Molecular Pathogenesis of Genetic and Inherited Diseases

A Multidisciplinary Evaluation of the Effectiveness of Cyclosporine A in Dystrophic Mdx Mice

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Chronic inflammation is a secondary reaction of Duchenne muscular dystrophy and may contribute to disease progression. To examine whether immunosuppressant therapies could benefit dystrophic patients, we analyzed the effects of cyclosporine A (CsA) on a dystrophic mouse model. Mdx mice were treated with 10 mg/kg of CsA for 4 to 8 weeks throughout a period of exercise on treadmill, a protocol that worsens the dystrophic condition. The CsA treatment fully prevented the 60% drop of forelimb strength induced by exercise. A significant amelioration ($P < 0.05$) was observed in histological profile of CsA-treated gastrocnemius muscle with reductions of nonmuscle area (20%), centronucleated fibers (12%), and degenerating area (50%) compared to untreated exercised mdx mice. Consequently, the percentage of normal fibers increased from 26 to 35% in CsA-treated mice. Decreases in creatine kinase and markers of fibrosis were also observed. By electrophysiological recordings *ex vivo*, we found that CsA counteracted the decrease in chloride conductance (gCl), a functional index of degeneration in diaphragm and extensor digitorum longus muscle fibers. However, electrophysiology and fura-2 calcium imaging did not show any amelioration of calcium homeostasis in extensor digitorum longus muscle fibers. No significant effect

was observed on utrophin levels in diaphragm muscle. Our data show that the CsA treatment significantly normalized many functional, histological, and biochemical endpoints by acting on events that are independent or downstream of calcium homeostasis. The beneficial effect of CsA may involve different targets, reinforcing the usefulness of immunosuppressant drugs in muscular dystrophy. (Am J Pathol 2005, 166:477–489)

Duchenne muscular dystrophy (DMD) is a fatal genetic disorder for which no definitive cure is available. The X-linked mutation of the dystrophin gene leads to the absence of dystrophin in skeletal muscle fibers, a biochemical defect also observed in the mdx mouse, the murine phenotype of DMD.¹ Dystrophin is a subsarcolemmal protein involved in the link between the contractile machinery and the extracellular matrix. It is generally accepted that the absence of dystrophin weakens the sarcolemma and impairs the transduction of the mechanical signal imposed by the contraction. This leads to a complex and still not fully understood network of interconnected pathogenic events responsible for progressive muscle degeneration; these events involve the increased entrance of calcium, the activation of proteases, and the occurrence of a functional ischemic state.^{1–4} Recent evidence suggests that a chronic inflammatory state is a secondary reaction that strongly contributes to the progression of the pathology. A significant overexpression of inflammatory and immune response genes has been described by microarray in muscle of dystrophic subjects.^{5,6} Also, activated helper and cytotoxic T cells have been found to be present in higher number in muscles of dystrophic mdx mice and to promote pathology in this phenotype.⁷ According to this view, immuno-

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suppressant therapies should prove beneficial for dystrophic subjects. In fact glucocorticoids, the sole drugs with a clinical interest for DMD until now, reduce symptoms and delay pathology in both patients and animal models of the disease.^{8,9} The interest toward immunosuppressant drugs for treating DMD is reinforced by the need of immunosuppression to increase the transfection rate of gene and cell therapy in dystrophic subjects.^{10–13} Within this frame we focused our attention on cyclosporine A (CsA), a drug with a wide immunosuppressant action because of its ability to inhibit the calcium/calmodulin-dependent phosphatase calcineurin and consequently the NF-AT-induced transcription of interleukin-2 in activated T lymphocytes, finally inhibiting their clonal expansion.¹⁴ In addition, CsA may directly and specifically act on some pathological events of dystrophic muscles, as supported by the finding that boys with DMD treated for 2 months with CsA showed an increased force in tibialis anterior muscle during both electrically evoked and voluntary contraction.¹⁵ In dystrophic muscle the calcium-dependent activation of calcineurin may directly contribute to the chronic inflammatory state, through activation of nuclear factor- κ B, and to apoptotic pathways, via activation of the proapoptotic Bcl-2 member BAD.^{16,17} Furthermore, CsA can exert calcineurin-independent effects on mitochondrial permeability pore, a mechanism effective in contrasting apoptosis in collagen VI-deficient muscular dystrophy and potentially important in view of the metabolic sufferance of DMD muscles.^{5,18} However, the pharmacological inhibition of calcineurin may have controversial outcome because of the physiological roles of the enzyme in skeletal muscle. Calcineurin controls calcium- and activity-dependent differentiation of the slow-twitch phenotype and plays a role in muscle growth, hypertrophy, and regeneration.^{19–21} Transgenic mdx mice overexpressing activated calcineurin show a better progression of the pathology, through a twofold increase in expression of the dystrophin-replacing protein utrophin.²² However, two studies dealing with the effects of CsA treatment in mdx mice led to controversial results. In both cases, 2-week-old mdx mice were used to match the first round of spontaneous degeneration; however, whereas the administration of CsA at 2 mg/kg/day for 30 days was without significant effect, a worsening of the pathology was reported by Stupka and colleagues²⁴ on the use of much higher dose (30 mg/kg, i.p.) for 2 weeks.^{23,24} In this latter study, the dose used was in the range for total inhibition of calcineurin activity in skeletal muscle,^{21,24} a situation of particular hazard for still developing muscles. Because of the narrow therapeutic index of this drug and the recognized role of its target for proper muscle development, in the present study we evaluated the effects of therapeutic doses of CsA on dystrophic mdx mice after the first month of postnatal life. The oral administration route was chosen being less invasive in long-term use. Possible variability in oral absorption, and thus the maintenance of therapeutic dose, was taken into account by using a dose of 10 mg/kg/day, well within the effective dose range (3 to 15 mg/kg).^{15,18,19} We also considered the available pharmacokinetic data that reported a longer clearance time for CsA

in rodents versus humans and a three-compartment kinetic model, for the extensive tissue distribution, leading to longer elimination times during chronic therapies.^{25–27} The CsA treatment has been performed on 4- to 5-week-old mdx mice for 4 to 8 weeks throughout a period of exercise on treadmill, a protocol that worsens the murine pathology by contrasting the spontaneous regeneration and allows to monitor the effectiveness of drug therapies both *in vivo* and *ex vivo*.^{9,28,29} *In vivo* we evaluated the ability of CsA to prevent the exercise-induced forelimb muscle strength loss. Creatine kinase level, histological analysis, and markers of fibrosis as well as the level of utrophin expression were determined as indexes of the ability of CsA treatment to contrast muscle damage. To gain insight in the mechanism of drug action, we recorded electrophysiologically the state of cellular parameters linked to the disease, such as the impairment of chloride channel function occurring spontaneously in diaphragm (DIA) muscle or induced by exercise in the hindlimb extensor digitorum longus (EDL) muscle.⁹ In addition calcium homeostasis was monitored functionally by electrophysiological recordings of the voltage threshold for mechanical activation and by FURA-2 imaging. The data provided evidence that CsA treatment contrasts, in a calcium-independent manner, some of the functional and morphological disease-related alterations, suggesting that the CsA targets contribute to the pathology progression in muscular dystrophy.

Materials and Methods

All experiments were conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conform with the European Community Directive published in 1986 (86/609/EEC).

In Vivo Experiments

Animal Groups and Drug Treatment

Twenty-two mdx and ten wild-type (WT) (C57/BL10) male mice 4 to 5 weeks old (IFFA Credo, France, and Jackson Laboratories, Bar Harbor, ME) were used. Initially, all of the animals were weighed and forelimb force was measured by means of a grip strength meter (see below). Each group was then subdivided into two further groups: exercised and sedentary. The group of exercised mdx mice was further subdivided into seven untreated and eight CsA-treated mice. The CsA treatment (10 mg/kg orally) started a few days before the beginning of the exercise protocol, and continued until the day of sacrifice. CsA was given with drug-enriched chow (0.01% in weight). The daily amount of food eaten by each animal ranged between 2 to 4 g. Thus the drug-enriched chow was given daily (for 6 days a week) up to reach the daily amount for the dose of 10 mg/kg. Then, the mice received the standard drug-free chow to avoid CsA overdose. Both standard and drug-enriched chow made under request was purchased from Eberle Nafag AG (Gossau, Switzerland). One of the CsA-treated mice

died few days after the beginning of the treatment. This event also occurred for one of the mdx mice belonging to the sedentary group and was therefore considered unrelated to the treatment and rather because of the degenerative phase of the disease at this age. During the CsA treatment, the mdx mice did not show any abnormal behavior or difference in macroscopic vital functions versus untreated groups. The six mdx mice, taken as sedentary, were left free to move in the cage, without additional exercise. The sedentary mdx mice as well as the exercised WT animals were monitored for *in vivo* and *in vitro* studies at the same time points of exercised counterparts, according to the experimental need.

Exercise Protocol and *in Vivo* Studies

The WT and mdx (both untreated and treated) mice belonging to the exercised groups underwent 30 minutes of running on an horizontal treadmill (Columbus Instruments, USA) at 12 meters/minute, twice a week, for 4 to 8 weeks.^{9,28} The training protocol started at the mouse age of 4 to 5 weeks. Approximately half of the mdx mice showed an avoidance behavior with respect to exercise, with a higher tendency to fatigue and had to be gently stimulated, or should be left resting, to complete the 30 minutes of running session. This behavior, never observed in WT animals, was not modified by either exercise or drug treatment. Every week all of the exercised mice were monitored for body weight and compared with related sedentary counterparts. The force of exercised mice (both controls and mdx) was evaluated before each training session by means of a grip strength meter (Columbus Instruments, USA). For this measurement the mice were allowed to grasp a triangular ring connected to a force transducer and then gently pulled away until the grip was broken. The transducer saved the force value at this point, which was a measure of the maximal resistance the animal can use with its forelimbs. Five measurements were taken from each animal within 2 minutes and the maximum values were used for statistical analysis.⁹ The sedentary mice, both WT and mdx, were monitored for muscle strength every week. At the end of the fourth week of exercise the electrophysiological *in vitro* experiments were started. The animals continued to be exercised until the day of sacrifice.

In Vitro Studies

Muscle Preparations

The *ex vivo* experiments were performed on different muscles collected from mice belonging to the various groups starting after the fourth week of either exercise or exercise plus treatment. The sedentary mice were used at corresponding time points. Thus the age of the animals at the time of experiment was 8 to 12 weeks. The animals were anesthetized with 1.2 g/kg urethane. Extensor digitorum longus (EDL) of one hindlimb was removed and rapidly placed in the recording chamber for the electrophysiological recordings. The contralateral EDL muscle

was either placed in normal physiological solution for surgical dissection to be used for fura-2 calcium imaging, or rapidly frozen in isopentane cooled with liquid nitrogen for immunohistochemical determination of fiber phenotyping. Gastrocnemius (GC) muscles were removed to be used for histological and/or biochemical experiments. To these aims the GC muscle of one hindlimb was dissected by surrounding tissue and fixed in 4% paraformaldehyde in phosphate-buffered saline or in a modified acetate-free Bouin fluid for 4 hours and then was routinely processed and paraffin wax-embedded. The contralateral GC muscle was washed in phosphate-buffered saline (PBS) and rapidly frozen in liquid nitrogen-cooled isopentane and stored at -80°C until use. Diaphragms (DIA) were rapidly removed, placed in normal physiological solution, and rapidly cleaned from connective tissue. The right-half side was used for the electrophysiological recordings, whereas the remaining half was rinsed in PBS, dried, and rapidly frozen in liquid N_2 . Samples were stored at -80°C until use for determination of utrophin content.

Morphometrical Analysis

Six- μm transversally cut sections of GC muscles were stained by hematoxylin and eosin (H&E), toluidine blue, and Azan-Mallory techniques, which allow to distinguish between healthy myofibers, showing peripheral nuclei (peripherally nucleated fibers), regenerating/regenerated myofibers, showing central nuclei (centrally nucleated fibers), and degenerating fibers, often present in areas where also small regenerating fibers and cell infiltrates were visible. Morphometrical analysis was performed on 10 cross-sections from each experimental group by using Image Analysis software (Olympus Italia, Rozzano, Italy). The following parameters were evaluated: 1) area and perimeter of peripherally and centrally nucleated fibers, 2) percentage of peripherally and centrally nucleated fibers referred to the total number, 3) percentage of total nonmuscle area (fibrotic or adipose tissue), and 4) percentage of degeneration tissue area (necrotic fibers and small regenerating/mononuclear inflammatory cells infiltrate).

Evaluation of Markers of Fibrosis

Area of Connective Tissue: The extent of connective tissue was measured on photographs of H&E-stained sections of GC muscles at $\times 20$ magnification using the NIH Image software. At least three fields from each animal were analyzed, the mean area was calculated for each group, and extracellular matrix was evaluated as percentage.

Immunohistochemical Evaluation of Collagen Types: First of all 5- μm -thick consecutive transverse GC muscle sections, were fixed in ice-cold acetone, incubated with a peroxidase block solution (DAKO, Glostrup, Denmark) for 5 minutes and then treated with a protein block solution (DAKO) for 10 minutes to reduce nonspecific bindings. All incubation steps were performed in humid chambers.

For the evaluation of type I and type III collagens, sections were incubated with two commercial polyclonal antibodies (Cedarlane and Abcam, respectively). The sections were incubated for 90 minutes, then rinsed in PBS. Samples were developed using secondary horseradish peroxidase-conjugated antibodies (60 minutes, DAKO), rinsed in PBS and successively developed with diaminobenzidine (DAKO) and counterstained with Mayer hematoxylin for 1 minute.

Determination of Transforming Growth Factor (TGF)- β 1 Level and Transcript: Total TGF- β 1 was determined by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems).³⁰ For determination of TGF- β 1 transcript, total RNA was extracted from 10 to 20 mg of muscle biopsy using RNazol B reagent (Ambion Inc., Austin, TX). One μ g of total RNA was reverse-transcribed according to the manufacturer's instructions and the cDNA stored at -20°C pending polymerase chain reaction (PCR) amplification. Transcripts of TGF- β 1 were assayed by the following semiquantitative method: a constant amount of cDNA (corresponding to 100 ng of total RNA) was amplified in a PCR reaction containing $1\times$ PCR buffer (Finnzymes), 0.1 mmol/L of each dNTP (PE Applied Biosystems), 1 U of DynaZyme DNA polymerase (Finnzymes), and 1 μ mol/L each of the two specific primers. Primer sequences were the following: TGF- β 1: forward 5'-AGTGTGGAGCAAC ATGTGGA-3', reverse 5'-GTGAGCGCTGAACGAAAG-3' (amplified fragment length, 181 bp). Amplification was performed with 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR product was separated by agarose gel electrophoresis and the fluorescence of the product quantitated using the Kodak Digital Science software. The expression of TGF- β 1 was normalized to that of β -actin. The results were expressed as ratios of band intensities and grouped according to the different animal subtypes.

Immunohistochemical Determination of Fiber Phenotype

Five- μ m cryostat sections of EDL muscles, mounted on silanated microscope slides, were kept in PBS for 10 minutes and preincubated with 0.1% gelatin in PBS for 15 minutes as blocking step. Then, sections were incubated with myosin skeletal slow (type I) or fast (both type IIa and IIb) mouse monoclonal antibodies (Sigma, St. Louis, MO) for 1 hour at room temperature. After being washed with PBS-gelatin, sections were incubated for 1 hour with fluorescein isothiocyanate-coupled goat anti-mouse antibody (diluted 1:1000, Molecular Probes) and then washed again. Sections were finally mounted in PBS/glycerol (1:1) containing 1% *n*-propylgallate, pH 8, and examined with a Leica DMRXA photomicroscope equipped for epifluorescence. Digital images were obtained with a Nikon DMX 1200 camera. For fiber quantification, 10 photographs of each section, taken at $\times 16$ magnification were randomly chosen for control and mdx EDL muscles. The number of slow and fast positive fibers was counted, and the results were expressed as a percentage of the stained cells on total cells on the photograph.

Evaluation of Utrophin Content

Muscle tissue was homogenized and proteins extracted as follows: frozen tissue specimens were disrupted in ice-cold extraction buffer (1% digitonin, 5 mmol/L ethylenediaminetetraacetic acid, 50 mmol/L Tris-HCl, pH 6.8, 500 mmol/L NaCl, supplemented with the protease inhibitors phenylmethyl sulfonyl fluoride, aprotinin, and leupeptin) using a cell disrupter device (2 pulses for 30 seconds at maximum speed; BIO 101, Savant). After disruption, debris were eliminated by centrifugation for 10 minutes at 14,000 rpm in a tabletop centrifuge at 4°C , and the supernatant was stored at -20°C . Protein concentration was determined by the BCA protein assay according to the manufacturer's recommendations (Pierce).

The utrophin protein content was measured by a two-site ELISA. For this, Costar EIA/RIA plates were coated overnight at 4°C with the rabbit anti-utrophin antibody anti-UT-31, directed against the mid-rod domain of utrophin (epitope comprising the amino acids 1754 to 2091, provided by Professor Schaub, University of Zurich, Zurich, Switzerland) in carbonate buffer (50 mmol/L, pH 9.6) and blocked with 2% fetal calf serum, 1% bovine serum albumin in PBS containing 0.1% Triton X-100. After washing, wells were incubated with the equivalent of 100 μ g of total protein extract for 6 hours at 4°C followed by repeated washing. Detection of antibody-bound utrophin protein was performed by reacting with a monoclonal anti-utrophin antibody (NCL-DRP2, diluted 1:1000 in blocking solution, overnight incubation at 4°C ; NovoCastrat) recognizing an epitope associated with amino acids 1 to 261 of utrophin. After additional washings, the wells were incubated with a peroxidase-conjugated AffiniPure donkey anti-mouse antibody (diluted 1:5000 in blocking solution; Jackson ImmunoResearch, West Grove, PA). On washing the amount of utrophin protein in muscle extracts was determined by fluorometric detection using the QuantaBlue kit (Pierce) according to the manufacturer's recommendations. As positive control and to determine the dynamic range of the assay and for calibration an extract of HEK cells expressing full-length mouse utrophin [amino acids 1 to 3429; NM.011682 (gi: 46575907)] was used in parallel.

Creatine Kinase Determination

Blood was collected from ventricular camera soon after animal death in ethylenediaminetetraacetic acid-rinsed centrifuge tubes. The blood was centrifuged at $3000\times g$ for 10 minutes and plasma was separated and stored at -20°C . Creatine kinase determination was performed by standard spectrophotometric analysis by using diagnostic kit (Sigma-Aldrich, Milan, Italy) within 7 days from plasma preparation.

Electrophysiological Recordings by Intracellular Microelectrodes

EDL muscle and diaphragm (DIA) were placed in the recording chamber at $30 \pm 1^{\circ}\text{C}$ and superfused with

normal and chloride-free physiological solutions. The normal physiological solution had the following composition (in mmol/L): NaCl, 148; KCl, 4.5; CaCl₂, 2.0; MgCl₂, 1.0; NaHCO₃, 12.0; NaH₂PO₄, 0.44; and glucose, 5.55. The chloride-free solution was made by equimolar substitution of methyl sulfate salts for NaCl and KCl and nitrate salts for CaCl₂ and MgCl₂. The solutions were continuously gassed with 95% O₂ and 5% CO₂ (pH = 7.2 to 7.4).³¹ The two intracellular microelectrode current clamp method was used to measure the membrane electrical properties of muscle fibers by evaluating the attenuation of the electrotonic potential in response to standard hyperpolarizing current pulse at two distances between the recording and the current-injecting electrode, according to the cable equation.³² Assuming constant value of fiber input resistance (R_{in}) of 140 and 200 Ω * cm for EDL and DIA muscle fibers,³¹ respectively, it was possible to calculate membrane cable parameters, among which was membrane resistance (R_m). The total membrane conductance (g_m) was calculated as 1/R_m in normal physiological solution, whereas 1/R_m calculated in a chloride-free solution was the potassium conductance g_K. Chloride conductance (g_{Cl}) was calculated as the mean g_m minus the mean g_K.^{31,32}

The mechanical threshold (MT) of the fibers was determined in EDL muscle fibers in normal physiological solution using a two microelectrode point voltage clamp method as previously described.^{9,33} In brief, the two microelectrodes were inserted within 5 μm of each other into the central region of a randomly selected superficial fiber that was continuously viewed using a stereomicroscope (×100 magnification). The holding potential was set at -90 mV and depolarizing command pulses of variable duration were given at a rate of ~0.3 Hz. Tetrodotoxin (3 μmol/L) was continuously present during recordings to prevent action potential generation.^{9,33} As a standard protocol the command-pulse duration was usually set sequentially to each of the following values: 500, 50, 5, 200, 20, 100, and 10 ms. At each duration, the command voltage was increased using an analogue control until contraction was just visible, and then backed down until the contraction just disappeared. A digital sample-and-hold millivoltmeter stored the value of the threshold membrane potential at this point. We estimated the uncertainty of any single measurement for a given fiber to be 1 to 2 mV.^{9,33} The threshold membrane potential V (mV) for each fiber was averaged at each pulse duration t (ms) and then mean values plotted against duration giving us a strength-duration relationship. A fit estimate of the rheobase voltage (R) and of the rate constant (1/τ) to reach the rheobase was obtained by nonlinear least square algorithm using the following equation: $V = [H - R \exp(t/\tau)]/[1 - \exp(t/\tau)]$ where H is the holding potential (mV), R, is the rheobase (mV), and τ is the time constant. In the fitting algorithm, each point was weighed by the reciprocal of the variance of that mean V and the best fit estimates of the parameters R and 1/τ were made.^{9,33} We used this procedure to be able to incorporate all of our determination points and their associated errors into our estimate of R under each condition.

Fura-2 Microfluorescence Analyses

Small bundles of 10 to 15 EDL muscle fibers arranged in a single layer were dissected length-wise, tendon to tendon, with the use of microscissors, as described elsewhere.^{29,34} Fluorescent measurements were made using a QuantiCell 900 integrated imaging system (VisiTech International Ltd., Sunderland, UK). Calcium measurements were performed using the membrane-permeant Ca²⁺ indicator fura 2-acetoxymethyl ester (fura2-AM, Molecular Probes). The loading of muscle fibers was performed for 1 hour at room temperature (23°C), in normal physiological solution containing 2.5 μmol/L fura2-AM and 0.02% Pluronic-F127 (Molecular Probes).^{29,34} Fura-2-loaded muscle fibers were mounted in a glass-bottomed RC-27NE experimental chamber (Warner Instrument Corp., Hamden, USA), modified by the authors. Tendons of the muscle preparations were attached by the means of hair loops, one extremity was clamped to a fixed tube and the other to a mobile one.^{29,34} The experimental chamber was placed on the stage of an inverted Eclipse TE300 microscope with a ×40 Plan-Fluor objective (Nikon, Japan).

Before fluorescence measurements and to check electrical integrity, preparations were stimulated by electric pulses (2-ms duration, 0.33 Hz) of increasing intensity, until visible contraction, with a pair of platinum-wire electrodes, one in the medium surrounding the fibers and the other close to muscle fibers (100 to 200 μm). Fibers that did not contract were not used for analysis. Then, the sarcomere length was set to ~2.4 to 2.5 μm as previously described.²⁹

Pairs of background subtracted images of the fura-2 fluorescence (510 nm) excited at 340 nm and 380 nm were acquired at rest and pixel-to-pixel radiometric images were calculated for each muscle fiber. Autofluorescence, determined in unloaded muscle fibers, was <2% of total signal and was not subtracted. Resting ratio values were used to calculate the resting cytosolic calcium using the equation: $[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) \times K_D$ × β where R is the ratio of fluorescence excited at 340 nm to that excited at 380 nm; K_D = 145 nmol/L; β, R_{min}, and R_{max} are constants according to Grynkiewicz and colleagues³⁵ and were determined experimentally *in situ* as previously described.^{29,35}

The manganese quench technique was used to estimate the sarcolemmal permeability to divalent cations.³⁶ Muscle preparations were first perfused for ~2 minutes with normal physiological solution containing 0.5 mmol/L Mn²⁺ as a surrogate of CaCl₂ (quenching solution). During the whole quenching protocol the fluorescence of fura-2 excited at 360 nm was acquired at 1 Hz. The quench rates were estimated using linear regression analysis of fluorescence signal and expressed as the decline per minute of the initial fluorescence intensity.

Statistics

All data are expressed as mean ± SEM or ±SD. The SE estimate for g_{Cl} were obtained as previously de-

Table 1. Effect of CsA Treatment on Body and Organ Weight of Exercised Mdx Mice

	<i>n</i>	Body weight (g), time 0	Body weight (g), time 4	Liver (g)	Kidney (g)	Spleen (g)	EDL muscle (g)
Wild type	7	17.8 ± 0.49	22.1 ± 0.54	0.982 ± 0.05	0.143 ± 0.004	0.064 ± 0.003	0.012 ± 0.001
Sed mdx	7	18.1 ± 1.1	24.2 ± 0.69*	1.271 ± 0.03	0.153 ± 0.003	0.074 ± 0.003	0.0113 ± 0.001
Exer mdx	6	17.9 ± 0.54	24.8 ± 0.63*	1.281 ± 0.07	0.161 ± 0.005	0.067 ± 0.002	0.0111 ± 0.0018
Exer mdx +CsA	7	18.6 ± 0.87	22.3 ± 0.9	1.329 ± 0.04	0.161 ± 0.005	0.072 ± 0.002	0.0115 ± 0.0005

The columns from left to right show as follows: experimental groups of mice used; *n*: number of animal per group; body weight in g at the beginning (time 0) and after 4 weeks (time 4) of either exercise (Exer MDX) or exercise + treatment (Exer MDX + CsA) or none (WT and sed MDX); Weight in g of organs that are possible target of the toxic action of CsA and in particular, liver, kidney, spleen, and skeletal muscle (EDL). The organs were removed when the animals were sacrificed for *ex vivo* experiments and have been therefore collected between week 4 and week 8.

The statistical analysis for multiple comparison by ANOVA showed a significant difference for body weight at time 4 ($F = 3.56$, $P = 0.03$), because of significantly higher values of body weight in *Sed mdx ($P = 0.043$) and *Exer mdx mice ($P < 0.025$). A statistical difference was observed also for liver, kidney, and spleen weights, the organs of WT mice being lighter with respect to those from mdx groups. However, no statistical difference in organ weight was observed between mdx mice belonging to the various groups.

scribed.^{9,31} Statistical analysis for direct comparison between two groups of data means was performed by unpaired Student's *t*-test, whereas multiple statistical comparison between groups was performed by one-way analysis of variance, with Bonferroni's *t*-test posthoc correction for allowing a better evaluation of intra- and inter-group variability and avoiding false positives. The MT values are expressed as both mean ± SEM for the absolute values of voltage threshold at each pulse duration. Fitted rheobase (*R*) and rate constant ($1/\tau$) parameters ± SE were determined from the variance-covariance matrix in the nonlinear least square fitting algorithm.^{9,33} For these fitted parameters, the statistical significance between groups was estimated by using the above tests using these standard errors and a number of degrees of freedom equal to the total number of threshold values determining the curves minus the number of means minus two for the free parameters.^{9,33}

Results

Effect of CsA on Body Weight and Forelimb Strength

At the beginning of the training protocol the body weight of 4- to 5-week-old mdx mice either belonging to the untreated or to the CsA-treated group did not show significant differences with respect to that of age-matched WT animals (Table 1). Four weeks of exercise did not modify the body weight gain of mdx mice versus sedentary counterparts. However in both mdx groups the body weight gain was significantly larger than that observed in WT animals. After 4 weeks of CsA treatment the body weight of mdx mice was lower than that observed in exercised untreated counterparts, being rather similar, as well as the 4-week gain, to that of WT animals. This allowed us to exclude the occurrence of a general detrimental effect of CsA reported by others.²⁴ As expected, no significant differences were observed in forelimb strength of the mice belonging to all groups at the beginning of the experimental section (time 0); however, in line to previous results⁹ after 4 weeks of exercise, the mdx mice appeared to be significantly weaker than the sedentary counterparts (Figure 1). On the absolute values of mouse strength, the treatment with CsA seems to be

rather ineffective. To minimize the influence of the different body weight gain observed between groups, for each mouse we normalized the forelimb strength to body weight at the beginning (time 0) and at the end of 4 weeks of exercise (time 4) and we considered the difference as normalized strength increment (Figure 1). By this approach it was evident that the 4 weeks of exercise protocol produced a significant drop in strength in mdx mice and that the CsA treatment significantly counteracted the deleterious effect of exercise on mouse force. In fact the normalized strength increment fully overlapped that observed in sedentary mdx and WT mice (Figure 1). No significant effect of the treatment was observed on the weight of organs known to be targeted by CsA, ie, liver, kidney, spleen, collected when the animals were sacrificed for *ex vivo* experiments (Table 1). Similarly no dif-

Figure 1. Effects of CsA treatment on *in vivo* forelimb muscle strength of exercised mdx mice. Each column is the mean ± SEM from six to seven animals belonging to the different experimental groups, and in particular WT (C57/BL10), mdx (MDX), exercised mdx (Exer MDX), and exercised mdx treated with CsA (Exer MDX + CsA). Each group of bars shows from left to right the absolute forelimb strength at time 0, the absolute forelimb strength at time 4 (ie, after 4 weeks of exercise or exercise + treatment or none), and the normalized force increment. This parameter (reduced by a scale factor of 10 for graphic purposes) has been calculated by normalizing, for each mouse, the force at time 0 and the force at time 4 with respect to the relative body weights, and considering the difference between these two normalized values. The analysis of variance test for multiple comparison between groups did not show statistical difference for the absolute strength values at time 0, whereas a statistical difference was found at time 4 ($F = 5.2$, $P = 0.00678$) and for the normalized force increment values ($F = 3.94$, $P = 0.0209$). Bonferroni *t*-test is as follows: significantly different with respect to: a, WT values ($0.0029 < P < 0.039$); b, sedentary mdx mice (MDX) ($0.0056 < P < 0.036$); and c, untreated exercised mdx mice (Exer MDX) ($P = 0.0214$).

Table 2. Effect of *in Vivo* CsA Treatment on Morphological Properties of Gastrocnemius Muscle of Exercised Mdx Mice

Conditions	Fiber area (μm^2)	Fiber perimeter (μm)	Centronucleated fibers (%)	Normal fibers (%)	Nonmuscle area (%)	Degenerating area (%)
WT	948 $\pm$ 189	126 $\pm$ 30	0	100	1 $\pm$ 0.8	0
Exer WT	800 $\pm$ 150	115 $\pm$ 45	0	100	1.9 $\pm$ 0.9	0
Sed mdx	525 $\pm$ 200	91 $\pm$ 27	56 $\pm$ 10	44 $\pm$ 8	7.8 $\pm$ 4	9 $\pm$ 3
Exer mdx	416 $\pm$ 30	83 $\pm$ 20	74 $\pm$ 4	26.4 $\pm$ 4	6.3 $\pm$ 3.5	20 $\pm$ 8
Exer mdx + CsA	492 $\pm$ 28*	92 $\pm$ 18	65.3 $\pm$ 8*	34.7 $\pm$ 2*	5.1 $\pm$ 1.78*	10 $\pm$ 4 [†]

The columns from left to right show the mean values $\pm$ SD obtained from 10 cross sections from each muscle from each experimental group. Dystrophic fibers have smaller area and perimeter, which further decreases in exercised group (Exer MDX). Centronucleated fibers are regenerated fibers that replace normal peripherally nucleated fibers and the percentage is an index of degeneration-regeneration cycles occurring in the animals. Nonmuscle area represents the amount of space between fibers or between groups of muscle fibers that is not stained as muscular tissue and is composed by connective and/or adipose tissue. Degenerating areas are composed of necrotic fibers and show a large infiltration of inflammatory mononucleated cells.

Significantly different by unpaired Student's *t*-test for **P* < 0.05 and [†]*P* < 0.001 versus Exer mdx mice.

ference was observed in the weight of the EDL muscle used for electrophysiological recordings between the various groups of mice (Table 1).

Effect of CsA Treatment on Markers of Muscle Damage: Histology and Plasma Creatine Kinase Level

In line with previous observations,⁹ although the exercise protocol was without damaging effect in WT muscles (Table 2 and Figure 2, A and D), the GC muscles of exercised mdx mice showed structural alterations that were similar, although quantitatively more serious, compared to sedentary mdx mice. In fact, extensive areas of degeneration, characterized by the presence of necrotic fibers and by their replacement with nonmuscle tissues, likely adipose and fibrotic ones, were observed in exercised mdx muscles (Table 2 and Figure 2; C, E, and G). Moreover, small centronucleated fibers, isolated or in clusters often nearby necrotic fibers, were present. These areas were also characterized by the presence of several infiltrates, resembling mononuclear inflammatory cells described in dystrophic muscle⁷ (Figure 2, C and E). The treatment of the mdx-exercised mice with CsA, induced a significant reduction of degenerating areas and a reduced percentage of the nonmuscle tissue replacing muscle fibers compared with untreated ones (Figure 2, D and H), indicating a greater preservation of viable contractile material. Moreover, the muscle tissue composed by both peripherally and centronucleated myofibers, showed a more regular shape and a more closely arrangement (Figure 2D).

The results of the morphometric analysis are shown in Table 2 and confirmed the histological observations. In fact, after CsA treatment, in the muscles of exercised mdx mice the number of peripherally nucleated cells as well as the area and perimeter of both normal and centronucleated fibers were significantly increased. Also the percentage of the area occupied by nonmuscle and degenerating tissue was reduced, as compared to the untreated mdx-exercised mice, thus indicating a recruitment of the contractile mass.

The histological evidence of the ability of CsA treatment to reduce the exercise-induced muscle damage in mdx animals has been confirmed by the values of plasma

creatine kinase (CK), a well known diagnostic marker for muscle injury (Figure 3). As expected, the mdx mice were characterized by high levels of CK and this increase was particularly evident in the exercised group. The increase observed in exercised mdx mice was clearly attributable

Figure 2. Morphological features of the GC muscles from exercised WT (A, B), untreated exercised mdx (C, E, G), and CsA-treated mdx mice (D, F, H), stained with toluidine blue (A, C, D, E, F) and Azan-Mallory (B, G, H). Closely packed and regularly sized myofibers (A) enveloped by a thin layer of connective tissue (B, arrow) characterize the control WT muscles. Necrotic fibers (C, arrow), and small centronucleated fibers (C, arrowhead) are present in exercised mdx muscle. The muscle fibers of untreated exercised mdx mice are also irregularly scattered and surrounded by adipose tissue (G, asterisk) or by areas of degenerating tissue with mononuclear infiltrate cells and/or small regenerating fibers (E, asterisk). CsA-treated mdx muscles showed more regular-sized centronucleated (D, arrow) and peripherally nucleated (D, arrowhead) fibers and a few connective tissues between normally packed peripherally and centronucleated myofibers (H, arrow), with less areas of mononuclear cell infiltrate (F, asterisk). Scale bars: 25 μm (A, B, D–H); 28.5 μm (C).

Figure 3. Plasma creatine kinase levels measured by standard spectrophotometric analysis. Each column is the mean $\pm$ SEM from five to seven animals. Bonferroni *t*-test after analysis of variance ($F = 3.05$, $P < 0.05$) was significantly different with respect to sedentary mdx (* $P = 0.04$) and significantly different with respect to Exer mdx (** $P = 0.008$).

to the effect of contractile stress on the dystrophic state, because the same protocol of exercise was without effect on plasma CK level of WT mice (Figure 3). Interestingly, the plasma levels of exercised mdx mice treated for 4 to 8 weeks with CsA was significantly lower with respect to that of the untreated group and even slightly lower with respect to that of sedentary counterparts.

Effect of CsA Treatment on Fibrosis, Utrophin Expression, and Fiber Phenotyping

CsA, as well as other immunosuppressants, has been reported to exert a profibrotic action in many tissues,^{30,37,38} a mechanism that may oppose to the amelioration of the histological picture. The connective tissue present in the mdx GC muscles belonging to the various groups was determined in H&E sections. As shown in Table 3, the area occupied by connective tissue was higher in GC muscle of exercised (11.5% with respect to the area examined) versus sedentary (8.5%) mdx mice. The CsA treatment significantly reduced the area of connective tissue to 5.9% of the standard area analyzed (Table 3). The staining for collagen type I was localized in the extracellular matrix between the muscle fibers and no significant difference was observed in the collagen distributions as a result of the drug treatment (data not shown).

We next evaluated the effect of CsA treatment on TGF- β 1, the major fibrotic cytokine. The transcript of TGF- β 1 (evaluated by semiquantitative PCR) as well as the level of the protein (evaluated by ELISA test), followed the same trend observed for the area of connective tissue. A clear increase was observed in the exercised mdx muscle with respect to the control, whereas the CsA-treated exercised muscle showed values markedly lower with respect to the untreated groups and rather similar to that of sedentary group (Table 3). Although the high inter-group variability did not allow reaching the statistical significance between the groups, the data clearly support the ability of the CsA treatment to decrease rather than increase the progression of fibrotic processes.

Recently, it has been proposed that calcineurin activity, important for maintaining the slow fiber phenotype, may exert a beneficial effect on dystrophic progression in mdx muscle by increasing the expression of utrophin.^{22,39} Thus it was important to establish the impact of calcineurin inhibition by the CsA treatment on both the level of utrophin and the fiber phenotype. The utrophin levels, detected by ELISA test in DIA of each experimental group are shown in Figure 4A. As can be seen comparable levels of the protein were detected in all groups. As far as the mdx genotype is concerned, a slight but significant increase in utrophin content was found in both sedentary and exercised DIA muscles versus WT groups. This is in line with earlier observations^{40,41} and may indicate a compensatory mechanism activated in surviving muscle fibers to withstand both pathology and exercise protocol. Interestingly, the CsA treatment did not increase nor decrease the utrophin content in a significant manner with respect to the mdx groups.

In parallel, as already described,²⁹ the exercise protocol was not intense enough to induce a fast-to-slow transition, because all of the EDL fibers of both genotypes expressed type II MHC, with a few percent of the fibers co-expressing the slow MHC isoform; this percentage was lower in exercised versus sedentary mdx mice (Figure 4B). The CsA treatment further reduced the fiber population co-expressing the slow MHC (Figure 4B). This data, in line with the physiological role of calcineurin in skeletal muscle, is an important evidence that some of the observed effects in treated animals were because of a muscle-based action of CsA.

Table 3. Effect of *in Vivo* CsA Treatment on Markers of Fibrosis of Gastrocnemius Muscle of Exercised Mdx Mice

Conditions	Area of connective tissue (cm ²)	TGF- β 1 transcript	TGF- β 1, pg/mg total protein
Sed mdx	0.644 $\pm$ 0.083	0.260 $\pm$ 0.115	0.278 $\pm$ 0.07
Exer mdx	0.871 $\pm$ 0.119	0.439 $\pm$ 0.052	0.631 $\pm$ 0.21
Exer mdx + CsA	0.447 $\pm$ 0.027*	0.263 $\pm$ 0.157	0.410 $\pm$ 0.007

The columns from left to right show the markers of fibrosis in sedentary, exercised, and exercised mdx mice treated with CsA. The area of connective tissue is the mean value $\pm$ SEM of the space occupied by connective tissue over a standard section area of 7.59 cm², determined in hematoxylin and eosin-stained sections (at least three sections for each muscle and three animals per group). TGF- β 1 transcript (expressed as ratio TGF- β 1/ β -actin) has been determined by semiquantitative PCR, whereas TGF- β protein has been determined by ELISA test. In both cases the data show the mean $\pm$ SE from at least three muscles per experimental group. The ANOVA analysis was significant only for the area of connective tissue ($F = 5.17$, $P = 0.049$), with the value of CsA group being significantly lower with respect to that of untreated exercised mdx (* $P < 0.02$ by Bonferroni's *t*-test). However a clear trend for higher transcript and protein levels of TGF- β 1 has been observed in untreated exercised mice versus both sedentary and CsA-treated exercised mdx mice.

Figure 4. A: Two-site ELISA quantification of the utrophin protein levels (relative units) in the DIA muscle of WT, exercised WT (Exer WT), mdx (MDX), exercised mdx (Exer MDX), and exercised mdx mice treated with CsA (Exer MDX+CsA). Note that the value of CsA-treated group is significantly different from both sedentary and exercised WT, but it is not different from both mdx groups. The data are expressed as mean $\pm$ SD ($n = 5$ to 7 mice measured in quadruplicate). Statistical analysis has been performed by unpaired Student's *t*-test. **B:** Percentage of EDL muscle fibers positive to slow type I MHC. Each bar is the mean $\pm$ SEM for 8 to 12 sections and two to three animals. Significantly different by unpaired Student's *t*-test with respect to sedentary mdx (* $P < 0.005$) and untreated exercised mdx muscles (** $P < 0.001$).

Effect of CsA on Functional Cellular Indexes of Dystrophic Degeneration in Exercised Mdx Mice: Component Ionic Conductances and Calcium Homeostasis

In agreement with previous observations^{9,31} the main electrophysiological index of degenerative events occurring in dystrophic muscle either spontaneously occurring or triggered by exercise was the decrease of macroscopic chloride conductance g_{Cl} , sustained by both activity and expression of muscle $ClC-1$ chloride channels (Figure 5A). The CsA treatment was able to significantly restore g_{Cl} of DIA muscle fibers to a value close to that of normal controls. As can be seen in Figure 5A, the value of g_{Cl} after CsA treatment was significantly higher with respect to that of untreated exercised mice ($P = 0.0031$ by Bonferroni's *t*-test) and almost overlapped that of WT DIA muscle fibers. Similarly, the CsA treatment fully counteracted the exercise-induced decrease in g_{Cl} occurring in mdx EDL muscle fibers, maintaining g_{Cl} to a value not significantly different with respect to that of WT animals. Actually the value of g_{Cl} in CsA-treated EDL muscle fibers was higher than that of WT, approaching the g_{Cl} value typical of regenerating sedentary mdx EDL muscle

fibers (data not shown).³¹ In exercised mdx muscle fibers, and in particular in DIA, the mean value of potassium conductance (g_K) was significantly higher with respect to the value of WT, an alteration not so evident in previously used exercised mdx strains.⁹ We did not characterize the channel underlying this increase, but we found that the *in vitro* application of 50 μ mol/L glybenclamide, a specific blocker of ATP-sensitive potassium channels, reduced g_K of exercised mdx EDL muscle fibers from $459 \pm 40 \mu S/cm^2$ (n fibers = 14) to $371 \pm 31 \mu S/cm^2$ ($n = 15$). A similar 20% decrease was also observed in DIA muscle fibers. Thus, an exercise-induced metabolic sufferance might in part account for the observed increase in g_K . No effect of CsA treatment was observed on the increased g_K of exercised mdx muscle fibers. This suggests that the alterations of g_K and g_{Cl} in dystrophic fibers occurs through different pathways differently sensitive to CsA actions.

The ameliorative effects of CsA on chloride channels function was not dependent on an effect on calcium homeostasis that is typically altered in mdx muscle fibers. In agreement with previous observations,^{9,29} the EDL muscle fibers from mdx muscle fibers were characterized by a shift of the voltage threshold for contraction (MT) toward more negative potentials (Figure 5B). In parallel, a higher level of cytosolic calcium was found by fura-2 calcium imaging (Figure 5C). The treatment with CsA did not produce any significant amelioration of the calcium homeostasis. In fact the strength-duration curve was only slightly shifted toward more positive potential with respect to that of untreated exercised EDL muscle fibers (Figure 5B). The rheobase voltage was -71.3 ± 0.99 mV, a value very close and not significantly different to that of untreated mdx mice (-72.5 ± 0.8 mV). Thus, the rheobase values of both mdx groups were significantly different with respect to WT value ($P < 0.001$ by Student's *t*-test). The rate constants ($1/\tau$) were 0.12 ± 0.013 seconds⁻¹ and 0.13 ± 0.01 seconds⁻¹ in CsA-treated and untreated exercised EDL muscle, respectively, both values accounting for a longer time constant to reach the rheobase with respect to WT muscle fibers ($1/\tau = 0.15 \pm 0.007$ seconds⁻¹, $P < 0.05$ versus CsA treated mdx mice). Accordingly, the fura-2 microspectrofluorimetric analysis showed a slight but not significantly lower value of resting calcium in CsA-treated versus untreated EDL muscle fibers (Figure 5C). In parallel, no significant difference was observed in the sarcolemmal permeability to calcium, estimated by the Mn^{2+} quench rate, between untreated and CsA-treated fibers (data not shown).

Discussion

In the present study we evaluated the potential direct benefit on skeletal muscle function and morphology of dystrophic mdx mice of a chronic treatment with therapeutic doses of CsA, a first choice drug for immunosuppression therapy. The rationale for this study was based on the proposed, but still debated, role of immune response in the progression of wasting condition in muscle affected by muscular dystrophy. The exercised mdx

mouse was used because it allows to evaluate drug effectiveness on disease-related alterations both spontaneously occurring and exacerbated by contractile stress.^{9,28} In fact, a chronic inflammatory response seems to be a hallmark of dystrophic skeletal muscle and inflammatory pathways are also exacerbated by mechanical stress.^{5,6,42} Strategies to contrast immune response,

such as irradiation or cytokine gene knockout in mdx mice have led to both encouraging and ambiguous results, whereas few data are available about the effectiveness of more suitable immunosuppressive pharmacological strategies other than the wide-acting immunosuppressant drugs such as steroids.^{7,9,23,43,44} Previous findings supported a beneficial effect of CsA in DMD patients.¹⁵ In addition Irwin and colleagues,¹⁸ found that CsA can counteract the mitochondrial dysfunction and the muscle degeneration in mice affected by collagen VI-deficient dystrophy, a data of interest in consideration of the reported failure of energy metabolism and mitochondria function found in DMD muscles by gene profile approach.⁵ We found that the CsA treatment was able to prevent the exercise-induced drop in forelimb strength *in vivo*. By means of a multidisciplinary approach *ex vivo* we found that the *in vivo* effect on strength was accompanied by specific amelioration in both histology and cellular function. The data supports the beneficial effect of the immunosuppressive therapy in contrasting the pathology progression, although the effectiveness of CsA may be brought about by the various mechanisms that overall define its pharmacological profile.

CsA Exerts a Protective Effect against Exercise-Induced Muscle Damage

The ability of CsA to decrease muscle damage was evidenced by both the histological analysis and by the reduction of plasma creatine kinase. In agreement with data available in the literature, the high number of fully regenerated and regenerating centronucleated fibers in mdx muscle and its further increase with exercise, is an index of ongoing degeneration-regeneration cycles.^{7,22}

Figure 5. Effect of CsA treatment on the functional cellular parameters of muscle fibers from exercised mdx mice. **A:** The mean values $\pm$ SEM of resting component ionic conductances to chloride (gCl) and potassium (gK) ions of EDL and DIA muscle fibers. Each column is the mean $\pm$ SEM from 29 to 67 fibers from four to five preparations for EDL and from 19 to 46 fibers from three to five preparations for DIA. The analysis of variance test for multiple comparison in EDL muscle was significant for both gCl ($F = 21$, $P = 0.00026$) and gK ($F = 5.08$, $P = 0.03$). Similarly, the analysis of variance showed significant differences in DIA for both gCl ($F = 13$, $P = 0.0016$) and gK ($F = 8.25$, $P = 0.011$). Bonferroni *t*-test is as follows: EDL: significantly different with respect to WT ($*P < 0.025$) and untreated exercised MDX mice ($^{\#}$ Exer MDX, $P = 8.3 \times 10^{-5}$). DIA: significantly different with respect to WT ($*P < 0.01$) and untreated exercised MDX mice ($^{\#}$ Exer MDX, $P = 0.0031$). **B:** The data, expressed as mean $\pm$ SEM from 15 to 30 values from three to five preparations, show the voltages for fiber contraction (MT) at each pulse duration in different experimental conditions and in particular in WT (open circles), untreated exercised mdx mice (Exer MDX, open squares), and CsA-treated exercised mdx mice (Exer MDX + CsA, open triangles). All threshold values of Exer-MDX muscle fibers were significantly more negative with respect to those of WT ones ($P < 0.001$). The values of Exer-MDX CsA-treated muscle fibers were slightly but not significantly less negative than those of untreated MDX ones, with the exception of the values at 5 and 10 ms ($P = 0.025$ and 0.05 , respectively), while they remained always significantly more negative than those of WT muscle fibers ($P < 0.05$ and less). For some data points the SE bar is not visible being smaller than symbol size. The values have been fitted to the equation shown in the Materials and Methods section to obtain the strength-duration curves and the fitted parameters of rheobase (R) and $1/\tau$, described in the text. **C:** The intracellular free calcium concentration measured in fura-2-loaded isolated muscle fibers using calibration parameters of the Grynkiewicz's equation determined *in situ*. A calibration was performed for each muscle used. Each bar is the mean $\pm$ SEM from 60 to 100 muscle fibers and three to five animals. Statistical difference between groups was determined by analysis of variance comparison ($F = 30.8$, $P < 9.6 \times 10^{-5}$) followed by Bonferroni's *t*-test. *, Significantly different with respect to WT with $P < 0.0001$.

Furthermore, this is accompanied in exercised muscles by a larger area of nonmuscle tissue and by the presence of small mononuclear cells resembling inflammatory infiltrates.⁷ The ability of CsA to reduce the percentage of centronucleated fiber as well as the areas of degeneration and nonmuscle tissue in exercised mdx muscles is indicative of a protective effect of the treatment against stress-induced worsening of the pathology. Despite the high regenerative potential of mdx mouse muscles, also supported in the present study by the large percentage of centronucleated fibers in all dystrophic groups, it is known that this mechanism can undergo a long-term failure because of an age-dependent fibrosis.^{1,45} Thus the decrease in connective tissue and in the level and expression of the profibrotic cytokine TGF- β 1 in CsA-treated muscle corroborate a potential ability of the drug to enhance the efficiency of regeneration on chronic treatments. Moreover, the levels TGF- β 1 presently found were, in absolute terms, lower than those reported by others in mdx DIA³⁷ and this is in line with the less severity of the disorder in hindlimb muscles and further support a possible involvement of TGF- β 1 in the exercise-induced fibrotic events occurring in dystrophic animals. It is important to underline that CsA is known to exert a profibrotic activity in many organs by enhancing the expression of TGF- β .^{30,37,38} Thus our findings suggest that the CsA-induced reduction of fibrotic events occurs through an indirect mechanism, ie, the reduction of the inflammatory state. Many of the parameters of mdx limb muscles worsened by exercise, including the impairment in chloride channel function observed in EDL muscle fibers (see below), were all similarly ameliorated by CsA treatment, suggesting that their alteration may be because of a general event aggravated by exercise and sensitive to CsA, such as inflammation induced by mechanical stress.⁴²

CsA Exerts Different Effects on Functional Cellular Parameters: A Basis for a Calcium-Independent Action

We observed *ex vivo* a specific ability of CsA treatment to contrast the impairment of chloride channel function, evidenced by the low gCl values, both in DIA, where this impairment is permanent and exercise-independent, and in EDL muscle, where, as opposite, it is an effect triggered by exercise.^{9,31} This evidence suggests that CsA is able to beneficially act on the different mechanisms responsible for gCl impairment in the two muscle types.⁹ Taking into account the pivotal role of calcineurin in maintaining the slow-twitch program and the phenotype-dependent expression of chloride channels,^{19,46} it is feasible that the CsA treatment can increase gCl by reducing the proportion of fibers belonging to the slow phenotype, as supported by the observation that the low percentage of EDL fibers co-expressing the slow isoform of myosin heavy chain, was further reduced in CsA-treated mice. However, although this mechanism may account for the CsA effect on gCl of mdx DIA, in which a compensatory fast-to-slow phenotype shift has been described, albeit

nonunanimously,^{47,48} it cannot explain the drug effect in mdx EDL muscle because we confirmed that no functionally significant phenotype transition occurs in this fast muscle in response to either genotype or exercise.²⁹ We previously hypothesized,⁹ that the decrease of gCl induced by exercise in EDL muscle fibers might be related to a short-term action of proinflammatory cytokines, produced in response to the damaging effect of exercise, on the function of chloride channel. Thus, the effect of CsA in EDL muscle can be because of its ability to reduce the immune response and consequently the production of cytokines by injured muscle tissue or proinflammatory cell infiltrates.^{14,43,44} Another intriguing, although purely speculative, hypothesis to explain the CsA effectiveness in both DIA and EDL muscle is that the expression of functional chloride channel may be controlled by mechanosensitive mitogen-activated protein kinase (MAPK) cascades that are enhanced in dystrophin-deficient muscle fibers and also involves activation of calcineurin.^{42,49,50} In fact this complex network of kinase/phosphatase pathways modulates different cellular targets either in a short term or through the modulation of gene expression, ie, by enhancing the transcription of inflammatory genes or controlling translational events of ion channels.^{42,50,51}

The CsA treatment did not ameliorate the alteration of calcium homeostasis that is a typical hallmark of dystrophic fibers and is worsened by exercise.^{2,4,29} CsA per se may even lead to a further increase in calcium mobilization;⁵² thus our finding leads to two main considerations: the first one is that the alteration in calcium homeostasis is rather independent on the inflammatory reaction. The second is that CsA acts on the sensitive parameters independently or downstream the alteration of calcium homeostasis. Interestingly, calcineurin is a calcium-dependent enzyme and its activation may also be involved in activation of nuclear factor- κ B and in apoptotic programs.^{16,17} Thus, in dystrophic muscle CsA may counteract calcineurin activity, and consequently the pathological cascade related to the enzyme, at a step following its overactivation by the increase in calcium level.

Specificity of CsA Effects: Possible Mechanisms

The lack of beneficial effect of CsA on calcium homeostasis, and thus its inability to contrast the other pathological events linked to this pathway, may explain why the CsA treatment was in fact partially effective. Accordingly, CsA may be ineffective on all of the pathological pathways that are strictly linked to the primary defect, ie, the absence of dystrophin. Another intriguing hypothesis to explain the partial effectiveness of CsA is the possible role that immune response pathways may play in muscle regeneration itself and supported by a recent microarray study showing a mitigated expression of inflammatory genes in DIA versus hindlimb mdx muscles.⁵³ In addition calcineurin pathway may play a role in muscle regeneration²⁰ and may be protective for dystrophic muscle through an enhanced expression of the compensatory protein utrophin.²² Although we cannot totally exclude this possibility, it is important to underline that no de-

crease in utrophin level has been observed by us as a result of CsA treatment. This data suggests that the positive effects of the CsA treatment observed on various *in vivo* (forelimb strength) and *ex vivo* (chloride channel function, CK levels, and morphology) parameters are not because of an utrophin-dependent mechanism, but also rule out a detrimental effect of our CsA treatment on utrophin expression. Furthermore, we did not observe the deleterious effects observed by Stupka and colleagues²⁴ in mdx mice treated with high doses of CsA (30 mg/kg i.p.), likely in relation of the more therapeutic dose used by us and the different ages of the mice undergoing the treatment. In their study 2-week-old mice were treated for ~2 weeks and the deleterious effect of CsA was associated to the proposed role of calcineurin in muscle growth and regeneration.^{21,24} This conclusion does not reconcile with the concomitant finding of a lower rather than higher enzyme level in mdx versus control and with a greater number of myogenin-positive fibers in CsA-treated muscles.²⁴ Interestingly, the overall effects observed by us after CsA treatment were similar to those produced by a treatment with insulin-like growth factor-1.⁹ Far from proposing that CsA may stimulate regeneration, this observation corroborates the hypothesis that the reduction of immune reaction and, consequently, of fibrosis (see above) may facilitate the action of regeneration-promoting factors. However, we agree that, because of both the physiological role of calcineurin for skeletal muscle fibers and the toxicity of the drug, the dose of CsA to perform a treatment during muscle development, ie, in very young dystrophic patients, should be monitored with attention.

Concluding Remarks

An enhanced immune reaction contributes to the pathology progression in muscular dystrophy. This is supported by the beneficial effect observed by us on dystrophic muscle by CsA, one of first choice drugs for immunosuppression,¹⁴ which may also prove beneficial through direct actions on skeletal muscle tissue, ie, the inhibition of calcineurin or the amelioration of impaired mitochondrial metabolism. The effectiveness of CsA for muscle function is an important point because the immunological reaction is also responsible for the rejection of the administered genetic material and a greater success of these approaches have been obtained in dystrophic animals that were immunosuppressed.^{10–13} Although the narrow therapeutic index of CsA shall be taken into account especially for early treatment of boys with DMD, our data generally strengthen the usefulness for muscle function of therapies addressed against immunoreaction in dystrophic patients.

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References

- Hoffman EP, Dressman D: Molecular pathophysiology and targeted therapeutics for muscular dystrophy. *Trends Pharmacol Sci* 2001, 22:465–470
- Alderton JM, Steinhardt RA: How calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *Trends Cardiovasc Med* 2000, 10:268–272
- Wehling M, Spencer MJ, Tidball JG: A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *J Cell Biol* 2001, 155: 123–131
- Iwata Y, Katanosaka Y, Arai Y, Komamura K, Miyatake K, Shigekawa M: A novel mechanism of myocyte degeneration involving the Ca^{2+} -permeable growth factor-regulated channel. *J Cell Biol* 2003, 161: 957–967
- Chen YW, Zhao P, Borup R, Hoffman EP: Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. *J Cell Biol* 2000, 151:1321–1336
- Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, Leahy P, Li J, Guo W, Andrade FH: A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum Mol Genet* 2002, 11:263–272
- Spencer MJ, Montecino-Rodriguez E, Dorshkind K, Tidball JG: Helper (CD4(+)) and cytotoxic (CD8(+)) T cells promote the pathology of dystrophin-deficient muscle. *Clin Immunol* 2001, 98:235–243
- Dubowitz V, Kinali M, Main M, Mercuri E, Muntoni F: Remission of clinical signs in early Duchenne muscular dystrophy on intermittent low-dosage prednisolone therapy. *Eur J Paediatr Neurol* 2002, 6:153–159
- De Luca A, Pierro S, Liantonio A, Cetrone M, Camerino C, Frayssé B, Mirabella M, Servidei S, Ruegg UT, Conte Camerino D: Enhanced dystrophic progression in mdx mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. *J Pharmacol Exp Ther* 2003, 304:453–463
- Kinoshita I, Vilquin JT, Guerette B, Asselin I, Roy R, Tremblay JP: Very efficient myoblast allotransplantation in mice under FK506 immunosuppression. *Muscle Nerve* 1994, 17:1407–1415
- Yuasa K, Sakamoto M, Miyagoe-Suzuki Y, Tanouchi A, Yamamoto H, Li J, Chamberlain JS, Xiao X, Takeda S: Adeno-associated virus vector-mediated gene transfer into dystrophin-deficient skeletal muscles evokes enhanced immune response against the transgene product. *Gene Ther* 2002, 9:1576–1588
- van Deutekom JC, van Ommen GJ: Advances in Duchenne muscular dystrophy gene therapy. *Nat Rev Genet* 2003, 4:774–783
- Cerletti M, Negri T, Cozzi F, Colpo R, Andreetta F, Croci D, Davies KE, Cornelio F, Pozza O, Karpatis G, Gilbert R, Mora M: Dystrophic phenotype of canine X-linked muscular dystrophy is mitigated by adeno-virus-mediated utrophin gene transfer. *Gene Ther* 2003, 10:750–757
- Matsuda S, Koyasu S: Mechanisms of action of cyclosporine. *Immunopharmacology* 2000, 47:119–125
- Sharma KR, Mynhier MA, Miller RG: Cyclosporine increases muscular force generation in Duchenne muscular dystrophy. *Neurology* 1993, 43:527–532
- Springer JE, Azbill RD, Nottingham SA, Kennedy SE: Calcineurin-mediated BAD dephosphorylation activates the caspase-3 apoptotic cascade in traumatic spinal cord injury. *J Neurosci* 2000, 20:7246–7251
- Alzuherri H, Chang KC: Calcineurin activates NF-kappaB in skeletal muscle C2C12 cells. *Cell Signal* 2003, 15:471–478
- Irwin WA, Bergamin N, Sabatelli P, Reggiani C, Megighian A, Merlini L, Braghetta P, Columbaro M, Volpin D, Bressan GM, Bernardi P, Bonaldo P: Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. *Nat Genet* 2003, 35:367–371
- Schiaffino S, Serrano A: Calcineurin signaling and neural control of skeletal muscle fiber type and size. *Trends Pharmacol Sci* 2002, 23:569–575
- Semsarian C, Wu MJ, Ju YK, Marciniak T, Yeoh T, Allen DG, Harvey RP, Graham RM: Skeletal muscle hypertrophy is mediated by a Ca^{2+} -dependent calcineurin signalling pathway. *Nature* 1999, 400: 576–581
- Mitchell PO, Mills ST, Pavlath GK: Calcineurin differentially regulates maintenance and growth of phenotypically distinct muscles. *Am J Physiol* 2002, 282:C984–C992

22. Chakkalakal JV, Harrison MA, Carbonetto S, Chin E, Michel RN, Jasmin BJ: Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice. *Hum Mol Genet* 2004, 13:379–388
23. Weller B, Massa R, Karpati G, Carpenter S: Glucocorticoids and immunosuppressants do not change the prevalence of necrosis and regeneration in mdx skeletal muscles. *Muscle Nerve* 1991, 14:771–774
24. Stupka N, Gregorevic P, Plant DR, Lynch GS: The calcineurin signal transduction pathway is essential for successful muscle regeneration in mdx dystrophic mice. *Acta Neuropathol (Berl)* 2004, 107:299–310
25. Sangalli L, Bortolotti A, Jiritano L, Bonati M: Cyclosporine pharmacokinetics in rats and interspecies comparison in dogs, rabbits, rats, and humans. *Drug Metab Dispos* 1988, 16:749–753
26. Biring MS, Fournier M, Ross DJ, Lewis MI: Cellular adaptations of skeletal muscles to cyclosporine. *J Appl Physiol* 1998, 84:1967–1975
27. Mehl ML, Kyles AE, Craigmill AL, Epstein S, Gregory CR: Disposition of cyclosporine after intravenous and multi-dose oral administration in cats. *J Vet Pharmacol Ther* 2003, 26:349–354
28. Granchelli JA, Pollina C, Hudecki MS: Pre-clinical screening of drugs using the mdx mouse. *Neuromuscul Disord* 2000, 10:235–239
29. Frayse B, Liantonio A, Cetrone M, Burdi R, Pierno S, Frigeri A, Pisoni M, Camerino C, De Luca A: The alteration of calcium homeostasis in adult dystrophic mdx muscle fibers is worsened by a chronic exercise in vivo. *Neurobiol Dis* 2004, 17:144–154
30. Hartel JV, Granchelli JA, Hudecki MS, Pollina CM, Gosselin LE: Impact of prednisone on TGF-beta1 and collagen in diaphragm muscle from mdx mice. *Muscle Nerve* 2001, 24:428–432
31. De Luca A, Pierno S, Conte Camerino D: Electrical properties of diaphragm and EDL muscles during the life of dystrophic mice. *Am J Physiol* 1997, 272:C333–C340
32. Bryant SH, Conte-Camerino D: Chloride channel regulation in the skeletal muscle of normal and myotonic goats. *Pfluegers Arch* 1991, 417:605–610
33. De Luca A, Pierno S, Liantonio A, Cetrone M, Camerino C, Simonetti S, Papadia F, Conte Camerino D: Alteration of excitation-contraction coupling mechanism in extensor digitorum longus muscle fibres of dystrophic mdx mouse and potential efficacy of taurine. *Br J Pharmacol* 2001, 132:1047–1054
34. Frayse B, Desaphy JF, Pierno S, De Luca A, Liantonio A, Mitolo CI, Conte Camerino D: Decrease in resting calcium and calcium entry associated with slow-to-fast transition in unloaded rat soleus muscle. *FASEB J* 2003, 17:1916–1918
35. Gryniewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985, 260:3440–3450
36. Parekh AB, Penner R: Store depletion and calcium influx. *Physiol Rev* 1997, 77:901–930
37. Waiser J, Dell K, Bohler T, Dogu E, Gaedeke J, Budde K, Neumayer HH: Cyclosporine A up-regulates the expression of TGF-beta1 and its receptors type I and type II in rat mesangial cells. *Nephrol Dial Transplant* 2002, 17:1568–1577
38. Sun BK, Li C, Lim SW, Jung JY, Lee SH, Kim IS, Kim YS, Kim J, Bang BK, Yang CW: Expression of transforming growth factor-beta-inducible gene-h3 in normal and cyclosporine-treated rat kidney. *J Lab Clin Med* 2004, 143:175–183
39. Gramolini AO, Belanger G, Thompson JM, Chakkalakal JV, Jasmin BJ: Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events. *Am J Physiol* 2001, 281:C1300–C1309
40. Pons F, Augier N, Leger JO, Robert A, Tome FM, Fardeau M, Voit T, Nicholson LV, Mornet D, Leger JJ: A homologue of dystrophin is expressed at the neuromuscular junctions of normal individuals and DMD patients, and of normal and mdx mice. Immunological evidence. *FEBS Lett* 1991, 282:161–165
41. Nguyen TM, Ellis JM, Love DR, Davies KE, Gatter KC, Dickson G, Morris GE: Localization of the DMDL gene-encoded dystrophin-related protein using a panel of nineteen monoclonal antibodies: presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles, and in proliferating brain cell lines. *J Cell Biol* 1991, 115:1695–1700
42. Kumar A, Boriek AM: Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. *FASEB J* 2003, 17:386–396
43. Gosselin LE, McCormick KM: Targeting the immune system to improve ventilatory function in muscular dystrophy. *Med Sci Sports Exer* 2004, 36:44–51
44. Grounds MD, Torrisi J: Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis. *FASEB J* 2004, 18:676–682
45. Passerini L, Bernasconi P, Baggi F, Confalonieri P, Cozzi F, Cornelio F, Mantegazza R: Fibrogenic cytokines and extent of fibrosis in muscle of dogs with X-linked golden retriever muscular dystrophy. *Neuromuscul Disord* 2002, 9:828–835
46. Pierno S, Desaphy JF, Liantonio A, De Bellis M, Bianco G, De Luca A, Frigeri A, Nicchia GP, Svelto M, Leoty C, George Jr AL, Conte Camerino D: Change of chloride ion channel conductance is an early event of slow-to-fast fibre type transition during unloading-induced muscle disuse. *Brain* 2002, 125:1510–1521
47. Petrof BJ, Stedman HH, Shrager JB, Eby J, Sweeney HL, Kelly AM: Adaptations in myosin heavy chain expression and contractile function in dystrophic mouse diaphragm. *Am J Physiol* 1993, 265:C834–C841
48. Gregorevic P, Plant DR, Leeding KS, Bach LA, Lynch GS: Improved contractile function of the mdx dystrophic mouse diaphragm muscle after insulin-like growth factor-I administration. *Am J Pathol* 2002, 161:2263–2272
49. Nakamura A, Yoshida K, Takeda S, Dohi N, Ikeda S: Progression of dystrophic features and activation of mitogen-activated protein kinases and calcineurin by physical exercise, in hearts of mdx mice. *FEBS Lett* 2002, 520:18–24
50. Kumar A, Khandelwal N, Malya R, Reid MB, Boriek AM: Loss of dystrophin causes aberrant mechanotransduction in skeletal muscle fibers. *FASEB J* 2004, 18:102–113
51. Yanagita T, Kobayashi H, Uezono Y, Yokoo H, Sugano T, Saitoh T, Minami S, Shiraishi S, Wada A: Destabilization of Na(v)1.7 sodium channel alpha-subunit mRNA by constitutive phosphorylation of extracellular signal-regulated kinase: negative regulation of steady-state level of cell surface functional sodium channels in adrenal chromaffin cells. *Mol Pharmacol* 2003, 63:1125–1136
52. Bultynck G, Vermassen E, Szlufcik K, De Smet P, Fissore RA, Callewaert G, Missiaen L, De Smedt H, Parys JB: Calcineurin and intracellular Ca^{2+} -release channels: regulation or association? *Biochem Biophys Res Commun* 2003, 311:1181–1193
53. Porter JD, Merriam AP, Leahy P, Gong B, Feuerman J, Cheng G, Khanna S: Temporal gene expression profiling of dystrophin-deficient (mdx) mouse diaphragm identifies conserved and muscle group-specific mechanisms in the pathogenesis of muscular dystrophy. *Hum Mol Genet* 2004, 13:257–269