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

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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 *mdx* 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.

statin | muscular dystrophy | fibrosis | inflammation | muscle force

Duchenne muscular dystrophy (DMD) is a degenerative muscle disease caused by the absence of dystrophin, a large protein that links the cytoskeleton to the surface membrane in muscle cells. Loss of dystrophin causes widespread effects on muscle signaling and metabolic pathways, leading to cell death and progressive replacement of functional muscle fibers with fibrotic connective tissue. This process results in profound muscle weakness, usually leaving DMD boys wheelchair-bound by their early teenage years and leading to death from the consequences of respiratory and/or cardiac muscle failure by age 20–30. Current treatments, such as corticosteroids, slow disease progression only marginally (1), whereas gene-based approaches, such as exon-skipping, although promising in preclinical studies, will need to overcome many technical and regulatory hurdles, as well becoming affordable, before they are a widely available therapy for DMD patients (2). Therefore, efficacious pharmaceutical agents that are cost-effective and already approved for human use are particularly attractive candidates for the current treatment of DMD.

In DMD patients and dystrophin-deficient (*mdx*) mice, muscle degeneration has been attributed to a number of pathogenic processes; however, chronic inflammation, oxidative stress, and fibrosis certainly have major impacts on the disease progression and functional impairment (3–5). Therefore, therapeutic approaches targeting these pathways would likely provide significant improvement in DMD muscles. HMG CoA-reductase inhibitors (statins) are the most commonly prescribed drugs for treating high blood LDL cholesterol levels and associated cardiovascular diseases. A number of studies have shown that statins improve the cardiovascular system both by lowering circulating LDL cholesterol levels and through cholesterol-independent or "pleiotropic" mechanisms that lead to reduced oxidative stress, inflammation, and fibrosis (6, 7). Therefore, we reasoned that if statins inhibited these same pathogenic pathways in dystrophic muscle, these drugs would result in reduced muscle damage and improved physiological function.

Before the present study, statins had never been tested in DMD or any other neuromuscular disease. Indeed, statins are typically avoided in patients with muscle diseases because of the rare, muscle-related side effects that occur with statin use in the general population. The overall prevalence of statin-induced myopathy is unclear; however, a recent systematic review of several clinical trials found the incidence of adverse muscle symptoms among statin users was <1% compared with placebo controls (8). Moreover, long-term follow-up studies of statin use in children 10 y or older—the most relevant patient cohort for DMD—indicated that statins are well tolerated and have minimal side effects, including those relating to skeletal muscle (9, 10). Indeed, many of the major risk factors for statin myopathy, such as female sex, older age, and intense exercise (11), are not relevant for DMD boys. The causes of statin-induced myopathy are unclear, although several mechanisms have been

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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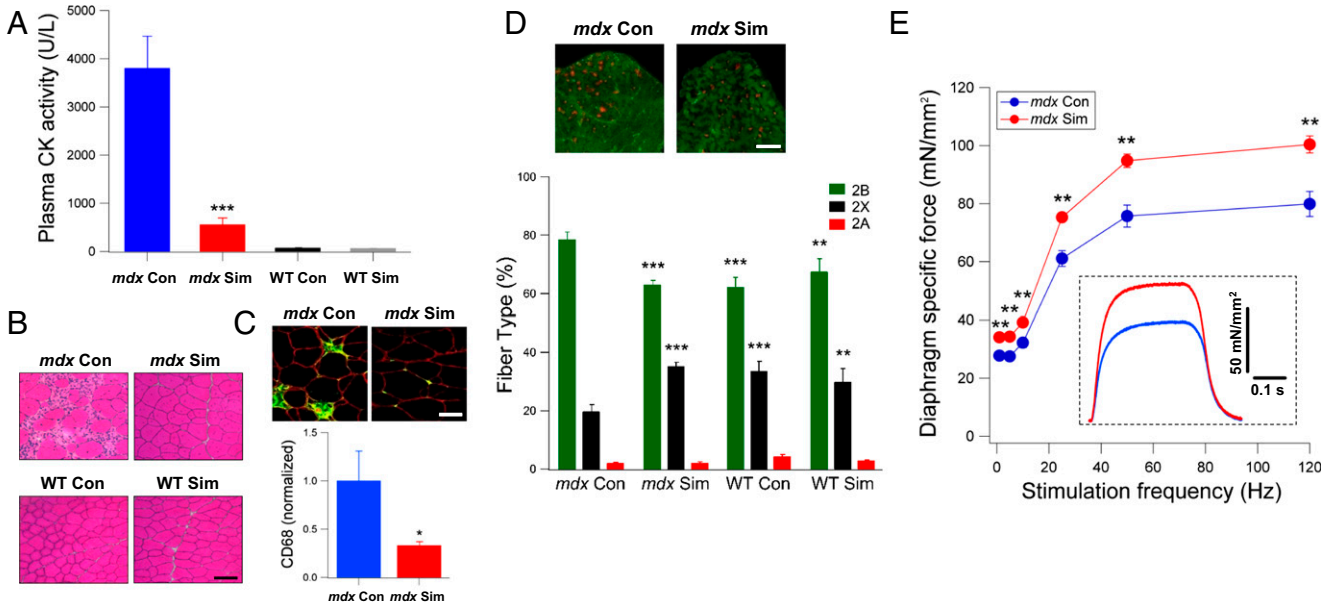

Fig. 1. Long-term simvastatin treatment minimizes muscle damage and inflammation, shifts fiber type, and increases diaphragm muscle force in *mdx* mice. In these experiments, mice were treated with simvastatin, starting from weaning (3 wk of age) for a total of 8 mo. (*A*) Whole-body muscle damage was measured by the levels of plasma CK activity. ***$P < 0.001$ for *mdx* Con ($n = 10$) compared with *mdx* Sim ($n = 9$). (*B*) Representative H&E-stained images of TA sections from *mdx* Con, *mdx* Sim, WT Con, and WT Sim mice. Note the inflammatory cell infiltration in the *mdx* Con section. (Scale bar: 50 μm.) (*C*, *Upper*) Representative images showing inflammation for *mdx* Con and *mdx* Sim using a CD68 antibody (green). The sarcolemma is labeled with a Caveolin-3 antibody (red). (Scale bar: 20 μm.) (*C*, *Lower*) Quantification of the CD68 levels for the two groups ($n = 9$) is shown. *$P < 0.05$. (*D*, *Upper*) Representative images showing myosin heavy chain 2B (green), myosin heavy chain 2A (red), and myosin heavy chain 2X (unstained, black) in an *mdx* Con and *mdx* Sim muscle section. (*D*, *Lower*) Pooled values for the percent of each fiber type are shown for *mdx* Con ($n = 8$), *mdx* Sim ($n = 7$), WT Con ($n = 5$), and WT Sim ($n = 5$) mice. ***$P < 0.001$; **$P < 0.01$ compared with *mdx* Con. (Scale bar: 200 μm.) (*E*) Diaphragm force normalized to cross-sectional area (specific force) was measured over a range of stimulation frequencies for *mdx* Con and *mdx* Sim mice. **$P < 0.01$ ($n = 9$ for both groups). *Inset* shows representative specific force traces from an *mdx* Con and *mdx* Sim mouse, during stimulation at 120 Hz.

postulated, including increased oxidative stress (12), activation of the atrogin-1 muscle atrophy pathway (13), and increased susceptibility to RyR1-induced Ca^{2+} leak in a malignant hyperthermia mouse model (14). Despite the fact that statins are often considered to be toxic to skeletal muscle, the opposite is true in ischemic limb diseases, such as diabetes, for which several studies have shown that statins provide substantial improvement to overall skeletal muscle health (15–19). These improvements are associated with reduced oxidative stress and inflammatory cell infiltration, which attenuates muscle necrosis (16–19). Given that dystrophic muscles are also subject to chronic oxidative stress (20), inflammation (21), and exercise-induced ischemia (22), all of which contribute to muscle damage and functional impairment, there is a strong rationale that statins could be efficacious in DMD.

In the present study, we treated DMD (*mdx*) mice with simvastatin, a lipophilic statin known to be effectively transported into skeletal muscle fibers. Using a simvastatin concentration that equates to a moderate daily dose in humans, we show that both long- and short-term treatment in *mdx* mice provides robust improvement in muscle pathology and physiological function, which was accompanied by reduced inflammation, oxidative stress, and fibrosis. We also provide evidence that statin 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.

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-y-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. 1*A*). This measure of improved muscle health was validated by histological assessment of the tibialis anterior (TA) muscle, which showed less inflammation in *mdx* Sim mice (Fig. 1*B*). Of note, simvastatin had no effect on CK levels or gross muscle histology of WT mice (Fig. 1 *A* and *B*), indicating that there was no measureable muscle damage in normal mice at this moderate dose. Next, we quantified the extent of inflammation—a key feature of the dystrophic muscle pathology—by immunofluorescence of TA muscle sections with CD68, a marker of macrophages and other inflammatory cells (20). As highlighted in Fig. 1*C*, simvastatin substantially reduced inflammatory cell levels by close to 70% compared with *mdx* Con. These results are

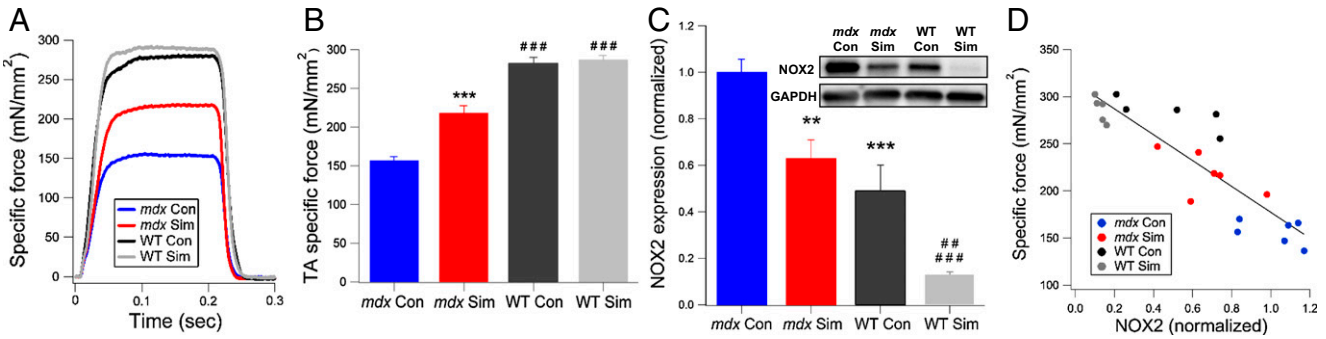

Fig. 2. Simvastatin treatment enhances TA muscle force and reduces NOX2 expression in *mdx* mice. In these experiments, mice were treated with simvastatin from 3 mo up to 6 mo of age. (*A*) Representative traces showing specific force values during 120-Hz isometric contractions at the optimum muscle length for *mdx* and WT mice, with or without simvastatin treatment. (*B*) Pooled values of TA-specific force for *mdx* Con ($n = 6$), *mdx* Sim ($n = 6$), WT Con ($n = 5$), and WT Sim ($n = 5$) mice. $^{***}P < 0.001$ compared with *mdx* Con; $^{###}P < 0.001$ compared with both *mdx* groups. (*C*) Representative Western blot showing NOX2 expression from TA (*Inset*) and the pooled values for each group. Values were normalized to GAPDH, which was used as a loading control. $^{**}P < 0.01$ compared with *mdx* Con; $^{***}P < 0.001$ compared with *mdx* Con; $^{###}P < 0.001$ compared with both *mdx* groups; $^{##}P < 0.01$ compared with WT Con. (*D*) Values for NOX2 expression plotted against TA-specific force for *mdx* and WT mice with or without simvastatin treatment. A linear regression line has been fitted to the data ($R^2 = 0.76$, $P < 0.001$).

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 results showed no additional increase in the expression levels of utrophin, other members of the dystrophin complex, or associated proteins such as nNOSμ 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. 1*D*, 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 *mdx* Sim and both WT groups (Fig. 1*D*). 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. 1*E*), 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. 2 *A* 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 muscle force production in specific disease conditions that are characterized by severe muscle damage and functional impairment.

Recent evidence by us and others has shown that oxidative stress due to increased reactive oxygen species (ROS) production by NADPH oxidase 2 (NOX2) is a major cause of muscle weakness in *mdx* mice (5, 31, 32). Because statins are known to inhibit 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 expression levels. NOX2 levels were also decreased in WT Sim mice, possibly suggesting a common inhibitory effect of simvastatin on skeletal muscle NOX2 protein levels (Fig. 2*C*). Importantly, there was a strong negative correlation between NOX2 levels and TA-specific force values ($R^2 = 0.76$; $P < 0.001$, Fig. 2*D*).

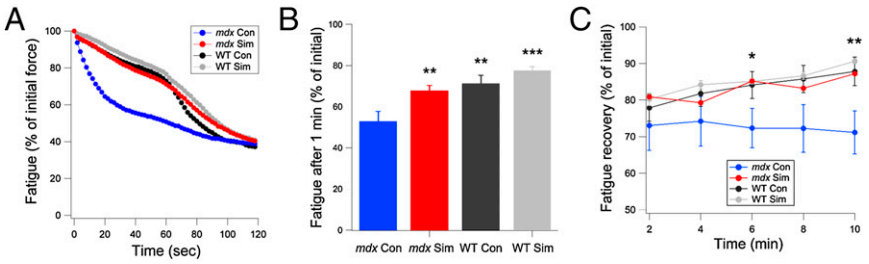

Fig. 3. Simvastatin protects against muscle fatigue and improves force recovery in *mdx* mice. In these experiments, mice were treated with simvastatin from 3 mo up to 6 mo of age. (*A*) Representative traces of TA muscle force for *mdx* and WT mice with or without simvastatin treatment during 2 min of fatiguing contractions (every 2 s). (*B*) Pooled values of TA force after 1 min of fatigue. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with *mdx* Con. (*C*) Pooled values of TA force recovery after fatigue from 2 to 10 min. $^{*}P < 0.05$; $^{**}P < 0.01$ for *mdx* Con vs. all other groups.

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. 3*A*). 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. 3*A*). 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. 3*B*). After 2 min, there was no difference between any groups, including WT mice (see Fig. 3*A*).

Recovery from fatigue was measured up to 10 min after fatigue (Fig. 3*C*). For *mdx* Con mice, the average recovery at 2 min was 73% of the initial, prefatigue 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 significantly 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. 2*C*), negatively correlated with muscle force after 1 min of fatigue ($R^2 = 0.48$, $P < 0.001$). 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 function 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. S3*A*). HDL cholesterol was not significantly different among any groups (Fig. S3*B*). 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. 1*A*), old *mdx* mice treated with simvastatin for 2 mo had significantly lower plasma CK levels, indicating protection against ongoing muscle damage (Fig. 4*A*). 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. 4*B*).

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. 4*C*), which revealed a dramatic (50%) attenuation of fibrosis in *mdx* Sim mice compared with *mdx* Con (Fig. 4*D*). Quantification of total collagen I levels in diaphragm muscles by hydroxyproline assay also indicated a 50% reduction in fibrosis for *mdx* Sim mice (Fig. 4*E*). 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. S4 *A* 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. S4*C*). 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,

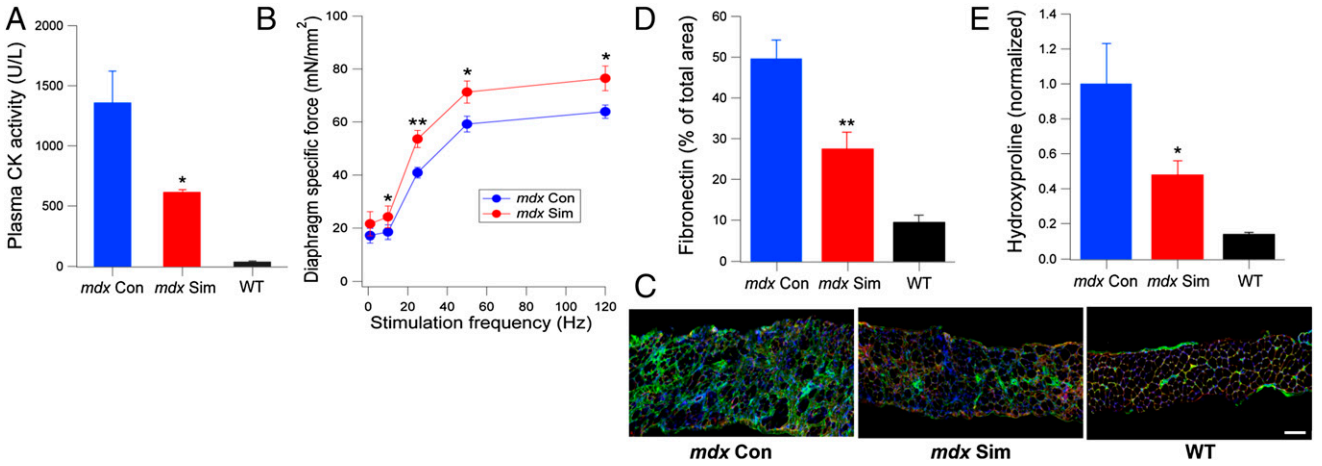

Fig. 4. Simvastatin treatment in old *mdx* mice attenuates muscle damage, improves diaphragm force, and reduces fibrosis. In these experiments, mice were treated with simvastatin starting at 12 mo of age for a total of 2 mo. (*A*) Whole-body muscle damage in old mice was measured by the levels of plasma 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. $*P < 0.05$; $**P < 0.01$ ($n \geq 6$). (*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.

organelles, and protein aggregates, which are then recycled by the cell for energy use. Impaired autophagy can trigger cell death, and in skeletal muscle, proper regulation of autophagy is essential for normal cellular and physiological function (43). Both *mdx* and DMD muscles show evidence of reduced 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. 5*A*, 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 (H_2O_2) in diaphragm muscle homogenates. We found that *mdx* Sim muscles had ∼30% less H_2O_2 compared with *mdx* Con (Fig. 5*B*), indicative of reduced oxidative stress. This finding highlights important differences between dystrophic muscle, where statins reduce oxidative stress derived from NOX2, and normal muscle susceptible to statin myopathy, where mitochondrial ROS increases oxidative stress (12).

Another pathway that has been implicated in statin myopathy is muscle atrophy mediated by atrogin-1, a ubiquitin-protein ligase that stimulates protein breakdown (13). Atrogin-1 is induced by statin treatment in animal models and humans with statin-induced myopathy (13). However, in DMD, atrogin-1 levels are consistently lower than in normal muscle and are not increased at any stage of the disease (21). We quantified mRNA 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. 5*C*). Interestingly, values for *mdx* Sim mice were also lower than WT and not significantly different from *mdx* Con (Fig. 5*C*). 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.

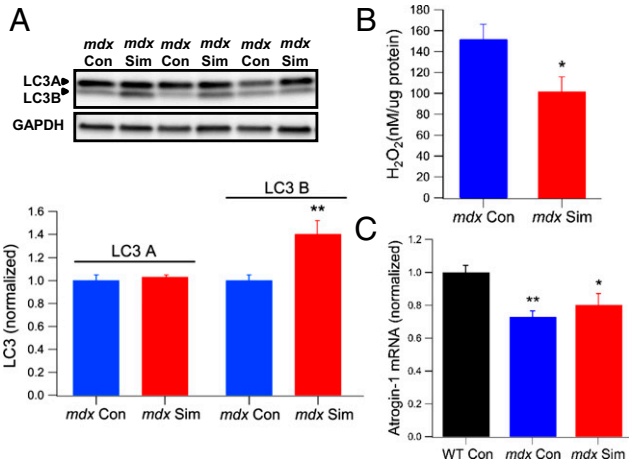

Fig. 5. Simvastatin treatment in old *mdx* mice enhances autophagy, attenuates ROS levels, and does not induce atrogin-1. In these experiments, mice were treated with simvastatin starting at 12 mo of age for a total of 2 mo. (*A*, *Upper*) Western blot showing the levels of the autophagy proteins LC3A (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 are shown. $**P < 0.01$ compared with *mdx* Con. (*B*) Hydrogen peroxide (H_2O_2) 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, our results reveal for 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 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 cholesterol 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 on 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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