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RESEARCH ARTICLE

Effects of PDE5 inhibition on dystrophic muscle following an acute bout of downhill running and endurance training

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Batra A, Vohra RS, Chrzanowski SM, Hammers DW, Lott DJ, Vandenborne K, Walter GA, Forbes SC. Effects of PDE5 inhibition on dystrophic muscle following an acute bout of downhill running and endurance training. *J Appl Physiol* 126: 1737–1745, 2019. First published April 4, 2019; doi:10.1152/japplphysiol.00664.2018.— Lack of sarcolemma-localized neuronal nitric oxide synthase mu (nNOSμ) contributes to muscle damage and fatigue in dystrophic muscle. In this study, we examined the effects of compensating for lack of nNOSμ with a phosphodiesterase type 5 (PDE5) inhibitor in *mdx* mice following downhill running and endurance training. Dystrophic mice (*mdx*) were treated with sildenafil citrate and compared with untreated *mdx* and wild-type mice after an acute bout of downhill running and during a progressive low-intensity treadmill running program (5 days/wk, 4 wk). Magnetic resonance imaging (MRI) and spectroscopy (MRS) transverse relaxation time constant (T_2) of hindlimb and forelimb muscles were measured as a marker of muscle damage after downhill running and throughout training. The MRI blood oxygenation level dependence (BOLD) response and ^{31}phosphorus MRS (^{31}P-MRS) data were acquired after stimulated muscle contractions. After downhill running, the increase in T_2 was attenuated ($P < 0.05$) in treated *mdx* and wild-type mice compared with untreated *mdx*. During training, resting T_2 values did not change in wild-type and *mdx* mice from baseline values; however, the running distance completed during training was greater ($P < 0.05$) in treated *mdx* (>90% of target distance) and wild-type (100%) than untreated *mdx* (60%). The post-contractile BOLD response was greater ($P < 0.05$) in treated *mdx* that trained than untreated *mdx*, with no differences in muscle oxidative capacity, as measured by ^{31}P-MRS. Our findings indicate that PDE5 inhibition reduces muscle damage after a single bout of downhill running and improves performance during endurance training in dystrophic mice, possibly because of enhanced microvascular function.

NEW & NOTEWORTHY This study examined the combined effects of PDE5 inhibition and exercise in dystrophic muscle using high-resolution magnetic resonance imaging and spectroscopy. Our findings demonstrated that sildenafil citrate reduces muscle damage after a single bout of downhill running, improves endurance-training performance, and enhances microvascular function in dystrophic muscle. Collectively, the results support the combination of exercise and PDE5 inhibition as a therapeutic approach in muscular dystrophies lacking nNOSμ.

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Duchenne muscular dystrophy; *mdx*; phosphodiesterase type 5 (PDE5) inhibitor; sildenafil citrate; skeletal muscle damage

INTRODUCTION

Mutations in the dystrophin gene lead to the absence or production of nonfunctional dystrophin protein (19). Dystrophin functions to anchor cellular contractile proteins to the sarcolemma, and lack of dystrophin results in diminished levels of neuronal nitric oxide synthase mu (nNOSμ) (6, 25). The importance of targeting nNOSμ as a potential therapeutic intervention in pathologies lacking dystrophin, such as Duchenne muscular dystrophy (DMD), is presently unclear. nNOSμ is localized at the sarcolemmal membrane and has been implicated in a number of important roles, including maintaining local muscle perfusion during and following activity (25, 45, 46), stimulating hypertrophy (15), enhancing mitochondrial biogenesis (4, 9), modulating ryanodine receptor Ca^{2+} release (55), and microtubule organization (9). Furthermore, in dystrophic muscle, lack of nNOSμ has been linked to enhanced inflammation and muscle damage (10, 15, 25).

A potential intervention aimed at compensating for lack of nNOSμ in dystrophic muscle is phosphodiesterase type 5 (PDE5) inhibitors, which function to attenuate the degradation of cyclic guanosine 3′,5′-monophosphate (cGMP), a downstream target of nitric oxide. Sildenafil citrate (Viagra) and tadalafil (Cialis) are commercially available PDE5 inhibitors that have been shown to have beneficial effects in both animal models and individuals with DMD. In animal models of DMD, benefits have included reduced muscle edema (25) and damage (38), improved muscle function (9), and extended life expectancy in zebrafish (23). Furthermore, PDE5 inhibition has been suggested to improve mitochondrial function and improve oxidative phenotype in *mdx* mice (9). In boys with DMD, both sildenafil and tadalafil were observed to restore functional sympatholysis, oxygenation, and blood flow during and following handgrip exercise (33). On the other hand, in a recent clinical trial of DMD (NCT 01865084), tadalafil (Cialis) failed to improve the primary end-point measure of performance during the 6-min walk test; however, this trial had some potential limitations, such as a heterogenous subject population and inclusion of subjects with limited muscle mass (50). Furthermore, given the close link between muscle contractions and nNOSμ (21, 35, 43), the benefits of PDE5 inhibition on

dystrophic skeletal muscle may be most effective when combined with regular exercise. Therefore, in this study, we combined PDE5 inhibition with a progressive low-intensity treadmill training protocol. Previous studies examining the effects of PDE5 inhibition in dystrophic muscle have not combined a PDE5 inhibitor intervention with regular daily aerobic exercise training (9, 25, 38).

Longitudinal changes in skeletal muscles can be evaluated noninvasively using magnetic resonance (MR) imaging (MRI) and spectroscopy (MRS), and a variety of MR methods have been widely used to monitor disease progression and treatment in dystrophic muscle (51, 53, 54). Muscle transverse relaxation time constant (T_2) is elevated after eccentric exercise (12, 29) and has been utilized as a marker of muscle damage in both healthy and dystrophic muscle (12, 30). Specifically, MR T_2 has been linked to processes associated with edema/inflammation (14) and sarcolemma integrity measured by Evans blue dye (30).

As a result, this longitudinal study examined the effect of PDE5 inhibition after an acute bout of downhill running and during progressive low-intensity endurance training in *mdx* mice monitored with T_2. We hypothesized that PDE5 inhibition (sildenafil citrate) will *1*) reduce muscle damage following an acute bout of eccentric-biased exercise (downhill running) and *2*) improve exercise performance during low-intensity treadmill training over 4 wk without exacerbating muscle damage in *mdx* mice. In addition, we hypothesized that the benefits of sildenafil citrate would be associated with an improved post-contractile MRI blood oxygenation level dependence (BOLD) response, an indicator of microvascular function (47), and faster phosphocreatine (PCr) recovery rate measured with ^{31}phosphorus MRS (^{31}P-MRS), an in vivo marker of muscle oxidative capacity (31, 34).

MATERIALS AND METHODS

Animals. The study was approved by the University of Florida (Gainesville, FL) Institutional Animal Care and Use Committee. A total of 28 *mdx* (C57BL/10ScSn-Dmd^{mdx}/J) and 10 wild-type adult mice (C57BL/10ScSnJ) were used for this study (13–48 wk of age). Mice were housed in a regulated Association for Assessment and Accreditation of Laboratory Animal Care accredited facility (12-h light/dark, 22°C, 42% humidity) and provided food ad libitum. Sildenafil citrate was administered as described previously (38). Briefly, sildenafil citrate (100-mg tablet; Viagra; Pfizer) was dissolved in water and administered via water bottles (400 mg/l) in their cages ad libitum at the start of the protocol after baseline measures and continued for the duration of the study (Fig. 1). This protocol has previously been observed to result in an average concentration of sildenafil in blood of 70 nM (SE 0.05) over 24 h in *mdx* mice (1, 37).

Exercise and training protocol. To examine the effect of PDE5 inhibition on an acute bout of muscle damage in vivo, *mdx* mice were randomized into a treated ($n = 14$) and untreated group ($n = 14$) and performed downhill running (14° downhill slope; speed 6–12m/min; maximum time: 60 min) using a motorized treadmill for small animals (Treadmill Simplex II; Columbus Instruments) (30). In addition, 10 wild-type mice performed the downhill running protocol. During treadmill running, an investigator continuously observed the mice to ensure the appropriate running speed was maintained. For those in the treatment group, sildenafil citrate was provided for 24 h before the downhill running protocol. MRI and MRS measures were acquired at baseline and 24 h after the downhill running protocol.

To examine the role of exercise training, subsets of *mdx* and wild-type mice underwent a low-intensity endurance treadmill training protocol (Fig. 1). There were a total of four groups ($n = 5$/group): *mdx* mice that trained (*mdx*^{train}), *mdx* that were administered sildenafil citrate (*mdx*^{sil}), *mdx* mice that both trained and were provided sildenafil citrate (*mdx*$^{train+sil}$), and wild-type mice that trained. The training incorporated a progressive interval-training regimen of horizontal running on a treadmill 5 days/wk over 4 wk (21). For the first wk, mice underwent a 30 min/day acclimatization period at a low speed (8 m/min) (Fig. 1). This acclimatization period was followed by 3 wk of interval training with increasing time of the bouts of exercise at a speed of 12 m/min separated by 1 min of rest. The duration of intervals systematically increased every 2–3 sessions (starting from 1-min intervals) with rest time between intervals remaining at 1 min. The running time of the intervals gradually increased with a goal of running 300 m (25 min) continuously during the final (fourth) wk of training. After completing the training, all mice underwent a bout of downhill running to volitional fatigue on a separate day (30).

MRI and MRS. MRI/MRS data were acquired using 4.7 T and 11.1T MR systems (Agilent, Santa Clara, CA). Animals were anesthetized before and during scanning using isoflurane (3% induction, 0.75%–1% maintenance). Respiratory rate and temperature were monitored throughout the scans to ensure maintenance of anesthesia, and a heating system with flexible water pads was used to keep the animal warm during the scans. A custom-built 200-MHz ^{1}H solenoid coil with 2-cm internal diameter was used to image both forelimbs and hindlimbs (separate scans and set-up). MR T_2 was acquired using two methods. First, a single voxel ^{1}H-MRS sequence was utilized with multiple echo times (TEs) to evaluate ^1H_2O T_2. This method provides a high-confidence measure but is limited in spatial coverage, as only a single region is acquired (Fig. 2). Second, an MRI T_2 multi-slice spin echo sequence was performed and enabled multiple muscles to be evaluated with a single acquisition. The details for these sequences are provided below.

^1H_2O T_2 was assessed using single voxel stimulated echo acquisition mode (STEAM) in the deep medial compartment (MC) of the lower hindlimbs and the anterior compartment (AC) of the upper forelimbs (voxel: $3 \times 3 \times 3$ mm^3; repetition time (TR): 9,000 ms; TE: 5–200 ms; 10–32 points exponentially spaced). Pilot studies showed these muscle regions to be sensitive to muscle damage after downhill running in *mdx* mice. Principal component analysis followed by mono-exponential nonlinear curve fitting analysis was performed on the spectroscopic data acquired using in-house developed software with Interactive Data Language (IDL, Exelis, version 8.5) (13, 49).

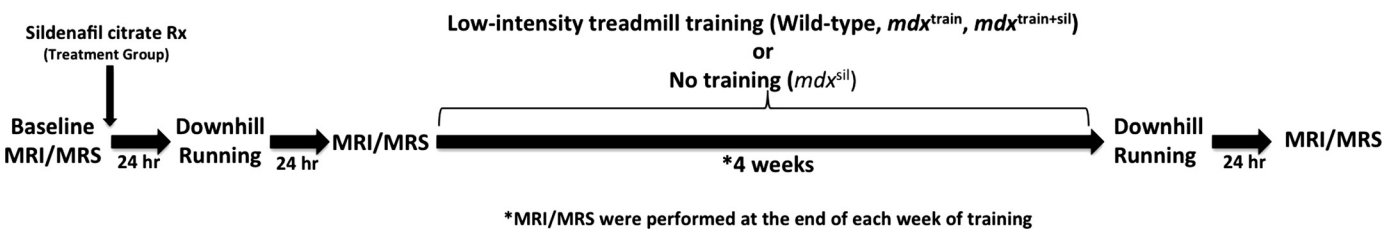

Fig. 1. Experimental timeline used to examine the effect of PDE5 inhibitor on downhill running and during progressive low-intensity treadmill training. MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; PDE5, phosphodiesterase type 5.

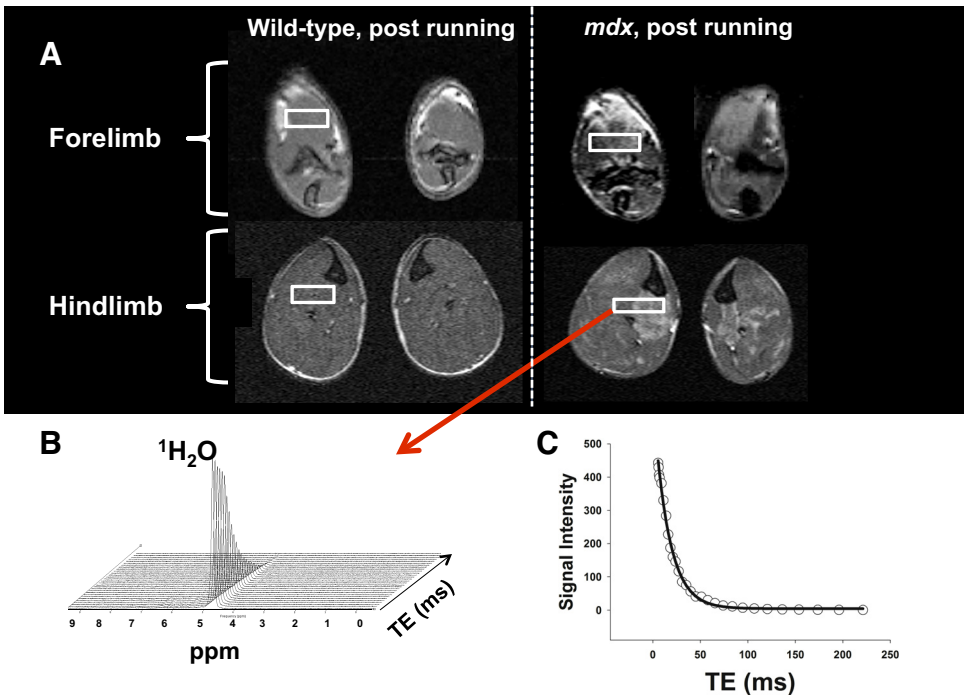

Fig. 2. *A*: representative transverse relaxation time constant (T_2)-weighted axial images (TR: 2 s; TE: 40 ms) of the upper forelimbs and lower hindlimbs of a wild-type and an untreated *mdx* mouse following downhill running. *B*: example single voxel ^{1}H-MRS spectra acquired from the deep medial region of the lower hindlimb. *C*: MR T_2 was calculated using a mono-exponential equation. MR, magnetic resonance; MRS, magnetic resonance spectroscopy; TE, echo time; TR, repetition time.

Proton-weighted T_2 spin echo images were acquired from mouse lower hindlimbs (TR: 2,000 ms; TE:14 and 40 ms; field of view: 10–15 mm × 10–15 mm; slices: 12; slice thickness: 10 mm; acquisition matrix: 128 × 256). Regions of interest representing the posterior compartment (PC), AC, and deep MC muscles were drawn over 5–8 slices using OsiriX software (Geneva, Switzerland) to calculate signal intensity (SI) (30, 51). T_2 was calculated assuming a single exponential decay curve (51) with the following equation:

$$T_2 = \frac{40\ \text{ms} - 14\ \text{ms}}{\ln\frac{SI_{14}}{SI_{40}}} \quad (1)$$

Post-contractile MRI-BOLD response. The MRI-BOLD response was measured in the posterior hindlimb muscles following tetanic contractions by using electrodes to stimulate the sciatic nerve (2 s, 100 Hz, 1-ms pulses, 8 V), similar to the set-up described previously (26). For the post-contractile BOLD measures, a spin echo sequence (TR: 2 s; effective TE: 19 ms; axial slice thickness: 1 mm; field of view: 40 mm (read) × 20 mm (phase); acquisition matrix: 128 × 32). A region of interest in the PC of the mouse hindlimb was traced using OsiriX software, and the SI following the muscle contractions was plotted over time to determine the peak post-contractile BOLD response normalized to baseline using Microsoft Excel for Mac 2011 (Version 14.7.7).

^{31}Phosphorus MRS. ^{31}P-MRS data were acquired using 11.1-T/470MHz Agilent system (Agilent, Palo Alto, CA) with VNMRJ 2.3 software. Mice were positioned supine with the hindlimb extended and right foot attached to a foot plate, with an oblong transmit/receive ^{31}P surface coil (6 mm × 12 mm) centered on the posterior region of the lower hindlimb. Also, a ^{1}H tuned surface coil (15 mm × 15 mm) was placed adjacent to the hindlimb for localized shimming of the posterior hindlimb region. ^{31}P-MRS data were acquired with the following parameters: TR 1 s; 15 number of signal averages (NSA); 2,048 data points; 8 k sweep width. Measures were obtained from the posterior lower hindlimb at rest and during a bout of muscle contractions at 2 Hz for 2 min with 10-min recovery. The PCr peak in the spectra was fit using principal component analysis, and the time constant of PCr recovery (PCτ) was calculated using a mono-exponential equation (11, 31). The relative concentration of inorganic phosphate (P_i) and PCr was quantified using the Advanced Method for Accurate, Robust and Efficient Spectral (AMARES) fitting algorithm of jMRUI (version 5.0). This analysis was performed in the time domain with zero and first order phasing, PCr set at 0 ppm for reference, and using estimated starting values and prior knowledge. In

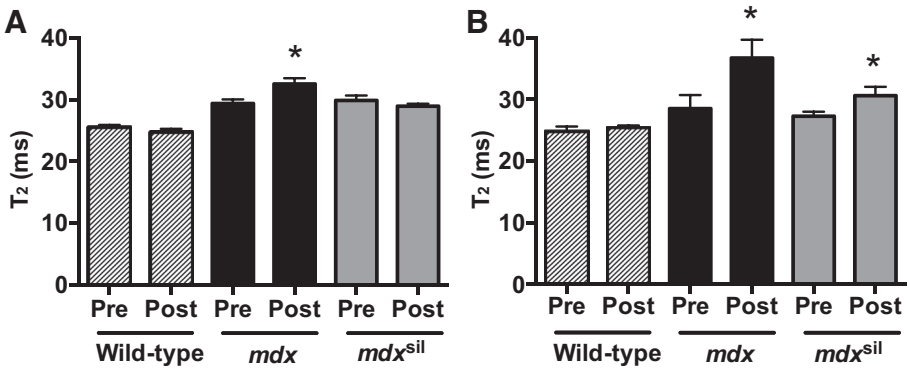

Fig. 3. *A*: ^{1}H-MRS ^1H_2O transverse relaxation time constant (T_2) in upper anterior forelimb muscles before and following an acute bout of downhill running in wild-type (n = 10), *mdx* (n = 14), and treated *mdx* (*mdx*^{sil}; n = 14) mice. *B*: ^{1}H-MRS ^1H_2O T_2 of the medial deep region of hindlimb muscles before and following downhill running in wild-type (n = 10), *mdx* (n = 14), and treated *mdx* (*mdx*^{sil}; n = 14) mice. Paired *t*-test with Bonferroni correction were used to examine differences in T_2 pre- and post-downhill running. *Significantly different from pre-downhill running at $P < 0.05$. MRS, magnetic resonance spectroscopy.

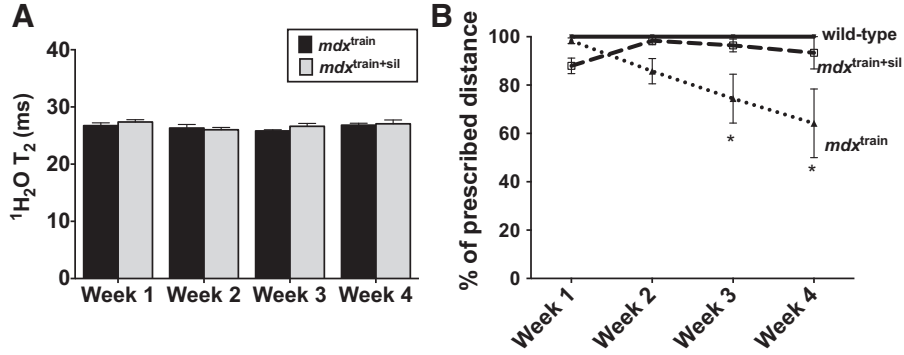

Fig. 4. *A*: MRS ^1H_2O transverse relaxation time constant (T_2) of the *mdx* mice measured weekly during training showed no changes in ^1H_2O T_2 from baseline. *B*: percentage of prescribed distance covered by wild-type and by *mdx* mice that trained without treatment (mdx^{train}) and with sildenafil citrate treatment ($mdx^{train+sil}$; $n = 5$/group). Note that running volume during training was reduced ($P < 0.05$) in untreated *mdx* mice compared with treated *mdx* and wild type. Two-way analysis of variance (treatment group vs. time) with Tukey post hoc analysis were performed. *Significantly different ($P < 0.05$) from wild type. Values are mean (SD). MRS, magnetic resonance spectroscopy.

addition, intracellular pH (pHi) was determined at rest and at the end of exercise using the relative chemical shift difference between Pi and PCr (44).

Histology. After 4 wk of training, gastrocnemius muscles were harvested, embedded in TissueTek optimum cutting temperature compound (Sakura Finetek, Torrence, CA), and frozen using isopentane chilled in liquid nitrogen. Using Leica cryostat, 7–10-μm frozen sections were obtained from the mid-belly region of gastrocnemius muscle. Multiple sections were taken and kept at room temperature for 20–30 min before staining for fibrosis using Masson's trichrome staining (Thermo Scientific 87019) and for inflammatory markers and macrophages using CD45 (Purified Rat Anti-Mouse CD45; BD PharMingen) and F4/80 antibodies (Thermo Fisher Scientific, catalog no. MF-48000, Research Resource Identifier (RRID) AB_10376289). Slides were imaged using a digital camera (Leica Microsystems, Solms, Germany) and analyzed using ImageJ 1.48v (National Institutes of Health) software.

Statistics. All statistical analyses were performed using Prism 6.0 (GraphPad Software, La Jolla, CA). Paired *t*-test with Bonferroni correction were used to examine differences in T_2 pre- and post-downhill running and running distance pre- and post-training. Two-way analysis of variance (treatment group vs. time) with Tukey post hoc analysis was performed to analyze T_2 and total distance over 4 wk. One-way analysis of variance was used to compare the post-contractile BOLD response and fibrosis at end of training. Significance levels were set at an alpha level of 0.05, and results are expressed as mean (SD).

RESULTS

Effect of acute bout of downhill running. At baseline, ^1H_2O T_2 was elevated ($P < 0.05$) in *mdx* compared with wild-type mice in both the hindlimb MC and forelimb AC (Fig. 3). Following downhill running, there was a significant increase in T_2 ($P < 0.05$) of untreated *mdx* mice compared with baseline in the hindlimb MC and forelimb AC (Fig. 3). In the *mdx* mice treated with sildenafil citrate and in wild-type mice, there was no increase in T_2 of the MC, indicating a beneficial effect of the treatment in the dystrophic mice. Also, although an increase in T_2 ($P < 0.05$) of the forelimb muscle AC after downhill running in treated *mdx* mice was observed, this increase was less (12%) than in the untreated *mdx* mice (33%; Fig. 3).

Effect of low-intensity treadmill training on muscle damage in mdx mice. After examining the effects of an acute bout of downhill running, we next examined the effects of 4 wk of treadmill running with and without sildenafil citrate treatment in *mdx* mice and in untreated wild-type mice. In comparison to wild-type mice, all *mdx* groups presented with greater T_2 values in both hindlimb and forelimb muscles at baseline and during training (Fig. 4, Table 1). Furthermore, there was no change within groups in T_2 of hindlimb muscles over 4 wk of training in any of the *mdx* or wild-type groups as measured by MRI and MRS (Fig. 4, Table 1).

Exercise performance was compared based on percent (%) of total prescribed distance covered each wk. Wild-type and $mdx^{train+sil}$ mice were able to complete nearly all the training (i.e., >90%) (Fig. 4). On the other hand, mdx^{train} mice were not able to complete the majority of the training sessions (67%), and their overall percentage of total distance covered each wk was lower ($P < 0.05$) than the other 2 groups (Fig. 4).

When comparing total distance run during the downhill running pre- and post- training, all groups performed significantly better after training (Fig. 5). This increase in distance was accentuated in *mdx* mice on sildenafil citrate treatment (Fig. 5). After training, there was no significant increase in hindlimb muscle MRI T_2 of the AC, MC, or PC after downhill running in the wild-type or *mdx* groups compared with before downhill running.

Microvascular function was estimated by examining the peak MRI-BOLD response following brief tetanic contractions among the wild-type and *mdx* groups. The peak BOLD response was observed to be lower in mdx^{train} compared with wild-type and $mdx^{train+sil}$ (Fig. 6).

^{31}P-MRS was used to measure high-energy phosphates and pHi in the posterior hindlimb muscles at rest and during and

Table 1. *MRI T_2 of hindlimb muscles over 4 wk of training*

	Week 1	Week 2	Week 3	Week 4
Tibialis anterior, ms				
Wild-type	24.5 (2.1)	22.8 (2.5)	23.3 (2.2)	23.1 (1.6)
mdx^{train}	26.3 (4.1)	25.5 (2.7)	24.7 (2.2)	25.1 (1.7)
$mdx^{train+sil}$	26.1 (2.3)	25.6 (1.8)	25.6 (2.6)	25.8 (3.3)
Medial compartment, ms				
Wild-type	24.3 (2.1)	24.1 (3.5)	23.7 (2.0)	24.2 (2.6)
mdx^{train}	26.1 (1.8)	25.2 (1.9)	25.3 (1.6)	26.3 (2.0)
$mdx^{train+sil}$	27.6 (2.2)	25.3 (1.5)	25.7 (1.6)	26.1 (2.1)
Gastrocnemius, ms				
Wild-type	24.3 (1.2)	23.8 (1.5)	23.5 (1.3)	24.3 (1.0)
mdx^{train}	26.4 (1.7)	24.9 (1.2)	25.5 (1.3)	26.0 (1.5)
$mdx^{train+sil}$	26.9 (1.8)	25.6 (1.0)	25.1 (1.0)	25.1 (1.3)

Values are mean (SD). mdx^{train}, *mdx* mice that trained without treatment; $mdx^{train+sil}$, *mdx* mice that trained with sildenafil citrate treatment; MRI, magnetic resonance imaging. Two-way ANOVA (group × time) was used to examine changes in muscle T_2 over 4 wk of progressive low-intensity treadmill training across three hindlimb muscle groups/region in wild-type ($n = 5$), mdx^{train} ($n = 5$), and $mdx^{train+sil}$ ($n = 5$). MRI transverse relaxation time constant (T_2) values were greater ($P < 0.05$) in *mdx* than wild-type mice, but no changes (>0.05) over time were evident.

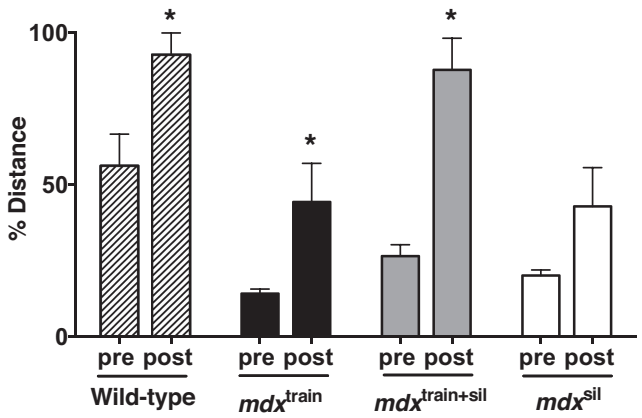

Fig. 5. Total distance run by mice during the downhill running protocol performed before and after training in wild-type and *mdx* mice without treatment (mdx^{train}) and that trained with sildenafil citrate treatment ($mdx^{train+sil}$) and in *mdx* mice treated with sildenafil citrate without training (mdx^{sil}; $n = 5$/group). Note that the mice in the treatment groups ($mdx^{train+sil}$ and mdx^{sil}) were administered sildenafil citrate before the pretraining downhill run. Paired *t*-tests with Bonferroni correction were used for comparisons of before and after training. Values are expressed as percent of maximum distance. *Significantly different from pretraining at $P < 0.05$.

following stimulated contractions. PCr/(Pi + PCr) and pH_i at rest and after muscle contractions were not different among the wild-type and treated and untreated *mdx* groups (Table 2). The PCr recovery time constant (τ) was reduced in wild-type compared with all *mdx* groups following training, with no differences observed among the treated and untreated *mdx* groups (Fig. 7).

To investigate the effect of training and sildenafil citrate treatment on muscle histopathology, muscle cryosections from all the groups of mice were stained with Masson's trichrome to quantify the accumulation of intramuscular fibrosis. All the *mdx* groups had greater ($P < 0.05$) fibrosis than wild type, with no significant differences among the *mdx* groups (Fig. 8*A*). Similar results were observed when normalizing the fibrotic tissue to training volume [mdx^{train}: 3.3% (SD 3.3); $mdx^{train+sil}$: 5.1% (SD 5.6); wild-type: 0.3% (SD 0.1)]. In addition, examination of inflammatory cell markers revealed all *mdx* groups had higher number of $CD45^+$ cells (Fig. 8*B*) and F4/80 cells per unit area (mm^2) than wild type, with no significant differences among *mdx* treatment/training groups.

DISCUSSION

This study is the first to examine the combined effects of exercise training and PDE5 inhibitor on muscle damage and microvascular function as measured by MRI/MRS in a dystrophic mouse model (*mdx*). The main findings were: *1*) PDE5 inhibition (sildenafil citrate) attenuated the increase in T_2 in forelimb and hindlimb muscles following an acute bout of downhill running in *mdx* mice, indicating reduced inflammation/edema and muscle damage; *2*) PDE5 inhibition enabled greater training volume without increasing skeletal muscle damage in dystrophic mice; and *3*) PDE5 inhibition increased the post-contractile BOLD response in trained *mdx* mice, indicating improved microvascular function.

Effects of sildenafil citrate on an acute bout of downhill running. In this study, we observed that sildenafil citrate reduced muscle damage in skeletal muscle 24 h after an acute bout of downhill running. Previous studies have reported a slow delayed rise in T_2 values that peak between 24 and 48 h after downhill running and correlate with Evans blue dye uptake, indicating sarcolemmal damage (29, 30). Based on these findings, we examined muscle T_2 24 h after downhill running and found a positive effect of PDE5 inhibition on T_2, indicating reduced events associated with muscle damage. These findings are consistent with Kobayashi et al. (25), who found reduced SI of MR images after a short downhill running protocol in treated *mdx* mice, although in that study quantitative T_2 values were not directly measured, as only a single TE was acquired. Also, in that study the MR acquisitions were made shortly (30 min) following the exercise, and the differences between *mdx* and wild type were attributed to impaired perfusion due to an inability to blunt sympathetic vasoconstriction. Therefore, the present study extends these findings by demonstrating T_2 is improved with PDE5 inhibition in dystrophic muscle over a more extended period of time (24 h) following eccentric-biased downhill running exercise.

Although there are many potential explanations for an increase in MR T_2, the increase in this study is consistent with events associated with inflammation and edema (12, 32). Another possible explanation is the shift in water compartments due to lactate and acidosis. However, given that the measures were acquired 24 h after exercise, it is unlikely that any potential increase in muscle lactate and acidosis would still be persistent at this time (16, 26). The magnitude of a 3-ms (hindlimb MC) or 8-ms (forelimb AC)

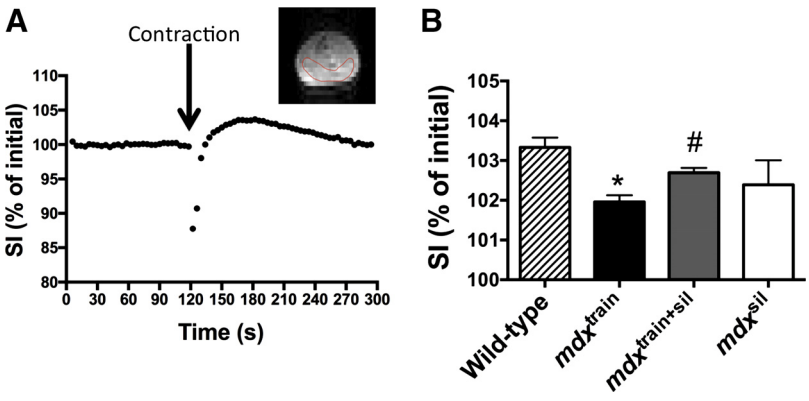

Fig. 6. *A*: example post-contractile MRI blood oxygenation level dependence (BOLD) response following brief (2 s) tetanic stimulated contractions in an *mdx* mouse (timing of the stimulated contractions is depicted with arrow). *B*: MRI peak BOLD response in wild-type mice that trained, in *mdx* mice that trained without treatment (mdx^{train}) and with sildenafil citrate treatment ($mdx^{train+sil}$), and in *mdx* mice treated with sildenafil citrate without training (mdx^{sil}; $n = 5$/group). One-way analysis of variance with Tukey multiple comparison test were used for comparisons. Values are mean (SD). *Significantly different from wild type; #significantly different from mdx^{train}. MRI, magnetic resonance imaging; SI, signal intensity.

Table 2. *Phosphorylated metabolites and intracellular pH in the lower posterior hindlimb of mice at rest and at the end of the stimulated contractions*

	Wild-type	*mdx*^{train}	*mdx*$^{train+sil}$	*mdx*^{sil}
Rest				
PCr/(Pi+PCr)	0.89 (0.04)	0.90 (0.05)	0.89 (0.03)	0.87 (0.01)
pH_i	7.20 (0.04)	7.22 (0.06)	7.20 (0.06)	7.24 (0.02)
End exercise				
PCr/(Pi+PCr)	0.31 (0.12)	0.42 (0.14)	0.36 (0.23)	0.39 (0.07)
pH_i	6.92 (0.06)	6.99 (0.08)	6.93 (0.11)	6.87 (0.06)

Values are mean (SD). *mdx*^{sil}, *mdx* mice treated with sildenafil citrate without training; *mdx*^{train}, *mdx* mice that trained without treatment; *mdx*$^{train+sil}$, *mdx* mice that trained with sildenafil citrate treatment; PCr, phosphocreatine; pHi, intracellular pH. No significant differences were observed among groups in PCr/(PCr+Pi) and pH_i at rest and at end exercise.

reduction in T_2 after downhill running appears to be relevant and clinically meaningful to human studies in DMD. For example, a drop in T_2 of 2–4 ms in lower leg muscles after initiation of corticosteroids was associated with an improvement in timed functional tests (2).

Our results are also consistent with a recent study that examined PDE5 inhibitors on running performance performed once per wk over 4 wk (9). In that study, tadalafil also improved performance and reduced muscle damage. The benefits were attributed to changes at multiple levels, including increased protein synthesis efficiency, an improved actin network organization of Z-disks, increased lipid metabolism, and a switch toward slow oxidative fibers (9). Another potential reason for the benefits of PDE5 inhibition is an increase in perfusion due to blocking cGMP breakdown (36). The combination of reduced perfusion and increased fragility of dystrophic muscle has become known as the "two-hit hypothesis" (3), and improving local blood flow with PDE5 inhibition may alleviate the susceptibility to muscle damage.

Effects of exercise training and PDE5 inhibition in dystrophic muscle. Although the benefits following a single bout of exercise are encouraging, ultimately an intervention will need to be initiated over a more extended period of time to demonstrate therapeutic efficacy. Therefore, in this study we evaluated the effect of PDE5 inhibitor (sildenafil citrate) in combination with low-intensity exercise (treadmill training) in *mdx* mice over 4 wk. We found that the *mdx* mice that received sildenafil citrate were able to complete nearly all of the training sessions (>90% of running distance) and perform significantly better on downhill running with no additional damage.

Also of note, untreated *mdx* mice showed significantly lower T_2 after downhill running and improved performance following training compared with pretraining, supporting that exercise alone can have beneficial effects and reduce the susceptibility to muscle damage in *mdx* mice. Exercise studies in muscular dystrophies are limited but have generally shown exercise to have no detrimental effect when prescribed at low intensities (20, 28). The positive effects of low-intensity exercise have been shown with voluntary wheel running, swimming, and treadmill training (7, 22). These exercise studies have reported improvement in contractile properties, reduced oxidative stress, and increase in grip strength and muscle function in *mdx* mice (18, 22). Though exercise has a beneficial effect, studies have also reported an inability to complete some training protocols (5, 7, 18). One of the potential contributing factors for this failure is due to an inability to blunt sympathetic vasoconstriction in dystrophic muscles from displaced and reduced levels of nNOSμ (25). Therefore, combining PDE5 inhibitors with exercise training may be a valuable therapeutic approach to maintaining and improving muscle function in muscular dystrophies.

The post-contractile BOLD response in skeletal muscle reflects the balance between oxygen delivery and utilization (47) and is influenced by blood volume and oxyhemoglobin saturation changes in small vessels after isometric contractions (8). There are a number of factors that could influence the BOLD response, including conduit artery bulk blood flow, the muscle pump (widening of the arterial-venous pressure gradient due to contractions), and vasodilators affecting endothelial function (47). This measure has shown to be sensitive to physical activity (48), aging (42), and certain disease conditions such as diabetes (40), and the magnitude of the BOLD response after a brief contraction appears to peak at ~60% of maximal voluntary contraction (52). In this study, the post-contractile BOLD response was used to evaluate the effects of PDE5 inhibition on microvascular function following training in in dystrophic and wild-type mice. We observed that the combination of PDE5 inhibition and training resulted in a greater BOLD response than training alone in dystrophic mice and was similar to wild-type mice. Although perfusion does not appear to be different at rest between wild type and *mdx* with and without sildenafil treatment (25, 45), the loss of sarcolemma-localized nNOSμ has been shown to cause vascular narrowing and inadequate muscle perfusion following mild exercise in *mdx*, and the impaired local blood flow has been linked to an exaggerated general fatigue response and drastic reductions in voluntary physical activity for a prolonged period of time (25). This has been attributed to *mdx* and nNOS-null mice lacking the normal ability to attenuate vasoconstriction during and after muscle contraction (45). Similarly, in children with DMD, an inability to blunt sympathetic vasoconstriction during sustained handgrip exercise has been reported (41). Furthermore, following single tetanic contractions, local blood flow was substantially impaired in *mdx* mice when red blood cell flux was measured (3). This lack of an increase in red blood cell flux in the microcirculation was

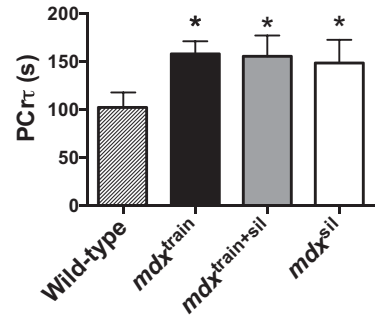

Fig. 7. The time constant (τ) for phosphocreatine (PCr) recovery after electrically stimulated contractions (2 Hz) in wild-type mice that trained, in *mdx* mice that trained without treatment (*mdx*^{train}) and with sildenafil citrate treatment (*mdx*$^{train+sil}$), and in *mdx* mice treated with sildenafil citrate without training (*mdx*^{sil}; n = 5/group). One-way analysis of variance with Tukey multiple comparison test were used for comparisons. *Significantly different from wild type.

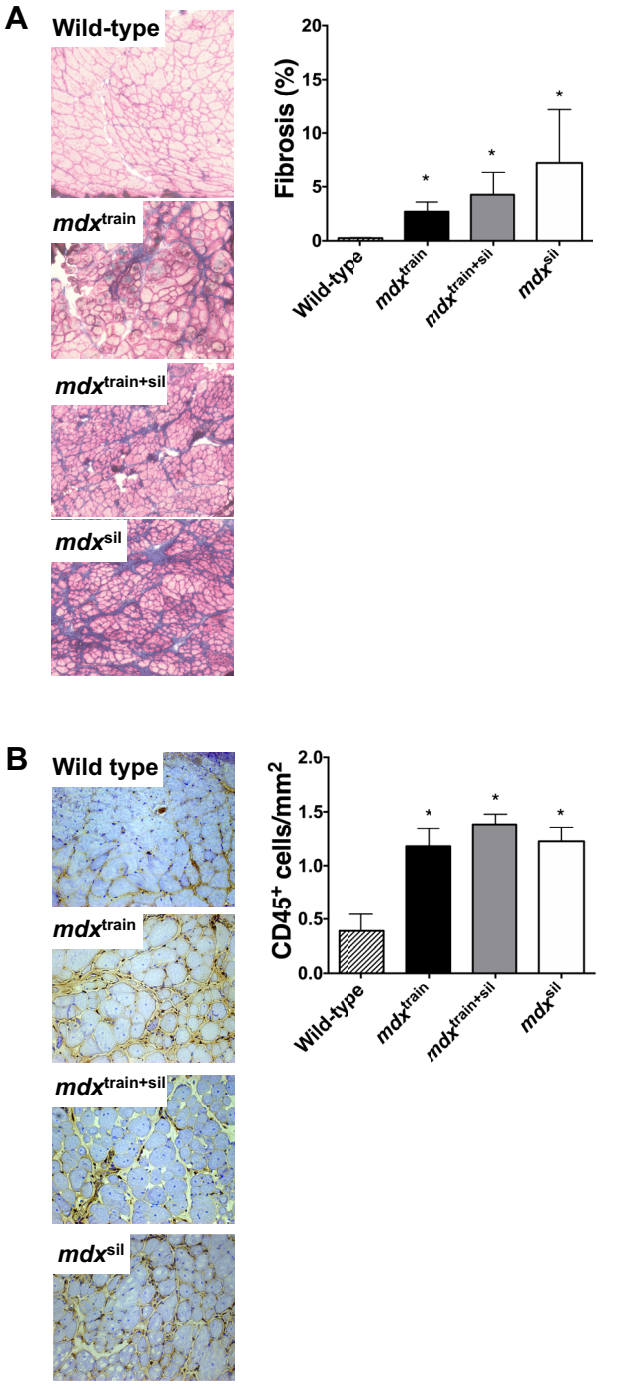

Fig. 8. *A*: representative images after Masson's trichrome staining of the gastrocnemius in wild-type mice that trained, in *mdx* mice that trained without treatment (mdx^{train}) and with sildenafil citrate treatment ($mdx^{train+sil}$), and in *mdx* mice treated with sildenafil citrate without training (mdx^{sil}; $n = 5$/group). *mdx* mice had significantly greater fibrosis in comparison to wild type, and no differences were observed among *mdx* groups. *B*: $CD45^+$ cells were greater in all *mdx* groups compared with wild type. One-way analysis of variance with Tukey multiple comparison test were used for comparisons. *Significantly different from wild type ($P < 0.05$).

reproduced in normal muscle by inhibiting NOS via the administration of N^{ω}-nitro-L-arginine-methyl ester (L-NAME) (3), which has been shown to enhance sympathetic vasoconstriction in the contracting hindlimbs of rats (46) and mice (45). Furthermore, exercise onset vasodilator kinetics were impaired with reduced NOS activity, but were improved to control levels with PDE5 inhibition (24). Therefore, these studies provide further support for the notion that lack of sarcolemma-localized nNOSμ in the *mdx* mice leads to inadequate O_2 delivery to the muscle and can be improved with PDE5 inhibition. The present study extends these findings by showing that a combination of exercise training and PDE5 inhibition enhances microvascular function in dystrophic mice, which may contribute to the enhanced performance observed in these mice.

Along with microvascular function, there are a number of other possible benefits of sildenafil citrate that may have contributed to improved performance, including satellite cell activation, enhanced regeneration, and a shift toward a more oxidative phenotype (9, 36, 39). In this study, we did not observe an improvement in functional oxidative capacity in trained *mdx* on PDE5 inhibitor compared with untreated trained *mdx* (mdx^{train}) or *mdx* mice treated with sildenafil that did not train (mdx^{sil}). The lack of an effect on mitochondrial function with sildenafil citrate is consistent with a previous study observing that *mdx* mice had impaired mitochondria function that was not alleviated with sildenafil citrate treatment (37). Furthermore, the lack of an effect on mitochondrial function from aerobic exercise training may be due to the relatively short training period (4 wk) and low intensity of exercise.

In addition to improved performance with sildenafil citrate, previous studies have shown PDE5 inhibitors to have a positive effect on reducing fibrotic accumulation (17, 38). In the present study, we did not observe treatment with sildenafil citrate and training to reduce fibrosis in *mdx* mice over the 4-wk protocol. This discrepancy may be attributed to the duration of treatment and age of the mice. For example, a study showing a positive effect of PDE5 inhibitors on fibrosis reported a treatment spanning over 14 wk, with an intervention starting at 3 wk of age (38). In the present study, the mice were older (average of 6 mo of age) and were only treated for 4 wk.

Conclusions. Our findings indicate that PDE5 inhibition reduces muscle damage following an acute bout of downhill running in dystrophic mice as measured with MR T_2. Furthermore, progressive low-intensity treadmill running combined with sildenafil citrate resulted in greater training volume over 4 wk, improved endurance performance, and enhanced microvascular function measured with MRI-BOLD in dystrophic mice. Overall, our findings support the use of PDE5 inhibitors in combination with exercise to improve muscle performance and possibly slow disease progression in dystrophic muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.B., D.J.L., K.V., G.A.W., and S.C.F. conceived and designed research; A.B., R.S.V., S.M.C., and S.C.F. performed experiments; A.B., D.W.H., and S.C.F. analyzed data; A.B., R.S.V., and S.C.F. interpreted results of experiments; A.B. and S.C.F. prepared figures; A.B., R.S.V., S.M.C., D.W.H., D.J.L., K.V., G.A.W., and S.C.F. drafted manuscript; A.B., R.S.V., S.M.C., D.W.H., D.J.L., K.V., G.A.W., and S.C.F. edited and revised manuscript; A.B., R.S.V., S.M.C., D.W.H., D.J.L., K.V., G.A.W., and S.C.F. approved final version of manuscript.

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