Multiple pathological events in exercised dystrophic mdx mice are targeted by pentoxifylline: outcome of a large array of in vivo and ex vivo tests

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Submitted 31 July 2008; accepted in final form 4 January 2009

Duchenne muscular dystrophy (DMD) is the most common and wasting form of muscular dystrophy and is characterized by progressive muscle degeneration and weakness. It is caused by an X-chromosome mutation leading to the absence of the protein dystrophin from muscle. A similar biochemical defect also occurs in the mdx mouse, the most widely used animal model of DMD (27). Dystrophin is a subsarcolemmal component of a multimolecular network (the dystrophin-glycoprotein complex) that ensures a physical linkage between the intracellular cytoskeleton and the extracellular matrix, providing mechanical stability to myofiber during contraction (27). The absence of dystrophin leads to myofiber injury and necrosis due to a cascade of events, which are possibly interconnected through complex and unresolved pathways. A central role can be played by the alteration of calcium homeostasis, sustained by the increased sarcolemmal influx of calcium ions through voltage-insensitive calcium channels (1, 28, 45). A compartmentalized elevation of calcium then activates proteolytic enzymes and/or apoptotic pathways (7, 41, 43). There are evidences of an early and self-sustained inflammatory response contributing to muscle degeneration (14, 26, 44), while profibrotic cytokines, such as transforming growth factor (TGF)-β1, can account for a progressive fibrosis (2, 14). In parallel, reactive oxygen species (ROS), either released by neutrophils or produced in situ by cytokine signaling, calcium, and/or functional ischemia, may cause oxidative stress with sarcolemmal damage and leakiness. A ROS-induced activation of nuclear factor-κB, a key transcription factor for inflammatory cytokines, may, in turn, reinforce the inflammatory state (4, 21, 27, 31, 38). A growing number of evidence supports the potential link between mechanical challenge, alteration of calcium homeostasis, and ROS-induced oxidative stress, as well as their role in triggering and sustaining muscle degeneration and dysfunction (for review see Refs. 4, 38, 49). However, the complexity of the pathology hampers the elucidation of the level of cross talk, as well as the threshold for these concurrent events to exert damage (4, 25). For instance, recent studies demonstrated that voluntary exercise on a wheel, combined with green tea extracts, which are known antioxidants, can ameliorate functional outcome of dystrophic mdx mice. This effect has been ascribed to the ability of both strategies to independently reduce oxidative stress (12). In parallel, Whitehead et al. (48) showed that a treatment of mdx mice with N-acetylcysteine is able to reduce the stretch-induced muscle

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impairment, linked to the enhancement of calcium entry, as well as the signs of oxidative stress in muscle. The amount of work load may be a crucial point. In fact, a chronic protocol of forced exercise on treadmill worsens the murine pathology, leading to in vivo weakness and to exacerbation of functional and morphological alterations in limb muscles ex vivo (10, 17–19). Among these alterations, we recently described an exercise-induced enhancement in the abnormal overactivity of a subset of voltage-insensitive calcium channels; such a biophysical mechanism may contribute to the further increase of calcium permeability and of the cytosolic calcium level in myofibers of exercised mdx mice (22, 40). In the same study (40), our laboratory also found that CAMP and the phosphodiesterase (PDE) inhibitor pentoxifylline contrast channel overactivity, disclosing a possible novel interest of this drug to ameliorate calcium homeostasis in dystrophic fibers. Pentoxifylline already received attention of clinicians [multicentric trials have been recently performed by Cooperative International Research Center (CINRG): http://www.cinrgresearch.org; http://clinicaltrials.gov] due to its strong efficacy in increasing mdx mouse strength in a preliminary screening of Granchelli et al. (23).

The interest toward pentoxifylline also resides in its wide anti-ischemic, antifibrotic, and anticytokine actions that may target the complexity of DMD pathology (3, 15, 16, 32). A limited number of studies in the mdx model suggest indeed the potential benefit of PDE inhibitors on the basis of their claimed anti-ischemic and antioxidant action (5, 20, 23, 29). The potential antioxidant/anti-inflammatory activity, along with the specific effect on calcium channels, reinforced our interest in characterizing in more details the pentoxifylline profile at preclinical level. To this aim, a chronic treatment with pentoxifylline has been performed in mdx mice (50 mg·kg\(^{-1}\)·day\(^{-1}\) for 4–8 wk). A large array of in vivo and ex vivo multidisciplinary approaches has been used to evaluate the general outcome of the treatment on disease-sensitive indexes, with particular attention to the effects on 1) the altered calcium homeostasis and excitation-contraction (E-C) coupling; and 2) plasma and muscle ROS production. To better assess the pentoxifylline effect in relation to the workload injury, as well as the potential cross talk between contraction-sensitive indexes, the treatment has been performed on either nonexercised or treadmill-trained adult mdx animals. Then, a comparative histological analysis of the effect of pentoxifylline on muscles that are possibly differently recruited by treadmill workload was also performed. The final goal was to better understand both the pathological cascade in mdx dystrophic fibers and the possible outcome of pentoxifylline use in DMD patients.

**METHODS**

Animal housing and all of the in vivo experiments, as well as most of the ex vivo studies, were done at the Department of Pharmacobiology, Faculty of Pharmacy, University of Bari, Bari, Italy, legally authorized to these experimental procedures by the Italian Minister of Health (authorization no. 219/95 - A 19/5/1995). The animal research protocol had been approved by local review board (approval for project GGP05130) in conformity with the Italian law for Guidelines for Care and Use of Laboratory Animals (D.L. 116/92), which conforms with the European Community Directive published in 1986 (86/609/EEC).

**In Vivo Experiments**

*Animal groups, treadmill running, and drug treatment.* A total of 29 mdx and 19 wild-type (WT; C57/BL10ScSn) male mice of 4–5 wk of age (IFFA Credo and Jackson Laboratories) and homogeneous for body weight were assigned to “exercised” and “sedentary” groups. The groups of exercised mice underwent a 30-min running on an horizontal treadmill (Columbus Instruments) at 12 m/min, twice a week (keeping a constant interval of 2–3 days between each trial), for 4–8 wk (17–19) and were composed of 10 untreated and 14 pentoxifylline-treated mdx mice. Exercised WT animals (treated or not) were also used for selected specific experimental purposes, as indicated in the text. After reviewing the available information, a dose of pentoxifylline (Trental) in the high therapeutic range (50 mg/kg/ip daily) was chosen to avoid false positive/negative and better correlate with the dose to be used in DMD patients (20, 23, 24). The treatment started 1 day before the beginning of the exercise protocol and continued until the day of death. The dose was formulated by proper dilution of the vials (100 mg/5 ml) with sterile water for intraperitoneal injection, so to have the desired drug amount in 0.1 ml/10 g body wt. Treatment was performed in conscious animals, as daily general anesthesia has been considered potentially life-threatening. Thus particular care in handling and environment was used to avoid any animal discomfort and stress during daily injection. Pentoxifylline-free animals were either injected with equal amount of vehicle or not injected. Since no significant differences were observed between these two groups, the data were pooled together. “Sedentary” mdx (treated or not) and WT mice were left free to move in the cage, without additional exercise, and monitored at the same time points of exercised counterparts, according to the experimental need. Every week, all mice were monitored for body weight and forelimb force by means of a grip strength meter (Columbus Instruments); the end of the 4th wk was considered for statistical analysis (17, 19). At this time, an exercise resistance test on treadmill was also performed. All mice were made to run on a horizontal treadmill for 5 min at 5 m/min and then increasing the speed of 1 m/min each minute. The total distance run by each mouse until exhaustion was measured. At the end of the 4th wk of exercise/treatment, the ex vivo experiments were also started. Due to the time-consuming nature of some of the ex vivo experiments, no more than one or two animals could be killed per day. This required us to prolong the experimental time window. Thus the animals continued to be exercised/treated until the day of death, but no longer than 8 wk in total. Due to the reduction in the number of animals in each group between the 4th and 8th wk, no statistical analysis was performed on the body weight and grip strength data collected in this time window to verify constant animal state over time.

**In Vitro Studies**

*Muscle preparations.* Animals of 8–12 wk belonging to the different groups were anesthetized with 1.2 g/kg ip urethane. Extensor digitorum longus (EDL) muscle of one hindlimb was removed and rapidly placed in the recording chamber for the electrophysiological recordings. The contralateral EDL muscle was used for fura 2 calcium imaging. Gastrocnemius (GC) and tibialis anterior (TA) muscles, removed for histological and/or biochemical experiments, were washed in PBS and rapidly frozen in liquid nitrogen-cooled isopentane and stored at −80°C until use. The same procedure was used for the left side of diaphragms (Dia), while the right side was used for isometric tension measurements. The flexor digitorum brevis (FDB) muscle was used for patch-clamp recordings.

**Electrophysiological recordings by intracellular microelectrodes.** EDL muscles were bathed at 30 ± 1°C in the following normal physiological solution (in mM): 148 NaCl, 4.5 KCl, 2.0 CaCl\(_2\), 1.0 MgCl\(_2\), 12.0 NaHCO\(_3\), 0.44 NaH\(_2\)PO\(_4\), and 5.55 glucose, continuously gassed with 95% O\(_2\) and 5% CO\(_2\) (pH = 7.2–7.4).

The mechanical threshold (MT) was determined in the presence of tetrodotoxin (3 μM) using a two microelectrode “point” voltage-clamp method (17, 19). In brief, the two microelectrodes (spaced ~50 μm) were inserted into the central region of a superficial fiber,
continuously viewed using a stereomicroscope (×100 magnification). Depolarizing command pulses of duration ranging from 500 to 5 ms (0.3 Hz) were progressively increased in amplitude from the holding potential (H) of −90 mV until visible contraction. The threshold membrane potential (V, in mV) was read on a digital sample-and-hold millivoltmeter for each fiber at the various pulse durations t (in ms); mean values at each t allowed the construction of a “strength-duration” curve. Rheobase voltage (R, in mV) and the rate constant (1/τ, s⁻¹) to reach the rheobase were obtained by a nonlinear least squares algorithm using the following equation: \( V = [H - R \exp(τ/t)][1 - \exp(τ/t)] \), formula (17, 18).

Isometric contraction. Strips of Dia, obtained from the abdominal right side, were securely tight at tendon insertion and placed in a muscle chamber containing the normal physiological solution (see composition above) continuously gassed with 95% O₂ and 5% CO₂ (pH = 7.2–7.4; 27 ± 1°C). The ribbon side was fixed to a 25-g isometric force transducer (FORT25, WPI) connected to a TCI 102 transducer interface and an MP 100 acquisition unit (Biopac Systems, Santa Barbara, CA), while the opposite site was fixed to a chamber hook. Electrical stimulation field was obtained by two axial platinum wires connected to a stimulator (LE 12406, 2Biological Instruments).

After an equilibration period (30 min), the preparation was stretched to its optimal length (L₀; measured with an external calliper), i.e., the length producing the maximal twitch to a 0.2-ms 40-V pulse. Tetanic contraction was elicited by applying a 2,000-ms train of 0.2-ms pulses at increasing frequencies (10–140 Hz) until maximal tension was reached. The tension was measured with a meter for each fiber at the various pulse durations t (in ms).

Table 1. Effect of PTX treatment on body weight and forelimb strength of mdx mice

<table>
<thead>
<tr>
<th>Body Weight, g</th>
<th>Force, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0</td>
<td>Time 4</td>
</tr>
<tr>
<td>WT (9)</td>
<td>18.4±0.64</td>
</tr>
<tr>
<td>mdx (12)</td>
<td>18.7±0.8</td>
</tr>
<tr>
<td>Exercised mdx (10)</td>
<td>18.9±0.56</td>
</tr>
<tr>
<td>Exercised mdx + PTX (14)</td>
<td>19.1±0.44</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of animals. Experimental groups of mice are as follows: wild type (WT), sedentary mdx (mdx); exercised mdx, and exercised mdx treated with pentoxifylline (PTX). Body weights (g) are given at the beginning (time 0) and after 4 wk (time 4) of exercise and/or PTX treatment. Force is forelimb strength (in kg) at time 0 and time 4. ΔF; forelimb strength increment between time 0 and time 4. For each parameter, the statistical significance between groups was evaluated by ANOVA test for multiple comparison (F values) and Bonferroni t-test post hoc correction. Statistical significance was achieved for P < 0.05 with respect to *WT and †Mdx as follows: Force time 4: F = 6.6; P < 0.001, *P < 0.02, †P < 0.0002 (exercised mdx) and †P < 0.005 (exercised mdx + PTX); ΔF: F = 5.138, P = 0.004, *P < 0.009, †P < 0.0006 (exercised mdx) and †P < 0.04 (exercised mdx + PTX).
by protein block solution (DAKO) for 10 min and then incubation for 90 min with primary antibody. The antibodies used were anti-neural cell adhesion molecule (anti-NCAM; Chemicon, Intern, Germany), a marker of cell adhesion indicative of satellite cell activation and thus of regenerating fibers (6, 11, 43) and anti-CD11b (BD Pharmingen, San Diego, CA) staining macrophages and neutrophils as marker of degeneration (44). Sections were then washed in PBS and incubated

San Diego, CA) staining macrophages and neutrophils as marker of

of regenerating fibers (6, 11, 43) and anti-CD11b (BD Pharmingen,

with anti-rat IgG biotinylated secondary antibody (Jackson ImmunoRe-

search Laboratories, West Grove, PA) for 1 h, followed by avidin

biotin horseradish peroxidase complex (ABC kit, Vector Laboratories,

Burlingame, CA) for 30 min. The chromogen was diaminobenzidine

(DAKO) applied for up to 10 min, followed by brief counterstaining

with Meyer’s hematoxylin. Sections were examined under a Zeiss

Axiophot microscope, and digital images were obtained with a Leica

DC-500 Firecam digital camera. At least three animals per group were

analyzed.

Dihydroethidium (DHE; Molecular Probes) was used to evaluate superoxide (O2•−) production in muscles in situ (48). DHE freely enters cells where, in the presence of O2•−, it is oxidized to ethidium bromide that intercalates within nuclear DNA and emits red fluorescence in proportion to the amount of O2•− present (excitation at 488 nm, emission at 610 nm). Unfixed, frozen TA muscles were cut into 10-μm-thick cross sections with an automated cryostat (Leica, Ben-

heim, Germany) and placed on a glass slide. Sections were prehy-

drated with PBS (10 min) and then incubated with DHE (2 μM, 30

min, 37°C) in a dark, humidified chamber. Sections were subse-

quently washed, stained with 4,6-diamidino-2-phenylindole dichloro-

hydrate (DAPI, Santa Cruz Biotech) to visualize cell nuclei, and

emission at 610 nm). Unfixed, frozen TA muscles were cut into

30-μm-thick cross sections with an automated cryostat (Leica, Ben-

heim, Germany) and placed on a glass slide. Sections were prehy-

hydrated with PBS (10 min) and then incubated with DHE (2 μM, 30

min, 37°C) in a dark, humidified chamber. Sections were subse-

quently washed, stained with 4,6-diamidino-2-phenylindole dichloro-

hydrate (DAPI, Santa Cruz Biotech) to visualize cell nuclei, and

mounted on a coverslip. Results were observed with an epifluorescent

Zeiss Axiocamert TS100 microscope with appropriate filters. Images

were captured with a charge-coupled device camera in conjunction

with AxioVision Software (Zeiss) using identical imaging settings for

each image acquisition. Densitometric analysis for merging of DHE

and DAPI fluorescence was performed using Axiovision Rel 4.6.3

software. Fluorescence was quantified by counting the number of

pixels in identical fields for each group and expressed as relative

increase with respect to unitary value from WT mice (36).

Determination of TGF-β1 level. TGF-β1 protein was measured by

enzyme-linked immunosorbent assay (ELISA), according to the man-

ufacturer’s instructions (RandD System, Minneapolis, MN). Briefly,

10–20 mg of tissue were homogenized in 500 μl of a solution

containing 1% Triton X-100, 20 mM Tris, pH 8.0, 137 mM sodium

chloride, 10% glycerol, 5 mM ethylendiaminetetraacetic acid, and 1

mM phenylmethylsulphonyl fluoride (2, 17). TGF-β1 levels were

expressed as picograms of TGF-β1 per microgram of total protein.

Fig. 1. A: the bars show the normalized force increment (∆normalized

strength) for wild-type (WT) and mdx mice undergoing or not the exercise

protocol [sedentary (sed) or exercise (exer)] and treated or not with pentoxi-

fylline (PTX). ∆Normalized strength has been calculated as follows: for each

mouse, the force at relaxation has been normalized to the respective body

weight both at the beginning (time 0) and after 4 wk (time 4) of the exercise/treatment protocol. The difference between the normalized strength

values at the two time points allowed us to calculate the normalized strength

increment for each mouse of each group. Each bar is the mean ± SE for 5–14

animals. To minimize the unpredictable variability due to the individual

differences, the size of the SE has been calculated from the SE of the mean

values of normalized strength at time 0 and time 4, as previously described

(18). Statistical significance between groups was evaluated by ANOVA test for

multiple comparison (F values) and Bonferroni t-test post hoc correction and

was as follows: F = 104, 84; P < 0.0001. The statistical difference between

untreated and PTX-treated exercised mdx mice is shown above the bars. Also

shown are the statistical differences with respect to WT (∗P < 0.005) and nonexercised mdx animals (∗P < 0.0001). B: the total distance (in meters)

run by mdx mice exercised or not (sed or exer) and treated or not with

PTX in an acute exhausting test on treadmill. Each bar is the mean ± SE for

5–6 animals. Statistical significance between groups was evaluated by

ANOVA test for multiple comparison (F values) and Bonferroni t-test post

hoc correction and was as follows: F = 10.97; P < 0.005. The statistical difference between untreated and PTX-treated exercised mdx mice is shown above the bars. Also shown are the statistical differences with respect to WT (∗P < 0.005) and nonexercised untreated mdx mice (∗P < 0.0001). C: normalized values of maximal isometric tetanic tension (2-s trains of 0.2-ms, 40-V pulses

at 100–120 Hz) of diaphragm strips from WT and mdx animals exercised or

not (sed or exer) and treated or not with PTX. Each bar is the mean ± SE for

3–5 animals per group. Statistical significance between groups was evaluated

by ANOVA test for multiple comparison (F values) and Bonferroni t-test post

hoc correction and was as follows: F = 4.11; P < 0.03. The statistical difference between untreated and PTX-treated exercised mdx mice is shown above the bars. Also shown are the statistical differences vs. WT

mice (∗P < 0.05).
Creatine kinase, ROS, and antioxidant level in plasma and/or blood. Blood was collected from ventricular camera soon after animal death in EDTA/heparin-rinsed centrifuged tubes. The blood was centrifuged at 3,000 g for 10 min, and plasma was separated and stored at −20°C. Creatine kinase (CK) determination was performed by standard spectrophotometric analysis by using diagnostic kit (Sentinel, Farmalab) within 7 days from plasma preparation. Free oxygen radicals [free oxygen radicals testing (FORT)] and total antioxidant activity [free oxygen radicals defense (FORD)] were measured in plasma and blood, respectively, using colorimetric tests using dedicated instruments and kits (FORM Plus, Callegari Spa, Catellani Group, Parma, Italy) (46). FORT is based on the properties of the amine derivative 4-amino-N-ethyl-N-isopropylaniline hydrochloride to produce the chromogen, a fairly long-lived radical cation, proportional to the concentration of hydroperoxyl molecules in the sample. The data are expressed in conventional units called FORT, corresponding to 0.26 mg/l of H₂O₂ (equivalent to 7.6 mmol/l). FORD test is based on the ability of all antioxidant molecules present in plasma to reduce a stable radical cation chromogen (4-amino-N,N-diethylaniline) formed at acidic pH in the presence of FeCl₃. The quenching of the color is proportional to their concentration in the sample. FORD results are express as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents (mmol/l). Absorbance is read at 505 nm.

Statistics

All data are expressed as means ± SE or SD. The SE estimate for the fitted rheobase (R) and rate constant (1/τ) values (and relative statistical analysis) were obtained as previously described (19). Statistical analysis for direct comparison between two means was performed by unpaired Student’s t-test. Multiple statistical comparisons between groups were performed by one-way ANOVA, with Bonferroni’s t-test post hoc correction for allowing a better evaluation of intra- and intergroup variability and avoiding false positive.

RESULTS

Effect of Pentoxifylline on Forelimb Strength and Resistance to Treadmill Exercise

Body weight and forelimb strength values for all groups of mice used are shown in Table 1. In agreement with previous studies (17, 19), the most remarkable change was the typical reduction in forelimb strength observed after 4 wk of exercise in the mdx animals. Minor effects were observed on the forelimb strength upon pentoxifylline treatment in exercised mdx animals (Table 1), as well as in WT and sedentary mdx mice (data not shown). However, to take into account the interindividual influence of body weight, for each mouse we normalized the forelimb strength to body weight both at the beginning (time 0) and at the end of 4 wk of exercise (time 4). The normalized force increment for each mouse was then calculated as the difference (normalized force time 4 − normalized force time 0). The mean values for all groups are

Fig. 2. A: representative traces of single-channel inward currents recorded in the cell-attached configuration of the patch-clamp technique at −40 mV in flexor digitorum brevis fibers from mice belonging to different groups: WT, exercised mdx (mdx exer), and exercised mdx treated with PTX (mdx exer PTX). c, The closed state of the channel. The traces shown are representative of 12–14 experiments. B: effects of PTX treatment on resting intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) of extensor digitorum longus muscle fibers of exercised mdx mice. Each bar is the mean ± SE from Nanimals/animals as follows: 3/100 (WT), 4/30 (mdx sed), 6/60 (mdx exer), and 5/111 fibers (mdx exer + PTX). The variability in fiber number between groups reflects the greater difficulty in obtaining proper intact bundles in more damaged muscles. A significant difference between individual means by Bonferroni’s t-test post hoc correction is shown in the graph. *Significantly different vs. WT values (P < 0.01 and less). C: effect of PTX treatment on mechanical threshold of extensor digitorum longus muscle fibers. The data, expressed as means ± SE for 11–22 values from 3–4 preparations, show the voltages for fiber contraction (mechanical threshold) at each duration of the depolarizing pulse in different experimental conditions and, in particular, in WT (○), untreated exercised mdx mice (mdx exer, □), and PTX-treated exercised mdx mice (mdx exer PTX, △). All threshold values of mdx exer muscle fibers were significantly more negative with respect to those of WT ones (P < 0.001). The strength duration curve obtained in PTX-treated mdx muscle fibers almost fully overlapped that of normal WT, with the voltage threshold data being significantly more positive with respect to the values of untreated mdx fibers (P < 0.05 or less by Student’s t-test) at each pulse duration, with the exception of that at 5 ms. For some data points, the SE bar is not visible, as it is smaller than symbol size. The values have been fitted to the equation shown in the METHODS section to obtain the strength-duration curves and the fitted parameters of rheobase (R) and 1/τ, shown in Table 2.
shown in Fig. 1A. The treatment with pentoxifylline significantly increased the normalized strength increment with respect to untreated exercised mdx mice; however, the increment was still significantly lower with respect to that observed in WT and unexercised mdx mice. No significant effects of pentoxifylline were observed in this latter group (Fig. 1A), as well as in WT mice (data not shown). To better verify the degree of functional amelioration by the drug, an acute test of resistance to treadmill exercise was performed in all animal groups under chronic study. The test was aimed at measuring the total distance run by each mouse, in a single bout, until exhaustion. As shown in Fig. 1B, mdx mice ran significantly less than WT, with the chronically exercised mdx being more fatigable than the sedentary ones. Pentoxifylline treatment significantly increased the resistance to exercise in both sedentary and exercised mdx mice; in the treated sedentary mdx mice, the distance run, although less, was not significantly different than that of WT mice.

No significant differences as a consequence of exercise and/or drug treatment were found in the weight of fast-twitch EDL and slow-twitch soleus muscles nor in organ weight (liver, kidney, heart and spleen) (both absolute and normalized to body weight values; data not shown).

Effect of Pentoxifylline on Tetanic Force of Dia Strips

As the resistance to exercise may also depend on contractile performance of respiratory muscles, the maximal tetanic force of Dia strips was measured by isometric contraction. Both the sedentary and exercised mdx animals showed a significant decrease in specific isometric force of Dia. The pentoxifylline treatment increased tetanic tension of Dia strips in both sedentary and exercised mdx, although the effect was only statistically significant in the exercised mdx-treated group (Fig. 1C).

Effect of Pentoxifylline on Altered Calcium Homeostasis and MT of Dystrophic Muscle Fibers

Our laboratory previously described the ability of cAMP and pentoxifylline to reduce the overactivity of voltage-independent and mechanosensitive cationic channel in mdx myofibers (40). A series of experiments were, therefore, focused on the impact of this effect on the altered calcium homeostasis in dystrophic muscle fibers. We first verified the occurrence of a channel effect by pentoxifylline in the present group of exercised mdx mice. To this aim, cell-attached patch-clamp recordings were performed in FDB muscle fibers with calcium as the sole cation in the pipette solution. The fibers from pentoxifylline-treated exercised animals showed a significant reduction of channel open probability toward the value of WT muscle fibers. This effect was accompanied by a decrease in channel occurrence, as demonstrated by close-to-normal ratio of silent patches over the patches showing channel activity (Fig. 2A, Table 2; data from sedentary mdx mice not shown). Then the outcome on calcium homeostasis was evaluated by fura 2 microspectrofluorimetric analysis. The resting cytosolic calcium was 40% lower in pentoxifylline-treated vs. untreated exercised mdx EDL muscle fibers, being markedly shifted toward the WT value (Fig. 2B). In parallel, the sarcolemmal permeability to calcium was reduced by the drug treatment to a value comparable to that of WT fibers (Table 2).

The pentoxifylline effects were then evaluated on the voltage threshold for contraction (MT), a calcium-dependent electrophysiological index of E-C coupling mechanism. A shift of

Table 2. Effect of PTX on parameters indicative of calcium homeostasis in exercised mdx mouse muscles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>Exercised mdx</th>
<th>Exercised mdx + PTX</th>
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<tbody>
<tr>
<td>Relative $P_{open}$</td>
<td>0.18±0.09 (3/13)</td>
<td>0.60±0.08* (3/15)</td>
<td>0.25±0.16 (3/16)</td>
</tr>
<tr>
<td>Silent/Active Patches</td>
<td>0.3</td>
<td>0.15</td>
<td>0.333</td>
</tr>
<tr>
<td>Sarcolemmal Calcium Permeability, %/min</td>
<td>4.99±0.38 (3/34)</td>
<td>6.17±0.17* (6/69)</td>
<td>4.61±0.28 (5/41)</td>
</tr>
<tr>
<td>Rheobase Voltage, mV</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>$1/r$, s⁻¹</td>
<td>0.15±0.007 (3/90)</td>
<td>0.11±0.007 (4/73)</td>
<td>0.18±0.02 (5/108)</td>
</tr>
</tbody>
</table>

Values are means ± SE for $N$ animals/fibers shown in parentheses. Groups of mice used are WT, exercised mdx, and exercised mdx treated with PTX. Relative $P_{open}$, open probability of calcium-permeable voltage-independent channel. Silent/active patches, ratio between the patches without and with channel activity. Sarcolemmal calcium permeability was measured as quench rate of fura 2 fluorescence by Mn²⁺. Rheobase voltage is the constant membrane voltage at which contraction occurs independently on depolarizing pulse duration, in mV. $1/r$ is the rate constant to reach the rheobase voltage. For each parameter, the statistical significance between groups was evaluated by ANOVA test for multiple comparison ($F$ values) and was as follows: $P_{open}$, $F_3,3.77, P < 0.05$; cytosolic calcium, $F_3,43.7, P < 0.0005$; sarcolemmal calcium permeability, $F_3,10.8, P < 0.0005$; rheobase voltage, $F_3,13.44, P < 0.00001$; $1/r$, $F_5,5.41, P < 0.005$. Bonferroni $t$-test post hoc correction was used to estimate statistical differences between individual mean values. The statistical significance of PTX-treated vs. untreated exercised mice is shown in the table below each value. *Statistical difference vs. WT values ($P_{open}$: $P < 0.05$; cytosolic calcium: $P < 0.0002$; sarcolemmal calcium permeability: $P < 0.02$; rheobase voltage: $P < 0.00001$).

Table 3. Effect of in vitro application of PTX on the voltage threshold for contraction of EDL muscle fibers of mdx mice

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPS</td>
<td>−56.8±0.9 (12)</td>
<td>−62.8±0.3 (12)</td>
<td>−68.9±0.4 (12)</td>
<td>−71.4±0.6 (13)</td>
<td>−72.9±0.3 (16)</td>
<td>−73.3±0.3 (13)</td>
<td>−74.7±0.27 (16)</td>
</tr>
<tr>
<td>PTX100μM</td>
<td>−55.7±0.5 (11)</td>
<td>−61.9±0.4 (13)</td>
<td>−67.2±0.3* (13)</td>
<td>−68.5±0.2* (12)</td>
<td>−70.9±0.4* (14)</td>
<td>−71.8±0.2* (15)</td>
<td>−71.8±0.2* (14)</td>
</tr>
</tbody>
</table>

Values are means ± SE of the no. of fibers shown in parentheses. For the experimental conditions, the fibers sampled are from extensor digitorum longus muscle from mdx mice of 8–12 wk of age. For each experimental condition are shown the threshold membrane potential values obtained with depolarizing command pulses of duration ranging from 5 ± 500 ms. These have been measured in normal physiological solution (NPS) and after the in vitro application of 100 μM PTX. *Significantly different with respect to the value in NPS by Student’s $t$-test ($P < 0.005$).
MT toward more negative potentials is a typical hallmark of EDL muscle fibers of mdx mice, being only slightly aggravated by the protocol of exercise (19). The threshold potential values of pentoxifylline-treated exercised mdx fibers were significantly shifted toward more positive potentials vs. those of untreated ones, at each pulse duration. Thus the strength-duration curve almost overlapped that of WT muscle fibers; the fitted values of rheobase and rate constant (1/\( t \)) were restored to the WT values (Fig. 2C and Table 2). A similar effect was also observed on MT of pentoxifylline-treated sedentary mdx mice (data not shown in Fig. 2). Since pentoxifylline fully counteracts the overactivity of the voltage-insensitive cation channel also upon acute in vitro application (40), we evaluated the potential influence of the acute channel effect on calcium permeability and E-C coupling. Mdx muscle fibers, incubated for 15 min with 100 \( \mu \)M pentoxifylline, showed a \( \text{Mn}^{2+} \) quench rate of 6.70 ± 0.36%/min (n = 28 fibers) vs. 9.47 ± 0.44%/min (n = 23) recorded in the absence of drug. The in vitro application of pentoxifylline produced only a slight shift of the MT toward more positive potentials, the rheobase voltage being −69.9 ± 1 vs. −72.2 ± 1 mV before the application of the drug (Table 3). Thus the amelioration of calcium homeostasis in dystrophic fibers depends on the long-term control of channel activity and calcium permeability. To verify the specificity of pentoxifylline action, we tested its effects on the physiological adaptive process to exercise. In fact, in WT EDL muscle fibers, the treadmill exercise causes a 6- to 10-mV shifts of the MT toward more negative potentials and increases cytosolic calcium, without signs of damage (19, 39). Interestingly, a minor effect of pentoxifylline was observed on MT of exercised WT mice, with the rheobase voltage being shifted only by 2 mV. In parallel, no effect of pentoxifylline treatment was observed on calcium level and permeability of exercised WT mice (data not shown). The data corroborates the specific efficacy of pentoxifylline on a pathology-based mechanism.

**Effect of Pentoxifylline Treatment on ROS and CK**

The level of plasmatic ROS was higher in mdx than in WT, and it significantly increased in exercised mdx mice (Fig. 3A). This latter was not related to an intrinsic damage due to exercise, as no change in ROS was observed in exercised WT mice (308 ± 50.5 FORT units; n = 4). The animals treated with pentoxifylline showed a clear reduction in ROS, with the effect being significant in the exercised animals. Plasma antioxidant level did not show significant differences in all of the experimental conditions (Fig. 3B), as well as in exercised WT mice (1.96 ± 0.5 mmol/trolox). In parallel, a decrease of plasma CK in pentoxifylline-treated exercised mdx mice was confirmed, while no significant effect was observed on the CK value of sedentary dystrophic animals (Fig. 3C). To test the ability of pentoxifylline to reduce ROS level in dystrophic

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**Fig. 3.** A: plasma level of radical oxygen species (ROS) from WT and mdx mice exercised or not (sed or exer) and treated or not with PTX. Each bar is the mean ± SE for 3–5 animals. FORT (free oxygen radicals testing) is an arbitrary unit corresponding to 0.26 mg/l of \( \text{H}_2\text{O}_2 \). The statistical difference between untreated and PTX-treated exercised mdx mice by Student’s t-test is shown above the bars. The value of ROS in exercised mdx mice was also significantly different with respect to WT (*\( P < 0.005 \)) and mdx sedentary (§\( P < 0.02 \)). B: blood antioxidant activity of WT and mdx mice exercised or not (sed or exer) and treated or not with PTX. Each bar is the mean ± SE for 3–5 animals. C: plasma creatine kinase activity measured by standard spectrophotometric analysis. Each bar is the mean ± SE for 5–10 animals. The statistical difference between untreated and PTX-treated exercised mdx mice by Student’s t-test is shown above the bars. All values were significantly higher vs. the value of WT mice (*\( P < 0.01 \)); the CK value of untreated exercised mdx mice was also significantly higher than that of nonexercised ones (§\( P < 0.05 \)).
muscles, the ROS-sensitive dye DHE was used on TA muscle cross sections. This permeable dye enters cells where, in the presence of \( \text{O}_2^- \), it is oxidized to ethidium bromide that intercalates within nuclear DNA and emits red fluorescence in proportion to the amount of \( \text{O}_2^- \) present. Thus specific fluorescence was evaluated by the magenta merging of DAPI and DHE signals. As shown in Fig. 4, a widespread and intense nuclear staining was observed in mdx animals, with a greater reactivity in exercised vs. sedentary ones. Reactivity was observed in both myonuclei and in nuclei of nonmuscle cells, likely inflammatory infiltrates. A reduction of reactivity was observed in pentoxifylline-treated animals. A densitometric analysis allowed quantifying the phenomenon in the different groups (36). By this approach, it was evident the significant increase related to exercise and the reduction observed in pentoxifylline-treated animals (Fig. 4). The pentoxifylline effect was significant in exercised mdx mice.

**Effect of Pentoxifylline on Histological Markers of Muscle Damage**

Both the alteration in calcium homeostasis and ROS can play an important role in dystrophic muscle degeneration and fibrosis. In consideration of the minor effects observed by pentoxifylline treatment in sedentary vs. exercised mdx animals, we focused on its effects on the histology profile of muscles excised from exercised mice. A comparative analysis was performed, including the rostral hindlimb muscle TA, the caudal hindlimb GC, and the respiratory Dia, to better evaluate the outcome on muscles possibly differently recruited by the training pattern. All three muscles showed structural alterations that were similar, although quantitatively more serious, compared with sedentary mdx mice, with extensive areas of degeneration and the presence of necrotic fibers and of nonmuscle tissue (Fig. 5A). Small centronucleated fibers (CNFs),...
isolated or in clusters often nearby necrotic fibers, were also present along with other infiltrates, resembling mononuclear inflammatory cells (Fig. 5A). This profile was observed in both GC, in which the effect of exercise was similar to that previously described (17, 18), as well as in TA and Dia muscle. The treatment with pentoxifylline led to a qualitative amelioration of the histological picture in all muscle tested, which showed a more regular architecture and an apparent greater preservation of muscle structure (Fig. 5A). The morphometric analysis lead to the following order of muscle damage: Dia > GC > TA in all groups of mdx animals. A constant trend toward an increase of damaged area was observed as a consequence of exercise, while the pentoxifylline-treated exercised muscles showed a profile more similar to that of sedentary ones (Fig. 5B). However, according to previous reports (17, 25), the high interindividual variability in histology profile did not allow for a statistical significance between groups to be reached. Thus we focused on the percent of CNFs. This index reflects the cyclic degeneration-regeneration events occurring in dystrophic muscle and may be significantly reduced by drugs contrasting myofiber degeneration (17, 18). The treatment with pentoxifylline did not exert any significant change in CNF of the muscle sampled, with a slight tendency toward reduction only being observed in TA muscles (Fig. 6A). To gain a better insight in this modest change in CNF, the area of active regeneration was then examined by NCAM immunostaining (6, 11, 43) (Fig. 6A). We found that the exercise markedly reduced the stained area in Dia, while the pentoxifylline treatment significantly increased it (Fig. 6A). A significant increase in NCAM-positive area, with respect to the value of untreated groups, was also detected in GC muscle. As opposite, the TA muscle showed the lowest percentage of NCAM-positive staining in the three experimental conditions (Fig. 6A). GC and TA muscles showed a comparable level of area occupied by connective tissue, which was significantly lower than that of Dia (P < 0.02 by Student’s t-test); this trend was similarly observed in muscles from exercised mdx animals (Table 4). In the pentoxifylline-treated group, a significant decrease in the percentage of connective tissue area vs. untreated counterpart was observed in TA muscle, while minor, if any, decrease was observed in Dia and GC muscles. A similar trend has been found in the level of the profibrotic cytokine TGF-β1, with
significant decrease by pentoxifylline being observed only in TA muscle (Table 4).

DISCUSSION

Pentoxifylline is an aspecific inhibitor of PDE enzymes, thus increasing cyclic nucleotide levels into cells; its large clinical spectrum is likely related to the little specificity toward PDE isoforms and the wide expression of more than one isoform in the different tissues (3, 8, 9). The ability of pentoxifylline to improve mdx mouse strength in a preliminary drug screening (23), as well as its wide anti-inflammatory, antioxidant, antifibrotic, and hemorrhologic effects on a variety of pathological states (3, 15, 16, 32), has raised attention for contrasting dystrophic progression at preclinical and clinical level. Our specific interest in this drug resides in its ability to contrast the overactivity of a channel population potentially involved in the enhanced calcium entry in dystrophic fibers (40). In fact, this mechanism may add positively to the antioxidant/anti-inflammatory effects mostly in relation to the susceptibility of dystrophic muscle to increased workload. This prompted us to evaluate in more details the effects of a chronic in vivo treatment on the mdx mouse, either with or without a chronic protocol of forced exercise, in the effort to better understand the potential therapeutic effect of pentoxifylline in relation to specific pathology-based events (17–19, 25).
Calcium Homeostasis and Pentoxifylline

Our laboratory’s previous results showed an increase in cytosolic calcium, worsened by in vivo forced exercise, in EDL muscle fibers of mdx mice. The alteration is paralleled by the increased permeability of calcium through transient receptor potential-like (TRP) leak/mechanosensitive channels, whereas the pathways involved in intracellular calcium handling mechanisms are little, if any, altered (22, 40). Thus the increased permeability to calcium is the most likely mechanism accounting for the alteration of the voltage threshold for mechanical activation observed in mdx muscle fibers. However, a direct evidence of this link was missing. We presently confirmed the ability of pentoxifylline to reduce the overactivity of a voltage-insensitive, calcium-permeable channels (40); in parallel we found a marked reduction of cytosolic calcium level and a full restoration of the MT. Few, if any, effects were produced by pentoxifylline in WT mice, suggesting its specific action on a disease-related mechanism. In addition, the results obtained after in vitro application of pentoxifylline imply that the amelioration of the altered calcium homeostasis requires the long-term drug effect on the specific targets. It is worth mentioning that pentoxifylline is one the most effective drugs in ameliorating MT/calcium homeostasis of mdx mice upon in vivo treatment (19). In fact, the alteration of calcium homeostasis in mdx myofibers is not contrasted by anti-inflammatory and immunosuppressive drugs and may require a >20% of de novo dystrophin expression for being ameliorated (17, 18). Thus the present findings support a clear link, never shown until now, between the function of TRP-like ion channels involved in passive calcium entry (1, 40, 45) and the long-term control of calcium homeostasis in mdx myofibers. Although we cannot rule out the effect of pentoxifylline on other structures involved with calcium handling, the data support the view that drugs acting directly on the channels involved in the altered calcium entry exert beneficial effects on calcium-dependent E-C coupling of dystrophic muscle.

Effect of Pentoxifylline on Plasma and Muscle ROS

Oxidative stress has been long claimed by Rando’s studies (38) to play a key role in the pathogenesis of DMD, and recent reviews focus on the main aspects of ROS production and their role in muscle wasting conditions (4, 38, 49). Increasing experimental evidences favor the interest of antioxidant compounds for contrasting dystrophic signs in the animal model (10, 12, 35, 49). Pentoxifylline has been claimed to have antioxidant activity, likely in relation to its ability to reduce proinflammatory cytokines, such as TNF-α (4, 16). We found that pentoxifylline treatment significantly reduced the exercise-induced increase in plasma ROS in mdx mice. The DHE staining demonstrated, for the first time, that our treadmill protocol significantly enhances O$_2^-$ formation in the dystrophic muscle, indicating that this pathway can play a pivotal role in the sensitivity of dystrophin-less muscle to workload. Pentoxifylline was able to significantly contrast the exercise-induced increase in muscle ROS, exerting minor effects on the basal level of sedentary sedentary mdx mouse muscle. This may be due to either a greater antioxidant effect on the pathways aggravated by the mechanical activity, or to the anti-inflammatory effect of the drug able to reduce ROS-producing inflammatory cells. Thus pentoxifylline seems particularly effective in protecting dystrophic muscle from the additional ROS and inflammation-induced damage brought about by the increased workload.

Various hypotheses can be proposed for the antioxidant effect of pentoxifylline. Apart from the well-known cross talk between ROS and proinflammatory signals that are the target of pentoxifylline action (3, 4, 16), ROS production could have been blunted by the amelioration of calcium homeostasis, considering the link between the alteration of calcium homeostasis and ROS production (4, 48, 49). In addition, it has been described as a direct effect of cAMP and protein kinase A in inhibiting NADPH oxidase activity, which is one of the major sources of O$_2^-$ in skeletal muscle (30, 49). The antioxidant action of pentoxifylline may also contribute to a decrease of membrane damage as corroborated by the reduction in plasma CK levels, an effect often observed with anti-inflammatory and antioxidant compounds (10, 17).

Effect of Pentoxifylline on In Vivo and Ex Vivo Functional Parameters

Pentoxifylline partially but significantly prevented the exercise-induced weakness and ameliorated the low resistance to treadmill running in mdx mice. An amelioration of isometric contraction of Dia was also observed in ex vivo experiments, suggesting an increased functional capacity of respiratory muscle as a consequence of the treatment. This is in line with the well-known activity of xanthines on respiratory performance. In addition, a pentoxifylline treatment has been found to be protective against the endotoxin-induced functional diaphragm-
matic deterioration due to its ability to reduce TNF-α and oxidative stress (16). This provides further evidence of a link between mechanical instability, prooxidative signals, and diaphragmatic impairment observed in the dystrophic pathology (31) and supports the interest of drugs, like pentoxifylline, able to target these pathology-related mechanisms.

**Pentoxifylline and Muscle Morphology: Role of Enhanced Regeneration**

Pentoxifylline has been described to exert a multisite antifibrotic action (32). Gosselin and Williams (24) found no significant amelioration of fibrosis nor a reduction in TGF-β1 expression in mdx Dia, upon the use of a lower dose of pentoxifylline (16 mg/kg) (24). Our findings using higher doses and longer treatment time on adult exercised mdx mice add new evidences about a rather modest and muscle-dependent antifibrotic action of pentoxifylline in muscular dystrophy. We found that the area occupied by connective tissue and the level of profibrotic cytokine TGF-β1 were significantly decreased by pentoxifylline solely in TA muscle. Conversely, a modest, if any, effect on markers of fibrosis was observed in more damaged muscles such as Dia and GC. The small antifibrotic effect can be indirectly related to the primary drug effect on ROS, considering the ability of ROS to control the activity of calpains, key enzymes in the activation of TGF-β1 (10, 49).

Although the high interindividual variability in the mdx phenotype makes difficult the statistical analysis of morphometric data (25), it has to be underlined that the qualitative amelioration of the histological picture in pentoxifylline-treated muscles was not paralleled by a significant reduction of damaged area nor by a decrease in CNFs in sampled muscle. CNFs are a sign of degeneration-regeneration cycles. Muscle regeneration efficiency, which is fairly high in mdx phenotype, is related to satellite cell activation and mainly triggered by muscle fiber damage (47). Drugs or other interventions able to prevent or counteract muscle damage ameliorate histological picture and reduce CNFs (17, 44). To gain more insight in the apparent lack of effect of pentoxifylline on CNF, we tried to temporally discriminate among the population of CNFs by NCAM immunostaining. This immunoglobulin adhesion molecule is expressed in activated satellite cells and during myogenic differentiation and is, therefore, a useful tool to assess active muscle regeneration following spontaneous and/or induced degeneration (6, 11, 43). Interestingly, the pentoxifylline treatment significantly increased the NCAM-positive area in both Dia and GC. Thus it is tempting to speculate a dual action of pentoxifylline: the drug may limit the progression of degeneration by ameliorating calcium homeostasis and reducing oxidative stress from one side, while concomitantly it stimulates regeneration. This may account for the improved histological picture, without significant reduction in the total number of CNFs. Importantly, cyclic nucleotides, such as cGMP, play a key role in the process of satellite cell activation induced by nitric oxide (37, 38). In addition, elevation of cAMP accelerates satellite cell activation and triggers a cAMP response element binding protein-modulated myogenic gene expression (13, 39). Thus pentoxifylline can exert similar effects as a consequence of PDE inhibition. Preliminary data on C2C12 cells support the potential ability of pentoxifylline to directly activate satellite cells and to promote their growth (A. De Luca, personal unpublished observations) and pushes toward a more detailed investigation on the molecular mechanisms underlying this effect. In support of this hypothesis, torbachyline, another xanthine derivative, has been found to stimulate muscle regeneration in an experimental model of myopathy (33).

**Final Considerations: Is There a Unifying Mechanism for the Action of Pentoxifylline?**

Although pentoxifylline apparently modulates many different targets, it is feasible to propose a unique mechanism of action. This may resides in its ability to inhibit aspecifically many isoforms of PDE in different tissues, thus controlling different cyclic-nucleotide-dependent processes, such as inflammation, calcium homeostasis, and regeneration. Also, a similar increase in cyclic nucleotides by pentoxifylline in smooth muscle cells can promote vasodilatation, attenuating ischemic sufferece of dystrophic muscle (5, 9, 27, 38). Accordingly, a cGMP-dependent mechanism has been claimed to be the basis for the cardioprotective effect of the PDE-5 selective inhibitor sildenafil in mdx mice in later phases of the disease (29). Thus, the PDE-mediated mechanism is able to explain many of the observed effects and remains the most likely one. In fact, pentoxifylline has little potency as an antagonist of adenine receptors (42), and the involvement of adenine system in muscular dystrophy is still unknown. As opposite, early findings showed that dystrophic muscles have a higher than normal activity of PDEs, predicting a failure of cyclic nucleotide-dependent transduction pathways (8).

The data open a certain expectation toward the clinical outcome of trials with pentoxifylline in DMD patients. Although no side effects were observed in the present study, as well as upon the use of higher doses (20, 23), caution has to be taken about dose and administration route in pediatric DMD patients, considering the housekeeping function of PDE enzymes and the key role of cyclic nucleotides for modulating vital functions.

**ACKNOWLEDGMENTS**

The authors thank Diana Conte Camerino for helpful comments.

**GRANT**

The financial support of Italian Telethon to the project no. GGP05130 is gratefully acknowledged. The contribution of the Industria Chimica Emiliana (ICE, s.r.l., Italy) to the fellowship of V. Splendorio, in the frame of the collaboration with B. Palmieri, is also acknowledged.

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