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Tadalafil alleviates muscle ischemia in patients with Becker muscular dystrophy

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Abstract

Becker muscular dystrophy (BMD) is a progressive X-linked muscle wasting disease for which there is no treatment. Like Duchenne muscular dystrophy (DMD), BMD is caused by mutations in the gene encoding dystrophin, a structural cytoskeletal protein that also targets other proteins to the muscle sarcolemma. Among these is neuronal nitric oxide synthase (nNOS μ), which requires certain spectrin-like repeats in dystrophin's rod domain and the adaptor protein α -syntrophin to be targeted to the sarcolemma. When healthy skeletal muscle is subjected to exercise, sarcolemmal nNOS μ -derived nitric oxide (NO) attenuates local α -adrenergic vasoconstriction thereby optimizing perfusion of muscle. We found previously that this protective mechanism is defective—causing functional muscle ischemia—in dystrophin-deficient muscles of the *mdx* mouse (a model of DMD) and of children with DMD, in whom nNOS μ is mislocalized to the cytosol instead of the sarcolemma. Here, we report that this protective mechanism also is defective in men with BMD in whom the most common dystrophin mutations disrupt sarcolemmal targeting of nNOS μ . In these men, the vasoconstrictor response, measured as a decrease in muscle oxygenation, to reflex sympathetic activation is not appropriately attenuated during exercise of the dystrophic muscles. In a randomized placebo-controlled cross-over trial, we show that functional muscle ischemia is alleviated and normal blood flow regulation fully restored in the muscles of men with BMD by boosting NO-cGMP signaling with a single dose of the drug tadalafil, a phosphodiesterase (PDE5A) inhibitor. These results further support an essential role for

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Competing interests: The authors declare no competing interests.

List of Supplementary Materials

Materials and Methods

Fig. S1. Equivalence of functional sympatholysis in dominant vs. non-dominant arms.

Fig. S2. Immunoblot experiments.

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sarcolemmal nNOS μ in the normal modulation of sympathetic vasoconstriction in exercising human skeletal muscle and implicate the NO-cGMP pathway as a putative new target for treating BMD.

Introduction

Becker muscular dystrophy (BMD) is a progressive X-linked muscle wasting disease for which there is no treatment (1–3). Like the closely related disease Duchenne muscular dystrophy (DMD), BMD is caused by mutations in the gene encoding the cytoskeletal protein dystrophin. Whereas DMD is caused by out-of-frame mutations yielding no functional dystrophin, BMD is caused by in-frame mutations yielding truncated or reduced dystrophin protein (4,5). Despite a more protracted clinical course than DMD and an almost normal life span, BMD is a debilitating disease with progressive muscle weakness culminating in loss of ambulation; there also is an increased risk of heart failure due to an associated cardiomyopathy (3). Thus, a therapeutic breakthrough is urgently needed. Although basic science on the dystrophinopathies has flourished, clinical translation has not (6).

Dystrophin is a large, rod-shaped, sarcolemmal protein that provides a physical link between the intracellular cytoskeleton and the extracellular matrix (7). With dystrophin deficiency, the sarcolemma is destabilized and the muscle fibers are susceptible to physical damage with repeated contraction (8). Dystrophin also is a scaffolding protein that targets other proteins to the sarcolemma. Among these is nNOS μ —a muscle-specific splice variant of the neuronal isoform of nitric oxide synthase (9,10)—which requires certain spectrin-like repeats in the mid-portion of dystrophin's rod domain and the adaptor protein α -syntrophin for sarcolemmal targeting (11). Dystrophin deficiency causes sarcolemmal nNOS μ deficiency: nNOS μ is reduced and the residual protein is misplaced from the sarcolemma to the cytosol (9–11).

With exercise of healthy skeletal muscle, sarcolemmal nNOS μ -derived nitric oxide (NO) attenuates local α -adrenergic vasoconstriction thereby optimizing perfusion to meet the metabolic demands of the active muscle (12–21). We previously found that this protective mechanism (termed functional sympatholysis) is lost in *mdx* mice (a model of BMD and DMD), nNOS null mice, and boys with DMD causing functional muscle ischemia (14,16). Repeated bouts of functional ischemia could accelerate use-dependent injury of muscle fibers already weakened by dystrophin deficiency (14,16,19).

In the *mdx* mouse, many features of the dystrophic phenotype can be improved by multiple strategies that boost NO signaling including transgenic expression of nNOS (22,23), transgenic expression of dystrophin mini-genes that restore sarcolemmal nNOS μ (and thereby restore functional sympatholysis) (11), administration of the NOS substrate L-arginine (24,25), treatment with NO-donating drugs (26,27), and phosphodiesterase (PDE5A) inhibition with the drugs tadalafil or sildenafil (28,29). These PDE5A inhibitors, which prolong the half-life of cGMP—the downstream target of NO in vascular smooth muscle—were shown in the *mdx* mouse to alleviate muscle ischemia, as well as injury and fatigue, after a brief bout of exercise (29). Also, these drugs were shown to improve cardiac dynamics in *mdx* mice (30,31) and to rescue dystrophic skeletal muscle and prolong survival in dystrophin-deficient zebrafish (32). However, this promising preclinical research has yet to be translated into treatment of human patients with muscular dystrophy.

Accordingly, the goal of this study was to test the hypothesis that boosting NO-cGMP signaling through PDE5A inhibition ameliorates functional muscle ischemia in patients with BMD. First, we conducted a case-control study to establish that exercise-induced attenuation

of reflex vasoconstriction (functional sympatholysis) is impaired in BMD. Most BMD patients have complete loss of sarcolemmal nNOS, largely because the most common in-frame mutations delete the exons encoding the spectrin-like repeats in dystrophin required for sarcolemmal targeting (11,33,34). Then, we conducted a randomized double-blind placebo-controlled crossover trial to determine if tadalafil would restore functional sympatholysis in the muscles of BMD patients.

Results

Of 15 patients screened for eligibility, five men were excluded: two were too weak to perform handgrip, one had reduced left ventricular ejection fraction (LVEF), one had hypertension, and one did not have BMD by mutational analysis. No patients with BMD were on nocturnal ventilator support.

Characteristics of the Study Groups

Baseline characteristics and indices of disease severity of the individual patients with BMD are shown in Table 1. All patients were ambulatory, though the most severely affected patient P10 often uses a wheelchair. Six of the ten patients (P1–P6) had exonal deletions encoding the spectrin-like repeats of dystrophin implicated in sarcolemmal targeting of nNOS μ (11,33,34). All ten subjects had elevated creatine kinase (CK) levels as expected.

As shown in Table 2, patients and controls were matched for age, body mass index (BMI), blood pressure (BP), heart rate, and LVEF. BMD patients, as expected, had lower grip strength (as measured by handgrip maximum voluntary contraction, MVC) in both arms.

Functional Sympatholysis Is Impaired in BMD

To compare muscle blood regulation in patients vs. controls, we assessed functional sympatholysis, which refers to exercise-induced attenuation of reflex sympathetic vasoconstriction—a protective mechanism that normally ensures perfusion of metabolically active skeletal muscle. Functional sympatholysis was assessed by applying the same reflex sympathetic stimulus—lower body negative pressure (LBNP), which simulates mild orthostatic stress and engages cardiac baroreceptors to trigger reflex sympathetic activation to the skeletal muscle circulation—with the subjects' forearm muscles either resting or performing light rhythmic handgrip exercise. Sympathetic constriction of skeletal muscle microvessels was measured as the decrease in forearm muscle oxygenation—oxygenated hemoglobin plus oxygenated myoglobin ($\text{HbO}_2 + \text{MbO}_2$) as measured by near infrared spectroscopy—in response to LBNP.

In resting forearm muscle, LBNP evoked comparable decreases in forearm muscle oxygenation ($\text{HbO}_2 + \text{MbO}_2$) in BMD patients and healthy control individuals, indicating comparable reflex vasoconstriction (Fig. 1A, B). During the increased oxygen consumption with handgrip exercise alone, muscle oxygenation decreased in both groups, quickly reaching a new steady state level. When LBNP was superimposed on handgrip in healthy controls, the reflex decrease in muscle oxygenation was attenuated by $60 \pm 8\%$ ($\Delta\text{HbO}_2 + \text{MbO}_2$: $-18 \pm 1\%$ at rest vs. $-7 \pm 2\%$ during handgrip, $P < 0.01$), indicating functional sympatholysis. However, no such attenuation was seen in the patients with BMD ($\Delta\text{HbO}_2 + \text{MbO}_2$: $-19 \pm 2\%$ at rest vs. $-17 \pm 2\%$ during handgrip, $P > 0.10$), indicating impaired sympatholysis and thus functional muscle ischemia (Fig. 1C, D).

Tadalafil Restores Functional Sympatholysis in BMD

Then, to test if tadalafil can restore sympatholysis in BMD, all 10 patients completed a double-blind randomized cross-over trial. Patients were randomly assigned by a research

pharmacist to have sympatholysis assessed after receiving either a single 20 mg capsule of tadalafil or a placebo capsule; after a two-week washout period, sympatholysis was re-assessed after the subject received the other treatment (the tadalafil group was switched by the research pharmacist to receive placebo and vice-versa). As shown in Figure 2, tadalafil treatment restored functional sympatholysis in BMD patients. When LBNP was superimposed on handgrip after tadalafil treatment, the reflex decrease in muscle oxygenation was attenuated by $52 \pm 12\%$ ($\Delta\text{HbO}_2 + \text{MbO}_2$: $-17 \pm 2\%$ at rest vs. $-9 \pm 2\%$ during handgrip, $P < 0.01$). In contrast, BMD patients treated with a placebo showed no effect: LBNP evoked comparable decreases in muscle oxygenation at rest and during exercise ($\Delta\text{HbO}_2 + \text{MbO}_2$: $-18 \pm 2\%$ at rest vs. $-17 \pm 2\%$ during handgrip; $P > 0.1$).

Comparing each patient's LBNP response during handgrip in the placebo arm of the treatment trial with that during the case-control study, the intraclass correlation was $r = 0.80$, demonstrating reproducibility of impaired functional sympatholysis in the absence of active drug treatment. Furthermore, the degree of functional sympatholysis in tadalafil-treated patients was indistinguishable from normal (healthy controls, $P > 0.1$). Hand dominance had no effect on the results of sympatholysis experiments (Figure S1).

Figure 3 shows the patient-specific responses to tadalafil and to placebo. Nine of ten patients with BMD had sympatholysis after tadalafil, whereas only one patient (P9) had sympatholysis with placebo. Deletion of exons 14–44 in this patient would be expected to result in a truncated dystrophin protein that could still target nNOS μ to the sarcolemma (35).

Figure 4 shows the results of immunohistochemistry experiments performed on muscle biopsy samples from P9 and, by comparison, one of the patients (P5) with deletion of exons 45–48. Sarcolemmal nNOS expression indeed was detected in P9 (at a reduced level) but not in P5 (as expected); cytoplasmic nNOS expression was detected in both patients. Dystrophin C-terminus staining on sections was similar to normal muscle in both patients, but immunoblot analysis showed truncated or decreased dystrophin characteristic of BMD in both patients (Figure S2). Peak tadalafil blood concentrations ranged from 170–310 ng/ml (mean: 260 ± 13 ng/ml). In the one patient (P10) with no sympatholysis after tadalafil, the peak blood concentration of tadalafil was 300 ng/ml.

Tadalafil had no effect ($P > 0.05$) on systolic blood pressure (116 ± 3 vs. 114 ± 4 mmHg, pre- vs. post-tadalafil), diastolic blood pressure (72 ± 2 vs. 73 ± 3 mmHg), or heart rate (84 ± 4 vs. 80 ± 4 beats per minute). There were no adverse events or side-effects with tadalafil; specifically, no patients experienced spontaneous penile erection, flushing, or visual disturbance either with the test dose or study dose of either tadalafil or placebo.

Discussion

A growing concern in the muscular dystrophy field is whether therapeutic strategies, including PDE5A inhibition, that have shown great promise in the *mdx* mouse (an important but imperfect model of the human disease) can be translated to benefit human patients with muscular dystrophy (6). Our report directly addresses this concern. We show that the PDE5A inhibitor tadalafil rescues the abnormal vascular phenotype in the muscle of patients with BMD, fully restoring NO-dependent modulation of reflex vasoconstriction in exercising human skeletal muscle.

Treatment trials focused on BMD are scarce, as BMD is rarer than DMD (1 in 19,000 vs. 1 in 3,500 live male births, respectively) and clinically far more heterogeneous (3,11,33,34,36). Yet, BMD provides an elegant experiment of nature that in many patients eliminates sarcolemmal nNOS μ from birth, affords the opportunity to test a preemptive

intervention in adult patients in an early stage of their disease, and provides insight into expected benefits from exon skipping which aims to convert DMD to BMD. Whereas most patients with DMD are treated with corticosteroids (deflazacort or prednisone) and many with prophylactic cardioprotective medication (1,2) that might affect sympatholysis, most of our BMD patients were taking no medication other than the study drug. We excluded BMD patients with heart failure or hypertension, which can impair sympatholysis via production of reactive oxygen species that destroy NO (19,20,37).

That functional sympatholysis was absent in nine of 10 adult ambulatory patients with BMD and normal LVEF—but was present in one patient shown to express sarcolemmal nNOS μ —provides new evidence in humans that sarcolemmal nNOS μ is essential for the normal modulation of sympathetic vasoconstriction in active skeletal muscle. In the absence of such modulation, the forearm muscles become ischemic when lightly exercised during mild orthostatic stress, simulating the common condition of a BMD patient performing repetitive arm activities of daily living while seated. The new data in adult patients with BMD extend our prior work showing that sympatholysis also is absent in pediatric patients with DMD (14) as well as in *mdx* mice (16), nNOS null mice (16), α -syntrophin null mice that lack sarcolemmal nNOS μ (19), and *mdx* transgenic mice expressing a dystrophin minigene which, like the common BMD mutations, produces a truncated dystrophin that cannot target nNOS μ to the sarcolemma (11).

A recent mouse study suggests that *mdx* mice have less sympathetic vasoconstriction to overcome during exercise due to impaired sympathetic neurotransmission, that is, a primary neural defect at the level of the sympathetic nerve terminals that is evident even in resting *mdx* skeletal muscle (38,39). Our data indicate that this finding in barbiturate-anesthetized mice is not applicable to conscious human patients with BMD, because the vasoconstrictor response to reflex sympathetic stimulation in resting BMD muscle is indistinguishable from normal. The reflex sympathetic vasoconstriction in resting DMD skeletal muscle also is not attenuated until the muscles are exercised (14).

The key finding of our study is that tadalafil alleviated microvascular ischemia and fully restored blood flow regulation in eight of nine BMD patients who lacked sympatholysis when given placebo. The tadalafil effect was both dramatic and immediate, occurring with a single dose. These findings do not constitute definitive proof but are consistent with our working hypothesis and that of Kobayashi et al. (29) that PDE5A inhibitors boost a residual NO-cGMP signal arising from nNOS μ misplaced to the cytosol of dystrophin-deficient skeletal muscle. Cytosolic nNOS has been found in most patients with BMD (40) and indeed was detected in our two patients in whom muscle biopsy tissue was available for study. That tadalafil affected neither reflex vasoconstriction in resting BMD skeletal muscle nor systemic blood pressure suggested that the exercise-specific action of tadalafil in this setting does not involve either endothelial NOS (eNOS)-derived NO or central inhibition of sympathetic outflow.

Because tadalafil, unlike other PDE5A inhibitors, is specific for cGMP (without affecting cAMP at clinical doses) (41,42), our human data confirm but also extend prior *mdx* mouse studies implicating vascular NO-cGMP signaling as a potential new drug target for BMD and DMD. Asai et al. (28) showed that the normal increase in microvessel flow with electrically-evoked muscle contraction is blunted in anesthetized *mdx* mice and that dystrophic muscle histology can be improved by prenatal treatment with tadalafil; however, the authors did not report whether the vascular dysregulation also was improved by tadalafil. Kobayashi et al. (29) showed that single-dose tadalafil dramatically eliminates spasm of intramuscular arterioles (as well as indices of muscle injury and fatigue) in *mdx* mice after a brief bout of eccentric (i.e., injury-producing) exercise. Whereas the optimal dose in the

mouse study of Kobayashi et al. was 500–1,000 times higher than the highest tadalafil doses used in clinical medicine, the 20 mg tadalafil dose that rescued functional muscle ischemia in our BMD patients is the same dose used clinically to treat men with erectile dysfunction and half the dose used to treat adult patients with pulmonary hypertension, the other Food and Drug Administration (FDA)-approved indication for tadalafil.

Our study has several limitations. The case-control study included no disease controls, but we previously showed that sympatholysis is well-preserved in weak patients with other muscle diseases in which skeletal muscle nNOS μ is plentiful (14). We do not know why tadalafil failed to restore sympatholysis in one BMD patient—whether the non-response is related to his severe dystrophin mutation or other factors. By excluding patients with heart failure, we likely biased our sample for certain dystrophin mutations (43); thus, without further study, the present results cannot be extrapolated to BMD patients with clinical cardiomyopathy. Because this was a single-dose study, we do not know if the positive outcome in BMD patients will be sustained with chronic administration. However, patients do not develop tolerance when chronic PDE5A inhibition is used to treat erectile dysfunction or pulmonary hypertension; in contrast, with chronic nitroglycerin (a short-acting NO donor), healthy subjects quickly develop nitrate tolerance caused by reactive oxygen species that impair NO-mediated sympatholysis (44).

Because forearm muscle oxygenation was this study's endpoint, the present data do not define the contribution of microvascular ischemia to the pathogenesis and progression of BMD. A recent mouse study (45) suggests that, whereas sarcolemmal nNOS μ mediates sympatholysis, a different splice variant nNOS β (which associates with the Golgi apparatus) is a more important regulator of muscle force and fatigue. On the other hand, increasing evidence by several groups shows that absence of nNOS μ at the sarcolemma is associated with more severe disease in BMD and other human muscle wasting diseases (40,46,47). The present study does not address whether restoring sympatholysis with PDE5A inhibition can slow disease progression and improve muscle strength. A longitudinal multicenter trial is needed to address these clinically meaningful outcomes and is being planned (48).

Despite these limitations, the present data are consistent with our hypothesis that sarcolemmal nNOS μ plays an essential role in the normal modulation of sympathetic vasoconstriction in exercising human skeletal muscle and constitutes a first-in-human study of PDE5A inhibition as a putative new therapeutic strategy in BMD. PDE5A inhibition will not cure BMD, but alleviating ischemic insult to vulnerable dystrophin-deficient muscle membranes may reduce use-dependent muscle injury, thereby slowing disease progression. Also, PDE5A inhibition may be an important adjunct to exon skipping, which is under investigation for DMD to produce a truncated BMD-like dystrophin that may or may not restore sarcolemmal targeting of NOS μ (49,50). Repurposing PDE5A inhibitors could quickly transform clinical practice in muscular dystrophy, as no new drug development is needed. As loss of sarcolemmal nNOS μ is common in severe cases of acquired and inherited human neuromuscular diseases (29,47)—as well as in mouse models of steroid myopathy and (47) disuse atrophy (51)—the findings of our study may extend beyond BMD to a broader patient population.

Materials and Methods

Subjects

We studied 10 ambulatory male patients with BMD, 18 to 55 years of age, with normal LVEF, and seven healthy male controls matched for age and BMI. All patients had a pre-existing clinical diagnosis of BMD, which we confirmed with direct sequencing analysis of the dystrophin gene (University of Utah Genome Center, Salt Lake City, UT).

Potential subjects (both cases and controls) were excluded from study if they had: a history of hypertension or measured blood pressure >140/90 mmHg; diabetes mellitus; heart failure by history, physical examination, elevated brain natriuretic peptide (BNP), or LVEF < 50% by echocardiography; nocturnal ventilator support; serum creatinine > 1.5 mg/d; any history of substance abuse (including alcohol) or other psychiatric illness; or contraindications to tadalafil (use of nitrates, α -adrenergic blockers, other PDE5A inhibitors, or potent inhibitors of cytochrome P450 3A4).

The study was approved by the Institutional Review Board at Cedars-Sinai Medical Center and each subject gave informed written consent to participate. A waiver for an investigational new drug application was granted by the FDA to use tadalafil in this trial. The study was registered with clinicaltrials.gov (identifier NCT01070511).

Direct Sequencing Analysis of the Dystrophin Gene

Mutation screening of the dystrophin gene was performed by multiplex ligation-dependent probe amplification (MLPA) of genomic DNA isolated from whole blood (52). The test surveyed single or multi-exon duplication in the dystrophin gene, using MLPA to screen for duplications in all 79 coding exons of the major mRNA transcript isoform in muscle (Dp427m, reference mRNA transcript NM 004006). MLPA was performed as described (53) with the P034 and P035 kits from MRC-Holland. These MLPA reactions were performed on genomic DNA. The extent of exonic deletion was confirmed by polymerase chain reaction (PCR) (54) using four independent primer pairs to verify the absence of each limiting exon.

Hemodynamic Measurements

Subjects were studied in the supine position. Heart rate was measured continuously by electrocardiography and BP by automated oscillometric sphygmomanometry (Welch Allyn Vital Signs Monitor 300 Series, Skaneateles Falls, NY).

Skeletal Muscle Oxygenation in NIR Spectroscopy

Forearm muscle oxygenation was measured with near-infrared (NIR) spectroscopy, which is based on the principle that laser light with wavelengths in the 700–900 nm range easily penetrates skeletal muscle, and is absorbed by the iron-porphyrin moieties in hemoglobin and myoglobin. Changes in NIR light absorption are proportional to changes in the relative concentrations of oxygenated hemoglobin and myoglobin (HbO₂ and MbO₂). Because of their nearly identical absorption spectra, individual contributions of HbO₂ and MbO₂ cannot be determined. The NIR signals reflect changes in oxygenation occurring mainly in the microvasculature, because vessels greater than one millimeter in diameter are maximal absorbers of photons due to the high extinction coefficient of blood. Thus, NIR spectroscopy provides continuous measurement of the adequacy of tissue oxygen delivery relative to its use.

To monitor the tissue absorption of NIR light, five optical fiber bundles (four emitting bundles and one detector bundle connected to a photomultiplier tube) housed in a customized flexible rubber casing were placed with adhesive on the skin over the flexor digitorum profundus muscle, the main muscle recruited during handgrip. Four fixed emitter-detector distances (1.5, 2.0, 2.5, and 3.0 cm) allowed direct calculation and, thus subtraction, of scatter from skin and subcutaneous non-muscle tissue (55). Each emitting bundle contained two laser-diode light sources, one at 830 nm, the wavelength at which the oxygenated and deoxygenated Hb/Mb species exhibit similar absorption coefficients, and the other at 690 nm, the wavelength at which light is absorbed primarily by the deoxygenated species. The difference between absorption at the two wavelengths is the

HbO₂ + MbO₂ (56). The NIR signals were sampled at a rate of five Hz, converted to HbO₂ + MbO₂ concentration using validated algorithms, displayed as the running average of 50 consecutive samples, and stored digitally for analysis (OxiplexTS, ISS, Inc. Champaign, IL). Before each experiment, absorption and scattering coefficients at each wavelength were calibrated against an external standard. After each experiment, a cuff was inflated on the upper arm to suprasystolic pressure of 250 mmHg to establish the “total labile signal” (TLS, the difference between the baseline and nadir in muscle tissue oxygenation). Changes in forearm tissue oxygenation were expressed as a percentage of TLS.

Handgrip Exercise

Handgrip exercise was performed using a dynamometer (Smedley Hand Dynamometer modified by Stoelting, Wood Dale, IL). To determine MVC, each subject was asked to grip the dynamometer as hard as possible. Force output was displayed on a computer screen to provide visual feedback for subjects. Subjects performed intermittent isometric handgrip (20 handgrips/minute, 50% duty cycle) at 20% MVC for seven minutes. This mild level of handgrip exercise alone does not activate sympathetic outflow to skeletal muscle (13, 57).

Reflex Sympathetic Activation with Lower Body Negative Pressure

The subject's lower body was enclosed in a negative pressure chamber to the level of the iliac crest as previously described (13). The pressure in the chamber was measured by a Satham transducer (Gould Inc., Oxnard, CA). LBNP at -20 mmHg simulates mild orthostatic stress (i.e., transition from the supine to the seated position). This technique mainly unloads the cardiopulmonary baroreceptors, producing highly reproducible reflex increases in sympathetic vasoconstrictor drive to the skeletal muscle vasculature without changing systemic arterial pressure (13,58–60).

Functional Sympatholysis

To measure exercise-induced attenuation of reflex vasoconstriction (i.e., functional sympatholysis), LBNP was applied (a) at rest, and then (b) superimposed on mild handgrip exercise. Reflex vasoconstriction was measured as the LBNP-induced decrease in forearm muscle oxygenation by NIR spectroscopy; this approach provides a valid measure of reflex neurogenic vasoconstriction under these conditions (13).

The BP, heart rate, handgrip force, and NIR signals were recorded in response to two minutes of LBNP applied at rest and during the third to fifth minutes of the seven-minute exercise period.

Protocol for the Case-Control Study

All subjects were screened for eligibility with a clinical examination, comprehensive blood chemistry panel, ECG, and echocardiogram. Patients with BMD also had blood drawn for CK, BNP, and DNA. In all eligible subjects, functional sympatholysis was measured in both arms (random order) on two separate days; all four measurements were averaged to calculate each subject's mean value.

Protocol for the Tadalafil Acute Treatment Trial

All BMD patients who completed the case-control protocol also completed the tadalafil treatment trial, which utilized a randomized, placebo-controlled, double-blind, crossover design with a two-week washout period before cross-over (to account for the 17.5-hour elimination half-life of tadalafil). Patients received a 10 mg test dose of oral tadalafil or placebo the night before the study (to test for adverse events) and, on the next morning, a 20 mg tablet of tadalafil or placebo at least three hours (to allow for peak tadalafil absorption)

before measurement of functional sympatholysis (in both arms). The order of tadalafil/placebo was random. The research pharmacist placed tadalafil tablets or lactose powder (placebo) in opaque capsules to blind patients and investigators, both for the test dose and study dose of drug or placebo. Patients were queried about potential tadalafil-specific side-effects both for safety monitoring and to assess whether blinding was maintained. Tadalafil blood levels were measured using high performance liquid chromatography/tandem mass spectrometry (NMS Labs Willow Grove, PA).

Immunohistochemistry

Needle muscle biopsies from two patients (P5 and P9) were obtained using standard technique and mounted in Optimal Cutting Temperature (OCT) compound and frozen in isopentane cooled in liquid nitrogen. Cryosections (6 μ m) were cut and mounted onto SuperFrost® Plus slides. Sections were incubated with monoclonal antibodies for nNOS (NCL-NOS-1, Novocastra, 1:400), and dystrophin C-terminus (NCL-DYS2, Novocastra, 1:1000). Immunodetection was carried out using a sensitive detection protocol (X-Cell-Plus HRP Detection - Menapath MP-XCPDAB-U100, according to the manufacturer's instructions). Sections were visualized with Liquid stable DAB (Menapath), counterstained in Carazzi's hematoxylin, dehydrated and permanently mounted. Primary antibody was omitted in negative controls. Patient biopsies were compared to a stored muscle sample with normal histology and protein expression previously obtained from a healthy individual. Hematoxylin & Eosin staining was performed with the standard protocol.

Statistics

Baseline characteristics of patients and controls were compared using Student's t test with the Welch variation for degrees of freedom applied to unequal variances. Exercise-induced attenuation of reflex vasoconstriction (functional sympatholysis) was assessed by comparing the LBNP-induced $\Delta\text{HbO}_2 + \text{MbO}_2$ (%TLS) at rest vs. the LBNP-induced decrease in $\Delta\text{HbO}_2 + \text{MbO}_2$ during handgrip. For each subject in the case-control study, the mean data from both arms on each study day were meaned to derive a single value for the LBNP response at rest and for the LBNP response during handgrip. These data were assessed for normality with the Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test. We tested for group differences in $\Delta\text{HbO}_2 + \text{MbO}_2$ during LBNP using a linear mixed effects model, with fixed effects for group (BMD patients or controls) and exercise condition (rest or handgrip) and random effects for individual subjects. In the tadalafil treatment trial, data from both arms were meaned to obtain patient-specific values for LBNP responses during placebo and during tadalafil. The main analysis was a linear mixed-effects model appropriate for a two-period and two-treatment randomized crossover design, containing fixed effects for sequence, period, drug treatment, exercise condition, baseline values, and subject within sequence as a random effect. Testing for carryover effects (using the sequence effect) and period effects was carried out; neither effect was statistically significant. The threshold of significance for the carryover effect was 0.1. For all other variables, two-sided *P* values of less than 0.05 were considered to indicate statistical significance. All analyses were performed in R, version 2.13.1. Data are expressed as mean $\pm$ standard error (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol.* 2010; 9:77–93. [PubMed: 19945913]
2. Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C. Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. *Lancet Neurol.* 2010; 9:177–189. [PubMed: 19945914]
3. Emery AE. The muscular dystrophies. *Lancet.* 2002; 359:687–695. [PubMed: 11879882]
4. Koenig M, Beggs AH, Moyer M, Scherpf S, Heindrich K, Bettecken T, Meng G, Muller CR, Lindlof M, Kaariainen H. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am J Hum Genet.* 1989; 45:498–506. [PubMed: 2491009]
5. Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics.* 1988; 2:90–95. [PubMed: 3384440]
6. Duan D. Duchenne muscular dystrophy gene therapy: Lost in translation? *Res Rep Biol.* 2011; 2011:31–42.
7. Blake DJ, Weir A, Newey SE, Davies KE. Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol Rev.* 2002; 82:291–329. [PubMed: 11917091]
8. Petrof BJ, Shrager JB, Stedman HH, Kelly AM, Sweeney HL. Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc Natl Acad Sci USA.* 1993; 90:3710–3714. [PubMed: 8475120]
9. Brenman JE, Chao DS, Xia H, Aldape K, Brecht DS. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell.* 1995; 82:743–752. [PubMed: 7545544]
10. Chang WJ, Iannaccone ST, Lau KS, Masters BS, McCabe TJ, McMillan K, Padre RC, Spencer MJ, Tidball JG, Stull JT. Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy. *Proc Natl Acad Sci USA.* 1996; 93:9142–9147. [PubMed: 8799168]
11. Lai Y, Thomas GD, Yue Y, Yang HT, Li D, Long C, Judge L, Bostick B, Chamberlain JS, Terjung RL, Duan D. Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest.* 2009; 119:624–635.
12. Chavoshan B, Sander M, Sybert TE, Hansen J, Victor RG, Thomas GD. Nitric oxide-dependent modulation of sympathetic neural control of oxygenation in exercising human skeletal muscle. *J Physiol.* 2002; 540:377–386. [PubMed: 11927694]
13. Hansen J, Thomas GD, Harris SA, Parsons WJ, Victor RG. Differential sympathetic neural control of oxygenation in resting and exercising human skeletal muscle. *J Clin Invest.* 1996; 98:584–596. [PubMed: 8755671]
14. Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor RG. Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. *Proc Natl Acad Sci USA.* 2000; 97:13818–13823. [PubMed: 11087833]
15. Thomas GD, Hansen J, Victor RG. Inhibition of alpha 2-adrenergic vasoconstriction during contraction of glycolytic, not oxidative, rat hindlimb muscle. *Am J Physiol.* 1994; 266:H920–H929.

16. Thomas GD, Sander M, Lau KS, Huang PL, Stull JT, Victor RG. Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. *Proc Natl Acad Sci USA*. 1998; 95:15090–15095. [PubMed: 9844020]
17. Thomas GD, Victor RG. Nitric oxide mediates contraction-induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. *J Physiol*. 1998; 506(Pt 3):817–826. [PubMed: 9503340]
18. Thomas GD, Zhang W, Victor RG. Impaired modulation of sympathetic vasoconstriction in contracting skeletal muscle of rats with chronic myocardial infarctions: role of oxidative stress. *Circ Res*. 2001; 88:816–823. [PubMed: 11325874]
19. Thomas GD, Shaul PW, Yuhanna IS, Froehner SC, Adams ME. Vasomodulation by skeletal muscle-derived nitric oxide requires alpha-syntrophin-mediated sarcolemmal localization of neuronal Nitric oxide synthase. *Circ Res*. 2003; 92:554–560. [PubMed: 12600881]
20. Vongpatanasin W, Wang Z, Arbique D, Arbique G, Adams-Huet B, Mithcell JH, Victor RG, Thomas GD. Functional sympatholysis is impaired in hypertensive humans. *J Physiol*. 2011; 589:1209–1220.
21. Wallin BG, Victor RG, Mark AL. Sympathetic outflow to resting muscles during static handgrip and postcontraction muscle ischemia. *Am J Physiol*. 1989; 256:H105–H110.
22. Wehling-Henricks M, Jordan MC, Roos KP, Deng B, Tidball JG. Cardiomyopathy in dystrophin-deficient hearts is prevented by expression of a neuronal nitric oxide synthase transgene in the myocardium. *Hum Mol Genet*. 2005; 14:1921–1933. [PubMed: 15917272]
23. Wehling M, Spencer MJ, Tidball JG. A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *J Cell Biol*. 2001; 155:123–131.
24. Barton ER, Morris L, Kawana M, Bish LT, Toursel T. Systemic administration of L-arginine benefits mdx skeletal muscle function. *Muscle Nerve*. 2005; 32:751–760. [PubMed: 16116642]
25. Voisin V, Sebric C, Matecki S, Yu H, Gillet B, Ramonaxo M, Israel M, De la Porte S. L-arginine improves dystrophic phenotype in mdx mice. *Neurobiol Dis*. 2005; 20:123–130. [PubMed: 16137573]
26. Brunelli S, Sciorati C, D'Antona G, Innocenzi A, Covarello D, Galvez BG, Perrotta C, Monopoli A, Sanvito F, Bottinelli R, Ongini E, Cossu G, Clementi E. Nitric oxide release combined with nonsteroidal antiinflammatory activity prevents muscular dystrophy pathology and enhances stem cell therapy. *Proc Natl Acad Sci U S A*. 2007; 104:264–269. [PubMed: 17182743]
27. Sciorati C, Miglietta D, Buono R, Pisa V, Cattaneo D, Azzoni E, Brunelli S, Clementi E. A dual acting compound releasing nitric oxide (NO) and ibuprofen, NCX 320, shows significant therapeutic effects in a mouse model of muscular dystrophy. *Pharmacol Res*. 2011; 64:210–217.
28. Asai A, Sahani N, Kaneki M, Ouchi Y, Martyn JA, Yasuhara SE. Primary role of functional ischemia, quantitative evidence for the two-hit mechanism, and phosphodiesterase-5 inhibitor therapy in mouse muscular dystrophy. *PLoS One*. 2007; 2:e806.
29. Kobayashi YM, Rader EP, Crawford RW, Iyengar NK, Thedens DR, Faulkner JA, Parikh SV, Weiss RM, Chamberlain JS, Moore SA, Campbell KP. Sarcolemma-localized nNOS is required to maintain activity after mild exercise. *Nature*. 2008; 456:511–515. [PubMed: 18953332]
30. Adamo CM, Dai DF, Percival JM, Minami E, Willis MS, Patrucco E, Froehner SC, Beavo JA. Sildenafil reverses cardiac dysfunction in the mdx mouse model of Duchenne muscular dystrophy. *Proc Natl Acad Sci USA*. 2010; 107:19079–19083.
31. Khairallah M, Khairallah RJ, Young ME, Allen BG, Gillis MA, Danialou G, Deschepper CF, Petrof BJ, Des RC. Sildenafil and cardiomyocyte-specific cGMP signaling prevent cardiomyopathic changes associated with dystrophin deficiency. *Proc Natl Acad Sci USA*. 2008; 105:7028–7033. [PubMed: 18474859]
32. Kawahara G, Karpf JA, Myers JA, Alexander MS, Guyon JR, Kunkel LM. Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proc Natl Acad Sci USA*. 2011; 108:5331–5336.
33. Chao DS, Gorospe JR, Brenman JE, Rafael JA, Peters MF, Froehner SC, Hoffman EP, Chamberlain JS, Bredt DS. Selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy. *J Exp Med*. 1996; 184:609–618. [PubMed: 8760814]

34. Torelli S, Brown SC, Jimenez-Mallebrera C, Feng L, Muntoni F, Sewry CA. Absence of neuronal nitric oxide synthase (nNOS) as a pathological marker for the diagnosis of Becker muscular dystrophy with rod domain deletions. *Neuropathol Appl Neurobiol.* 2004; 30:540–545.
35. Wells KE, Torelli S, Lu Q, Brown SC, Partridge T, Muntoni F, Wells DJ. Relocalization of neuronal nitric oxide synthase (nNOS) as a marker for complete restoration of the dystrophin associated protein complex in skeletal muscle. *Neuromuscul Disord.* 2003; 13:21–31.
36. Romfh A, McNally EM. Cardiac assessment in duchenne and becker muscular dystrophies. *Curr Heart Fail Rep.* 2010; 7:212–218. [PubMed: 20857240]
37. Zhao W, Swanson SA, Ye J, Li X, Shelton JM, Zhang W, Thomas GD. Reactive oxygen species impair sympathetic vasoregulation in skeletal muscle in angiotensin II-dependent hypertension. *Hypertension.* 2006; 48:637–643.
38. Bagher P, Duan D, Segal SS. Evidence for impaired neurovascular transmission in a murine model of Duchenne muscular dystrophy. *J Appl Physiol.* 2011; 110:601–609. [PubMed: 21109597]
39. Lombard JH. Microcirculation in a mouse model of Duchenne muscular dystrophy: another blow to the vascular hypothesis? *J Appl Physiol.* 2011; 110:587–588. [PubMed: 21212249]
40. Gentil C, Leturcq F, Ben YR, Kaplan JC, Laforet P, Penisson-Besnier I, Espil-Taris C, Voit T, Garcia L, Pietri-Rouxel F. Variable phenotype of del45-55 Becker patients correlated with nNOSmu mislocalization and RYR1 hypernitrosylation. *Hum Mol Genet.* 2012; 21:3449–3460.
41. Palit V, Eardley I. An update on new oral PDE5 inhibitors for the treatment of erectile dysfunction. *Nat Rev Urol.* 2010; 7:603–609.
42. Ravipati G, McClung JA, Aronow WS, Peterson SJ, Frishman WH. Type 5 phosphodiesterase inhibitors in the treatment of erectile dysfunction and cardiovascular disease. *Cardiol Rev.* 2007; 15:76–86.
43. Kaspar RW, Allen HD, Ray WC, Alvarez CE, Kissel JT, Pestronk A, Weiss RB, Flanigan KM, Mendell JR, Montanaro F. Analysis of dystrophin deletion mutations predicts age of cardiomyopathy onset in becker muscular dystrophy. *Circ Cardiovasc Genet.* 2009; 2:544–551.
44. Fadel PJ, Farias IM, Gallagher KM, Wang Z, Thomas GD. Oxidative stress and enhanced sympathetic vasoconstriction in contracting muscles of nitrate-tolerant rats and humans. *J Physiol.* 2012; 590:395–407. [PubMed: 22106180]
45. Percival JM, Anderson KN, Huang P, Adams ME, Froehner SC. Golgi and sarcolemmal neuronal NOS differentially regulate contraction-induced fatigue and vasoconstriction in exercising mouse skeletal muscle. *J Clin Invest.* 2010; 120:816–826.
46. Anthony K, Cirak S, Torelli S, Tasca G, Feng L, Arechavala-Gomez V, Armaroli A, Guglieri M, Straathof CS, Verschuuren JJ, Aartsma-Rus A, Helderma-van den Enden P, Bushby K, Straub V, Sewry C, Ferlini A, Ricci E, Morgan JE, Muntoni F. Dystrophin quantification and clinical correlations in Becker muscular dystrophy: implications for clinical trials. *Brain.* 2011; 134:3547–3559.
47. Finanger Hedderick EL, Simmers JL, Soleimani A, Andres-Mateos E, Marx R, Files DC, King L, Crawford TO, Corse AM, Cohn RD. Loss of sarcolemmal nNOS is common in acquired and inherited neuromuscular disorders. *Neurology.* 2011; 76:960–967. [PubMed: 21403107]
48. [Last Accessed 9-26-2012] NIH RePORT, Planning a Multicenter Trial of PDE5A Inhibition for Duchenne Muscular Dystrophy. http://projectreporter.nih.gov/project_info_description.cfm?aid=8295579&icde=13915249&ddparam=&ddvalue=&ddsub=&cr=1&csb=default&cs=ASC
49. Cirak S, Arechavala-Gomez V, Guglieri M, Feng L, Torelli S, Anthony K, Abbs S, Garraida ME, Bourke J, Wells DJ, Dickson G, Wood MJ, Wilton SD, Straub V, Kole R, Shrewsbury SB, Sewry C, Morgan JE, Bushby K, Muntoni F. Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet.* 2011; 378:595–605. [PubMed: 21784508]
50. Cirak S, Feng L, Anthony K, Arechavala-Gomez V, Torelli S, Sewry C, Morgan JE, Muntoni F. Restoration of the Dystrophin-associated Glycoprotein Complex After Exon Skipping Therapy in Duchenne Muscular Dystrophy. *Mol Ther.* 2012; 20:462–467.
51. Suzuki N, Motohashi N, Uezumi A, Fukada S, Yoshimura T, Itoyama Y, Aoki M, Miyagoe-Suzuki Y, Takeda S. NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. *J Clin Invest.* 2007; 117:2468–2476. [PubMed: 17786240]

52. Zeng F, Ren ZR, Huang SZ, Kalf M, Mommersteeg M, Smit M, White S, Jin CL, Xu M, Zhou DW, Yan JB, Chen MJ, van BR, Huang SZ, den DJ, Zeng YT, Wu Y. Array-MLPA: comprehensive detection of deletions and duplications and its application to DMD patients. *Hum Mutat.* 2008; 29:190–197. [PubMed: 17854090]
53. Lalic T, Vossen RH, Coffa J, Schouten JP, Guc-Scekic M, Radivojevic D, Djuricic M, Breuning MH, White SJ, den Dunnen JT. Deletion and duplication screening in the DMD gene using MLPA. *Eur J Hum Genet.* 2005; 13:1231–1234.
54. Flanigan KM, von NA, Dunn DM, Alder J, Mendell JR, Weiss RB. Rapid direct sequence analysis of the dystrophin gene. *Am J Hum Genet.* 2003; 72:931–939. [PubMed: 12632325]
55. Gratton E, Fantini S, Franceschini MA, Gratton G, Fabiani M. Measurements of scattering and absorption changes in muscle and brain. *Philos Trans R Soc Lond B Biol Sci.* 1997; 352:727–735. [PubMed: 9232861]
56. Jobsis FF. Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters. *Science.* 1977; 198:1264–1267. [PubMed: 929199]
57. Victor RG, Seals DR, Mark AL. Differential control of heart rate and sympathetic nerve activity during dynamic exercise. Insight from intraneural recordings in humans. *J Clin Invest.* 1987; 79:508–516. [PubMed: 3805279]
58. Jacobsen TN, Morgan BJ, Scherrer U, Vissing SF, Lange RA, Johnson N, Ring WS, Rahko PS, Hanson P, Victor RG. Relative contributions of cardiopulmonary and sinoaortic baroreflexes in causing sympathetic activation in the human skeletal muscle circulation during orthostatic stress. *Circ Res.* 1993; 73:367–378. [PubMed: 8330379]
59. Victor RG, Leimbach WN Jr. Effects of lower body negative pressure on sympathetic discharge to leg muscles in humans. *J Appl Physiol.* 1987; 63:2558–2562. [PubMed: 3436888]
60. Vissing SF, Scherrer U, Victor RG. Relation between sympathetic outflow and vascular resistance in the calf during perturbations in central venous pressure. Evidence for cardiopulmonary afferent regulation of calf vascular resistance in humans. *Circ Res.* 1989; 65:1710–1717. [PubMed: 2582598]
61. Anderson LV, Davison K. Multiplex Western blotting system for the analysis of muscular dystrophy proteins. *Am J Pathol.* 1999; 154:1017–1022. [PubMed: 10233840]

Becker muscular dystrophy (BMD) results from abnormal dystrophin and presents with variable but progressive skeletal muscle wasting disease about which there has been little research and no treatment. Preclinical research in the dystrophin-deficient *mdx* mouse model shows that phosphodiesterase (PDE)5 inhibitors, drugs that boost cGMP—the downstream target of nitric oxide (NO) in vascular smooth muscle—dramatically alleviate many features of the dystrophic phenotype including vasospasm of skeletal muscle microvessels as a vascular mechanism of use-dependent muscle injury and fatigue. The challenge is to determine if this compelling mouse work can be translated to benefit human patients with muscular dystrophy. In 10 BMD patients and seven age-matched healthy male controls, we assessed exercise-induced attenuation of reflex sympathetic vasoconstriction—a protective mechanism that optimizes perfusion to meet the metabolic demands of exercising skeletal muscle. Reflex vasoconstriction was induced by simulated orthostatic stress, measured as the decrease in forearm muscle oxygenation with near infrared spectroscopy, and performed when the forearm muscles were rested or lightly exercised with rhythmic handgrip. Here we show first that exercise-induced attenuation of reflex vasoconstriction was defective in nine of 10 patients with BMD in whom the common dystrophin mutations disrupt sarcolemmal targeting of nNOS μ —the main source of skeletal muscle-derived NO—but well-preserved in one patient with biopsy-proven sarcolemmal nNOS. Then, in a double-blind randomized placebo-controlled cross-over trial, we show that normal blood flow regulation was fully restored in eight of nine patients with initially abnormal regulation by a single oral 20 mg dose of the drug tadalafil, a specific PDE5 inhibitor. These findings support an essential role for sarcolemmal nNOS μ in modulating sympathetic vasoconstriction in exercising human skeletal muscle and implicate PDE5 inhibition as a putative novel treatment for BMD. Alleviating ischemic insult to vulnerable dystrophic muscles could protect patients from use-dependent muscle injury.

Fig. 1.

Functional sympatholysis is impaired in patients with Becker muscular dystrophy (BMD).

(A) As shown in the representative trace from a healthy control subject, the lower body negative pressure (LBNP)-induced decrease in forearm muscle oxygenation ($\text{HbO}_2 + \text{MbO}_2$) is greatly attenuated during mild handgrip exercise, demonstrating functional sympatholysis (i.e., normal exercise-induced attenuation of reflex vasoconstriction; grey oval). (B) The representative trace from a patient with BMD shows that sympatholysis is impaired, because handgrip fails to attenuate the LBNP response (red oval) indicating functional muscle ischemia. At the end of each experiment, an arm cuff was inflated to suprasystolic pressure to occlude the forearm circulation, producing a maximal decrease in muscle oxygenation to calculate total labile signal (TLS). (C, D) Summary data for 7 healthy controls (C) and 10 patients with BMD (D) expressed as a percentage of TLS (mean $\pm$ SEM). * $P < 0.01$ rest vs. exercise using a linear mixed-effects model.

Fig. 2.

Tadalafil alleviates functional muscle ischemia in a patient with Becker muscular dystrophy (BMD). (A) Tadalafil restores functional sympatholysis in a patient with BMD, as shown by the exercise-induced attenuation of the decrease in muscle oxygenation ($\text{HbO}_2 + \text{MbO}_2$) with lower body negative pressure (LBNP) (grey oval). (B) In contrast, a placebo is without effect in the same patient, as shown by comparable LBNP responses during rest and exercise (red oval). (C, D) Summary data expressed as a percentage of total labile signal (TLS) (mean $\pm$ SEM) from 10 patients with BMD all of whom completed the double-blind cross-over trial on tadalafil (C) and then placebo (D) or vice versa. $*P < 0.01$, treatment condition $\times$ exercise condition interaction using a linear mixed-effects model.

Fig. 3.

Patient-specific data showing the consistency of the tadalafil effect. (A) With tadalafil, functional sympatholysis (exercise-induced attenuation of reflex vasoconstriction) is evident in nine of 10 patients with Becker muscular dystrophy (BMD), because the lower body negative pressure (LBNP)-induced decrease in forearm muscle oxygenation ($\text{HbO}_2 + \text{MbO}_2$) is less during exercise (grey bars) than at rest (black bars). The exception was patient 10 (P10) in whom an adequate tadalafil blood concentration was achieved. (B) With placebo, the LBNP response is virtually identical during rest and exercise for each patient, except patient P9 in whom sympatholysis is clearly evident without tadalafil and possibly in patient P2 in whom sympatholysis is greatly augmented by tadalafil. Data are expressed as a percentage of total labile signal (TLS) (mean $\pm$ SEM).

Fig. 4.

Immunohistochemistry of muscle biopsies from BMD patients. Muscle biopsy sections stained with haematoxylin and eosin (upper panel) from patient P5 (deletion of exons 45–48) and patient P9 (deletion of exons 14–44) show typical dystrophic hallmarks including variation in muscle fiber size, internal nuclei, fibrosis, and increase in fat tissue. Staining for dystrophin C-terminus (DYS-C) is present in muscle from both P5 and P9. Cytoplasmic staining for nNOS is evident in both P5 and P9 but not in control muscle. Sarcolemmal nNOS labeling is present in P9 but not detected in P5. Bottom panel shows higher magnification of fields enclosed in rectangles. Scale bars are 100 μ m.

Baseline characteristics of patients with Becker muscular dystrophy

Table 1

Exon Deletion	Age, at study, years	Age at onset of symptoms, years	Age at loss of ability to run, years	Maximum voluntary contraction (grip strength)		CK level, IU/L	Frequency of wheelchair/scooter use	Medications	
				right arm, kg	left arm, kg				
P1	45-48	32	5	18	32	22	1883	never	none
P2	45-48	33	5	22	23	29	1247	never	none
P3	45-48	42	30	30	42	39	719	never	none
P4	45-55	38	12	26	29	23	2006	never	none
P5	45-48	42	28	32	29	33	1453	rarely	none
P6	48-49	38	25	27	35	33	2058	never	none
P7	67	50	32	38	36	42	2247	rarely	oxybutynin
P8	20-29	30	12	15	13	10	3745	never	deflazacort
P9	14-44	50	33	35	21	17	707	never	none
P10	3-7	19	6	8	13	12	2448	often	none

P, patient; CK, creatine kinase

Table 2

Baseline comparison of the two study groups

Group	n	Age, years	BMI, kg/m ²	MVC right arm, kg	MVC left arm, kg	SBP, mmHg	DBP, mmHg	HR, beats per min	LVEF, %
Patients with BMD	10	37 ± 3	25.0 ± 1.6	27 ± 3*	26 ± 3*	118 ± 3	78 ± 3	79 ± 5	60.7 ± 1.8
Healthy controls	7	34 ± 3	25.0 ± 1.7	54 ± 2	47 ± 3	114 ± 2	71 ± 3	78 ± 2	65.7 ± 1.3

BMI, body mass index; MVC, maximum voluntary contraction; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; LVEF, left ventricular ejection fraction.

* $P < 0.05$ versus controls.