Evidence for impaired neurovascular transmission in a murine model of
Duchenne Muscular Dystrophy

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Running Head: Impaired neurovascular transmission in \textit{mdx} mice

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Abstract

Duchenne muscular dystrophy (DMD) is a muscle wasting disease caused by mutations in the dystrophin gene. Little is known about how blood flow control is affected in arteriolar networks supplying dystrophic muscle. We tested the hypothesis that mdx mice, a murine model for DMD, exhibit defects in arteriolar vasomotor control. The cremaster muscle was prepared for intravital microscopy in pentobarbital anesthetized mdx and C57BL/10 control mice (n ≥ 5 per group). Spontaneous vasomotor tone increased similarly with arteriolar branch order in both mdx and C57BL/10 mice (pooled values: first-(1A), 6%, second- (2A), 56% and third-(3A) order, 61%) with no difference in maximal diameters between groups measured during equilibration with topical 10 µM sodium nitroprusside (pooled values: 1A:70±3, 2A: 31±3 and 3A: 19±3 µm, respectively). Concentration-response curves to acetylcholine (ACh) and norepinephrine added to the superfusion solution did not differ between mdx and C57BL/10 mice, nor did constriction to elevated (21%) oxygen. In response to local stimulation from a micropipette, conducted vasodilation to ACh and conducted vasoconstriction to KCl were also not different between groups however constriction decayed with distance (P<0.05) whereas dilation did not.

Remarkably, arteriolar constriction to perivascular nerve stimulation (PNS) at 2, 4, and 8 Hz was reduced by ~25-30% in mdx mice compared to C57BL/10 mice (P < 0.05). With intact arteriolar reactivity to agonists, attenuated constriction to PNS indicates impaired neurovascular transmission in arterioles controlling blood flow in mdx mice.

Keywords: Arteriole, cremaster muscle, microcirculation, perivascular nerves, functional sympatholysis
Introduction

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder that occurs in 1 of 3500 newborn boys and results in progressive muscle weakness which is ultimately lethal (7). The genomic locus disrupted in DMD encodes dystrophin, a 427 kDa cytoskeleton protein (10, 15). In concert with dystroglycans and sarcoglycans, dystrophin forms a trans-membrane complex which provides mechanical stability of the sarcolemma while localizing neuronal nitric oxide synthase to the membrane (nNOS) (17). In humans and wild-type mice dystrophin is expressed in striated muscles, as well as in vascular smooth muscle cells (VSMCs) of arteries and veins (22, 24). mdx mice which have a mutation in dystrophin (29) are the most commonly utilized model for studying DMD, although their disease pathology only partially mimics DMD in humans (3, 13). Since loss of dystrophin was identified as the defect underlying DMD pathology, many studies have focused on identifying changes in gene expression and cellular function in dystrophic muscle. For example, the loss of dystrophin is associated with loss of nNOS and the upregulation of utrophin at the sarcolemma, a homologous protein, that may play a compensatory role for dystrophin in maintaining cellular structural integrity (2, 17, 30).

Despite the dependence of skeletal muscle on blood flow for its functional integrity, little is known of how the microcirculation may be affected in dystrophic muscle. Blood flow control in arteriolar networks results from the interplay between VSMCs, the intimal monolayer of endothelial cells (ECs) and perivascular sympathetic nerves (27, 31). In concert, these three functional components of the arteriolar wall determine spontaneous vasomotor tone (i.e., the level of SMC contraction under resting baseline conditions) and the regulation of peripheral vascular resistance in response to physiological stimuli. Findings from the cremaster muscle in mdx mice have indicated that arteriolar dilations in response to rhythmic muscle contractions (18) or to elevations in luminal shear stress (26) were attenuated relative to control (C57BL/10) mice, with the defect in NO production (associated with loss of nNOS) thought to contribute to these functional deficits (18, 26). Impaired ability to overcome α-adrenergic vasoconstriction in hindlimbs of mdx mice versus C57BL/10 mice has also been attributed to loss of nNOS (12). Consistent with findings in arterioles, mesenteric and carotid arteries of mdx mice demonstrated defective flow-mediated vasodilation (22) while mesenteric arteries were unable to adapt to
chronic increases or decreases in blood flow (21). These studies collectively suggest impairment of endothelium-dependent mechanical transduction in vessels of mdx mice.

In addition to releasing NO in response to shear stress, the endothelium can induce relaxation of the surrounding VSMCs through electrical signaling. For example, conducted vasodilation entails the initiation and spread of hyperpolarizing signals through gap junctions along the endothelium and into VSMCs to promote relaxation (6, 20). In a complementary manner, conducted vasoconstriction reflects the spread of depolarization promoting VSMC contraction.

In turn, the activation of periarteriolar sympathetic nerves releases norepinephrine (NE) resulting in α-adrenergic contraction of VSMCs (11, 16). Whereas the ability of ECs, VSMCs and perivascular nerves to conduct and propagate vasomotor responses along arteriolar networks has been evaluated in wild-type (e.g., C57BL/6) mice (11), it has not been determined how these respective control processes are affected in DMD. Providing such insight for mdx mice in vivo was the goal of the present study. We hypothesized that there are functional defects in vasomotor signaling along arteriolar networks controlling blood flow to skeletal muscle of mdx mice. By using stimuli that are selective for respective control elements of the vessel wall, we investigated the functional integrity of arteriolar ECs, VSMCs and perivascular nerves of mdx mice relative to C57BL/10 mice (wild-type background strain of mdx mice).

**Materials and Methods**

**Animal care and use**

All procedures and protocols were approved by the Animal Care and Use Committee of the University of Missouri and performed in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were housed on a 12 hour light/12 hour dark cycle at ~22 °C with fresh water and food available *ad libitum*. Male mice (C57BL/10, n= 15; 39 ± 1 g, 6-13 months old, Jackson Laboratory, Bar Harbor Maine; mdx, n=19; 38 ± 1 g, 6-9 months old bred at the University of Missouri) were studied. Each mouse was anesthetized using pentobarbital sodium [60 mg/kg intraperitoneal injection (i.p.)]. Anesthesia was maintained via...
supplemental administration (10-20% of initial injection, i.p.). The mouse was placed in a supine position on a Plexiglas platform and esophageal temperature was maintained at 37 °C by positioning the mouse on an aluminum platform maintained at ~39 °C. At the end of each day’s experimental procedures, the anesthetized mouse was killed with an overdose of pentobarbital (i.p. injection) followed by cervical dislocation.

Cremaster muscle preparation.

The left or right cremaster muscle was prepared as described for C57BL/6 mice (11). Briefly, a ventral incision was made through the scrotal skin. The cremaster muscle was carefully exposed and freed of surrounding connective tissue. A longitudinal incision was made through the ventral surface of the muscle from the base to the apex and an orchiectomy performed. The cremaster muscle was spread radially and pinned at the edges to a transparent pedestal (Sylgard® 184; Dow-Corning, NY, USA) with great care taken to minimize disruption of the vascular supply. First-(1A), second-(2A), and third-order (3A) arterioles located in the central region of the preparation were studied to minimize any effects of damaged tissue edges. The muscle was superfused continuously (3-6 ml min⁻¹) with a bicarbonate-buffered (pH, 7.4) physiological salt solution (PSS; composition in mM: 137 NaCl, 4.7 KCl, 1.2 MgSO₄, 2 CaCl₂, 18 NaHCO₃) equilibrated with 5% CO₂ / 95% N₂ and maintained at 34-35 °C.

Video microscopy

Upon completion of surgery, the preparation was transferred to a custom-built intravital microscope (ACM; Zeiss) and equilibrated for 30 minutes. During equilibration, arteriolar networks were scanned and sites for data collection were identified in 1A, 2A and 3A branches. The preparation was viewed using Köhler illumination [condenser numerical aperture (NA), 0.35]. The image was acquired with a Nikon 20X SLWD objective (NA=0.35), projected to a video camera (C2400, Hamamatsu Photonics, Japan) and viewed on a monitor (PVM 133, Sony) at a final magnification of ~580X (field of view along arterioles, ~500 µm). Vessel diameter was measured at the edges of the vessel lumen using a video caliper with a spatial resolution of ~1
µm. Data were acquired at 40 Hz using a PowerLab system (model 800; AD Instruments, Colorado Springs, CO, USA) coupled to a personal computer.

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Stock solutions [tetrodotoxin (TTX), acetylcholine (ACh), sodium nitroprusside (SNP), NE and KCl] were prepared in ultrapure deionized (18.2 MΩ) water. For superfusion, vasoactive compounds were diluted to their final working concentration in fresh PSS.

Micropipettes

Micropipettes were pulled (P-97; Sutter Instruments, Novato, CA, USA) from borosilicate glass capillaries (G120F-4; Warner Instruments, Hamden, CT, USA) and backfilled with filtered (0.22 µm) solutions. Micropipette tip sizes are described in context of experimental procedures.

Experimental procedures

Oxygen sensitivity and vasomotor tone. At the beginning of an experiment the sensitivity of arterioles to changes in ambient PO2 was tested by increasing the oxygen tension over the cremaster muscle (11, 16). Control baseline diameters in 1A, 2A and 3A were recorded while equilibrating the superfusion solution with 5% CO2 / 95% N2. The superfusate was then equilibrated with 21% O2 (5% CO2, balance N2) for 8 minutes and respective diameters were recorded. The superfusate was then returned to 5% CO2 / 95% N2 for the remainder of experimental procedures. At the end of each experiment the maximum diameter of each arteriole studied was recorded during equilibration with SNP (10 µM) in the superfusion solution. Spontaneous vasomotor tone (expressed as % maximum diameter) was calculated as: [(maximum diameter - resting baseline diameter)/maximum diameter] X 100.
Cumulative concentration-response curves. Vasoconstriction in response to activating α-adrenoreceptors on VSMCs was tested by performing cumulative concentration-response curves to NE added to the superfusion solution. The entire preparation was exposed to a given concentration of NE for at least 2 minutes until a stable diameter was recorded for 1A, 2A and 3A, then the next concentration was administered. After the final concentration, the preparation recovered during a 15 minute wash with control PSS. Using a similar protocol, responses to incremental concentrations of ACh in the superfusion solution were evaluated to test endothelium-dependent vasodilation mediated through G-protein coupled receptor (muscarinic, M3) activation. In 3 (of 15) experiments, responses to ACh were evaluated prior to NE.

Conducted vasomotor response. To optimize studying vasomotor responses to discrete stimuli delivered from micropipettes these experiments were performed in preparations separate from those used for cumulative concentration-response curves. Vessels must have tone under resting conditions in order to observe vasodilation (34). The 2A and 3A segments studied here exhibited robust spontaneous vasomotor tone (Table 1). In contrast, vasoconstriction is most readily resolved in arterioles lacking tone, which corresponded to the larger 2A and 1A segments. Because larger arterioles exhibit less-frequent branching, conducted vasoconstriction was typically studied along a single vessel segment. To evaluate conducted vasodilation for similar distances along smaller branches with greater tone required stimulating a daughter branch (3A) and observing responses spreading into its parent 2A. Arterioles studied for conducted vasoconstriction were not different between mdx and C57BL/10 mice, nor were those studied for conducted vasodilation (Table 1). In each case, sites upstream from the stimulus were observed to avoid the possibility of stimulus convection in the flow stream.

Conducted Vasodilation. Changes in the membrane potential of ECs and SMCs are integral to vasomotor control of tissue blood flow (6, 19, 35). In response to local delivery of ACh from a micropipette, the conduction of vasodilation reflects the initiation and transmission of hyperpolarization along the endothelium and into VSMCs to produce relaxation along the arteriolar wall (5, 8, 35). A glass micropipette [tip internal diameter (I.D.) ~1 µm] was filled with ACh (1M), secured in a micromanipulator and connected to a microiontophoresis programmer (Model 260; World Precision Instruments, Sarasota, Fl, USA). A silver wire secured at the edge
of the preparation served as the reference electrode. Preliminary experiments determined that a 500 ms, 500 nA pulse of ACh provided a submaximal stimulus evoking highly reproducible responses (data not shown).

The micropipette containing ACh was positioned with its tip adjacent to a 3A branch at a site located ~600 µm downstream from where it bifurcated from the parent 2A (illustrated in Figure 3). Diameter responses were recorded at the site of stimulation (‘local’) and at remote sites (500, 1000 and 2000 µm) upstream. Thus remote sites at 1000 and 2000 µm were located along the parent 2A. Distances were measured using a calibrated eyepiece reticule with reference to anatomical landmarks. For each observation site, a separate stimulus was delivered at the local site with ~2 minutes recovery between stimuli to restore resting diameter.

Conducted Vasoconstriction. Local delivery of concentrated KCl induces vasoconstriction by depolarizing ECs and SMCs through a Nernst effect (35). Our preliminary experiments established that direct access of the KCl micropipette to the arteriolar wall was essential for reproducible vasomotor responses. For this purpose a small incision (~250 µm) was made through skeletal muscle fibers adjacent to the designated 1A or 2A. Retraction of muscle fibers thereby exposed the abluminal arteriolar surface to the micropipette (tip I.D., 3 µm) containing KCl (1M). A 1-second pulse of KCl was ejected by applying pressure (35 kPa) to the back of the micropipette (PLI-1000 Harvard Apparatus, Holliston, MA, USA). To avoid data collection immediately adjacent to injured muscle fibers, arteriolar diameter responses were recorded ~250 µm upstream of the stimulus micropipette (referred to as ‘local’ for these experiments; as determined visually during recording, responses at 250 µm were not different from those adjacent to the micropipette). Conducted vasoconstriction was evaluated at remote sites (500, 1000 and 2000 µm) further upstream.

Perivascular nerve stimulation (PNS). Activation of perivascular nerves that invest cremasteric arterioles underlies sympathetic vasoconstriction, a key element in the regulation of peripheral vascular resistance (11, 16). A segment (~250 µm) of a 1A or 2A branch was exposed using a small incision (~250 µm, as above) to provide access for a saline-filled microelectrode (tip internal diameter ~2 µm) to the arteriolar wall. Perivascular sympathetic nerves were stimulated (1 ms
pulses; 2, 4 & 8 Hz for 10 s at 45 V). A silver wire secured at the edge of the preparation served as the reference electrode. Stimuli were delivered through a stimulus isolation unit (SIU5; Grass Instruments, Quincy, MA, USA) coupled to a monophasic square-wave stimulator (S48, Grass). A viable site for PNS was confirmed by arteriolar constriction along the entire field of view that was maintained throughout PNS. Upon termination of PNS arteriolar diameter recovered within 2 minutes. As with KCl microejection, diameter responses were measured ~250 µm upstream to avoid collecting data immediately adjacent to injured muscle fibers.

**Immunolabeling.** Along with cremaster muscles, the rectus abdominus muscle was collected from *mdx* mice and C57BL/10 mice, embedded in OCT (Tissue Tek, Sakura Finetek, Torrence, CA, U.S.A.) and flash-frozen in liquid N₂. Tissue cross-sections (thickness, 8 µm) were obtained using a cryostat. Expression profiles of dystrophin (Dys-2, clone Dy8/6C5, IgG1; Novocastra; 1:30), and utrophin (mouse monoclonal against the N-terminal domain; VP-U579, clone DRP3/20C5, IgG1; Vector Laboratories; 1:20) were examined as independent markers to ensure that *mdx* mice were compared with C57BL/10 control mice in studying the microcirculation.

**Data Analysis**

One arteriolar network was studied per cremaster preparation. Each network was treated as a separate experiment. Data were analyzed using One-way Analyses of Variance with post-hoc comparisons performed using Tukey’s test. Software included SigmaStat (Version 3.5, Systat Software, San Jose, CA), Prism (Version 5; GraphPad, La Jolla, CA) or JMP software (SAS Analytics, Cary, NC). Summary data represent at least 5 vessels from as many different animals and are presented as means ± S.E. Results were considered statistically significant with *P* < 0.05.

**Results**

**Immunolabeling**
As a positive control for dystrophic tissue, we examined muscles for expression of dystrophin and utrophin. Consistent with previous reports (14, 32), dystrophin was highly expressed at the periphery of muscle fibers of C57BL/10 control mice, which otherwise did not stain for utrophin (Figure 1). In contrast, \textit{mdx} mice lacked dystrophin expression while expressing utrophin at the periphery of skeletal muscle fibers (Figure 1). Similar expression profiles were observed in fibers of the cremaster and rectus abdominus muscles; however due to the criss-crossed orientation of cremaster muscle fibers, these typically sectioned at oblique angles. Therefore, cross-sections of the parallel-fibered rectus abdominus muscle are presented for clarity.

**Oxygen sensitivity and vasomotor tone.** Spontaneous vasomotor tone increased with arteriolar branch order but was not significantly different between \textit{mdx} and C57BL/10 mice (3A > 2A > 1A; Table 1). Raising superfusate oxygen from 0 to 21% reduced 2A and 3A diameters to a similar extent in both \textit{mdx} and C57BL/10 mice (Table 1).

**Concentration-response curves.** In response to either ACh (Figure 2A-C) or NE (Figure 2A-C), diameter changes were not significantly different between \textit{mdx} and C57BL/10 mice. Because 3A constricted to closure when exposed to 0.1 µM NE, response curves were terminated at this agonist concentration.

**Conducted vasodilation.** Arteriolar networks used to evaluate conducted vasodilation had similar levels of spontaneous vasomotor tone between C57BL/10 (64 ± 2%) and \textit{mdx} (67 ± 2%) mice. A 500-ms pulse of ACh onto a 3A evoked vasodilation that conducted rapidly upstream into the parent 2A branch (Figure 3A). With no difference in resting baselines, diameter changes at respective sites were not significantly different between \textit{mdx} and C57BL/10 mice with conducted vasodilation well-maintained for at least 2 mm in both groups (Figure 3B).

**Conducted vasoconstriction.** Delivery of a KCl pulse initiated conducted vasoconstriction along 1A and 2A branches in both C57BL/10 and \textit{mdx} mice. With no difference in resting diameters (Figure 4A) or changes in diameter (Figure 4B), arterioles in both C57BL/10 and \textit{mdx} mice exhibited a similar decrement in conducted vasoconstriction with distance ($P < 0.05$). In each group, several arterioles (C57BL/10, n=5/8; \textit{mdx}, n=7/12) had no response at 2000 µm upstream.
from the site of stimulation. Results from all vessels were included in the summary data for each site (Figure 4B). Removing the data for non-responders at 2000 µm elevated mean values at this site by 2-3 µm but otherwise did not alter the pronounced decay of conducted vasoconstriction.

Perivascular nerve stimulation. Focal stimulation of a 1A or 2A segment with a microelectrode resulted in reproducible vasoconstriction along the entire field of view. Baseline diameters between C57BL/10 (40 ± 2, n=17) and mdx (46 ± 3, n=17) mice prior to PNS were not significantly different. Nevertheless, arteriolar responses to PNS in mdx mice were consistently attenuated by ~25-30% (P<0.05) compared to C57BL/10 mice (Figure 5). Control experiments (n=3 per group) in which tetrodotoxin (1 µM; a selective inhibitor of voltage-activated Na⁺ channels) was equilibrated in the superfusion solution for 15-30 minutes inhibited vasoconstriction to PNS, confirming that perivascular nerves were activated by PNS (11).

Discussion

In light of the paucity of information regarding the functional properties of the microcirculation in dystrophic skeletal muscle, the goal of the present study was to evaluate the functional integrity of signaling along arterioles in skeletal muscle of mdx mice in vivo relative to C57BL/10 wild-type controls. Confirming previous reports (14, 32), mdx mice had no detectable dystrophin while utrophin was upregulated in their muscle fibers (Figure 1). These data confirm that our studies of the microcirculation were being performed on animals with dystrophic and control muscles, respectively. When applied to the entire cremaster preparation, no differences were observed between respective groups in vasoconstriction to NE or vasodilation to ACh. When discrete sites within arteriolar networks were stimulated using micropipettes, neither conducted vasodilation nor conducted vasoconstriction were different. However, arteriolar constriction in response to PNS was impaired consistently in mdx mice compared to C57BL/10 controls. With no difference in responses to topical NE, our findings therefore suggest that the attenuated response to PNS in mdx mice may be attributable to a defect in effective sympathetic neurotransmission.
There were no significant differences between mdx mice and C57BL/10 mice with respect to resting baseline diameters, maximal diameters or spontaneous vasomotor tone under resting conditions. The constrictor response of arterioles to an increase in superfusate PO2 is a sensitive index of the integrity of the microcirculation when prepared for intravital microscopy (11, 16). As shown in Table 1, vasoconstriction of 2A and 3A segments in response to equilibration with 21% O2 was not different between groups. These findings suggest that the mutation in mdx mice did not affect the viability of preparations studied here. Furthermore, the similarities in dose-response curves between mdx and C57BL/10 mice illustrate that vasomotor responses to the neurotransmitters ACh (an EC-dependent vasodilator) and NE (a vasoconstrictor acting on α-adrenergic receptors of VSMCs), remained intact for mdx mice. These data exclude mdx-related defects in EC and VSMC responses to muscarinic and adrenergic receptor activation, respectively.

Consistent with the present findings in mdx mice that were studied when 6-9 months old, cremasteric arterioles in mdx mice and C57BL/10 mice studied at 2-3 months of age had similar levels of spontaneous vasomotor tone and vasodilations in response to topical ACh and SNP (26). Nevertheless, flow-mediated vasodilation in response to elevated luminal shear stress was impaired (26). When mesenteric and carotid arteries of mdx mice and C57BL/10 mice were isolated and studied in vitro, a similar defect in flow-mediated vasodilation was observed (21, 22). Further, unlike the remodeling observed in C57BL/10 mice, mesenteric arteries of mdx mice were unable to remodel during chronic (2 week) increases or decreases in blood flow (21). Nevertheless, and consistent with the present findings, mesenteric and carotid arteries from mdx mice and C57BL/10 mice responded similarly to ACh, SNP and phenylephrine (an α1-selective adrenergic receptor agonist), as well as to increases in transmural pressure (22). Thus, given normal spontaneous vasomotor tone in arterioles (18, 26) (and Table 1 of present study) and maintenance of myogenic responsiveness in arteries (22) of mdx mice, these earlier findings (21, 22, 26) collectively suggest an inherent defect in the ability of ECs from mdx mice to respond appropriately to mechanical stimulation by luminal shear stress.
Conducted vasodilation and vasoconstriction

Once initiated from a local stimulus, electrical signals spread from cell to cell along the vessel wall through gap junctions (4, 6, 8). The resulting conducted vasomotor responses can encompass individual arterioles as well as spread among daughter, parent and sister branches of microvascular resistance networks (28). The endothelium has been recognized as the primary cellular pathway for conducted vasodilation (6, 9, 20). In response to muscarinic receptor activation, the release of inositol trisphosphate liberates Ca\textsuperscript{2+} from the endoplasmic reticulum to activate intermediate (K\textsubscript{Ca3.1}) - and small (K\textsubscript{Ca2.3}) - conductance K\textsuperscript{+} channel (5, 19). The ensuing hyperpolarization travels through gap junctions along the endothelium and into SMCs to promote relaxation [e.g., by closing voltage-gated (L-type) Ca\textsuperscript{2+} channels and lowering intracellular Ca\textsuperscript{2+}] (5, 6, 20). Arteriolar networks with similar diameters and levels of spontaneous vasomotor tone were examined between \textit{mdx} and C57BL/10 mice, thereby ensuring that both groups were studied under similar conditions. Our finding that conducted vasodilation to ACh was not different between \textit{mdx} mice versus C57BL/10 mice (Figure 3) indicates that the underlying intercellular signaling events were preserved in \textit{mdx}.

In turn, conducted vasoconstriction reflects the intercellular spread of depolarization to effect SMC contraction (e.g., via promoting Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels) along the arteriolar wall (11, 35). To test conducted vasoconstriction in \textit{mdx} mice, a high concentration of KCl (1M) was delivered adjacent to an arteriole to initiate depolarization (11, 35). Conducted vasoconstriction was not significantly different between \textit{mdx} mice and control at any site. Whereas the “local” site for conducted vasoconstriction was recorded ~250 µm upstream from the stimulus pipette (to avoid collecting data adjacent to damaged muscle fibers) there was no perceptible difference in response along this distance. Remarkably, whereas conducted vasodilation showed little decay over 2000 µm, the conduction of vasoconstriction exhibited pronounced decay along the same distance. These findings are consistent with previous reports illustrating that, for conducted responses, constriction decays more rapidly than dilation (4, 8, 11, 28). Collectively the present findings imply that the initiation and conduction of hyperpolarization and depolarization, of cell-to-cell signaling through gap junctions, and of the coupling between changes in membrane potential and arteriolar diameter remain intact in \textit{mdx}.
mice. The integrity of these responses contrasts sharply with deficits in flow-mediated vasodilation reported previously for arterioles and arteries in \textit{mdx} mice (21, 22, 26).

\textit{Adrenergic vasoconstriction and perivascular nerve stimulation}

During exercise, sympathetic nerve activity (SNA) increases concomitant with contractile activity (31). Although an increase in SNA induces arteriolar constriction, these responses are inhibited during skeletal muscle activity. Numerous mechanisms have been proposed by which such “functional sympatholysis” can induce vasodilation during exercise, including NO release from nNOS activation in skeletal muscle fibers (30, 31). In response to intra-arterial delivery of NE, vasoconstrictor responses in resting hindlimbs were similar between \textit{mdx} and C57BL/10 mice (12, 30). This behavior is consistent with the present findings using topical NE delivered in the superfusion solution (Figure 2). To model functional sympatholysis \textit{in vivo}, hindlimb muscles were stimulated to contract while NE was infused into the arterial supply (12, 30).

Vasodilation mediated by NO release was attenuated in \textit{mdx} mice compared to C57BL/10 controls and was similar to responses observed in nNOS\(^{-/-}\) mice (12, 30), suggesting that reduced expression of nNOS in \textit{mdx} mice attenuated functional sympatholysis. This conclusion was strengthened by finding that specific restoration of nNOS expression in VSMCs partially restored NO-mediated opposition of SNA (12). However, intravascular or topical delivery of NE does not address the functional properties of perivascular nerves \textit{in vivo}. The experiments presented here are the first to test perivascular nerve function in \textit{mdx} mice directly \textit{in vivo} using electrical stimulation of arterioles. In so doing, our findings indicate that mutations in the dystrophin gene are associated with an attenuated neurovascular transmission (Figure 5).

Because vasoconstriction to exogenous NE was not impaired in \textit{mdx} mice, adrenoreceptor activation and downstream signaling events appear to remain intact. Thus the defect we observed for sympathetic vasoconstriction is likely to be neuronal in nature. In turn, our findings imply that there may be less sympathetic vasoconstriction to antagonize in dystrophic muscles during exercise.

\textit{Impaired neurotransmission}
Neurotransmission can be regulated pre- and post-synaptically with defects at both levels linked to a deficiency in dystrophin in mdx mice. Post-synaptic defects in mdx mice have been found in the neuromuscular junction of somatic motor nerves and are associated with aberrant expression and variable function of nicotinic cholinergic receptors at the motor end plate (23, 25). Myenteric neurons of mdx mice have enlarged synaptic vesicles, an increase in the number of recycling vesicles and a ‘spongy’ enlargement of the endoplasmic reticulum (33). These structural data suggest a presynaptic defect in neurotransmitter packaging. In the gastric fundus of mdx mice, smooth muscle cells responded normally to stimulation with agonists, however their relaxant response to electrical field stimulation was impaired (1). The latter findings are consistent with the present observations of attenuated vasoconstriction to electrical activation of perivascular nerves and suggest that neurotransmitter packaging and/or release may contribute to the attenuated response to PNS in mdx mice. The specific mechanism(s) underlying attenuated responses to PNS remains an exciting question to address in future studies.

Conclusion

With an increase in the use of genetically modified mice and the establishment of mouse models of human disease comes an increased demand to understand the phenotypes associated with these genetic variations. In light of arteriolar networks controlling blood flow to skeletal muscle, and recognizing that the pathology of muscular dystrophy is manifest in skeletal muscle, we sought to examine possible deficiencies in blood flow regulation by investigating arteriolar function in the cremaster muscle of mdx mice in vivo compared to C57BL/10 controls. Our findings here illustrate that endothelium-dependent arteriolar dilation to ACh and constriction of arteriolar VSMCs to NE are well-preserved when these agents are applied topically to the tissue. Furthermore, the intercellular conduction of vasodilation and of vasoconstriction remained intact demonstrating that functional cell-to-cell coupling through gap junctions along the arteriolar wall is preserved in mdx mice. In contrast, arteriolar constrictions in response to direct stimulation of perivascular nerves were depressed consistently in mdx mice relative to C57BL/10 mice. These findings provide the first evidence to implicate impaired neurotransmission in perivascular nerves of mdx mice. In turn, there may be less sympathetic vasoconstriction to antagonize during
exercise of the intact organism thus impaired functional sympatholysis may have a lesser role in the pathology of DMD than has been suggested in previous studies.

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Disclosures

The authors do not have any financial disclosures or other conflicts of interests.
References


Table 1. Arteriolar diameters, oxygen sensitivity and spontaneous vasomotor tone.

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<td>1A</td>
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</tr>
<tr>
<td>3A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>69 ± 6</td>
<td>32 ± 6</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>2A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A</td>
<td></td>
<td></td>
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<tr>
<td><strong>Vasomotor Tone</strong></td>
<td>5 ± 1%</td>
<td>55 ± 2%</td>
<td>54 ± 3%</td>
</tr>
</tbody>
</table>

Diameters of 1A, 2A, and 3A at rest under control conditions (superfusate equilibrated with 5% CO₂ / 95% O₂) and during equilibration with elevated oxygen (21% O₂ / 5% CO₂ / 95% O₂). Maximum diameters determined during superfusion with 10 µM SNP. Spontaneous vasomotor tone calculated as: [(maximum diameter - resting diameter)/maximum diameter] x 100 in mdx; higher values indicate greater vasomotor tone. Summary data are means ± S.E. (n = 6-11 per cell). There were no significant differences between mdx mice and C57BL/10 mice.
Figure Legends

Figure 1. Dystrophin is absent and utrophin is upregulated in skeletal muscle of mdx mice.
Representative immunofluorescence staining for dystrophin (left column) and utrophin (right column) of rectus abdominus muscle fibers C57BL/10 (top row) and mdx mice (bottom row). Alternate serial sections (thickness, 8 µm) were immunolabeled for dystrophin and utrophin. Dystrophin was expressed at the perimeter of skeletal muscle fibers from C57BL/10 mice but was absent in mdx mice. Utrophin was not expressed in muscle fibers of C57BL/10 mice but was expressed at the perimeter of muscle fibers in mdx mice. Scale bar applies to all images.

Figure 2. Dilation to ACh and constriction to NE are intact in arterioles of mdx mice.
Cumulative concentration-response curves in 1A, 2A and 3A arterioles of mdx mice (■) and C57BL/10 (○) mice to A) ACh and B) NE. Resting baseline (B) and maximum (SNP) diameters shown for reference. Note difference in ordinate scales between arteriolar branch orders. Responses to ACh and NE were not significantly different between mdx (n=7) and C57BL/10 mice (n=6).

Figure 3. Conducted vasodilation is intact in mdx mice. Using microiontophoresis, ACh was delivered from a micropipette positioned on a 3A as illustrated at top; direction of blood flow indicated by arrow. Diameter changes were evaluated locally and at observation sites (indicated by “*”) located 500 µm upstream in the 3A and at 1000 and 2000 µm upstream in the parent 2A. A) Baseline diameter at rest. B) Diameter change at local and remote sites. Vasomotor tone was not significantly different between mdx and C57BL/10 mice (C57BL/10, 64 ± 2; mdx, 67 ± 2 %). Neither resting diameters, nor diameter changes were significantly different between mdx mice and C57BL/10 mice (n=5 per group).

Figure 4. Conducted vasoconstriction is intact in mdx mice. Using pressure ejection, KCl was delivered onto an arteriole from a micropipette positioned on an unbranched 2A segment as illustrated at top; direction of blood flow indicated by arrow. Diameter was recorded locally and at observation sites (indicated by “*”) located 500, 1000 and 2000 µm upstream. A) Baseline diameters at rest. B) Diameter change at local and remote sites. Neither resting diameters nor
diameter changes were significantly different between mdx mice and C57BL/10 mice (n=8 for C57BL/10; n=12 for mdx). There was a main effect of distance on the amplitude of vasoconstriction, which progressively decreased from the site of stimulation (†P < 0.01).

**Figure 5. Arteriolar constriction to perivascular nerve stimulation is attenuated consistently in mdx mice.** A) Representative records of arterioles from mdx and C57BL/10 stimulated at 8 Hz. B) Vasoconstriction during PNS increased with stimulus frequency and was attenuated consistently in mdx compared to C57BL/10 mice. *mdx significantly different from C57BL/10, P < 0.05. There was a main effect of frequency on the amplitude of vasoconstriction (†,P < 0.01).
Figure 1

Utrophin

Dystrophin

C57BL/10

mx
Figure 3
Figure 4

**A**

Baseline Diameter (μm)

- C57BL/10
- mdx

Distance (μm)

Local 500 1000 2000

**B**

Diameter Change (μm)

Local 500 1000 2000

Figure 4
Figure 5

A

C57BL/10  mdx

Diameter (µm)

Frequency (Hz)

10 s

B

Diameter Change (µm)

2 4 8

C57BL/10  mdx

*  *  *

†