Disease-modifying effects of orally bioavailable NF-κB inhibitors in dystrophin-deficient muscle

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Introduction

Duchenne muscular dystrophy (DMD) is a devastating muscle disease characterized by progressive muscle deterioration and replacement with an aberrant fatty, fibrous matrix. Chronic upregulation of nuclear factor κB (NF-κB) is implicated as a driver of the dystrophic pathogenesis. Herein, 2 members of a novel class of NF-κB inhibitors, edasalonexent (formerly CAT-1004) and CAT-1041, were evaluated in both mdx mouse and golden retriever muscular dystrophy (GRMD) dog models of DMD. These orally bioavailable compounds consist of a polyunsaturated fatty acid conjugated to salicylic acid and potently suppress the pathogenic NF-κB subunit p65/RelA in vitro. In vivo, CAT-1041 effectively improved the phenotype of mdx mice undergoing voluntary wheel running, in terms of activity, muscle mass and function, damage, inflammation, fibrosis, and cardiac pathology. We identified significant increases in dysferlin as a possible contributor to the protective effect of CAT-1041 on sarcolemmal damage. Furthermore, CAT-1041 improved the more severe GRMD phenotype in a canine case study, where muscle mass and diaphragm function were maintained in a treated GRMD dog. These results demonstrate that NF-κB modulation by edasalonexent and CAT-1041 is effective in ameliorating the dystrophic process and these compounds are candidates for new treatments for DMD patients.
muscle, where chronic activation of cNF-κB occurs in the muscle of dystrophic mice (8, 9) and DMD patients (10, 11). In agreement with NF-κB–dependent pathogenesis, genetic haploinsufficiency experiments in the mdx mouse model of DMD have confirmed that reduction of p65, but not p50, improves the dystrophic phenotype and affects both the muscle fibers and immune infiltrate (8). Accordingly, inhibition of NF-κB in dystrophic muscle via gene therapy with a dominant-negative IKKα or IKKβ (12) or peptide-based IKKγ inhibitors (8, 13, 14) has impressive therapeutic potential; however, both of these strategies are problematic for immediate translation.

The current report details the investigation of a novel class of orally bioavailable NF-κB inhibitors for the treatment of dystrophic muscle. These compounds are composed of a polyunsaturated fatty acid (PUFA) and salicylic acid, molecules individually known to inhibit the activation of cNF-κB (15, 16), conjugated together by a linker that is only susceptible to hydrolysis by intracellular fatty acid hydrolase (17). Herein, we present data that demonstrate that 2 compounds of this class (edasalonexent [formerly CAT-1004] and CAT-1041) potently inhibit cNF-κB activation in vitro, and that long-term treatment improves the phenotype of both the mdx mouse and golden retriever muscular dystrophy (GRMD) dog models of DMD. These results suggest that this class of NF-κB inhibitors can serve as an effective treatment to slow disease progression in DMD patients.

Results
We first verified the increases in NF-κB component proteins in our mouse model of dystrophic muscle by comparing 4-month-old WT and mdx mouse quadriceps for both cNF-κB and altNF-κB components (Figure 1, A–C). As expected, every measured component of the cNF-κB pathway, including p65, p105, p50, and IκBα, was significantly elevated in mdx muscle (Figure 1B). Absolute phosphorylation of p65 on Ser536 is unchanged in dystrophic muscle; thus, the phosphorylated to total ratio is significantly reduced due to large increases in total p65. RelB and p100 of the altNF-κB pathway were also increased; however, variation in

Figure 1. NF-κB components are elevated in stabilized mdx muscle. (A) Representative immunoblot images of 16-week-old WT (n = 3) and mdx (n = 3) quadriceps with accompanying quantifications for (B) canonical and (C) alternative NF-κB component protein levels. Protein levels were normalized to Ponceau red–visualized loading (Loading) and quantified relative to WT values. Data are displayed as box-and-whisker plots, indicating first and third quartiles, median, minimum, and maximum values, and were analyzed using 2-tailed Welch’s t test with effect size displayed as Cohen’s d.
diomyopathies in DMD are a leading cause of patient mortality (20). Results suggest that CAT-1041 actually improves the dystrophic heart phenotype. This is important, as car-
diomyopathy. Indeed, these
damage to the quadriceps, as visualized by sarcolemmal permeability to serum proteins (Figure 4, D and E). Reductions in intramuscular fibrosis of the quadriceps, EDL, and diaphragm (Figure 4C), and resulted in hypertrophy of the limb muscles, relative to the untreated group (Figure 4A), while body weight was not affected (Figure 4B). Treatment improved ex vivo function of the EDL and diaphragm (Figure 4C), and resulted in hypertrophy of the limb muscles, relative to the untreated group (Figure 4A, D and E). Reductions in intramuscular fibrosis of the quadriceps, EDL, and diaphragm (Figure 5, A and B), and damage to the quadriceps, as visualized by sarcolemmal permeability to serum proteins (Figure 5, C and D), were evident with CAT-1041 treatment. Immunoblotting of quadriceps revealed that CAT-1041 reduced the expression of the NF-κB components p65, RelB, and IκBα, p52 content suggests that the formal activation of the alternate pathway (i.e., p100 processing) is not similarly increased (Figure 1C). Elevations of NF-κB, as well as the characteristic myofiber hypertrophy of mdx mice, were absent in 3-week-old mdx quadriceps (Figure 2, A–C), which is prior to the degenerative/inflammatory phase that occurs at 4 to 5 weeks of age in mdx mice. This indicates that total cellular NF-κB elevation is coincident with or subsequent to the severe degeneration and inflammation, and hence is not a direct consequence of dystrophin loss.

As PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and salicylic acid inhibit the activation of cNF-κB individually (15, 16), we evaluated the novel, orally bioavailable compounds edasalonexent and CAT-1041, which consist of either DHA or EPA, respectively, conjugated to salicylic acid (chemical structures shown in Figure 3A). This approach results in the accumulation and simultaneous intracellular release of 2 separate NF-κB inhibitors, leading to a more potent inhibition of NF-κB activity in RAW 264.7 (murine) macrophages stably expressing an NF-κB luciferase reporter than the components alone or in equimolar combination can achieve, as well as inhibition of LPS- and TNF-α-mediated p65 transcription in human peripheral blood mononuclear cells (Figure 3, B–D). Indeed, 5-month pilot trials of either edasalonexent or CAT-1041 in mdx mice resulted in improved resistance of extensor digitorum longus (EDL) muscles to eccentric damage (Figure 3E) with no apparent adverse effects. As edasalonexent, the DHA-conjugated compound, is in clinical development for the treatment of DMD (NCT02439216) and the 2 compounds demonstrated nearly identical in vitro and in vivo efficacy, we continued evaluation of the preclinical CAT-1041 compound for the treatment of dystrophinopathic muscle.

In a 6-month trial of CAT-1041 on individually housed mdx mice with ad libitum access to a voluntary running wheel, a model used to exacerbate the relatively mild dystrophic phenotype of mdx mice (18, 19), the CAT-1041 treatment group consistently ran greater distances than the untreated mice (Figure 4A), while body weight was not affected (Figure 4B). Treatment improved ex vivo function of the EDL and diaphragm (Figure 4C), and resulted in hypertrophy of the limb muscles, relative to the untreated group (Figure 4, D and E). Reductions in intramuscular fibrosis of the quadriceps, EDL, and diaphragm (Figure 5, A and B), and damage to the quadriceps, as visualized by sarcolemmal permeability to serum proteins (Figure 5, C and D), were evident with CAT-1041 treatment. Immunoblotting of quadriceps revealed that CAT-1041 reduced the expression of the NF-κB components p65, p105, p50, RelB, and p100, while p52 expression trended towards a reduction and the RelB/p65 ratio trended towards an increase (Figure 5E). In agreement with a decrease of the NF-κB system, lower levels of the inflammatory markers osteopontin, IL-6, IL-4, and MMP2, as well as the fibrosis markers FSP-1 and fibronectin were found (Figure 5F). CAT-1041 treatment in this cohort also resulted in reduced cardiac mass (Figure 6, A and B), fibrosis (Figure 6, C and D), and signaling of the Akt and ERK pathways (Figure 6, E and F), despite higher mouse activity, suggesting the potent skeletal muscle benefits do not exacerbate the mdx cardiomyopathy. Indeed, these results suggest that CAT-1041 actually improves the dystrophic heart phenotype. This is important, as cardiomyopathies in DMD are a leading cause of patient mortality (20).

While the benefits of canonical NF-κB inhibition on the dystrophic phenotype are likely multifaceted through reductions in inflammation (8), we were intrigued by the observed resilience to sarcolemmal damage
by the treatment with these compounds (Figure 3F and Figure 5, C and D). Protein levels of utrophin, integrin α7, and integrin β1, stabilizers of dystrophin-deficient sarcolemma (21–23), were unchanged by CAT-1041 treatment (Figure 7, A and B). As shifts in cardiomyocyte NF-κB signaling towards RelB-mediated transcription causes upregulation of the membrane repair protein dysferlin (24), we sought to determine whether increases in dysferlin may be involved in the resiliency to damage by CAT-1041 treatment. Indeed, we found significant increases in full-length dysferlin protein, as detected with both Romeo (N-terminal) and Hamlet (C-terminal) monoclonal antibodies to dysferlin, in CAT-1041–treated quadriceps (Figure 7, C and D). The C72 C-terminal cleavage product (25, 26) was also increased with treatment, suggesting active membrane repair was enhanced in treated mice. While total Dysf gene expression was nearly 2-fold higher in treated mice (Figure 7E), expression of the canonical (calcium sensitive) C2A isoform of Dysf was significantly reduced. Instead, we found a nearly 14-fold increase in the expression of the C2A variant 1 (C2Av1), which is largely calcium insensitive and has a high affinity for phospholipids (27). A 40% increase in expression of Dysf containing exon 40a, which expresses the calpain-susceptible cleavage site to release the C72 fragment (25), was also found. In agreement with the increase in dysferlin C2Av1 expression, immunofluorescence shows greater intensity and homogeneity of dysferlin localization to the sarcolemma in CAT-1041–treated quadriceps (Figure 7F). These data suggest that a potentially novel component of cNF-κB inhibition by CAT-1041 involves an upregulation of dysferlin, possibly improving sarcolemma repair potential in the absence of dystrophin.

Figure 3. Edasalonexent and CAT-1041 are potent NF-κB inhibitors. (A) Chemical structures of edasalonexent (aka CAT-1004) and CAT-1041. (B) Edasalonexent and (C) CAT-1041 inhibit LPS-induced p65-dependent transcriptional activity in RAW 264 cells (mouse macrophage cell line). (D) CAT-1041 inhibits LPS- and TNF-α-stimulated p65 binding in human peripheral blood mononuclear cells. Data are displayed as box-and-whisker plots, indicating first and third quartiles, median, minimum, and maximum values. *P < 0.05, ***P < 0.001, ****P < 0.0001 for respective comparisons. (E) The treatment of mdx mice with either CAT-1041 or edasalonexent for 20 weeks results in reduced susceptibility of the extensor digitorum longus muscle to eccentric contraction–induced injury. Values are indicated as mean ± SEM; contraction-matched values not connected by a letter are significantly different by Tukey honest significant difference post-hoc test (α = 0.05).
Our group has previously reported dysferlin to be elevated in \textit{mdx} muscle (28), so we further explored this observation in the context of localization and isoform expression. Immunofluorescent staining for dysferlin in \textit{mdx} quadriceps revealed a heterogeneous localization pattern compared with WT muscle (Figure 8A), characterized by high levels of dysferlin abundance at the sarcolemma of small, regenerating fibers and low levels in many larger fibers. Gene expression of total \textit{Dysf} and \textit{Dysf} exon 40a were elevated in \textit{mdx} (compared with WT) muscle; however, no difference exists in the C2A isoforms (Figure 8B). Thus, the increase in the C2Av1 isoform of dysferlin is not a normal phenomenon in \textit{mdx} muscle, and its upregulation by CAT-1041 is unique and a potential modifier of the dystrophinopathic muscle phenotype.

CAT-1041’s efficacy was also tested in the GRMD dog model of DMD, which better recapitulates pathology and progression of DMD patients than the \textit{mdx} mouse. As a case study, an affected GRMD dog received daily oral CAT-1041 treatment for 9 months, starting at 3 months of age. Serial MRI measurements show that the treated dog exhibited substantially larger muscle volume of the anterior hind limb compartment (consisting of the tibialis cranialis [TC] and EDL muscles; outlined in Figure 9A), compared with age-matched untreated GRMD dogs, as soon as 2 months following treatment initiation (Figure 9B). In vivo ventilatory function testing revealed that at 12 months of age, the CAT-1041 treatment results in larger tidal volume (Figure 9C) and better inspiratory compliance (Figure 9D), both indicators of improved diaphragm function, than untreated dogs. CAT-1041 also reduced histopathology in limb muscles, diaphragm, and heart (Figure 9E), decreased fibroblast prevalence in the TC and diaphragm (Figure 9F), improved homogeneity of diaphragm fiber size (Figure 9G), and increased homogeneity of sarcolemmal dysferlin in the diaphragm (Figure 9H). Thus, CAT-1041 has the potential to ameliorate the severe phenotype of GRMD muscle.

**Discussion**

DMD affects \(~1\) in 3,500 boys, and with potential cures, such as genetic and cellular therapies, still far away from widespread clinical use, a large focus of therapeutic development should be devoted towards therapies that can immediately benefit patients by slowing disease progression. Such therapies may have potential as monotherapies as well as adjunct therapies to complement genetic and/or cellular therapeutic approaches. The current report places the NF-\(\kappa\)B inhibitors, edasalonexent and CAT-1041, into this category. These orally bioavailable conjugates of a PUFA and salicylic acid improve the severe dystrophic phenotype found in both mechanically damaged \textit{mdx} mice and a GRMD dog, and create an environment that can support more successful muscle regeneration.
Figure 5. Phenotype of mdx muscle is improved by CAT-1041 treatment. Dystrophic mdx mice fed either control (n = 7) or CAT-1041–containing chow (n = 6) were provided in-cage voluntary running wheels for 25 weeks. (A) Representative images of Masson’s trichrome staining and (B) accompanying quantification of intramuscular fibrosis of the quadriceps, extensor digitorum longus (EDL), and diaphragm as performed using 5 independent fields of view for each sample. (C) Muscle damage to the quadriceps was visualized by immunofluorescence (IF) staining muscle cross sections for laminin and endogenous mouse IgGs to label serum-permeable muscle fibers. The dotted lines demonstrate labeling of damaged fibers, which was (D) quantified as percentage of total area using 4 independent fields of view for each sample. Scale bars: 100 μm. Immunoblot quantifications for (E) NF-κB components and (F) markers of inflammation, including osteopontin (OPN), IL-6, IL-4, and matrix metalloproteinase (MMP) 2, and fibrosis, including fibroblast-specific protein 1 (FSP-1) and fibronectin (Fn), as measured by immunoblotting. Protein contents are normalized to Ponceau red–visualized loading. Data are displayed as box-and-whisker plots, indicating first and third quartiles, median, minimum, and maximum values, and were analyzed using 2-tailed Welch’s t test with effect size displayed as Cohen’s d.
Canonical NF-κB inhibition has demonstrated great promise as a therapeutic target in dystrophin-deficient skeletal muscle. In particular, the work of Acharyya et al. provided evidence that cNF-κB is involved in mdx pathology by demonstrating that both p65 haploinsufficiency and administration of an NF-κB–inhibitory peptide (IKKγ/NEMO-binding domain; NBD) fused with a protein transduction domain (PTD; promotes cell entry) reduced the dystrophic phenotype of mdx mice (8). However, the translational limitations of this peptide-based approach were realized when long-term intravenous treatments with NBD provoked immune responses in GRMD dogs (29). An attractive feature of the compounds used in the current study is that they are orally bioavailable small molecules with low risk of toxicity that exhibit substantial NF-κB inhibitory effects and demonstrate the capacity to improve the dystrophic muscle phenotype in 2 animal models, with no signs of adverse events. In fact, a recently completed phase 1 trial with edasalonexent demonstrated high safety profiles in subjects after 2 weeks of administration (30), with plasma exposure levels that exceeded those observed in mice.

The beneficial effects of CAT-1041 on the dystrophic phenotype are likely multifaceted, as both inflammatory and fibrotic markers are reduced with long-term treatment in mdx mice (Figure 5). However, a surprising finding in this study was the significant increase in dysferlin content with CAT-1041, which coincides with a large increase in the gene expression of the C2Av1 splice variant of dysferlin. Taken together with the greater homogeneity of dysferlin staining at the sarcolemma, this suggests that CAT-1041 may protect dystrophic muscle from damage by upregulating a dysferlin variant that constitutively resides near the sarcolemma despite elevated intracellular Ca²⁺ concentrations. As muscle-specific transgenic overexpression of (presumably canonical) dysferlin fails to improve the muscle phenotype of Scgd⁻/⁻ mice (31) and actually causes a myopathy at very high levels (32), the specific induction of the C2Av1 isoform appears to be key for this hypothesized protective effect in the model utilized in the current study. This warrants further investigation of the C2Av1 dysferlin variant as a potential modifier of mechanical muscle damage, which may be additive to other sarcolemma-stabilizing strategies, such as dystrophin replacement or utrophin overexpression.

In addition to the robust skeletal muscle benefit of CAT-1041 treatment, this compound appears to protect the mdx hearts from cardiac pathology, as cardiac hypertrophy, fibrosis, and associated signaling pathways were reduced with treatment. The reduction in cardiac fibrosis was also found in the treated GRMD dog heart. Unfortunately, cardiac function was not evaluated in this study, as the terminal time points selected for skeletal muscle phenotype were earlier than the typical age of cardiomyopathy onset in both mdx and GRMD models (discussed in ref. 33). Future studies focused on cardiac function are
needed to determine if treatment with CAT-1041 or edasalonexent prevents or slows the onset of functional cardiomyopathy in our models of DMD.

In summary, the data reported in the current study demonstrate that a new class of orally bioavailable NF-κB inhibitors improves the phenotype of dystrophic muscles. These data suggest that edasalonexent is potentially a treatment option to improve both quantity and quality of DMD patient muscles.

Figure 7. Dysferlin content is increased in CAT-1041–treated mdx mice. (A) Representative immunoblots and (B) quantifications of utrophin, integrin α7, and integrin β1 in the quadriceps lysates from mdx mice fed either control (n = 7) or CAT-1041–containing chow (n = 6) and provided in-cage voluntary running wheels for 25 weeks. (C) Representative immunoblots and (D) quantifications of full-length dysferlin, as detected with both Romeo (N-terminal) and Hamlet (C-terminal) antibodies, and the C72 dysferlin cleavage product, as detected with Hamlet antibody. Protein contents are normalized to Ponceau red–visualized loading. Gene expression of total Dysf and isoforms containing the canonical C2A isoform, C2A variant 1 (C2Av1), and exon 40a in the quadriceps of untreated (n = 4) and CAT-1041–treated (n = 4) mdx mice, using Gapdh as the ΔΔCt normalization gene. (F) Immunofluorescence (IF) staining for N-terminal dysferlin in stabilized regions of untreated and CAT-1041–treated quadriceps cross sections acquired under identical conditions. Scale bar: 100 μm. Data are displayed as box-and-whisker plots, indicating first and third quartiles, median, minimum, and maximum values, and were analyzed using 2-tailed Welch’s t test with effect size displayed as Cohen’s d.

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Methods

Mice. The experimental procedures used in this study were approved by the University of Pennsylvania IACUC using male mdx or WT mice on the C57BL/10 background. Drug treatment protocols entailed feeding individually housed mice a specialty control chow or chow containing either CAT-1041 or edasalonexent (0.75% w/w) ad libitum starting at 4 weeks of age. Average drug consumption typically ranged between 0.75 and 1 mg/g body weight per day. The 24-hour plasma exposures at this dosage were 450 and 270 ng hr/ml for edasalonexent and CAT-1041, respectively. These values are at the lower end of clinical exposures observed in phase 1 studies in adults and boys with DMD (30), suggesting that efficacy is obtained in the mdx mouse at clinically relevant exposures. Moreover, the exposure of edasalonexent was 7.2-fold greater in the gastrocnemius muscle compared with plasma and 4.0-fold higher in muscle than plasma for CAT-1041, demonstrating that both molecules are able to effectively reach and accumulate in skeletal muscle. In running wheel arms of this study, mice were provided ad libitum access to running wheels (11-cm diameter) for 25 weeks beginning at 6 weeks of age, with revolution counts and body weights monitored daily.

NF-κB reporter assay. RAW 264.7 macrophages stably transfected with 3xNF-κB-luciferase reporter were the gift of Steve Shoelson (Joslin Diabetes Center, Boston, Massachusetts, USA). One day prior to the assay, cells were scraped from 75-cm² tissue culture flasks and seeded onto 96-well plates at a density of 30,000 cells per well. Edasalonexent, CAT-1041, EPA, and the combination of both EPA and salicylate were prepared as 100 mM stocks in ethanol, which were then diluted 1:100 into FBS. These 1 mM FBS stocks were then serially diluted 1:2 in FBS supplemented with 1% ethanol, and all dilutions were sonicated for 30 minutes. Medium from the 96-well plate was aspirated and replaced with 90 μl sera-free DMEM. To these cells, 10 μl of FBS/compound dilutions were added to the wells, in triplicate, to create a dilution series ranging from 100 μM to 0.78 μM in 10% FBS/0.1% ethanol. In the case of salicylate only, a stock solution of 10 mM sodium salicylate was made in DMEM containing 10% FBS/0.1% ethanol by directly dissolving sodium salicylate powder into the medium. This solution was serially diluted 1:2 in DMEM/10%FBS/0.1% ethanol. Medium from the 96-well plate was aspirated, and 100 μl of DMEM/10% FBS/0.1% ethanol/salicylate dilutions were added. Cells were incubated at 37°C for 3 hours before stimulation with 5 μl of 4 μg/ml LPS (Sigma-Aldrich, L2630) in AlamarBlue viability reagent (Invitrogen, DAL1100) or 5 μl AlamarBlue only for unstimulated control cells. Cells were then incubated at 37°C for an additional 2 hours. Cell viability was assessed using a Victor X5 plate reader (PerkinElmer), measuring fluorescence with an excitation filter of 550 nm and an emission filter of 590 nm. Wells were then aspirated, and 100 μl of AlamarBlue only for unstimulated control cells. Cells were then incubated at 37°C for an additional 2 hours. 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Figure 9. GRMD phenotype is improved by CAT-1041 in a canine case study. An affected golden retriever muscular dystrophy (GRMD) dog was treated with CAT-1041 daily, starting at 3 months of age, for 9 months. (A) Serial magnetic resonance imaging (MRI) to measure the (B) muscle volume of the anterior compartment of the hind limb (consisting of the tibialis cranialis [TC] and extensor digitorum longus [EDL] muscles) in the treated and age-matched, untreated GRMD dogs (n = 3). At the end of the 9-month treatment protocol, the dogs were evaluated for ventilatory function, as measured by (C) conscious inspiratory tidal volume and (D) dynamic lung compliance (20 breaths per dog). Dotted lines indicate minimum reference values for size-matched normal dogs. (E) Representative Masson’s trichrome staining of untreated and CAT-1041–treated TC, diaphragm (Dp), and left ventricle (LV). (F) Immunohistochemical (IHC) staining of the fibroblast marker prolyl 4-hydroxylase in the TC and Dp. (G) Dp fiber size distribution and (H) N-terminal dysferlin immunofluorescence (IF) of normal, untreated GRMD, and CAT-1041–treated GRMD dogs. Scale bars: 100 μm. Data are displayed as (B) mean ± SD of left and right limbs, (C and D) box-and-whisker plots, indicating first and third quartiles, median, minimum, and maximum values of total breaths measured, or (G) percentage total muscle fiber population (n = 200–400 fibers per sample).
counted using a hemacytometer, and brought up to a density of $1 \times 10^6$ cells/ml. Cells (1 ml) were then proportioned into each well of a 24-well plate. For compound treatment, CAT-1041 was solubilized in 100% ethanol to generate a 50 mM stock solution. This stock solution was then diluted 1:100 into FBS and sonicated for 30 minutes (along with a matched FBS control containing 1% ethanol). FBS/ethanol and the FBS/CAT-1041 mixtures were then diluted 1:10 over the PBMCs such that the final concentrations were 0.1% ethanol/10% FBS ± 50 μM CAT-1041. Control- and CAT-1041–treated cells were incubated at 37°C in a humidified CO₂ incubator for 2 hours before stimulating with either 250 ng/ml final LPS or 20 ng/ml TNF-α for 1 hour. Cells were then washed 2 times in PBS and lysed in Passive Lysis Buffer (Promega, E194). Lysate (10 μl) was then used for chemiluminescent p65 DNA-binding ELISA following the manufacturer’s protocol (Thermo Scientific, 89859). A Bradford assay was then performed on the remaining lysates to quantify protein. Data are expressed as normalized CPS divided by total protein (in μg). Error bars represent standard deviation (SD) of experiments performed in triplicate.

Muscle mechanics. Maximal tetanic tension and eccentric damage assessments of the EDL and diaphragm were evaluated as previously described (34) by the Muscle Physiology Core at the University of Pennsylvania. Briefly, the muscles of anesthetized mice were dissected and placed in physiological Ringer’s solution gas equilibrated with 95% O₂/5% CO₂. After determining optimal length, muscles were subjected to 3 isometric contractions (stimulated at 120 Hz for 500 ms) to determine maximal tetanic tension (Po). Subsequently, a series of 5 eccentric contractions with (stimulated at 80 Hz for 700 ms) a stretch of 10% optimal length was imposed on the muscle in the last 200 ms of each contraction. Each contraction was separated by a 5-minute rest period. Following experimental procedures, muscles were weighed, frozen embedded in OCT or snap-frozen, and stored at –80°C until further use. Force measurements and dissections were performed by investigators blind to treatment groups.

Histology, immunofluorescence, and immunohistochemistry. OCT- or paraffin-embedded samples were sectioned and stained with Masson’s trichrome (Polysciences, Inc.). Slides were viewed using a Leitz DMRBE microscope (Leica) and imaged with a Leica DCF480 digital camera. Fibrosis was quantified using k-means segmentation in ImageJ software (NIH) by investigators blind to treatment groups.

For immunofluorescence, frozen, OCT-embedded samples were sectioned at 10 μm, fixed in ice-cold acetone, blocked in 5% BSA-PBS, and incubated with anti-dysferlin (Romeo Rb mAb; Abcam; catalog ab124684), and/or anti-laminin (Acris, catalog BM6046P) primary antibodies overnight at 4°C. Following PBS washes, slides were incubated in donkey anti–rabbit Alexa-568 (catalog A10042), donkey anti–rabbit Alexa-488 (catalog A-21208), and/or donkey anti–mouse Alexa-568 (catalog A10037) secondary antibodies (all Life Technologies), washed with PBS, coverslipped with VectaShield (Vector Labs), and imaged using a Leitz DMRBE microscope with a Leica DCF480 digital camera. Lipofuscin-dependent autofluorescence was eliminated from sections using 1% Sudan black B dissolved in 70% ethanol following second-ary antibody incubation. Comparative images were stained, acquired, and processed under identical conditions. Quantifications were performed using ImageJ software on multiple images per sample.

For immunohistochemistry, paraffin-embedded samples were sectioned at 5 μm, deparaffinized, antigen-retrieved in heated 1 mM citrate buffer (pH 6), treated for endogenous peroxidase activity, blocked with 5% goat serum, and incubated with anti–prolyl 4-hydroxylase (Proteintech, catalog 11245-1-AP) primary antibody for 1 hour at room temperature. Following PBS washes, sections were blocked with avidin/biotin (Biocare), incubated with goat anti–rabbit biotinylated secondary antibody (Vector Labs, catalog BA-1000), washed, and incubated with HRP-conjugated streptavidin (Vector Labs). Sections were developed with DAB reagent (Dako), counterstained with hematoxylin (Dako), coverslipped, and imaged using a Leitz DMRBE microscope with a Leica DCF480 digital camera.

Immunoblotting. Snap-frozen samples were finely crushed and homogenized in T-PER buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Thermo Scientific). Protein concentration of resulting supernatant was determined using Bio-Rad Protein Assay (Bio-Rad). Protein samples were boiled in 4× sample buffer, resolved by SDS-PAGE using 4%–12% SDS-polyacrylamide gels (Life Technologies), and transferred to nitrocellulose membranes using the iBlot system (Life Technologies). Membranes were blocked in 5% milk-TBST and incubated with primary antibody overnight at 4°C. Following TBST washes, membranes were incubated in the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature, washed, incubated for 5 minutes in ECL reagent (Thermo Scientific), and imaged using the LI-COR C-DiGit (LI-COR Biosciences) imaging system. Primary antibodies used in this study include: p65 (catalog 4764), p-p65 (Ser536, catalog 3033), RelB (catalog 4922), IxBα (catalog
placed over each dog’s muzzle and attached to the sensor of a CO2SMO mainstream capnograph/pulse oximeter. Once the dogs had calmed and were breathing room air normally, inspiratory and expiratory tidal volume, peak inspiratory and expiratory flow rates, and end-tidal carbon dioxide (EtCO2) were collected. Following this data collection, the dogs were allowed to breathe spontaneously while intubated and con-
nected to the CO₂SMO. Inspiratory and expiratory tidal volumes and peak inspiratory and expiratory flow rates were collected over 20 spontaneous breaths. Following all data collection, the dogs were extubated and recovered uneventfully.

Statistics. Two-tailed Welch’s t test (effect size displayed as Cohen’s d) and 1-way ANOVA (Tukey honest significant difference post-hoc tests) were used to analyze data, where appropriate (P < 0.05 is considered significant). Values are represented as mean ± SEM, mean ± SD, or box-and-whisker plots of the 5 statistical summary (first and third quartiles, median, minimum, and maximum), where indicated.

Study approval. All animal procedures were approved by the IACUC of the University of Pennsylvania.

Author contributions
Research and study design was contributed by DWH, MMS, MRJ, GAW, and HLS. DWH, MMS, SCF, CCC, MZ, and GAW conducted experimental procedures and data acquisition. All authors were involved in data analysis, interpretation, and manuscript writing.

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