denervated gastrocnemius muscle. Since ubiquitin-proteasome-mediated protein degradation is a major pathway activated by denervation and the caspase-3-knockout mice were partially protected from denervation atrophy, we sought to determine whether there were differences between caspase-3-knockout and wild-type mice in the total levels of protein ubiquitination and proteasome activation within muscle. Ubiquitination assays revealed significantly increased total ubiquitination levels in the denervated gastrocnemius muscle of caspase-3-knockout and wild-type mice. GAPDH was shown as a loading control. Representative Western blots are shown. B: 14-kDa actin fragment chemiluminescent signal in denervated muscle was quantified and is expressed as fold change relative to control muscle. The 14-kDa fragment was significantly increased in denervated muscle of caspase-3-knockout (1.9 ± 0.6-fold change, n = 9, P < 0.05) and wild-type mice (1.5 ± 0.2-fold change, n = 8, P < 0.05) mice, but there was no difference in the magnitude of increase between the 2 cohorts of mice.
ference, however, in the magnitude of the increase of these transcripts between caspase-3-knockout and wild-type mice.

Finally, we carried out a functional analysis to measure proteasome activity in muscle lysates (Fig. 4). The 26S proteasome consists of a 20S catalytic core and two 19S regulatory subunits. We measured fluorescence of a fluorogenic substrate cleaved by the 20S catalytic core in vitro in muscle lysates. Proteasome activity was increased in the

Fig. 4. Caspase-3-knockout and wild-type mice demonstrate equivalent increases in ubiquitination and proteasome activity in denervated gastrocnemius muscle. A: SDS-PAGE and Western blotting with anti-ubiquitin antibodies of denervated and control gastrocnemius soluble muscle protein lysates, subjected to ubiquitination assay, from caspase-3-knockout and wild-type mice. Ubiquitinated products appear as a high-molecular-weight smear. Western blotting for GAPDH demonstrates equal loading of protein lysates. Representative Western blots are shown. Chemiluminescent signal of ubiquitinated proteins was quantified and normalized to GAPDH and is expressed as fold change in denervated relative to control muscle. Ubiquitinated proteins were significantly increased in denervated muscle of caspase-3-knockout (2.1 ± 0.6-fold change, n = 7, P < 0.050) and wild-type (2.2 ± 0.7-fold change, n = 6, P < 0.050) mice, but there was no significant difference in the magnitude of increase between the 2 cohorts of mice.

B: atrogin-1 and muscle-specific ring finger protein 1 (MuRF1) transcript levels were determined in gastrocnemius muscle by relative quantitative real-time RT-PCR. Hydroxymethylbilane synthetase served as housekeeping gene. Transcript levels in denervated gastrocnemius muscle are expressed as fold change relative to contralateral control muscle. Atrogin-1 mRNA levels were increased in denervated gastrocnemius muscle of caspase-3-knockout (1.9 ± 0.4-fold change, n = 4, P < 0.050) and wild-type (1.7 ± 0.5, n = 5, P < 0.050) mice, but there was no difference in the magnitude of the increase between the 2 cohorts of mice. Similarly, MuRF1 mRNA levels were increased in denervated gastrocnemius muscles of caspase-3-knockout (3.1 ± 0.9, n = 4, P < 0.050) and wild-type (2.0 ± 0.5, n = 5, P < 0.050) mice, but there was no difference in the magnitude of the increase between the 2 cohorts of mice.

C: quantitative in vitro analysis of 20S proteasome activity was determined in gastrocnemius muscle lysates by measurement of hydrolysis of the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-amino-4-methylcoumarin in the presence and absence of the proteasome inhibitor MG132 over 1 h. Activity was increased in denervated muscle of wild-type mice compared with control contralateral limb (2.6 ± 1.5-fold change, n = 3, P < 0.05) and in caspase-3-knockout denervated muscle compared with control muscle (2.0 ± 0.5, n = 3, P < 0.05), but there was no difference in the magnitude of increased proteasome activity between the caspase-3-knockout and wild-type mice. Experiments were repeated 3 times.
denervated gastrocnemius muscle of caspase-3-knockout and wild-type mice, but there was no difference in the magnitude of the increase between the two cohorts of mice. Overall, this series of experiments demonstrates the equivalent upregulation of ubiquitin-proteasome-mediated protein degradation in the denervated muscle of caspase-3-knockout and wild-type mice.

Denervated muscle of caspase-3-knockout mice demonstrates diminished apoptosis compared with wild-type mice. Since caspase-3 is a proapoptotic protease and programmed cell death contributes to denervation atrophy, we sought to determine whether diminished apoptosis could account for the protective effect against denervation-induced muscle atrophy in the caspase-3-knockout mice. We performed TUNEL staining on fixed cross sections of the gastrocnemius muscle and found an increased percentage of TUNEL-positive apoptotic nuclei in the denervated gastrocnemius muscle of wild-type, but not caspase-3-knockout, mice (Fig. 5).

Apoptotic signaling downstream of caspase-3 is altered in caspase-3-knockout mice. Since others demonstrated that mitochondria-associated apoptotic signaling is specifically activated by muscle denervation (40, 41), we rationalized that signaling downstream of caspase-3 in this pathway would be inhibited in caspase-3-knockout mice, while signaling upstream of caspase-3 should remain intact. Indeed, using a combination of SDS-PAGE and Western blot analysis of subcellular fractions and in vitro functional enzymatic assays, we demonstrate that the upstream event of cytochrome c release from the mitochondria into the cytosol occurred to the same extent in the denervated muscle of wild-type and caspase-3-knockout mice (Fig. 6A). We also demonstrate equivalent increases in the translocation of Bax nuclei in the denervated gastrocnemius muscle of wild-type, but not caspase-3-knockout, mice (Fig. 5).

**Fig. 5.** Denervated muscle of caspase-3-knockout mice demonstrates diminished apoptosis compared with wild-type mice. A: TdT-mediated dUTP nick end label (TUNEL) staining of formalin-fixed transverse sections of gastrocnemius muscle. TUNEL-positive apoptotic nuclei stain brown (example nuclei are indicated with arrows). Hematoxylin was used as counterstain. Representative sections are shown. B: Denervation induced a significant increase in percentage of apoptotic nuclei in gastrocnemius of wild-type mice (n = 4) compared with contralateral control muscle (9.0 ± 1.0% vs. 1.2 ± 0.4%, P < 0.05). Denervated muscle of caspase-3-knockout mice (n = 4) did not demonstrate increased TUNEL reactivity compared with contralateral control muscle (1.6 ± 0.3% and 1.4 ± 0.5%, respectively, P > 0.05). A total of 400 nuclei in 6 fields of view (×40 magnification) were counted for each section by 2 independent reviewers.
to the mitochondria and equivalent activation of the upstream caspase-9 in the denervated gastrocnemius muscle of wild-type and caspase-3-knockout mice (Fig. 6, B and C). In contrast, we found that PARP, a caspase-3 cleavage target, was differentially handled in the wild-type and caspase-3-knockout mice. Denervation induced PARP cleavage in wild-type and caspase-3-knockout gastrocnemius muscles, as indicated by the detection of the 85-kDa PARP cleavage fragment, but the magnitude of the increase was significantly greater in the wild-type mice (Fig. 7).

**DISCUSSION**

Ubiquitin-proteasome-mediated protein degradation is a key proteolytic pathway activated in atrophying skeletal muscle across many disease states, including denervation-induced muscle injury (for review see Refs. 3, 12, 20, 22, 24). Ubiquitin-proteasome-mediated protein degradation is a key proteolytic pathway activated in atrophying skeletal muscle across many disease states, including denervation-induced muscle injury (for review see Refs. 3, 12, 20, 22, 24).

**Fig. 6.** Caspase-3-knockout and wild-type mice demonstrate equivalent activation of apoptotic signaling upstream of caspase-3. A: SDS-PAGE and Western blotting for cytochrome c in cytosolic fraction of gastrocnemius muscle protein lysates demonstrated expression in denervated and control muscle of caspase-3-knockout and wild-type mice. Absence of mitochondrial contamination of cytosolic fractions was ensured by Western blotting for mitochondria-specific marker F1F0-ATP synthase (complex V) α-subunit, which is not visible in cytosolic fractions. F1F0-ATP synthase (complex V) α-subunit is an inner mitochondrial membrane protein that is not released into the cytoplasm during apoptosis. GAPDH served as loading control. Representative Western blots are shown. Chemiluminescent signal was quantified, and cytochrome c levels are expressed as fold change in denervated relative to control muscle. Cytochrome c was significantly increased in denervated muscle of caspase-3-knockout (1.6 ± 0.2-fold change, n = 4, P < 0.05) and wild-type (1.5 ± 0.1-fold change, n = 4, P < 0.05) mice, but there was no difference in the magnitude of increase between the 2 cohorts of mice.

**B**: SDS-PAGE and Western blotting for Bax in mitochondrial fraction of gastrocnemius muscle protein lysates demonstrates expression in denervated and control muscle of caspase-3-knockout and wild-type mice. Mitochondrial marker F1F0-ATP synthase (complex V) α-subunit served as a loading control. Representative Western blots are shown. Chemiluminescent signal was quantified, and Bax levels are expressed as fold change in denervated relative to control muscle. Mitochondrial expression of Bax was significantly increased in denervated muscle of caspase-3-knockout (5.5 ± 2.7-fold change, n = 4, P < 0.05) and wild-type (4.3 ± 1.8-fold change, n = 4, P < 0.05) mice, but there was no difference in the magnitude of increase between the 2 cohorts of mice.

**C**: quantitative in vitro analysis of caspase-9 activity was determined in gastrocnemius muscle lysates by measurement of hydrolysis of fluorogenic substrate Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin over 2 h. Activity was increased in denervated muscle of wild-type mice compared with control contralateral limb (2.8 ± 1.6-fold change, n = 3, P < 0.05) and in caspase-3-knockout denervated muscle compared with control muscle (1.6 ± 0.1, n = 3, P < 0.05), but there was no difference in the magnitude of caspase-9 activation between caspase-3-knockout and wild-type mice. Experiments were repeated 3 times.
ABSENCE OF CASPASE-3 PROTECTS FROM SKELETAL MUSCLE ATROPHY

ubiquitin is a posttranslational process whereby ubiquitin moieties are covalently attached to lysine residues within target proteins, marking the proteins for degradation by the 26S proteasome (13). Ubiquitination can also modify protein trafficking by influencing sorting and endocytosis and regulates gene transcription and DNA repair (15). A series of enzymes conjugate the ubiquitin moieties to the target protein. E1 (ubiquitin-activating enzymes) and E2 (ubiquitin carrier/conjugating proteins) prepare ubiquitin for conjugation, but the key enzymes that confer specificity to the system and link ubiquitin to targets are the E3, or ubiquitin, ligases (13). Atrogin-1 and MuRF1, two muscle-specific ubiquitin ligases, are key mediators of skeletal muscle atrophy (6, 14). Knockout mice lacking either of these proteins were initially shown to be partially protected from denervation-induced muscle atrophy (6), and subsequent investigations demonstrated that atrogin-1 and MuRF1 were also critical to the development of skeletal muscle atrophy arising from fasting, unloading, chronic uremia, diabetes, and glucocorticoid administration (16, 23, 44).

In this study, we sought to delineate whether the absence of caspase-3 protected against the loss of muscle mass following denervation injury by inhibiting actin degradation and subsequent ubiquitin-proteasome-mediated proteolysis, as it does under catabolic conditions. Our work demonstrates a role for caspase-3 in the development of denervation-induced skeletal muscle atrophy. We confirm the activation of caspase-3 in gastrocnemius muscle following denervation and observe that mice lacking caspase-3 are partially protected from denervation injury, manifesting retained gastrocnemius muscle mass and larger type II fibers after tibial nerve transection, compared with caspase-3 wild-type mice. Yet the absence of caspase-3 did not affect ubiquitin-proteasome-mediated proteolysis as anticipated. There was no difference in the extent of accumulation of 14-kDa actin fragments in the denervated muscle between caspase-3-knockout and wild-type mice. Ostensibly, in the absence of caspase-3 in vivo, another unidentified enzyme(s) possess(es) the ability to break down actinomyosin in a redundant pathway, in preparation for proteasome-mediated degradation, and caspase-3 is not the sole mediator of this process. In addition, although we observed significant increases in total ubiquitination and proteasome activation in the denervated gastrocnemius muscles of wild-type and caspase-3-knockout mice following denervation injury, the magnitude of the increase was not affected by the absence of caspase-3. Similarly, the absence of caspase-3 did not influence denervation-induced upregulation of atrogin-1 or MuRF1, the ubiquitin ligases critical to the development of muscle atrophy. Thus, although caspase-3 plays a role in denervation-induced skeletal muscle atrophy, it is not necessary for the process of actin degradation and ubiquitin-proteasome-mediated proteolysis in muscle after denervation.

A role for apoptosis in the progression of denervation-induced muscle atrophy must be considered in a discussion of...
the partial protection from denervation-induced atrophy in animals lacking caspase-3. Caspase-3 is an effector caspase in the apoptotic cascade, and apoptosis has been demonstrated to contribute to the development of denervation-induced muscle atrophy (1, 19, 30, 38, 46). Siu and Alway (40, 41) investigated the apoptotic signal transduction pathways that result in loss of skeletal muscle mass after denervation and determined that denervation stimulated activation of Bax/Bcl2 signaling and the mitochondria-associated apoptotic/cell death pathway.

Mitochondria act as central modulators for activation of apoptosis in response to numerous apoptotic stimuli (8, 10, 26, 48). Bax translocation to, and permeabilization of, the mitochondrial membrane by the insertion of Bax-bax homodimers result in the release of several proapoptotic factors, including cytochrome c. Cytochrome c complexes with procaspase-9 in the cytosol and Apaf-1 to form the apoptosome, which subsequently cleaves caspase-9 to its active form (26). Procaspase-3 is recruited to the apoptosome and is cleaved and activated by caspase-9. Caspase-3 is an apoptotic effector that induces DNA fragmentation and cell death via interaction with a variety of target substrate molecules, one of which is PARP (21). PARP is a regulator of DNA repair, and, during the execution of apoptosis, PARP is cleaved into 24- and 85-kDa fragments by caspase-3 (37, 50). Siu and Alway (40, 41) demonstrated that denervation-induced apoptosis of rat gastrocnemius muscle entailed Bax translocation to the mitochondria, mitochondrial cytochrome c release, activation of caspase-9 and caspase-3, and cleavage of PARP.

We found that apoptotic signaling upstream of caspase-3 was intact in the denervated gastrocnemius muscle of caspase-3-deficient mice, as demonstrated by an equivalent translocation of Bax to the mitochondria and subsequent release of mitochondrial cytochrome c into the cytosol and caspase-9 activation, between the denervated wild-type and caspase-3/-/- muscle. However, apoptotic signaling downstream of caspase-3 was inhibited by the absence of the protease, inasmuch as we found diminished PARP cleavage and generation of the 85-kDa fragment in caspase-3-knockout mice, consistent with the fact that PARP is a caspase-3 substrate.

PARP is an abundant nuclear protein that is activated in response to DNA damage, and cleavage by caspase-3 into 85- and 24-kDa fragments diminishes its enzymatic activity (37). Cytosolic expression of PARP cleavage fragments occurs during the induction of apoptosis (37). Indeed, Siu and Alway (40) demonstrated increased expression of the cleaved 85-kDa PARP fragment in the cytosolic and nuclear extracts of apoptotic denervated muscle but found the cytosolic expression to be much greater. Similarly, we were better able to detect PARP in the cytosolic than in the nuclear lysate. Although the role of PARP in apoptosis is unclear, the general consensus suggests that PARP plays an antiapoptotic role by protecting DNA from single-strand breaks and enhancing DNA repair by other base excision repair enzymes (50). Nevertheless, the diminished generation of PARP cleavage fragments in the caspase-3-deficient mice demonstrates that the loss of the protease inhibits its downstream apoptotic signaling. Consistent with these findings is the observation of increased apoptotic nuclei quantitated using TUNEL assays in wild-type denervated compared with caspase-3-deficient muscle.

The absence of caspase-3 may also influence other signaling networks that contribute to the loss of muscle mass after denervation, in addition to apoptosis. Calpains are proteases that have been shown to contribute to muscle proteolysis (for review see Refs. 12 and 20). Preliminary work from our laboratory revealed that the protein expression levels of calpain-1, calpain-2, and a calpain inhibitor, calpastatin, were the same in caspase-3-deficient and wild-type denervated muscle (data not shown), suggesting that the loss of caspase-3 does not influence this signaling network in the development of denervation atrophy.

In conclusion, we have demonstrated that the absence of caspase-3 partially protects against denervation-induced skeletal muscle atrophy. Caspase-3 activation is a common phenomenon in the ubiquitin-proteasome pathway and apoptosis. Although caspase-3 seems to contribute to the development of muscle atrophy in catabolic conditions by cleaving actinomysin and providing substrate for the 26S proteasome, we show, using an in vivo genetic model, that the activation of caspase-3 in the development of denervation-induced muscle atrophy does not influence actinomysin degradation or proteasome-mediated proteolysis of muscle. Instead, caspase-3 regulates denervation-induced signaling via the mitochondria-associated cell death/apoptotic pathway to result in loss of muscle mass. Our work demonstrates specificity to the molecular signaling networks that mediate skeletal muscle atrophy, dependent on the etiology of the atrophy. This knowledge is important to allow the development of therapeutic interventions to counteract muscle atrophy, to improve patient function, quality of life, and workplace productivity, and to contain health resource utilization and cost.

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REFERENCES


