A new therapeutic effect of simvastatin revealed by functional improvement in muscular dystrophy

Nicholas P. Whitehead1, Min Jeong Kim, Kenneth L. Bible, Marvin E. Adams, and Stanley C. Froehner

Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195

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Duchenne muscular dystrophy (DMD) is a lethal, degenerative muscle disease with no effective treatment. DMD muscle pathogenesis is characterized by chronic inflammation, oxidative stress, and fibrosis. Statins, cholesterol-lowering drugs, inhibit these deleterious processes in ischemic diseases affecting skeletal muscle, and therefore have potential to improve DMD. However, statins have not been considered for DMD, or other muscular dystrophies, principally because skeletal-muscle-related symptoms are rare, but widely publicized, side effects of these drugs. Here we show positive effects of statins in dystrophic skeletal muscle. Simvastatin dramatically reduced damage and enhanced muscle function in dystrophic (mdx) mice. Long-term simvastatin treatment vastly improved overall muscle health in mdx mice, reducing plasma creatine kinase activity, an established measure of muscle damage, to near-normal levels. This reduction was accompanied by reduced inflammation, more oxidative muscle fibers, and improved strength of the weak diaphragm muscle. Shorter-term treatment protected against muscle fatigue and increased hindlimb muscle force by 40%, a value comparable to current dystrophin gene-based therapies. Increased force correlated with reduced NADPH Oxidase 2 protein expression, the major source of oxidative stress in dystrophic muscle. Finally, in old mdx mice with severe muscle degeneration, simvastatin enhanced diaphragm force and halved fibrosis, a major cause of functional decline in DMD. These improvements were accompanied by autophagy activation, a recent therapeutic target for DMD, and less oxidative stress. Together, our findings highlight that simvastatin substantially improves the overall health and function of dystrophic skeletal muscles and may provide an unexpected, novel therapy for DMD and related neuromuscular diseases.

Significance

Duchenne muscular dystrophy (DMD) is a lethal, degenerative muscle disease for which there is no effective treatment. Statins have been used for decades to improve cardiovascular health. In addition to lowering blood cholesterol levels, statins also reduce inflammation, oxidative stress, and fibrosis. These pathogenic processes all contribute to functional decline in DMD muscles. Therefore, we reasoned that statins could be a beneficial treatment for dystrophic muscles. In this study, we show that simvastatin dramatically improves muscle strength and fatigue resistance in DMD (mdx) mice. This result was accompanied by significantly reduced inflammation, oxidative stress, and fibrotic deposition in old, degenerated mdx muscle. These findings indicate that simvastatin is a promising, novel therapeutic approach for DMD and related muscle disorders.

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1To whom correspondence should be addressed. Email: npwhite@uw.edu.

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Results and Discussion
Long-Term Simvastatin Treatment Protects Dystrophic Muscle from Damage and Inflammation While Improving Diaphragm Function.

DMD is a chronic, progressively degenerative disease, and consequently, potential treatments need to be effective over many years. Therefore, we first evaluated long-term simvastatin treatment in mdx mice both to determine its effectiveness on the disease pathogenesis and assess any side effects. Simvastatin was orally administered for 8 mo starting from 3 wk of age, which is just before the onset of muscle damage in mdx mice (5). The calculated dose given to the mice was between 5 and 10 mg/kg per d. This dose would equate to ~20–40 mg/d for a 10-yr-old DMD boy weighing 30 kg (23), based on mouse-to-human equivalence calculations (24), which is within the recommended dose range of statins for children (10). Whole-body muscle health was dramatically improved in simvastatin-treated mdx (mdx Sim) compared with control mdx mice (mdx Con), as evidenced by an 85% reduction in plasma creatine kinase (CK) activity level, a widely used clinical marker of muscle damage (Fig. 1A). This measure of improved muscle health was validated by histological assessment of the tibialis anterior (TA) muscle, which was accompanied by reduced inflammation, oxidative stress, and fibrosis. We also provide evidence that simvastatin treatment in dystrophic mice does not impede muscle regeneration or induce pathways thought to cause statin myopathy in humans. To our knowledge, these results demonstrate for the first time that statins are beneficial in a degenerative skeletal muscle disease. This finding suggests that statins have great potential to improve muscle health and function in DMD and possibly related neuromuscular diseases.
consistent with the known anti-inflammatory effects of statins in skeletal muscles of ischemic limbs (17, 18).

The dystrophin homolog utrophin is up-regulated and expressed along the sarcolemma in mdx muscles. An increase in utrophin can partially compensate for the loss of dystrophin and provide protection against muscle damage, making it a therapeutic target for DMD (25). Therefore, we sought to determine whether the beneficial effects of simvastatin were related to enhanced expression of utrophin and/or other members of the dystrophin protein complex (DPC). However, Western blotting showed no additional increase in the expression levels of utrophin, other members of the dystrophin complex, or associated proteins such as nNOSp and Caveolin-3 (Fig. S1). These data indicate that the beneficial effects of simvastatin are not attributable to increased expression of utrophin or the dystrophin-associated protein complex.

Recent evidence has shown that metabolic changes can provide protection against damage in dystrophic muscle. In particular, shifting muscle fiber type from glycolytic type 2B fibers to more oxidative type 2A/X or very oxidative type 1 fibers by pharmacological or genetic approaches reduces muscle damage and improves physiological function (26, 27). Therefore, we measured the fiber type composition of TA muscle sections from mdx Con and mdx Sim mice. As shown in Fig. 1D, compared with mdx Con mice, TA muscles of mdx Sim mice displayed a significant fiber type shift of 15% from fast glycolytic 2B fibers to more oxidative, type 2X fibers. In addition, there was no difference in fiber type composition between WT Con and both WT groups (Fig. 1D). Interestingly, voluntary wheel running in mice also causes a fiber-type shift from 2B to 2A/X, which is associated with improved endurance and enhanced oxidative metabolism (28). Therefore, although the mechanism for the fiber type shift in mdx Sim mice is currently unclear, it likely contributes to the improvement in overall muscle health in these mice.

In mdx mice, the diaphragm is the most severely affected skeletal muscle (4), and diaphragm dysfunction is a major cause of respiratory failure in DMD. Therefore, we tested whether simvastatin could improve diaphragm strength in mdx mice. Using isolated diaphragm muscle strips, specific muscle force (force normalized to muscle cross-sectional area) was significantly higher (20–25%) in mdx Sim mice compared with mdx Con, over the full range of stimulation frequencies (Fig. 1E), indicating a robust improvement in diaphragm physiological performance. As expected, diaphragm force of WT mice was significantly higher compared with mdx, but there was no difference between values for WT Con and WT Sim (Fig. S2).

**Simvastatin Treatment Enhances mdx Hindlimb Muscle Force, Which Correlates with Reduced NADPH Oxidase 2 Expression.** Muscle degeneration and loss of function in DMD begins very early in the disease and progressively worsens over time (3). Consequently, useful therapeutic agents must be effective when administered at various stages of the disease. Therefore, we investigated whether simvastatin could improve muscle function when given several months after the onset of muscle damage in mdx mice. Simvastatin treatment was started when mice were 3 mo of age, and measurements were performed 3 mo later. Hindlimb (TA) muscle physiology was measured in situ. This method has the advantages of direct nerve stimulation and intact blood circulation, which provides an essentially in vivo approach for measuring muscle function. Remarkably, specific muscle force increased by 40% ($P < 0.001$) for mdx Sim compared with mdx Con mice (Fig. 2A and B), a dramatic increase for a pharmacological agent. In fact, this 40% increase in specific force with simvastatin is comparable to that provided by the most effective gene-based therapeutic approaches, including a minidystrophin gene therapy construct containing the neuronal NOS (nNOS) binding region (29) and antisense oligonucleotides (exon skipping), which led to homologous expression of a slightly truncated dystrophin protein throughout mdx TA muscle (30). Therefore, our data demonstrate that, as a nongenetic approach, simvastatin provides a substantial improvement in contractile performance of dystrophic muscle. Interestingly, statin treatment also increases skeletal-muscle-specific force in an animal model of hindlimb ischemia (19), again emphasizing the point that statins augment NOX2-derived ROS in the cardiovascular system (33, 34), we measured the NOX2 expression levels in TA muscles. Compared with mdx Con, mdx Sim mice had a significant reduction in NOX2 levels. This data (Fig. 2C) indicates a common inhibitory effect of simvastatin on skeletal muscle NOX2 protein levels (Fig. 2C). Importantly, there was a strong negative correlation between NOX2 levels and TA-specific force values ($R^2 = 0.76; P < 0.001$, Fig. 2D).
These data are consistent with a recent finding showing improved force production by mdx muscle after genetic ablation of a NOX2 regulatory subunit (32).

Simvastatin Improves Resistance to Muscle Fatigue in mdx Mice. In addition to the loss of specific force, increased muscle fatigue and slowed force recovery are significant causes of muscle weakness in DMD (4, 35). Muscle fatigue in TA was measured during repetitive tetanic contractions (every 2 s) for a total of 2 min. For mdx Con mice, force declined rapidly during the early part of the fatigue and then much less steeply for the remainder of the contractions (Fig. 3A). In contrast, the force decline for mdx Sim was very similar to the WT groups, with a slow force drop over the first minute and then a greater decline over the last minute (see Fig. 3A). After 1 min of fatigue, mdx Sim had significantly greater force than mdx Con mice (68% vs. 53% of initial force, P < 0.05; Fig. 3B). After 2 min, there was no difference between any groups, including WT mice (see Fig. 3A).

Recovery from fatigue was measured up to 10 min after fatigue (Fig. 3C). For mdx Con mice, the average recovery at 2 min was 73% of the initial, pretetanic force, compared with 80.9% for mdx Sim mice. By 10 min, there was no further recovery for mdx Con, but values for mdx Sim mice increased to 86.9% and were statistically different (P < 0.01). Values for mdx Sim mice were not statistically different from both WT groups, indicating that simvastatin normalizes muscle fatigue and recovery to WT levels in dystrophic mice. The cellular mechanisms of muscle fatigue are complex; however, ROS are known to be an important cause of force loss during muscle fatigue (36). As for specific force, we also found that NOX2 levels (see Fig. 2C), negatively correlated with muscle force after 1 min of fatigue (R² = 0.48, P < 0.001). Therefore, it is likely that reduced ROS production from NOX2 contributes to the improved fatigue resistance in simvastatin-treated mdx mice.

Plasma LDL Cholesterol Is Higher in mdx Mice but Not Reduced by Simvastatin. In contrast to humans, statins are usually ineffective at reducing the naturally low, circulating LDL cholesterol levels in mice, except when specific genetic and/or dietary changes are made (37). Nevertheless, to determine whether the beneficial effects of simvastatin on TA muscle force were associated with reduced circulating cholesterol levels, we measured the plasma LDL and very-low-density lipoprotein (VLDL) as well as HDL cholesterol concentrations. Interestingly, mdx Con mice had significantly higher LDL/VLDL levels than WT mice, in accordance with elevated serum cholesterol levels in DMD individuals (38). However, simvastatin did not lower LDL/VLDL in mdx or WT mice (Fig. S3A). HDL cholesterol was not significantly different among any groups (Fig. S3B). These data indicate that the improved muscle function in mdx Sim mice is not attributable to a plasma cholesterol-lowering effect of simvastatin.

Simvastatin Improves Diaphragm Muscle Function in Old mdx Mice and Reverses Fibrosis. In mdx mice, the diaphragm most closely recapitulates the functional deficits and fibrotic deposition that occur in DMD (4). Therefore, we treated old mdx mice with simvastatin to determine whether it could improve diaphragm force and reduce or reverse preexisting fibrosis. As with younger mice (see Fig. L4), old mdx mice treated with simvastatin for 2 mo had significantly lower plasma CK levels, indicating protection against ongoing muscle damage (Fig. 4A). Diaphragm-specific force was also significantly improved by 20–30% over a wide range of stimulation frequencies (10–120 Hz), compared with untreated mdx mice (Fig. 4B).

The replacement of muscle fibers with fibrotic connective tissue is a major cause of impaired muscle force generation in DMD (3) and therefore an important therapeutic target. First, we evaluated fibrosis by fibronectin immunofluorescence of diaphragm sections (Fig. 4C), which revealed a dramatic (50%) attenuation of fibrosis in mdx Sim mice compared with mdx Con (Fig. 4D). Quantification of total collagen I levels in diaphragm muscles by hydroxyproline assay also indicated a 50% reduction in fibrosis for mdx Sim mice (Fig. 4E). Because diaphragm connective tissue deposition is already extensive in mdx mice at this age, our data suggest that simvastatin likely reversed some of the preexisting fibrosis, consistent with findings of statin treatment in fibrotic cardiac muscle (39).

Physiological Concentrations of Simvastatin Do Not Impair Muscle Regeneration or Myogenesis in mdx Muscle. It has been suggested that statins, including simvastatin, impair muscle regeneration by impeding myoblast differentiation (40). However, the statin concentrations required to induce these deleterious effects in vitro are typically 1 μM or greater. These concentrations are considerably (100–1,000 times) higher than those found in vivo in mice and humans (41). In the present study, we measured the plasma levels of simvastatin in treated mdx mice, which were, on average, 403 ± 108 nM (n = 7). In treated rats, the simvastatin concentration in skeletal muscle relative to plasma is 30% (42). Therefore, we would expect the muscle levels of simvastatin in our mice to be ∼120 nM. At this concentration, we found that muscle regeneration in vivo was unaffected in mdx Sim mice, because they had a comparable number of centrally nucleated (regenerated) muscle fibers to mdx Con mice after long-term (8-mo) treatment (Fig. S4A and B). We then carried out an in vitro experiment using an immortalized mdx myoblast cell line (kindly provided by Terry Partridge, Children’s National Medical Center, Washington). At the start of differentiation, we treated the cells with a range of simvastatin concentrations for 3 d and found that muscle differentiation, in terms of myotube formation, appeared similar to untreated cells at concentrations ranging from 50 to 500 nM (Fig. S4C). In accord with previous studies, at doses of 1 μM or higher, simvastatin became more toxic, and the number of viable cells progressively decreased over 3 d of treatment. Again, this finding highlights the point that simvastatin concentrations within the normal, in vivo physiological range do not impair myogenesis in dystrophic muscle cells, and deleterious effects only occur with exposure to much higher doses.

Simvastatin Enhances Autophagy and Reduces Oxidative Stress but Does Not Simulate Atrogin-1 in mdx Muscle. Autophagy is an important cellular pathway for degrading damaged proteins,
muscles were determined by the Hydroxyproline assay. 

Increased at any stage of the disease (21). We quantified mRNA levels are consistently lower than in normal muscle and are not statin-induced myopathy (13). However, in DMD, atrogin-1 is a gase that stimulates protein breakdown (13). Atrogin-1 is in- 
ductive from NOX2, and normal muscle susceptible to statin myopathy, dystrophic muscle, where statins reduce oxidative stress derived (12). 

This finding highlights important differences between mitochondiral ROS increases oxidative stress (12). 

Autophagy, and treatment of mdx mice with a low-protein diet triggered autophagy, which reduced inflammation and fibrosis and enhanced muscle function (43). Simvastatin was recently shown to enhance autophagy in arterial myocytes (44), and therefore, we postulated that increased autophagy might contribute to the improved muscle health in mdx mice. A key protein marker of autophagic flux is the microtubule-associated protein 1A/1B light chain 3 (LC3), which has a cytosolic form (LC3A) and a lipidated form (LC3B). An increased level of LC3B relative to LC3A is indicative of enhanced autophagic flux (43). We measured LC3A and LC3B expression in the diaphragm of simvastatin-treated and untreated mdx mice, using an antibody that detects both protein isoforms. As shown in Fig. 5A, the levels of LC3A were not different between the groups; however, LC3B was significantly increased by 40% in mdx Sim mice (P < 0.01), signifying enhanced autophagy. This result is consistent with the increased levels of LC3B by simvastatin in arterial myocytes (44).

Interestingly, recent evidence revealed that NOX2-derived ROS play a key role in reducing autophagy in mdx muscle (32). Therefore, our data showing reduced NOX2 expression by simvastatin is consistent with its autophagy enhancement of dystrophic muscle. To further explore this idea, we also measured the levels of the ROS (H2O2) in diaphragm muscle homoge- 

Fig. 4. Simvastatin treatment in old mdx mice attenuates muscle damage, improves diaphragm force, and reduces fibrosis. In these experiments, mice were treated with simvastatin starting at 12 mo of age for a total of 2 mo. (A) Whole-body muscle damage in old mice was measured by the levels of plasma CK activity. *P < 0.05 compared with mdx Con. (B) Pooled specific force values of diaphragm muscle strips as measured at different stimulation frequencies for mdx Con and mdx Sim mice. *P < 0.05; **P < 0.01 (n ≥ 6). (C) Representative sections showing connective tissue levels in diaphragm muscles by fibronectin (green) immunostaining. The sarcolemma is outlined by Caveolin3 (red), and nuclei are stained with DAPI (blue). (Scale bar: 100 μm.) (D) Quantification of Fibronectin immunofluorescence from diaphragm muscle cross-sections. **P < 0.01 compared with mdx Con. (E) Collagen I levels in homogenized diaphragm muscles were determined by the Hydroxyproline assay. *P < 0.05 compared with mdx Con.

levels of atrogin-1 by real-time quantitative RT-PCR (qPCR) in quadriceps muscles of old mice and also found that levels in mdx Con were significantly lower than for WT (Fig. 5C). Interestingly, values for mdx Sim mice were also lower than WT and not significantly different from mdx Con (Fig. 5C). These data indicate that atrogin-1 is not induced in dystrophic muscle by simvastatin. Again, this finding emphasizes the opposite effect of simvastatin on a pathogenic pathway in dystrophic skeletal muscle compared with normal muscle.

Fig. 5. Simvastatin treatment in old mdx mice enhances autophagy, attenuates ROS levels, and does not induce atrogin-1. In these experiments, mice were treated with simvastatin starting at 12 mo of age for a total of 2 mo. (A, Upper) Western blot showing the levels of the autophagy proteins LC3A (upper band) and LC3B (lower band) for mdx Con and mdx Sim. GAPDH is shown as a loading control. (A, Lower) Pooled data for LC3A and LC3B. Hydrogen peroxide (H2O2) levels in diaphragm muscle homogenates, as quantified with a fluorescent amplex red assay. *P < 0.05 compared with mdx Con. (C) Atrogin-1 mRNA levels in quadriceps muscles were quantified by qPCR and normalized to the internal control (HPRT). *P < 0.05; **P < 0.01 compared with WT Con.
Conclusions
In summary, to our knowledge, this is the first time that treatment of dystrophic mdx mice with simvastatin provides a dramatic reduction in inflammation, oxidative stress, and fibrosis, key pathogenic pathways that mediate skeletal muscle damage and functional impairment in DMD. Most importantly, these mechanistic effects translated into a substantial improvement in skeletal muscle physiological function, both in terms of specific force production and protection from muscle fatigue. Although our results may initially seem unexpected, based on the general perception that statins can be myotoxic, they are accordant with extensive evidence demonstrating statin-mediated inhibition of these pathogenic pathways in both the cardiovascular system and ischemic skeletal muscle. Thus, our data are consistent with the idea that statins are highly beneficial to skeletal muscles afflicted with an underlying disease that involves ischemia, oxidative stress, and inflammation. Further studies are now required to delve into both the cellular and molecular mechanisms that mediate these positive effects. From a clinical perspective, several statins, including simvastatin, are already FDA-approved for the treatment of familial hypercholesterolemia in children as young as 10 y of age. Thus, our novel findings indicate that simvastatin and possibly other statins have great potential to provide a readily available therapy for DMD and related neuromuscular diseases.

Methods
Detailed methods can be found in SI Methods. Details of assays for Western blotting, immunostaining, hydroxyproline, RT-PCR, and plasma CK and CK-MB are available in SI Methods. Protocols for simvastatin treatment, muscle function, cell culture, and plasma simvastatin measurements are also available in SI Methods.

Male dystrophin-deficient (mdx) and WT mice at the C57BL/10ScSn background were used for all experiments, which were approved by the Institutional Animal Care and Use Committee at the University of Washington.

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Supporting Information

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SI Methods

Simvastatin Treatment. Simvastatin powder (TCI America) was formulated as a treatment in two ways: in the drinking water and in the food. The inactive form of simvastatin contains a lactone group and is relatively insoluble in water. Therefore, for the drinking-water experiments (see data for Fig. 1), simvastatin (50 mg) was initially dissolved in 1 mL of ethanol and then mixed for several minutes into 1 L of alkaline water (pH ∼10), which hydrolyzes the lactone group, enabling the active (hydroxy acid) form of simvastatin to be solubilized in water. Hydrochloric acid was then added to lower the pH to ∼2.5–3.0, which was the pH of the drinking water given to the control (untreated) mice. For formulation into the food (see data for Figs. 2–4), simvastatin (lactone form) was mixed into a standard rodent diet (D12450B) at a concentration of 80 mg/kg (Research Diets).

Skeletal Muscle Contractile Function. Diaphragm function, ex vivo, was measured as described (4). Briefly, diaphragm strips (∼2–3 mm wide) were perfused with a physiological solution bubbled with 95% O2/5% CO2. Stimulation was provided by two platinum electrodes, attached along the sides of the chamber. A length-force curve, established by using 120-Hz isometric contractions (300-ms duration) spaced 1 min apart, established the optimum length (length producing maximum tetanic force), which was later used to calculate the specific force (force divided by cross-sectional area) (4).

TA muscle function was measured in situ. Mice were anesthetized with an i.p. injection of Avertin (625 mg/kg) and placed on a heated metal platform (∼37 °C). The distal TA tendon was dissected free and sutured to the lever arm of the force and length control system (305C-LR; Aurora Scientific). Muscle contraction was provided by stimulation of the sciatic nerve using bipolar electrodes, which were kept moist during the experiment with PBS (pH 7.4). The knee bone was firmly anchored to the platform via a steel pin. A length-force curve was generated by stimulating the muscle at 120 Hz (200 ms duration), every 90 s, from short to long muscle lengths. Isometric force at the optimum length was used to calculate the specific force, by dividing force by cross-sectional area, as described (4). After establishing the optimum length, the muscle then underwent a fatigue protocol, in which it was stimulated at 120 Hz (200-ms duration) every 2 s for a total of 2 min. Fatigue recovery was measured every 2 min up to 10 min after fatigue.

Western Blotting. Western blotting was performed as described (5). Briefly, muscles were homogenized in a PBS buffer (pH 7.4) containing EDTA (5 mM), protease (Thermo Scientific) and phosphatase (Roche) inhibitor mixtures, and 1% Triton X-100. Samples were loaded onto 4–15% gradient gels (Bio-Rad) and transferred on PVDF membranes (Millipore). Membranes were blocked for 1–2 h with 5% (wt/vol) skim milk in PBS containing 0.1% Tween 20 (PBST) (pH 7.4) or 2.5% (wt/vol) BSA in PBST for phosphorylated proteins and then incubated with primary antibodies in blocking buffer for 1 h at room temperature or overnight at 4 °C. Primary antibodies used were as follows: NOX2, α-dystrobrevin 1 and 2, and caveolin 3 (BD Biosciences); LC3A/B (Bio-Rad), utrophin and α-syntrophin (noncommercial antibodies from Froehner laboratory); nNOS (Invitrogen); β-dystroglycan (Novocastra); and GAPDH (Santa Cruz Biotechnology). HRP-labeled secondary antibodies were then incubated for 1 h at room temperature. Membranes were incubated with enhanced chemiluminescence reagent (Amersham), and bands were detected by the FluorChem M imaging system (Protein Simple).

Immunofluorescence Images. Muscles were imbedded in optimal cutting temperature compound and frozen in isopentane cooled in liquid nitrogen. Cryosections (10-μm thick) were placed on a glass slide. Sections were fixed with either ice-cold methanol or 2% (vol/vol) paraformaldehyde for 5–10 min. Some sections were stained with hematoxylin and eosin (H&E) using standard procedures. For immunofluorescence, sections were incubated in blocking buffer (0.8% BSA and 1% fish gelatin in PBS) for 45 min. Primary antibodies—Fibronectin (Sigma); CD68 (Abcam); Caveolin-3 (BD Biosciences); and myosin heavy chain 1, 2A, and 2B (Developmental Studies Hybridoma Bank)—were added for 1 h and 30 min at room temperature. Alexa Fluor-conjugated secondary antibodies (Life Technologies) were then added for 1 h and 30 min. In some experiments, DAPI was also added to detect nuclei. Sections were mounted with anti-fade Gold reagent (Life Technologies), coveredslipped, and imaged with a Zeiss LSM510 confocal microscope (W. M. Keck Center, University of Washington) or a Zeiss Axioscope 2 fluorescent microscope. For fluorescence quantification (CD68, fibronectin, and myosin heavy chains), images were converted to grayscale in ImageJ, and a threshold was applied to calculate the area of fluorescence as a percent of the total area of the section. Fibers with central nuclei were expressed as a percent of total fibers with detectable nuclei (i.e., both central and peripheral).

Hydroxyproline Collagen I Fibrosis Assay. To quantify muscle fibrosis, Collagen I content was measured by using the hydroxyproline assay. Muscles were homogenized in the same PBS–Triton X-100 buffer (pH 7.4) used for Western blotting. Samples were centrifuged at 9,500 × g for 10 min. The pellet was used for hydroxyproline measurement. The supernatant was used to measure the total protein concentration (BCA assay) for later normalizing hydroxyproline values. The insoluble pellets, containing Collagen I, were placed into glass tubes with 200 μL of 6M HCl. Samples were boiled for 24 h at 120 °C in a heat block. After vortexing and centrifuging at 9,500 × g for 3 min, the supernatants were used for the hydroxyproline assay, using a commercial kit (Chondrex). Hydroxyproline values (micrograms per microliter) were normalized to the total protein content of the muscle.

Plasma CK Activity. Plasma CK was evaluated as a measure of whole-body muscle damage. Blood was drawn via submandibular puncture, collected into EDTA-coated tubes (BD Microtainer), and centrifuged at 2,400 × g for 12 min at 4 °C. Plasma was then used to measure CK activity with a standard assay kit, according to the manufacturer’s instructions (StanBio).

Cell Culture Experiments. Immortomouse mdr myoblasts were kindly provided by Terry Partridge (Children’s National Medical Center). Myoblasts were grown on gelatin-coated plates and until ∼70% confluence in DMEM supplemented with 10% (vol/vol) horse serum (HS), 20% (vol/vol) FBS, 0.5% chicken embryo extract, and 20 units/mL of γ-IFN. To induce differentiation into myotubes, cells were incubated with DMEM containing 5% (vol/vol) HS, with or without different concentrations of simvastatin for 3 d. Myotubes were washed in PBS (pH 7.4), fixed in ice-cold methanol for 10 min, and H&E-stained. Images of myotubes were taken with a Nikon inverted microscope (W. M. Keck Center, University of Washington).
Real-Time PCR. Total RNA was isolated from quadriceps of WT, mdx, and simvastatin-treated mdx mice by using TRIzol (Life Technologies) and further purified by using an RNAeasy Kit (Qiagen). qPCR was performed by using TaqMan chemistry and the ABI 7000 sequence detection system with 50 ng of RNA, ABI Fast Virus 1-Step Master Mx reagents, and primer sets specific for atrogin-1 and HPRT (Applied Biosystems). Data were obtained from five mice in each treatment group and normalized to the internal HPRT control.

Plasma Cholesterol Measurements. By using a commercial assay kit (Abcam), LDL/VLDL and HDL fractions were separated from plasma and mixed with an enzyme reaction solution containing a fluorescent probe to quantify the amount of cholesterol in the sample. Fluorescence levels were measured with a spectrophotometer in a 96-well plate. Cholesterol values for each sample were determined from a cholesterol standard curve.

Simvastatin Concentrations in mdx Mouse Plasma. Simvastatin concentrations in the plasma of mdx mice treated from 3 to 6 mo of age were measured and analyzed by Josefin Koehn and Rodney Ho (Department of Pharmaceutics, University of Washington). Simvastatin was extracted from plasma samples with methanol and cyheptamide. Briefly, 20 μL of plasma was mixed with 50 μL of methanol and 5 μL of cyheptamide (250 μg/mL in methanol) was added. Samples were vortexed for 2 min, followed by centrifuging for 10 min at 18,600 × g. The supernatant was collected and used for analysis by HPLC/UV. The separation was performed on a Zorbax XDB-C8 column (50 × 2.1 mm; particle size, 5 μm) (Agilent). The mobile phase consisted of A, H2O; and B, MeOH. The flow rate was set to 0.6 mL/min, and simvastatin was detected at a UV wavelength of 238 nm. Plasma samples from untreated mice were used as a negative control and for setting up the standard curve.

Statistical Analysis. All data are presented as means ± SEM. The significance level for all experiments was set at \( P < 0.05 \). Unpaired Student’s t test was used to compare values between two groups. One-way ANOVA with least significant difference post hoc test was used for analysis of more than two groups. For analysis of CD68 immunostaining, the nonparametric Mann–Whitney \( U \) test was applied. For some variables, a linear regression analysis was performed. The statistical program used was Data Desk.

**Fig. S1.** Simvastatin does not increase utrophin or other dystrophin complex proteins in mdx muscle. In these experiments, mice were treated with simvastatin starting from weaning (3 wk of age) for a total of 8 mo. (A) Representative Western blots showing expression levels of utrophin and other members of the DPC in quadriceps muscles from mdx Con and mdx Sim mice. (B) Pooled data showing the expression levels of the proteins shown in A.
**Fig. S2.** Diaphragm-specific force for WT mice. In these experiments, mice were treated with simvastatin starting from weaning (3 wk of age) for a total of 8 mo. Diaphragm force normalized to cross-sectional area (specific force) was measured over a range of stimulation frequencies for WT Con and WT Sim mice ($n = 5$ for both groups).

**Fig. S3.** Plasma cholesterol measurements in simvastatin-treated and untreated mdx and WT mice. In these experiments, mice were treated with simvastatin from 3 mo up to 6 mo of age. Plasma LDL and VLDL (LDL/VLDL) and HDL fractions were separated and quantified by using a fluorescent assay. LDL/VLDL concentration (A) and HDL concentration (B) are shown. ***$P < 0.001$ vs. mdx Con; **$P < 0.01$ vs. mdx Sim; ###$P < 0.001$ vs. both mdx groups.
Fig. S4. Simvastatin does not impair muscle regeneration or myogenesis in mdx muscle. (A) Representative images showing Caveolin-3 (green) and DAPI (red) from mdx Con and mdx Sim muscles after 8 mo of simvastatin treatment. In both groups, note the large number of muscle fibers with one or more central nuclei. (Scale bar: 20 μm.) (B) Pooled data showing the number of fibers with central nuclei as a percentage of the total fibers (central and peripheral nuclei). (C) Representative H&E images of differentiated myotubes, after 3 d of treatment without or with simvastatin concentrations ranging from 0.05 to 0.5 μM. (Scale bar: 50 μm.)