Skeletal muscle denervation causes skeletal muscle atrophy through a pathway that involves both Gadd45a and HDAC4

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WHEN SKELETAL MUSCLES ARE denervated, they undergo atrophy.

MATERIALS AND METHODS

Mouse protocols. All mice were 8- to 12-wk-old males. Mice were housed in colony cages at 21°C with a 12:12-h light-dark cycle. Mice had ad libitum access to standard chow (Harlan Teklad 7013) except during fasting experiments. Muscle-specific ATF4 knockout (ATF4−/−;MCK-Cre(0/0)) and littermate control mice lacking the MCK-Cre transgene [ATF4−/−;MCK-Cre(0/0)] were generated and genotyped as described previously (8). C57BL/6 mice

ure, loss of independence, and mortality (10, 16, 27, 28, 32). In patients with diabetic neuropathy, localized denervation of small foot muscles leads to foot deformities, a major risk factor for ulcers and amputations (1, 2, 13, 19, 30). However, despite the prevalence and severity of denervation-induced muscle atrophy, its molecular pathogenesis remains incompletely understood, which hinders development of pharmacological therapies.

In a recent study, we found that the small nuclear protein growth arrest and DNA damage-inducible 45a (Gadd45a) mRNA in skeletal muscle. Previous studies established that muscle denervation strongly induces Gadd45a mRNA, which increases Gadd45a, a small myonuclear protein that is required for denervation-induced muscle fiber atrophy. However, the mechanism by which denervation increases Gadd45a mRNA remained unknown. Here, we demonstrate that histone deacetylase 4 (HDAC4) mediates induction of Gadd45a mRNA in denervated muscle. Using mouse models, we show that HDAC4 is required for induction of Gadd45a mRNA during muscle denervation. Conversely, forced expression of HDAC4 is sufficient to increase skeletal muscle Gadd45a mRNA in the absence of muscle denervation. Moreover, Gadd45a mediates several downstream effects of HDAC4, including induction of myogenin mRNA, induction of mRNAs encoding the embryonic nicotinic acetylcholine receptor, and, most importantly, skeletal muscle fiber atrophy. Because Gadd45a induction is also a key event in fasting-induced muscle atrophy, we tested whether HDAC4 might also contribute to Gadd45a induction during fasting. Interestingly, however, HDAC4 is not required for fasting-induced Gadd45a expression or muscle atrophy. Furthermore, activating transcription factor 4 (ATF4), which contributes to fasting-induced Gadd45a expression, is not required for denervation-induced Gadd45a expression or muscle atrophy. Collectively, these results identify HDAC4 as an important regulator of Gadd45a in denervation-induced muscle atrophy and elucidate Gadd45a as a convergence point for distinct upstream regulators during muscle denervation and fasting.

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http://www.ajpendo.org
were obtained from the National Cancer Institute. Unilateral hindlimb denervation was performed as described previously (18); mice were anesthetized with an intraperitoneal injection of 91 mg/kg ketamine and 9.1 mg/kg xylazine, and then one sciatic nerve was isolated, ligated, and transected near the head of the femur. Transfection of mouse skeletal muscle with plasmid DNA was performed as described previously (9); mice were anesthetized with ketamine-xylazine, hindlimbs were shaved, and the tibialis anterior muscles (TAs) were injected with 30 μl of 0.4 U/μl bovine placental hyaluronidase (Sigma) resuspended in sterile 0.9% saline. Two hours later, mice were reanesthetized. The TAs were then injected with 30 μl plasmid DNA in sterile saline, coated with ultrasound jelly, and subjected to ten, 20-ms pulses of 175 V/cm using an ECM-830 electroporator (BTX Harvard Apparatus). Importantly, electroporation transfects differentiated muscle fibers, but not satellite cells or connective tissue cells (26). Mice were fasted by removing food but not water. With the exception of experiments in the right panel of Fig. 1B, all experiments utilized TA muscles. The Institutional Animal Care and Use Committee of the University of Iowa approved all mouse procedures.

**Plasmids.** p-Gadd45a-FLAG was described previously (8) and encodes wild-type mouse Gadd45a with three copies of the FLAG epitope tag at the NH3-terminus, under control of the cytomegalovirus (CMV) promoter. p-HDAC4-FLAG encodes wild-type mouse histone deacetylase 4 (HDAC4) with three copies of the FLAG epitope tag at the NH3-terminus, under control of the CMV promoter. To generate p-HDAC4-FLAG, we obtained a full-length mouse HDAC4 cDNA from Open Biosystems (catalog no. MMM1013–202859554) and then subcloned the cDNA into the pXFLAG-CMV10 vector (Sigma). p-miR-Control was described previously (8) and encodes emerald green fluorescent protein (EmGFP) and a nontargeting pre-miRNA vector (Sigma). p-miR-Control was described previously (8) and encodes emerald green fluorescent protein (EmGFP) and a nontargeting pre-miRNA vector (Sigma). p-miR-Control was described previously (8) and encodes emerald green fluorescent protein (EmGFP) and a nontargeting pre-miRNA vector (Sigma).

**Quantitative real-time RT-PCR.** Skeletal muscles were placed in RNAlater (Ambion), and RNA was extracted with TRIZol (Invitrogen) and treated with DNase (Turbo DNA-free kit; Ambion). First-strand cDNA was synthesized in a 20-μl reaction containing 2 μg RNA, RNase inhibitor, random hexamer primers, and components of the High-Capacity cDNA reverse transcription kit (Applied Biosystems). All quantitative real-time RT-PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems) using Taqman Gene Expression Assays (Applied Biosystems) for mRNAs encoding Gadd45a, HDAC4, myogenin, the α1-, β1-, δ-, γ-, and ε-nicotinic acetylcholine receptor subunits (nAChR), atrogin-1/MAFbx, and MuRF1. mRNA encoding 36B4 was used as the invariant control. Samples were run in triplicate, cycle threshold (Ct) values were averaged, and the ΔΔCt method was used to calculate fold changes.

**Immunoblot analysis.** Skeletal muscles were snap-frozen in liquid nitrogen and homogenized in 1 ml ice-cold homogenization buffer [50 mM HEPES, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, complete Mini protease inhibitor mixture (Roche Applied Science), 25 mM sodium fluoride, 1% (vol/vol) Triton X-100, and a 1:100 dilution of phosphatase-free n-dodecyl-β-D-maltopyranoside, 1% (wt/vol) β-mercaptoethanol, and 0.5% (wt/vol) 2-mercaptoethanol] and heated at 95°C for 5 min. An equal amount of protein from each sample was subjected to SDS-PAGE, and then transferred to Hybond-C extra nitrocellulose filters (Milli-
Interestingly, denervation also strongly induced Gadd45α mRNA in mouse skeletal muscle. As expected, muscle denervation increased Gadd45α mRNA 15-fold in wild-type skeletal muscle (Fig. 1A). Interestingly, denervation also strongly induced Gadd45α mRNA in ATF4 mKO muscles, and there was no difference between the two genotypes (Fig. 1A). Consistent with their capacity to fully induce Gadd45α during denervation, ATF4 mKO muscles exhibited no resistance to denervation-induced skeletal muscle atrophy (Fig. 1, B–D). These data indicated that distinct upstream pathways increase Gadd45α mRNA during fasting and muscle denervation; fasting increases Gadd45α mRNA through an ATF4-dependent pathway, whereas muscle denervation increases Gadd45α mRNA through an ATF4-independent pathway.

**HDAC4 is an ATF4-independent factor that increases Gadd45α mRNA during muscle denervation.** The finding that ATP4 was not required for denervation-induced Gadd45α expression led us to consider other potential upstream regulatory factors. HDAC4 is perhaps the best-studied molecular pore. Immunoblots were performed at 4°C for 16 h using a 1:3,000 dilution of mouse anti-FLAG monoclonal antibody (no. F1804; Sigma) or a 1:35,000 dilution of polyclonal anti-actin antiserum (no. A2103; Sigma).

**Histological analysis of mouse skeletal muscle.** Skeletal muscles were fixed in 4% (wt/vol) paraformaldehyde for 16 h at 4°C, incubated in 30% sucrose (wt/vol) for 24 h, and embedded in tissue freezing medium. A Microm HM 505E cryostat (Instrumedics) was used to prepare 10-μm sections from the midbelly of the muscle. Sections were washed three times with PBS, mounted with Vectashield (Vector Laboratories), and then imaged on an Olympus IX-71 microscope with a DP-70 camera. Image analysis was performed with ImageJ, and transfected fibers were defined as fibers having a mean fluorescence ≥25 arbitrary units above background, as described previously (9). The diameters of ≥150 muscle fibers/muscle were measured using the lesser diameter method, as recommended elsewhere (7).

**Statistical analysis.** Paired t-tests were used to compare within-subject samples, and unpaired t-tests were used for all other comparisons.

**RESULTS**

Muscle denervation increases Gadd45α mRNA through an ATF4-independent pathway. ATF4 mKO mice lack ATF4 expression in differentiated skeletal muscle fibers (8). During fasting, ATF4 mKO skeletal muscles cannot maximally induce Gadd45α expression and thus exhibit resistance to fasting-induced skeletal muscle atrophy (8). To test the hypothesis that ATF4 might also be responsible for increasing Gadd45α mRNA during muscle denervation, we denervated one hindlimb in ATF4 mKO mice and in wild-type littermate control mice. The contralateral hindlimb remained innervated and served as an intrasubject control. As expected, muscle denervation increased Gadd45α mRNA ≈15-fold in wild-type skeletal muscle (Fig. 1A).
mediator of denervation-induced muscle atrophy (6, 22, 31). Muscle denervation increases HDAC4 expression and activity in skeletal muscle fibers (3, 6), and muscle-specific HDAC4 knockout mice are resistant to denervation-induced muscle atrophy (22). To begin to examine the potential role of HDAC4 in denervation-induced Gadd45a expression, we performed a time course study of HDAC4 and Gadd45a mRNA expression in denervated muscle. We found that both transcripts significantly increased within the first 1–2 days after muscle denervation; thus, induction of HDAC4 mRNA temporally correlated with induction of Gadd45a mRNA (Fig. 2A).

To test the hypothesis that HDAC4 might increase Gadd45a mRNA, we transfected the TA muscle of wild-type (C57BL/6) mice with plasmid encoding HDAC4. In each mouse, the contralateral TA was transfected with empty plasmid and served as a negative control. Both TA muscles remained innervated throughout the experiment. As expected, transfection of HDAC4 plasmid increased HDAC4 protein, which was detectable within 1 day and maximal by 2 days (Fig. 2B, top). Moreover, this increase in HDAC4 protein was accompanied by a rise in Gadd45a mRNA that was detectable at 1 day and maximal by 2 days (Fig. 2B, bottom). In contrast, transfection of plasmid encoding Gadd45a increased Gadd45a protein (Fig. 2C, top) but not HDAC4 mRNA (Fig. 2C, bottom). These results identified HDAC4 as a potential mediator of Gadd45a induction during muscle denervation.

Because muscle denervation increases Gadd45a mRNA through an ATF4-independent mechanism (Fig. 1A), we hypothesized that HDAC4 might increase Gadd45a mRNA in the absence of ATF4. To test this, we transfected ATF4 mKO mice and wild-type littermates with plasmid encoding HDAC4. As shown in Fig. 3, the absence of ATF4 did not diminish the capacity of HDAC4 to increase Gadd45a mRNA. Thus, HDAC4, like denervation, increases Gadd45a mRNA through an ATF4-independent mechanism.

To test the hypothesis that HDAC4 increases Gadd45a mRNA during muscle denervation, we generated plasmids encoding miR-HDAC4 #1 and miR-HDAC4 #2, artificial miRNAs that specifically target two independent regions of HDAC4 mRNA and reduce the level of HDAC4 protein (Fig. 4A). We transfected TA muscles of wild-type (C57BL/6) mice with plasmids encoding miR-HDAC4 #1, miR-HDAC4 #2, or miR-Control (a

Fig. 4. HDAC4 is required for induction of Gadd45a mRNA and muscle fiber atrophy during muscle denervation. A: TA muscles of C57BL/6 mice were transfected with 10 μg of p-HDAC4-FLAG plus 20 μg p-miR-Control, 20 μg p-miR-HDAC4 #1, or 20 μg p-miR-HDAC4 #2, as indicated. Muscles were harvested 4 days later for immunoblot analysis with anti-FLAG and anti-actin antibodies. B: bilateral TA muscles of C57BL/6 mice were transfected with 20 μg p-miR-Control, 20 μg p-miR-HDAC4 #1, or 20 μg p-miR-HDAC4 #2. Seven days later, mice underwent unilateral muscle denervation. Two days later, bilateral TAs were harvested for qPCR analysis of HDAC4 and Gadd45a mRNA levels. mRNA levels in denervated muscles were normalized to levels in contralateral control muscles. Data are means ± SE from 8 mice/condition. *P ≤ 0.05. C: bilateral TAs of C57BL/6 mice were transfected with 20 μg p-miR-Control, 20 μg p-miR-HDAC4 #1, or 20 μg p-miR-HDAC4 #2 and then subjected to unilateral muscle denervation. Seven days later, bilateral TAs were harvested for quantification of muscle fiber size. Top left, representative images. Bottom left, mean fiber diameter ± SE. Right, fiber size distributions. n = 5–6 muscles/condition. *P ≤ 0.05.
nontargeting control miRNA; see Ref. 8) and then denervated the TA muscles. In denervated muscles, miR-HDAC4 #1 and miR-HDAC4 #2 prevented induction of HDAC4 mRNA (Fig. 4B, left) and significantly blunted the induction of Gadd45a mRNA (Fig. 4B, right), indicating that HDAC4 is required for denervation-induced Gadd45a expression. Importantly, and consistent with the reduction in Gadd45a mRNA, miR-HDAC4 #1 and miR-HDAC4 #2 reduced muscle fiber atrophy in denervated muscles (Fig. 4C). Moreover, miR-HDAC4 #1 and miR-HDAC4 #2 did not alter muscle fiber size under basal (innervated) conditions (Fig. 4C), likely because HDAC4 and Gadd45a expression is low under basal conditions. Collectively, these data identified HDAC4 as an ATF4-independent factor that increases Gadd45a mRNA during muscle denervation.

Gadd45a is a key downstream mediator of HDAC4 in denervated skeletal muscle. Because Gadd45a induces muscle fiber atrophy (8), we hypothesized that HDAC4 might cause muscle fiber atrophy by increasing Gadd45a mRNA. Consistent with its capacity to increase Gadd45a mRNA, transfection of HDAC4 plasmid induced skeletal muscle fiber atrophy in C57BL/6 mice (Fig. 5, A and B) and in ATF4 mKO mice (Fig. 5C). To determine whether Gadd45a is required for HDAC4-mediated muscle fiber atrophy, we cotransfected wild-type (C57BL/6) TA muscles with HDAC4 plasmid and plasmids encoding either miR-Gadd45a #1 or miR-Gadd45a #2, artificial miRNAs that target two independent regions of Gadd45a mRNA and decrease denervation-induced muscle fiber atrophy (8). miR-Gadd45a #1 and miR-Gadd45a #2 did not reduce HDAC4 mRNA (Fig. 6A) or HDAC4 protein (Fig. 6B). However, both miR-Gadd45a #1 and miR-Gadd45a #2 significantly reduced HDAC4-mediated muscle fiber atrophy (Fig. 6, C–F). These data suggest that HDAC4 causes muscle fiber atrophy at least in part by increasing Gadd45a.

HDAC4 stimulates muscle atrophy at least in part by increasing expression of myogenin, a bHLH transcription factor (22). Consistent with previous reports (6, 11, 14, 22, 31), we found that muscle denervation and forced expression of HDAC4 significantly increased myogenin mRNA, as well as the expression of myogenin target genes encoding the embryonic form of the nAChR (α1-, β1-, γ-, and δ-nAChR subunits) (Fig. 7, A and B). In contrast, denervation and HDAC4 did not alter the level of mRNA encoding the ε-nAChR subunit, which is specific for the adult nAChR and does not increase during muscle denervation (11, 14, 21, 25) (Fig. 7, A and B). Because Gadd45a was required for HDAC4-mediated muscle fiber atrophy (Fig. 6, C–F), we hypothesized that Gadd45a might contribute to myogenin expression. We found that forced expression of Gadd45a mimicked the effects of denervation and HDAC4, increasing expression of mRNAs encoding myogenin and embryonic nAChR subunits (α1-, β1-, γ-, and δ-nAChR), but not ε-nAChR (Fig. 7C). Conversely, miR-Gadd45a #1 and miR-Gadd45a #2 significantly blunted induction of mRNAs encoding myogenin and γ-nAChR during muscle denervation (Fig. 7, D and E). These data suggest that Gadd45a contributes to at least some of the effects of HDAC4 on skeletal muscle gene expression and provide further evidence that Gadd45a is an important downstream mediator of HDAC4.

Myogenin also stimulates transcription of genes encoding atrogin-1/MAFbx and MuRF1, E3 ubiquitin ligases that promote muscle atrophy (22). However, Gadd45a overexpression...
does not increase atrogin-1 or MuRF1 mRNAs (8). Similarly, we found that HDAC4 overexpression did not increase atrogin-1 or MuRF1 mRNAs (Fig. 7F). In contrast, muscle denervation significantly increased not only mRNAs encoding myogenin and the embryonic nAChR (Fig. 7A) but also atrogin-1 and MuRF1 mRNAs (Fig. 7G). Collectively, these findings suggest that additional factors, not regulated by HDAC4 or Gadd45a, are required for myogenin-mediated induction of atrogin-1 and MuRF1.

HDAC4 is not required for Gadd45a induction or muscle fiber atrophy during fasting. The finding that HDAC4 mediates Gadd45a induction during muscle denervation led us to investigate whether HDAC4 might contribute to Gadd45a induction during fasting. To test this, we transfected wild-type (C57BL/6) muscles with plasmids encoding miR-HDAC4 #1, miR-HDAC4 #2, or miR-Control and then fasted the mice for 24 h. As expected, fasting induced muscle fiber atrophy, reducing mean muscle fiber diameter by 11 ± 1% (P < 0.01), similar to our previous reports (8, 9, 18). Interestingly, neither miR-HDAC4 #1 nor miR-HDAC4 #2 reduced induction of Gadd45a mRNA during fasting (Fig. 8A). Moreover, although miR-HDAC4 #1 and miR-HDAC4 #2 reduced denervation-induced muscle fiber atrophy (Fig. 4C), they did not diminish fasting-induced muscle fiber atrophy (Fig. 8B). These data indicate that HDAC4 is not required for Gadd45a induction or muscle fiber atrophy during fasting and suggest that HDAC4 may specifically regulate Gadd45a during muscle denervation.

**DISCUSSION**

Although denervation-induced skeletal muscle atrophy is both common and serious, its molecular mechanisms are not well understood. From previous studies, we knew that muscle denervation strongly induces Gadd45a mRNA (8, 12, 29), which increases Gadd45a, a small myonuclear protein that is required for denervation-induced muscle fiber atrophy (8). However, the mechanism by which denervation increases Gadd45a mRNA remained unknown. Thus, in the current study, we investigated how muscle denervation increases Gadd45a mRNA.
Our results identify HDAC4 as an important regulator of Gadd45a expression during muscle denervation. This conclusion is supported by our findings that: 1) HDAC4 is required for maximal induction of Gadd45a mRNA during muscle denervation; and 2) HDAC4 is sufficient to induce Gadd45a mRNA in the absence of muscle denervation. The way in which HDAC4 increases Gadd45a mRNA remains to be determined. Although HDAC4 is a histone deacetylase, it also regulates nonhistone proteins such as MEKK2 (5), and it can influence gene transcription independently of its deacetylase domain (4). Because histone deacetylation typically represses gene transcription (15, 23, 24), we speculate that HDAC4 may repress a gene whose product either represses Gadd45a transcription or stimulates Gadd45a mRNA turnover. However, many other possibilities exist, and this is an important area for future investigation. Our data also suggest that HDAC4 may not be the only factor that increases Gadd45a mRNA during muscle denervation. Although denervation increased Gadd45a mRNA 15- to 40-fold (Figs. 1A and 2A), forced expression of HDAC4 increased Gadd45a mRNA only 2- to 4-fold (Figs. 2B and 3). This difference could reflect the existence of another factor that contributes to Gadd45a induction during muscle denervation, or an inhibitory factor in innervated muscle that limits the effect of HDAC4 on Gadd45a expression.

Our results also elucidate Gadd45a as a convergence point for distinct upstream regulators during muscle denervation and fasting. Although HDAC4 promotes Gadd45a expression during muscle denervation, it is not required for Gadd45a expres-
sion during fasting. Conversely, ATF4, which mediates Gadd45a expression during fasting (8, 9), is not required for Gadd45a expression during muscle denervation. Thus, muscle denervation and fasting utilize distinct pathways to increase Gadd45a mRNA and cause muscle atrophy; when muscle innervation is present but nutrients are not, Gadd45a is induced by ATF4, and, when nutrients are present but muscle innervation is not, Gadd45a is induced by HDAC4 (Fig. 9). These results help to explain how two very different conditions (muscle denervation and fasting) generate similar effects in skeletal muscle and have potential implications for patients suffering from lower motor neuron disorders or malnutrition.

Our results also identify Gadd45a as an important downstream mediator of HDAC4 during muscle denervation. This conclusion is supported by our findings that Gadd45a is required for HDAC4-mediated muscle fiber atrophy, and sufficient to generate several well-established effects of HDAC4 on skeletal muscle gene expression, including induction of mRNAs encoding myogenin and the embryonic nAChR. These findings are consistent with previous findings that Gadd45a, like HDAC4, is required for denervation-induced muscle fiber atrophy, and sufficient to induce muscle fiber atrophy in the absence of muscle denervation (8). Thus, HDAC4 and Gadd45a are both key components of the same molecular pathway to skeletal muscle atrophy in denervated muscle.

Although myogenin is required for denervation-induced muscle atrophy (20, 22), it is unlikely that Gadd45a promotes muscle atrophy solely by inducing myogenin. In contrast to HDAC4 and Gadd45a, myogenin is not sufficient to induce muscle atrophy (22). Moreover, Gadd45a generates hundreds of positive and negative changes in skeletal muscle mRNA expression, leading to stimulation of protein breakdown and reductions in anabolic signaling, protein synthesis, and mitochondrial biogenesis (8). Altogether, Gadd45a generates ~40% of the changes in skeletal muscle gene expression that occur during muscle denervation (8).

In summary, the current study identifies an important pathway in denervation-induced muscle atrophy, mediated by both HDAC4 and Gadd45a. The current study also demonstrates that muscle denervation and fasting utilize distinct proximal signaling pathways that converge on Gadd45a to cause skeletal muscle atrophy. Inhibition of the HDAC4/Gadd45a pathway could be considered as a potential therapeutic approach in denervation-induced muscle atrophy, which currently lacks a pharmacological therapy.

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DISCLOSURES

Christopher Adams is a co-founder and officer of Emmyon, Inc.

AUTHOR CONTRIBUTIONS

REFERENCES


25. Sanes JR, Lichtman JW. Induction, assembly, maturation and mainte-


27. Schiffman PL, Belsh JM. Pulmonary function at diagnosis of amyotro-


