Poloxamer 188 reduces the contraction-induced force decline in lumbrical muscles from \textit{mdx} mice

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Running title:

P188 reduces injury in \textit{mdx} muscle

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ABSTRACT

Duchenne Muscular Dystrophy is a genetic disease caused by the lack of the protein dystrophin. Dystrophic muscles are highly susceptible to contraction-induced injury, and following contractile activity, have disrupted plasma membranes that allow leakage of calcium ions into muscle fibers. Because of the direct relationship between increased intracellular calcium concentration and muscle dysfunction, therapeutic outcomes may be achieved through the identification and restriction of calcium influx pathways. Our purpose was to determine the contribution of sarcolemmal lesions to the force deficits caused by contraction-induced injury in dystrophic skeletal muscles. Using isolated lumbrical muscles from dystrophic (mdx) mice, we demonstrate for the first time that P188, a membrane sealing poloxamer, is effective in reducing the force deficit in a whole mdx skeletal muscle. A reduction in force deficit was also observed in mdx muscles that were exposed to a calcium-free environment. These results, coupled with previous observations of calcium entry into mdx muscle fibers during a similar contraction protocol, support the interpretation that extracellular calcium enters through sarcolemmal lesions and contributes to the force deficit observed in mdx muscles. The results provide a basis for potential therapeutic strategies directed at membrane stabilization of dystrophin-deficient skeletal muscle fibers.

Keywords: muscular dystrophy, P188, sarcolemmal lesions
INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease caused by a mutation in the dystrophin gene. As a result, muscles from patients with DMD lack dystrophin, a 427 kDa protein located beneath the cytoplasmic surface of the plasma membrane, the sarcolemma, of muscle fibers (5). Dystrophin is required for the assembly of the dystrophin-associated glycoprotein complex that is embedded in the sarcolemma (26). The dystrophin-glycoprotein complex links the actin cytoskeleton to the basement membrane and is thought to provide mechanical stability to the sarcolemma (19; 28).

Although the exact function of dystrophin is still unknown, the pathology demonstrated by skeletal muscles of young males that lack dystrophin is dramatic. Boys with DMD experience progressive muscle weakness beginning at about 2-5 years of age, are wheelchair bound by age 12, and die in their mid-twenties from respiratory or cardiac failure (17).

The *mdx* mouse, discovered in 1984 (9), does not express dystrophin and consequently provides an important animal model for studying the effects of dystrophin deficiency. Studies performed on muscles from the *mdx* mouse, hereafter termed *mdx* muscles, have documented impairments in structure and function, including a high susceptibility to contraction-induced injury (11; 15; 21). We have shown previously that force deficits produced by contraction-induced injury to *mdx* muscles are associated with an influx of extracellular calcium into muscle fibers (11). Stretch-activated, store-operated and calcium leak channels have been implicated as entry sites responsible for the influx of extracellular calcium (6; 13; 35). However, these ion channels are unlikely to be entirely
responsible for the calcium influx since bulky, membrane impermeable dyes and enzymes also traverse the sarcolemma of dystrophic muscles (1; 29; 30). These observations suggest the involvement of larger, nonspecific calcium entry pathways in the membrane, such as sarcolemmal lesions.

Poloxamer 188 (P188) is an 8.4 kDa amphiphilic polymer that localizes into lipid monolayers (33) and damaged portions of membranes (22). When applied to injured cells, P188 repairs disrupted membranes and enhances the recovery of skeletal muscle (20), fibroblasts (25), cardiac myocytes (34) and the spinal cord (7) from a variety of injury-inducing protocols. Our purpose was to determine, through the application of P188, the extent to which sarcolemmal lesions are responsible for the increased susceptibility of dystrophic skeletal muscles to contraction-induced injury. To minimize concerns regarding non-uniform intramuscular distribution of the applied compounds, we utilized the lumbrical (LMB) muscle, a very small whole muscle located in the forepaw of the mouse. LMB muscles were treated with P188 and then subjected to an isometric contraction protocol in vitro that produced a force deficit in untreated mdx muscles. We hypothesized that the force deficits would be highest in untreated dystrophic muscles, intermediate in dystrophic muscles treated with P188, and lowest in wild-type muscles

MATERIALS AND METHODS

Specific-pathogen-free male mdx mice (C57BL/10ScSn-mdx stock #001801) 2-3 months of age and wild-type (WT) C57BL/10 mice 2-5 months of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific-pathogen-free
barrier facility at the University of Michigan. All experimental procedures were approved by the University of Michigan Committee on the Use and Care of Animals and in accordance with the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. 85–23 (NIH), Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892].

**LMB muscle as a model to study contraction-induced injury in vitro**

The use of a small muscle such as the LMB minimizes the demands made on diffusion processes that may arise when using a larger whole muscle. The shorter diffusion paths of the small muscle facilitate the movement of metabolites to and from its core, maintaining its viability *in vitro*. Throughout the course of the experiments, LMB muscles of WT mice exhibited a sustained ability to maintain force, a positive indication of the overall stability of the muscle. The initial experimental design included a parallel set of experiments on the more commonly used extensor digitorum longus muscle (EDL). During these experiments, the EDL exhibited fatigue, with a ~10% loss in force after 10 isometric contractions followed by a full recovery of force after a rest period of 10 min (see Supplementary Materials). Since our goal was to investigate contraction-induced injury in the absence of confounding factors such as loss of force due to fatigue, the experiments on the EDL muscles were discontinued.

The shorter diffusion distance associated with small muscles is also advantageous in drug-based experiments, particularly when macromolecules such as P188 are tested. This is because the time required for the concentration of a compound at the core of the
muscle to reach 50% of its concentration in the bathing medium is proportional to the square of the radius of the muscle (16). For example, the LMB muscle with a typical radius of 150\(\mu\)m would require a diffusion time that is 16-fold less than the EDL muscle that has a typical radius of 600\(\mu\)m (10).

Intact single muscle fibers circumvent diffusion-based problems and have been used to study the effects of membrane-targeting compounds in \textit{mdx} muscles (35). Despite advantages for drug distribution, the behavior of an isolated single muscle fiber without interactions with adjacent muscle fibers might not provide an accurate representation of the function of a whole muscle. A small whole muscle such as the LMB retains some of the diffusion benefits and visualization advantages normally accorded to single muscle fiber preparations, while maintaining a more accurate representation of \textit{in vivo} whole muscle function.

**Operative procedure**

Mice were anesthetized with an intraperitoneal injection of Avertin (tribromoethanol 400mg/kg). Supplemental doses of Avertin were administered as required to keep the mouse unresponsive to tactile stimuli. The front paws were severed from the mice and the LMB muscles dissected free from the third digit. The mice were subsequently euthanized by an overdose of Avertin followed by a thoracotomy. Dissections were performed in a chilled bathing solution (approx. 8\(^\circ\)C), composition in mM: 137 NaCl, 11.9 NaHCO\(_3\), 5.0 KCl, 1.8 CaCl\(_2\), 0.5 MgCl\(_2\), 0.4 NaH\(_2\)PO\(_4\). Based on physical dimensions, LMB muscle mass was estimated to be approximately 0.2 mg. The isolated
LMB muscle was mounted horizontally in a custom-fabricated chamber with the distal
tendon attached to a force transducer (Aurora Scientific, Inc., modified Model 400A) and
the proximal tendon to a servomotor (Aurora Scientific, Inc., Model 318B). The ties were
composed of 10-0 monofilament nylon suture. Bath temperature was maintained at 25°C
and the chamber was perfused continuously with Tyrode solution (composition in mM:
121 NaCl, 24 NaHCO₃, 5.0 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaH₂PO₄). A pH of 7.3 was
maintained by bubbling with a 95%/5% O₂/CO₂ mixture.

**Isometric contraction protocol**

LMB muscles were stimulated electrically by current passed between two platinum plate
electrodes. The constant-current stimulation pulses were 0.5 ms in duration and their
magnitude was adjusted to elicit a maximum twitch response. Optimum length (Lₒ) of
each muscle was determined by adjusting muscle length until maximum twitch force was
attained. To achieve a maximum isometric tetanic contraction, the muscle was stimulated
with supramaximal intensity and frequency using pulses of alternating polarity. The
protocol used to induce a force deficit consisted of 20 maximum isometric contractions,
each lasting 1 second and separated by 1 minute. The one minute rest period between
contractions was necessary to eliminate fatigue, thereby ensuring that any decline in the
force generating capability of the muscles during and after the 20 contractions was
attributable to contraction-induced injury. To facilitate comparisons among groups of
muscles that varied in mass, the absolute isometric force of each muscle during the
contraction protocol was normalized to the maximum isometric force (Pₒ) produced by
the muscle during the 20-contraction protocol.
Treatment groups

LMB muscles from WT mice were divided into two groups. One group was exposed to normal Tyrode solution and the other group to a nominally calcium-free Tyrode solution. LMB muscles from *mdx* mice were divided into 5 groups according to their treatment with: (i) P188, (ii) streptomycin, (iii) P188 and streptomycin, (iv) nominally calcium-free Tyrode, or (v) normal Tyrode. Streptomycin is an inhibitor of stretch-activated channels that reduces the magnitude of the contraction-induced force deficits in EDL muscles from *mdx* mice (32; 35). Experiments with streptomycin were included in the present study to validate the relatively novel LMB muscle preparation. Concentrations of P188 (Bayer, NJ) and streptomycin (Sigma, #S1277) in Tyrode solution were 1 mM and 200 μM, respectively. For all treatments, muscles were allowed to incubate in the chamber for 15 minutes prior to commencement of the contraction protocol. Pilot experiments performed on WT muscles exposed to P188 (1 mM) or streptomycin (200 μM) indicated that these compounds caused no decline in the P₀ of the muscles when used at these concentrations.

For nominally calcium-free experiments, CaCl₂ was omitted from the Tyrode solution and MgCl₂ was increased to 2.3 mM to maintain the concentration of divalent cations.

Force deficits that arise from calcium-free experiments have two potential origins: a contraction-induced force deficit and an “environmental” force deficit caused by prolonged exposure of the muscle to a non-physiological environment. To separate the contraction-induced from the environmental force deficit, we assumed that the calcium-free environment had an effect that was equally deleterious to both WT and *mdx* muscles.
and consequently normalized the force responses of \textit{mdx} muscles in calcium-free environments to those of WT muscles in the same calcium-free environments. At the end of the contraction protocol, isometric tetanic force of \textit{mdx} muscles, expressed as a percentage of \(P_0\), was divided by the isometric tetanic force of WT muscles, also expressed as a percentage of \(P_0\). This procedure for the normalization of the data isolated the contraction-induced force deficits, allowing comparisons between the \textit{mdx} muscles in calcium-free and normal environments.

\textbf{Statistics}

Data are presented as a mean value ± SEM. Statistical analyses were performed using analysis of variance (ANOVA) with the level of significance set \textit{a priori} at \(P < 0.05\). When significance was detected, the Holm-Sidak post hoc comparison was applied.

\textbf{RESULTS}

\textbf{Histology and isometric force production}

LMB muscles were approximately 300 μm in diameter and consisted of 200 to 250 fibers (Fig 1A, B). Cross-sections from \textit{mdx} muscles displayed typical dystrophic features (8) including areas of mononuclear cell infiltration and a population of fibers with central nuclei (Fig. 1C). The mean absolute \(P_0\) of untreated \textit{mdx} muscles (10.8 ± 0.4 mN, \(n=8\)) was less than that of WT muscles (14.8 ± 0.9 mN, \(n=6\)). Treatment of \textit{mdx} muscles with streptomycin, or P188, or with both streptomycin and P188 simultaneously, did not affect the absolute \(P_0\) (data not shown). In the nominally calcium-free Tyrode solution, the absolute \(P_0\) of both WT and \textit{mdx} muscles decreased by ~30% to 10.8 ± 0.5 mN (\(n=3\)) and
7.2 ± 1.3mN (n=4), respectively. This decrease in force is likely due to an impairment in the excitation-contraction coupling process caused by removal of calcium from the extracellular buffer (18).

**Force production of WT and mdx muscles during the contraction protocol**

Forces generated by LMB muscles from WT mice remained constant throughout the contraction protocol with no signs of fatigue or injury (Fig 2A & B). In contrast, untreated mdx muscles displayed a steady decline in force production as the protocol progressed (Fig 2B). Comparisons between mdx and WT muscles at individual contraction intervals revealed differences between the two genotypes for each contraction after the 7th. After a recovery period of 10 minutes, the magnitude of force was unchanged, indicating that the force deficits observed in mdx LMB muscles were not caused by muscle fatigue but by contraction-induced injury (8).

**Effects of P188 and streptomycin**

At the end of the isometric contraction protocol, normalized force values were highest in the WT group and lowest in the untreated mdx group, at 98% and 69% of P_o, respectively (Fig 3). The mdx muscles in the P188+streptomycin group and in the calcium-free group generated normalized forces that were not different from muscles in the WT group (Fig 3). Treatment of mdx muscles with either P188 or streptomycin alone resulted in force values that were intermediate between the untreated mdx group and the WT group (Fig 3).
DISCUSSION

The increased potential for permeability of the sarcolemma of dystrophic muscle fibers to extracellular calcium is likely to contribute to the increased susceptibility of dystrophic fibers to contraction-induced injury. In agreement with this hypothesis, we have shown previously that during a contraction protocol similar to the one used here, the contraction-induced mechanical failure of LMB muscles from the hindpaw of \textit{mdx} mice was largely attributable to the influx of extracellular calcium into muscle fibers (11). Because of the direct relationship between increased intracellular calcium concentration and muscle dysfunction (3; 12; 31), therapeutic outcomes may be achieved through the identification and restriction of calcium influx pathways. In the present study, we confirm that stretch-activated channels (SAC) (32; 35) are one such pathway and report the additional involvement of sarcolemmal lesions as contributors to contraction-induced force deficit in \textit{mdx} skeletal muscles.

Previous studies have established P188 as a membrane-patching polymer that interacts directly with monolayers (33) and disrupted membranes (22). P188 is effective in stabilizing membranes and enhances the recovery of a variety of cell types from an array of injury-inducing protocols (7; 20; 25; 34). In the present study, P188 reduced the contraction-induced force deficit in \textit{mdx} muscles by approximately 50\% (Fig 3). A reduction in force deficit was also observed in \textit{mdx} muscles that were exposed to a calcium-free environment. These results, coupled with a direct observation of calcium entry into dystrophic LMB muscle fibers during a similar contraction protocol (11),
support the interpretation that the contraction protocol used in the present study results in sarcolemmal lesions that allow an influx of extracellular calcium, and that these entry pathways contribute significantly to the magnitude of the force deficit observed in \textit{mdx} muscles. Despite the evidence of sarcolemmal lesions in \textit{mdx} muscles, the mechanisms responsible for their formation remain uncertain. Given the vulnerability of the dystrophin-deficient sarcolemma (24; 27), lesions could have arisen from mechanical stress associated with contractile activity (23; 29), or alternatively, sarcolemmal lesions could result as a secondary consequence of Ca$^{2+}$ entry into the muscle fibers (32). In this scenario, excessive influx of Ca$^{2+}$ triggers the activity of lipid-damaging pathways (14) that induce lesions in the sarcolemma.

A treatment that combined both P188 and streptomycin produced only a marginal improvement over singly treated \textit{mdx} muscles (Fig 3). The lack of a clear additive effect when P188 and streptomycin were used together suggests that, while both SAC and membrane lesions contribute to increased intracellular calcium, blockage of either pathway alone is sufficient to reduce the magnitude of the observed force deficit. Several potential explanations could account for this observation. One possibility is that the magnitude of calcium influx through either SAC or membrane lesions alone does not exceed the capacity of the muscle fibers to maintain intracellular calcium homeostasis. That is, endogenous calcium buffering, sequestration and extrusion pathways (4), coupled with a rapid membrane repair mechanism (2), might enable the muscle fibers to maintain normal levels of intracellular calcium as long as one of the calcium entry pathways is blocked. However, when calcium entry is occurring through both SAC and membrane
lesions, the capacity of the calcium removal mechanisms might be exceeded, resulting in a calcium-induced force deficit. Another possibility is that the majority of sarcolemmal lesions repaired by P188 were a result of SAC activity. In this case, inhibition of SAC by streptomycin or repair of sarcolemmal lesions by P188 would be sufficient to rescue the muscle, and the combination of the two would not yield a pronounced improvement. Finally, we cannot exclude the possibility that P188 also acts as an inhibitor of SAC.

Using an isolated lumbrical muscle preparation, we have demonstrated for the first time that P188, a membrane sealing poloxamer, is effective in the reduction of contraction-induced force deficits in a whole *mdx* skeletal muscle. This observation supports the interpretation that the contractions cause sarcolemmal lesions that permit the entry of extracellular calcium into muscle fibers. These results provide the basis for potential therapeutic strategies directed at membrane stabilization in dystrophin-deficient skeletal muscles.
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GRANTS

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**FIGURE LEGENDS**

**Fig 1.** Lumbrical muscle from the 3rd digit of the forepaw of a mouse. (A) WT muscle is shown beside a 30G hypodermic needle. (B) Hematoxylin and eosin stained cross-section of a WT lumbrical muscle. Muscles typically consist of 200-250 fibers and are approximately 300 μm in diameter. (C) *mdx* muscle. Dystrophic features include presence of central nuclei and mononuclear cell infiltration. All scale bars are 200μm and panels B and C share the same scale.
Fig 2. **Force production of WT and mdx lumbrical muscles.** (A) Example records of isometric force production. For clarity, only records of the first (1) and last (20) isometric contractions are shown. (B) Force production of WT (n=6) and mdx (n=8) lumbrical muscles during a protocol of 20 isometric contractions in normal Tyrode solution. Break in x-axis denotes a 10-minute rest period. A two-way ANOVA was performed to determine effects of dystrophin deficiency and contraction number on the force deficit. # indicates difference between WT and mdx muscles at matching time points (P < 0.05).

Fig 3. **Force production of mdx and WT lumbrical muscles at the end of 20 isometric contractions.** A one-way ANOVA was performed followed by 2 separate post hoc comparisons between groups. In the first comparison, all groups were compared against the untreated mdx group (Power > 0.8). In the second, all groups were compared against the WT group (Power > 0.8). * and # indicate difference from WT and untreated mdx group, respectively (P < 0.05).
Figure 2

A

Contraction Number

Isometric Force (% of $P_0$)

Wild Type

$mdx$

B

Isometric Force (% of $P_0$)

Contraction Number

Wild Type

$mdx$
Figure 3

Isometric Force (% of $P_o$)

- mdx
- mdx + step
- mdx + P188
- mdx + step + P188
- mdx + 0 Ca
- WT

$n=8$ 6 6 5 4 6

∗,#, # # # #