

Animal Model

Proteasome Inhibitor (MG-132) Treatment of mdx Mice Rescues the Expression and Membrane Localization of Dystrophin and Dystrophin-Associated Proteins

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Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, is absent in the skeletal muscle of DMD patients and mdx mice. At the plasma membrane of skeletal muscle fibers, dystrophin associates with a multimeric protein complex, termed the dystrophin-glycoprotein complex (DGC). Protein members of this complex are normally absent or greatly reduced in dystrophin-deficient skeletal muscle fibers, and are thought to undergo degradation through an unknown pathway. As such, we reasoned that inhibition of the proteasomal degradation pathway might rescue the expression and subcellular localization of dystrophin-associated proteins. To test this hypothesis, we treated mdx mice with the well-characterized proteasomal inhibitor MG-132. First, we locally injected MG-132 into the gastrocnemius muscle, and observed the outcome after 24 hours. Next, we performed systemic treatment using an osmotic pump that allowed us to deliver different concentrations of the proteasomal inhibitor, over an 8-day period. By immunofluorescence and Western blot analysis, we show that administration of the proteasomal inhibitor MG-132 effectively rescues the expression levels and plasma membrane localization of

dystrophin, β -dystroglycan, α -dystroglycan, and α -sarcoglycan in skeletal muscle fibers from mdx mice. Furthermore, we show that systemic treatment with the proteasomal inhibitor 1) reduces muscle membrane damage, as revealed by vital staining (with Evans blue dye) of the diaphragm and gastrocnemius muscle isolated from treated mdx mice, and 2) ameliorates the histopathological signs of muscular dystrophy, as judged by hematoxylin and eosin staining of muscle biopsies taken from treated mdx mice. Thus, the current study opens new and important avenues in our understanding of the pathogenesis of DMD. Most importantly, these new findings may have clinical implications for the pharmacological treatment of patients with DMD. (Am J Pathol 2003, 163:1663–1675)

Duchenne muscular dystrophy (DMD) is one of the most prevalent and severe inherited diseases of childhood, characterized by progressive muscular wasting and weakness. The deficient gene product, dystrophin,¹ is a peripheral membrane protein of ~426 kd, which is expressed in muscle tissues and the brain. At the plasma membrane, dystrophin associates with a large multimeric complex, termed the dystrophin-glycoprotein complex (DGC).² The DGC is composed of two subcomplexes: the dystroglycan complex (α and β subunits) and the sarcoglycan complex (α , β , γ , and δ subunits). The N-terminal region of dystrophin interacts directly with the cytoskeletal protein actin, while the dystrophin C-terminal domain binds to the plasma membrane through interactions with β -dystroglycan. As such, dystrophin is thought

Supported by grants from the National Institutes of Health (NIH), the Muscular Dystrophy Association (MDA), the American Heart Association (AHA), and the Breast Cancer Alliance (BCA), as well as a Hirsch/Weil-Caulier Career Scientist Award (all to M. P. L.).

C. M. was supported by grants from Telethon-Italia and the Italian Ministry of Health (G. Gaslini Institute, Ricerca Finalizzata).

Accepted for publication June 24, 2003.

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to provide a mechanical linkage between the intracellular cytoskeleton and the extracellular matrix. The dystrophin complex also interacts with neuronal-type nitric oxide synthase (nNOS), whose biological product, NO, regulates contraction in skeletal muscle.^{3,4} Another protein associated with the DGC, although not essential for the biogenesis of the complex itself, is caveolin-3 (Cav-3), a member of the caveolin protein family.⁵ Caveolins are the main structural components of caveolae, which are cholesterol- and sphingolipid-rich vesicular invaginations of the plasma membrane.^{6,7}

Research on DMD has greatly benefited from the availability of a naturally occurring mouse model, known as mdx, in which a non-sense mutation (premature stop codon) in the dystrophin gene ablates the expression of the dystrophin protein product.^{8,9} The mdx mouse is viable and fertile, and exhibits histological lesions typical of muscular dystrophy. Although the mdx mouse is a valuable model for DMD, muscular wastage progresses in a much milder fashion than as compared with humans. This difference could be due to compensatory mechanisms, such as increased muscle regeneration, or the functional replacement of dystrophin by utrophin. Utrophin, the ubiquitous homologue of dystrophin, is normally expressed at the sarcolemma of skeletal muscle fibers during fetal development, but is restricted to the neuromuscular and myotendinous junctions in adult skeletal muscle.¹⁰

The complete loss of dystrophin perturbs the structural composition of the DGC, such that all members of the DGC complex are greatly reduced in skeletal muscle fibers from DMD patients and from mdx mice.¹¹ The only exception is Cav-3, which was shown to be up-regulated by ~2-fold in dystrophin-deficient skeletal muscle.^{12,13} A lack of dystrophin is thought to cause sarcolemmal instability, which may render the dystrophin-glycoprotein complex more susceptible to proteolytic degradation.¹⁴

Similarly to other tissues, skeletal muscle has at least three different pathways for protein degradation: 1) proteolysis by lysosomal proteases, such as the cathepsins, 2) proteolysis by non-lysosomal Ca²⁺-dependent proteases, such as calpain, and 3) proteolysis by non-lysosomal ATP-ubiquitin-dependent proteases, eg, the multicatalytic protease complex (or proteasome). The ubiquitin-proteasome pathway is the major proteolytic system present in all eukaryotic cells, and degrades the substrates marked by attachment of many molecules of ubiquitin, a small 8-kD protein. The resulting ubiquitinated proteins are then recognized and degraded by a 2.4-MDa proteolytic complex, the 26S proteasome. The proteasome consists of a cylindrical 20S catalytic core particle, capped by two 19S regulatory complexes that control the access of substrates to the proteolytic chamber.¹⁵

Several lines of evidence have suggested that enhanced activation of proteolytic degradation pathways underlies the pathogenesis of various diseases, including skeletal muscle atrophy and muscular dystrophy.^{16–19} Combaret and colleagues²⁰ have demonstrated that increased protein degradation in skeletal muscle from mdx mice and DMD patients correlates with elevated expression of the non-lysosomal protease calpain, but not with elevated mRNA levels of components of the proteasomal pathway. Con-

versely, Kumamoto and colleagues²¹ have provided preliminary evidence that, in DMD patients, muscle fiber degradation is due to concomitant activation of the non-lysosomal calpain-mediated pathway and of the non-lysosomal ATP-ubiquitin dependent proteasome system, as assessed by immunohistochemical staining. As such, the role of the proteasomal pathway in dystrophin-deficient skeletal muscle degeneration still remains controversial.

Over the last several years, an increasing body of evidence has emerged highlighting the function of the proteasomal machinery in maintaining normal muscle size and capacity, and has suggested that dysregulation of the proteasomal pathway might result in muscle pathology. The discovery of two muscle-specific ubiquitin ligases, which target proteins for degradation by the proteasomal pathway, has provided a greater understanding of the mechanisms underlying muscle atrophy. For example, adenovirus-mediated over-expression of these muscle-specific ubiquitin ligases produces muscle atrophy, whereas their genetic ablation resulted in resistance to muscle atrophy.²²

Previous studies from our laboratory²³ have demonstrated that proteasomal degradation may be involved in the pathogenesis of a form of muscular dystrophy in humans, Limb-Girdle Muscular Dystrophy (LGMD-1C). LGMD-1C is an autosomal dominant form of muscular dystrophy caused by heterozygous mutations in the caveolin-3 gene.²⁴ In a heterologous cell system, LGMD-1C mutants of Cav-3 behave in a dominant-negative fashion, causing the retention of wild-type Cav-3 at the level of the Golgi complex.²⁵ Further analysis has demonstrated that LGMD-1C mutants of Cav-3 undergo ubiquitination and proteasomal degradation. Treatment of cultured cells with MG-132 could effectively block the dominant negative effect of these mutants and rescue the expression levels and the subcellular localization of wild-type Cav-3.²³

The discovery of many synthetic and natural inhibitors of the proteasomal pathway has been extremely useful in the comprehension of the mechanisms underlying intracellular protein degradation.¹⁵ Most importantly, these new drugs may have very important *in vivo* applications, and may be instrumental in the pharmacological treatment of various diseases.

Here we assess whether activation of the ubiquitin-proteasome proteolytic pathway underlies the rapid loss of muscle proteins in dystrophin-deficient skeletal muscle *in vivo*. Our findings may have implications for the pharmacological treatment of patients with DMD.

Experimental Procedures

Materials

Monoclonal antibodies directed against β -dystroglycan (NCL-b-DG), α -sarcoglycan (NCL-a-sarco) and dystrophin (NCL-DYS3) were purchased from Novocastra (Newcastle, United Kingdom). A monoclonal antibody for α -dystroglycan was from Upstate Biotechnology (Lake Placid, NY); a polyclonal antibody against neuronal nNOS was purchased from BD Transduction Laboratories; and anti-caveolin-3 IgG (mAb 26⁵) was the gift of Dr.

Roberto Campos-Gonzalez from BD Transduction Laboratories. The proteasome inhibitor MG-132 (CBZ-leucyl-leucyl-leucinal) was from Calbiochem (San Diego, CA); Evans blue dye (EBD) was from Sigma (St. Louis, MO); Alzet Minipumps were purchased from Alza Corp. (Palo Alto, CA). The calpain activity kit (using a fluorogenic substrate) was from Oncogene Research Products (San Diego, CA).

Laboratory Animals

Six-month-old male mdx (C57BL/10ScSn DMD mdx) mice, purchased from The Jackson Laboratory (JAX mice; Bar Harbor, ME), were used throughout this study. Skeletal muscle tissues were quickly dissected, flash-frozen in 2-methyl butane (isopentane) cooled in liquid nitrogen, and stored at -80°C until use.

Administration of MG-132

Localized administration was performed by injection of MG-132 into the gastrocnemius muscles of mdx mice. To visualize the injected muscle, MG-132 (final concentration of $20\text{ }\mu\text{mol/L}$) was pre-mixed with 1% India ink in phosphate-buffered saline (PBS) for a total volume of $100\text{ }\mu\text{l}$. Mice were sacrificed 24 hours after injection, and skeletal muscles were quickly isolated for further analysis.

To systemically administer MG-132, we subcutaneously implanted Alzet Minipumps in the anterior back region of mdx mice. Experiments were conducted on 6-month-old mdx mice. For 8 days, we administered either different concentrations of MG-132 (delivered at rate of either $1\text{ }\mu\text{g}$, or $5\text{ }\mu\text{g}$ or $10\text{ }\mu\text{g/kg/24 hours}$) or the inhibitor-diluent (PBS only), as a negative control. Skeletal muscle tissues were collected from untreated (PBS only) and MG-132-treated mdx mice for further analysis.

Immunostaining of Murine Skeletal Muscle Tissue Sections

Tissue samples were isolated from the gastrocnemius muscle, rapidly frozen in liquid nitrogen-cooled isopentane, and stored at -80°C . Unfixed sections ($6\text{-}\mu\text{m}$ thick) of frozen skeletal muscle were blocked in PBS with 1% bovine serum albumin (BSA) for 1 hour at room temperature. Then, sections were incubated with the primary antibody for 1 hour at room temperature (diluted in PBS containing 1% BSA). After three washes with PBS (5 minutes each), sections were incubated with the secondary antibody for 30 minutes at room temperature: either a lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody ($5\text{ }\mu\text{g/ml}$) or a fluorescein isothiocyanate-conjugated goat anti-mouse antibody ($5\text{ }\mu\text{g/ml}$). Finally, the sections were washed three times with PBS (10 minutes each wash). Slides were mounted with Slow-Fade anti-fade reagent (Molecular Probes, Inc., Eugene, OR) and observed under an Olympus IX 70 inverted microscope.

Immunoblot Analysis

Skeletal muscle tissues were harvested, minced with a scissors, homogenized in a Polytron tissue grinder for 30 seconds at a medium-range speed, and solubilized in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, and 60 mmol/L octyl glucoside for 45 minutes at 4°C . Samples were centrifuged at $13,000 \times g$ for 10 minutes at 4°C to remove insoluble debris. Soluble proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) and transferred to nitrocellulose membranes. Blots were blocked for 1 hour in Tris-buffered saline Tween (TBST) (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.2% Tween 20) containing 4% powdered skim milk and 1% BSA. Then, the membranes were incubated for 1 hour with a given primary antibody (or an overnight incubation with the anti-dystrophin antibody), and diluted in TBST/1%BSA. After three washes with TBST, the blots were incubated for 30 minutes with horseradish peroxidase (HRP)-conjugated secondary antibodies, diluted in TBST/1%BSA. Antibody-bound proteins were detected using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Histological and Histochemical Analysis

Skeletal muscle frozen sections were subjected to hematoxylin and eosin (H&E) staining, essentially as described.²⁶

Vital Staining with EBD

To detect damaged muscle fibers, EBD (stock solution: 10 mg/ml in PBS) was injected (0.1 ml/10 g body weight) into the right side of the peritoneal cavity, as previously described.^{27,28} Then, animals were returned to their cages and were sacrificed 20 hours postinjection. Diaphragm and gastrocnemius muscles were dissected. After isolation, diaphragms were rinsed in PBS, fixed in 10% formalin and evaluated macroscopically. Gastrocnemius frozen sections were simply mounted with Slow-Fade anti-fade reagent (Molecular Probes, Inc.), and observed under an Olympus IX 70 inverted microscope to detect the EBD red autofluorescence signal.

Calpain Activity Assay

Calpain activity was assayed using a fluorimetric assay (Oncogene Research Products), as per the manufacturer's instructions. The assay measures the ability of calpain to digest the synthetic substrate Suc-LLVY-AMC. Released free AMC was measured fluorometrically at an excitation of $360\text{--}380\text{ nm}$ and an emission of $440\text{--}460\text{ nm}$.

Frozen samples ($25\text{--}40\text{ mg}$) of gastrocnemius muscle, collected from untreated (PBS only) and MG-132-treated mdx mice, were quickly minced, immediately diluted in lysis buffer provided by the manufacturer, and homogenized on ice. Homogenates were centrifuged at $14,000 \times g$ in a pre-cooled table-top microcentrifuge. After deter-

mination of the protein content using the BCA protein assay (Pierce), aliquots of the supernatants were incubated with the fluorogenic substrate. A fluorimeter (BMG Labtechnologies, Inc., Durham, NC) was used to measure the hydrolysis of peptides.

Results

Localized Treatment with the Proteasomal Inhibitor MG-132

Previous studies have shown that the expression of dystrophin and of dystrophin-associated proteins is absent or greatly reduced in skeletal muscles from mdx mice. The lack of dystrophin is thought to cause sarcolemmal instability, which may render the dystrophin-glycoprotein complex more susceptible to proteolytic degradation.¹⁴ However, the role of the ubiquitin-dependent proteasomal pathway in muscle fiber degeneration in dystrophin-deficient muscles remains controversial.

We reasoned that inhibition of the proteasomal pathway should rescue the expression and subcellular localization of dystrophin-associated proteins. For this purpose, we treated mdx mice with the potent, reversible, and cell-permeable proteasomal inhibitor CBZ-leucyl-leucyl-leucinal (MG-132).^{23,29}

We first performed localized treatment, by injecting MG-132 (at a final concentration of 20 μ mol/L) into the gastrocnemius muscle of mdx mice. To visualize the injected muscle, we pre-mixed MG-132 with a blue dye (1% India Ink in PBS). The gastrocnemius muscle from the other hindlimb of each animal served as an internal control. After 24 hours, skeletal muscle tissues were harvested from untreated and MG-132-treated hindlimbs. Frozen skeletal muscle tissue sections were prepared and examined by immunofluorescence (Figure 1). Immunostaining was performed with antibodies directed against β -dystroglycan (Figure 1A), α -dystroglycan (Figure 1B), α -sarcoglycan (Figure 1C), dystrophin (Figure 1D), and nNOS (Figure 1E). As expected, all these proteins were absent or expressed at very low levels in skeletal muscle fibers from untreated mdx muscles (Figure 1, A to E, upper panels). Interestingly, treatment with the proteasomal inhibitor MG-132 could efficiently rescue the expression level and subcellular localization of β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin. Similar results were obtained using other structurally related proteasome inhibitors, such as MG-115 (CBZ-leucyl-leucyl-norvalinal) and ALLN (N-acetyl-leucyl-leucyl-norleucinal).

The lower panels of Figure 1, A to D, show that β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin were all clearly detectable at the plasma membrane and partially in the cytoplasm of muscle fibers from MG-132-treated skeletal muscles. However, nNOS expression levels were only slightly increased after MG-132 treatment (Figure 1E, lower panel).

To independently verify these observations, tissue lysates from untreated and MG-132-treated mdx gastrocnemius muscle were subjected to Western blot analysis

with antibodies directed against β -dystroglycan, α -dystroglycan, α -sarcoglycan, dystrophin, and nNOS. Figure 2 demonstrates that the expression levels of β -dystroglycan, α -sarcoglycan, and dystrophin are greatly increased in MG-132-treated skeletal muscles, as compared with the untreated controls. Immunoblotting with the α -dystroglycan antibody shows that MG-132 treatment results in the specific augmentation of a band, that we believe to be the precursor form of α -dystroglycan (Figure 2A). The heavily-glycosylated mature form of α -dystroglycan does not appear to be increased in MG-132-treated skeletal muscles, *versus* the untreated muscles. Previous results have shown that the binding of β -dystroglycan to α -dystroglycan occurs independently of α -dystroglycan glycosylation.³⁰ In support of this notion, our current data show that unglycosylated α -dystroglycan binds β -dystroglycan, as both are properly targeted to the plasma membrane (Figures 1B and 2A). Consistent with what we observed by immunofluorescence analysis (Figure 1E), nNOS expression levels were only moderately increased in MG-132-treated skeletal muscle (Figure 2B).

The observation that MG-132 treatment is able to rescue dystrophin expression in mdx skeletal muscle fibers is noteworthy itself (Figures 1D and 2B). Interestingly, dystrophin migrates as a band of approximately 115 kd, instead of 426 kd expected for the full-length dystrophin. It has been previously shown that the mdx mouse lacks dystrophin expression due to a premature stop codon in the dystrophin gene. As a result, the dystrophin protein is produced as a truncated protein⁹ which, however, retains the N-terminal domain and is expected to migrate at \sim 120 kd, as we observe here.

It is important to note that, in this study, to detect dystrophin expression, we used a monoclonal antibody raised against the amino-terminal domain of dystrophin (NCL-DYS3). However, proteins with a premature termination codon are usually subjected to degradation.^{31,32} Localized treatment with the proteasomal inhibitor could block the degradation of dystrophin, and restore its robust expression in skeletal muscle fibers from mdx mice. Even if truncated, dystrophin might still retain residual function with a beneficial role for skeletal muscle functioning.

Importantly, the results obtained by immunoblot analysis are fully consistent with the results achieved by immunofluorescence analysis. Taken together, these data indicate that treatment of mdx skeletal muscles with the proteasomal inhibitor is able to prevent the degradation of dystrophin and other members of the DGC, and to rescue their expression and localization pattern. It is also remarkable that these effects are observed after only a 24-hour period of treatment.

Systemic Treatment with MG-132

Given the effectiveness of localized treatment, we next systemically administered MG-132 to mdx mice for an extended period. For this purpose, osmotic pumps (Alzet Minipumps) were implanted subcutaneously in the ante-

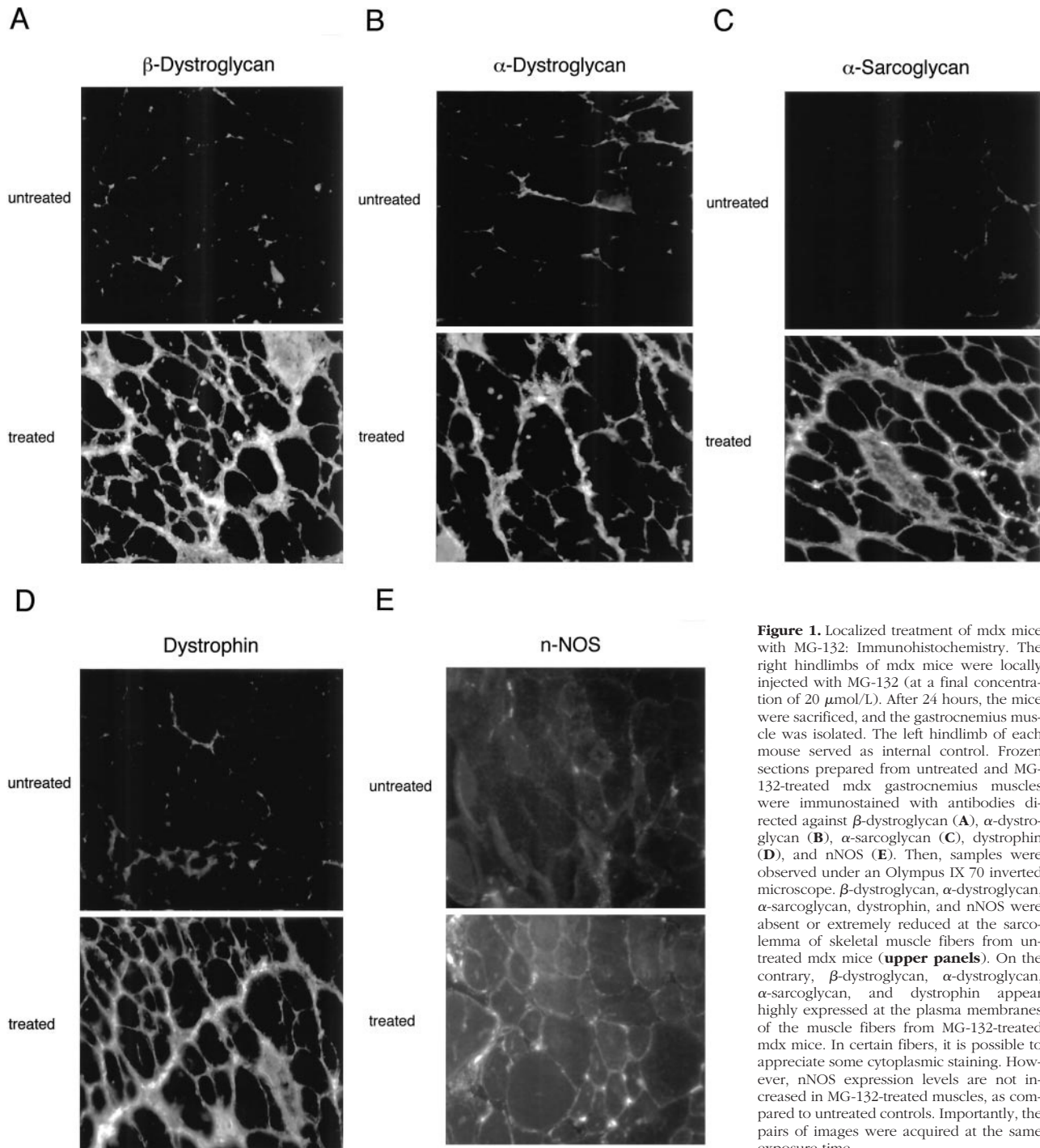


Figure 1. Localized treatment of mdx mice with MG-132: Immunohistochemistry. The right hindlimbs of mdx mice were locally injected with MG-132 (at a final concentration of 20 $\mu\text{mol/L}$). After 24 hours, the mice were sacrificed, and the gastrocnemius muscle was isolated. The left hindlimb of each mouse served as internal control. Frozen sections prepared from untreated and MG-132-treated mdx gastrocnemius muscles were immunostained with antibodies directed against β -dystroglycan (**A**), α -dystroglycan (**B**), α -sarcoglycan (**C**), dystrophin (**D**), and nNOS (**E**). Then, samples were observed under an Olympus IX 70 inverted microscope. β -dystroglycan, α -dystroglycan, α -sarcoglycan, dystrophin, and nNOS were absent or extremely reduced at the sarcolemma of skeletal muscle fibers from untreated mdx mice (**upper panels**). On the contrary, β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin appear highly expressed at the plasma membranes of the muscle fibers from MG-132-treated mdx mice. In certain fibers, it is possible to appreciate some cytoplasmic staining. However, nNOS expression levels are not increased in MG-132-treated muscles, as compared to untreated controls. Importantly, the pairs of images were acquired at the same exposure time.

rior portion of the backs of mdx mice. Using this approach, we could continuously deliver MG-132 to mdx mice over an 8-day period.¹⁹ We administrated different concentrations of MG-132 (delivered at rates of either 1 μg , 5 μg , or 10 $\mu\text{g/kg/24 hours}$), or the inhibitor-diluent (PBS only), as an important negative control. After 8 days of treatment, the mice were sacrificed. Skeletal muscle tissues were dissected and were subsequently subjected to immunofluorescence and Western blot analysis.

To assess whether systemic treatment with MG-132 would ameliorate the membrane damage of muscle fibers, we first examined the status of the diaphragm, which is the muscle most affected in mdx mice.^{33,34} For this reason, the diaphragm is useful for monitoring the degree of prevention of the dystrophic process achieved by treatment with the proteasomal inhibitor. To visualize muscular membrane damage, 20 hours before the end of the treatment, we injected EBD intraperitoneally into wild-

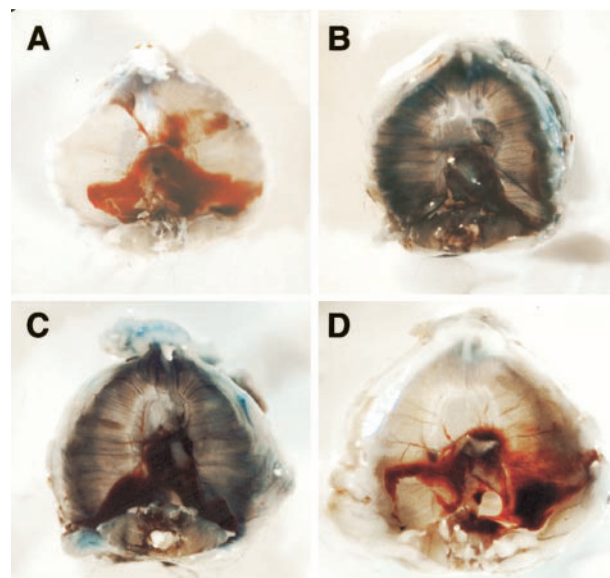
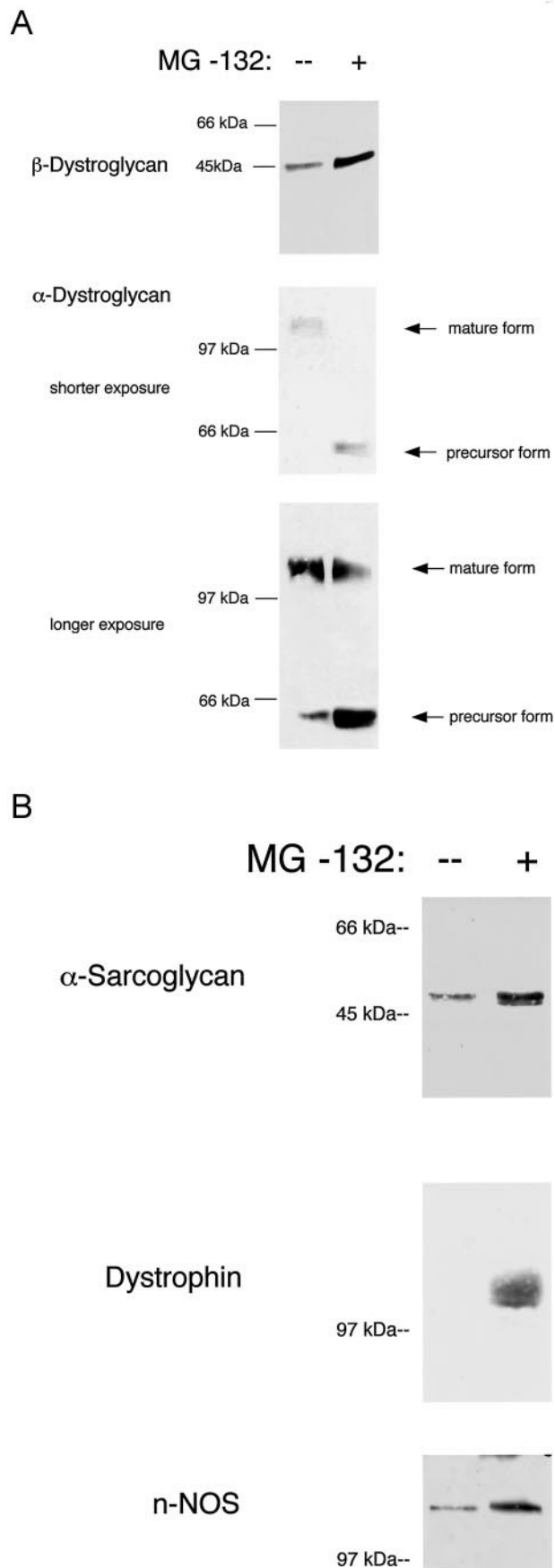


Figure 3. EBD staining of the diaphragm in mdx mice after systemic treatment with MG-132. To systemically administrate MG-132, we subcutaneously implanted osmotic pumps in 6-month-old mdx mice. Using this approach, we were able to deliver either MG-132 at a constant rate (either 1 μg , 5 μg , or 10 $\mu\text{g}/\text{kg}/24$ hours), over an 8-day period. As a negative control, 6-month-old mdx mice were implanted with osmotic pumps containing inhibitor-diluent (PBS). To evaluate muscle fiber damage, 20 hours before the end of treatment we injected EBD intraperitoneally into wild-type mice, untreated (PBS only) mdx mice and MG-132-treated mdx mice. EBD is used as marker of damaged fibers. After isolation, the diaphragms were rinsed in PBS, fixed in 10% formalin, and evaluated macroscopically. No evident uptake of the blue tracer was detected in fibers of the diaphragm from wild-type mice (**A**), indicating that the muscle fibers are not damaged, as expected. In contrast, many blue fibers were detected in the diaphragms of the untreated mdx mice (**B**), and from MG-132-treated mdx mice (delivered at a rate of 5 $\mu\text{g}/\text{kg}/24$ hours) (**C**). However, the diaphragms from MG-132-treated mdx mice (delivered at a rate of 10 $\mu\text{g}/\text{kg}/24$ hours) exhibit little or no blue fibers, suggesting that systemic administration of the proteasomal inhibitor can effectively improve the integrity of the skeletal muscle fiber plasma membrane in mdx mice (**D**).

type mice, control (PBS only) mdx mice, and MG-132-treated mdx mice. As it penetrates, EBD stains only damaged muscle fibers and is useful for evaluating the extent of muscle fiber degeneration.^{28,35} After isolation, the diaphragms were rinsed in PBS, fixed in 10% formalin, and evaluated macroscopically.

As shown in Figure 3A, diaphragms from WT mice do not show any blue-stained fibers, indicative of well-preserved normal fibers. On the contrary, we macroscopically observed many blue dye-stained fibers in the diaphragms from untreated mdx mice (Figure 3B) and from

Figure 2. Localized treatment of mdx mice with MG-132: Western blot analysis. The right hindlimbs of mdx mice were locally injected with MG-132 (at a final concentration 20 $\mu\text{mol}/\text{L}$). After 24 hours, the mice were sacrificed, and gastrocnemius muscle was isolated. The left hindlimb of each mouse served as internal control. Protein lysates were prepared from untreated and MG-132-treated mdx skeletal muscles. Equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose. Then, the blots were incubated with specific antibodies directed against β-dystroglycan, α-dystroglycan, α-sarcoglycan, dystrophin, and nNOS. Note that proteasomal inhibitor treatment induces an increase of the expression levels of β-dystroglycan, α-dystroglycan, α-sarcoglycan, and dystrophin in skeletal muscles from mdx mice. Interestingly, MG-132 treatment causes the accumulation of the precursor form of α-dystroglycan in skeletal muscle. Consistent with the results obtained from immunofluorescence analysis, nNOS expression levels are only moderately increased in MG-132-treated skeletal muscles, as compared with untreated controls.

treated mdx mice (MG-132 delivered at rate of 5 $\mu\text{g/kg/24 hours}$) (Figure 3C). Similar results were obtained from observation of the diaphragms from mdx mice, in which MG-132 was delivered at rate of 1 $\mu\text{g/kg/24 hours}$ (not shown). These results are suggestive of ongoing muscular damage. However, we could detect very few EBD-positive fibers in diaphragms from mdx mice which were infused with MG-132 at rate of 10 $\mu\text{g/kg/24 hours}$ (Figure 3D). These data suggest that the treatment with MG-132 (delivered at rate of 10 $\mu\text{g/kg/24 hours}$) may ameliorate muscle cell membrane damage, and possibly improve the muscle pathology. In addition, these results suggest that the effect is dependent on the drug dosage, since we could observe a positive change in fiber integrity only at the maximum dosage of the proteasomal inhibitor that we delivered.

We also attempted to evaluate whether improvement of muscle fiber lesions was restricted to the diaphragm, or whether the phenomenon was perceptible in other muscles. Since EBD emits red autofluorescence, we next examined frozen sections of gastrocnemius muscles from untreated (PBS only) and MG-132-treated mdx mice (delivered at rate of 10 $\mu\text{g/kg/24 hours}$) by fluorescence microscopy. As shown in Figure 4A, we could detect a strong red autofluorescence EBD signal in the skeletal muscle fibers from untreated mdx mice, suggesting that muscular fibers are indeed damaged. In contrast, skeletal muscle fibers from MG-132-treated mdx mice did not show any red autofluorescence signal (Figure 4B). These findings are fully consistent with our results using the diaphragm, and further corroborate the idea that the treatment with MG-132 delivered at rate of 10 $\mu\text{g/kg/24 hours}$ may improve the integrity of muscle fibers.

As treatment with a proteasomal inhibitor seemed to ameliorate the cell membrane lesions, as judged by EBD staining of the diaphragm and the gastrocnemius muscle, we next evaluated whether MG-132 treatment would improve the overall muscle pathology. Thus, we performed H&E staining of frozen gastrocnemius sections from untreated (PBS only) mdx mice and MG-132-treated (delivered at rate of 10 $\mu\text{g/kg/24 hours}$) mdx mice. Skeletal muscle biopsies from untreated mdx mice showed clear signs of myopathic changes, including muscle fiber regeneration with many central nuclei, variability in fiber diameter, lymphocytic invasion, and lipidic vesicles (Figure 5, A and B). Interestingly, analysis of skeletal muscle biopsies from MG-132-treated mdx mice revealed few centrally nucleated muscle fibers, more uniform fiber diameters, and no signs of lymphocytic invasion, suggesting that the inflammatory process was reduced and that signs of muscular dystrophy were greatly reduced (Figure 5, A and B). These results further corroborate the idea that, over an 8-day period, systemic treatment with MG-132 may significantly improve the muscle pathology.

To evaluate whether MG-132 systemic treatment would rescue the expression level, and the localization pattern of dystrophin and other members of the DGC, we examined skeletal muscle biopsies from untreated (PBS only) mdx mice and MG-132-treated mdx mice by immunofluorescence analysis.

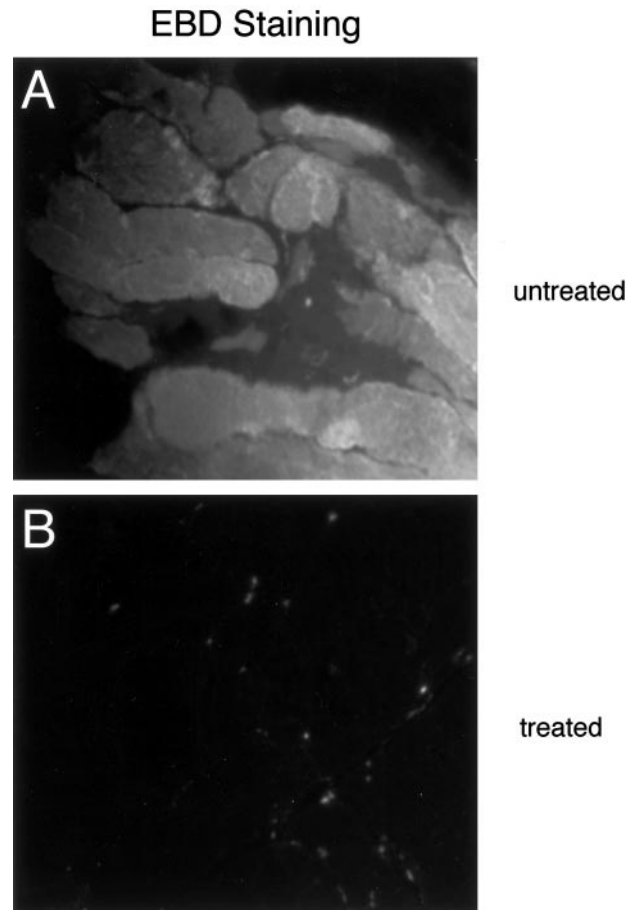


Figure 4. EBD staining of mdx gastrocnemius muscle biopsies after systemic treatment with MG-132. We systemically administered the proteasomal inhibitor in 6-month-old mdx mice, by subcutaneous implantation of osmotic pumps. Using this approach, we were able to deliver MG-132 at constant rate (either 1 μg , or 5 μg or 10 $\mu\text{g/kg/24 hours}$) or inhibitor diluent (PBS) for a period of 8 days. To evaluate muscle fiber damage, 20 hours before the end of the treatment, we intraperitoneally injected EBD into wild-type mice, untreated (PBS only) mdx mice and MG-132-treated mdx mice. EBD staining is a marker of damaged muscle fibers. As EBD emits red autofluorescence, we examined frozen sections of gastrocnemius muscle biopsies taken from wild-type mice, untreated (PBS only) mdx mice and MG-132-treated mdx mice by fluorescence microscopy. **A:** Strong red autofluorescent signal is present in myofibers from untreated mice. **B:** In contrast, myofibers from MG-132-treated mice (delivered at rate of 10 $\mu\text{g/kg/24 hours}$) did not show any red fluorescent signal; similar results were obtained with wild-type mice (not shown). These results suggest that MG-132 systemic treatment effectively ameliorates the skeletal muscle fiber damage.

Frozen sections of gastrocnemius muscles were immunostained using antibodies directed against β -dystroglycan (Figure 6A), α -dystroglycan (Figure 6B), α -sarcoglycan (Figure 6C), dystrophin (Figure 6D), nNOS (Figure 6E), and Cav-3 (Figure 6F). As expected, β -dystroglycan, α -dystroglycan, α -sarcoglycan, dystrophin, and nNOS were absent or greatly reduced in skeletal muscle fibers from untreated (PBS only) mdx mice (Figure 6, A to E, upper panels). Interestingly, the expression levels of β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin were significantly augmented in skeletal muscle fibers from MG-132-treated mdx mice. Figure 6, A to D (lower panels) show that β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin were able to properly reach the plasma membrane in skeletal muscle fibers

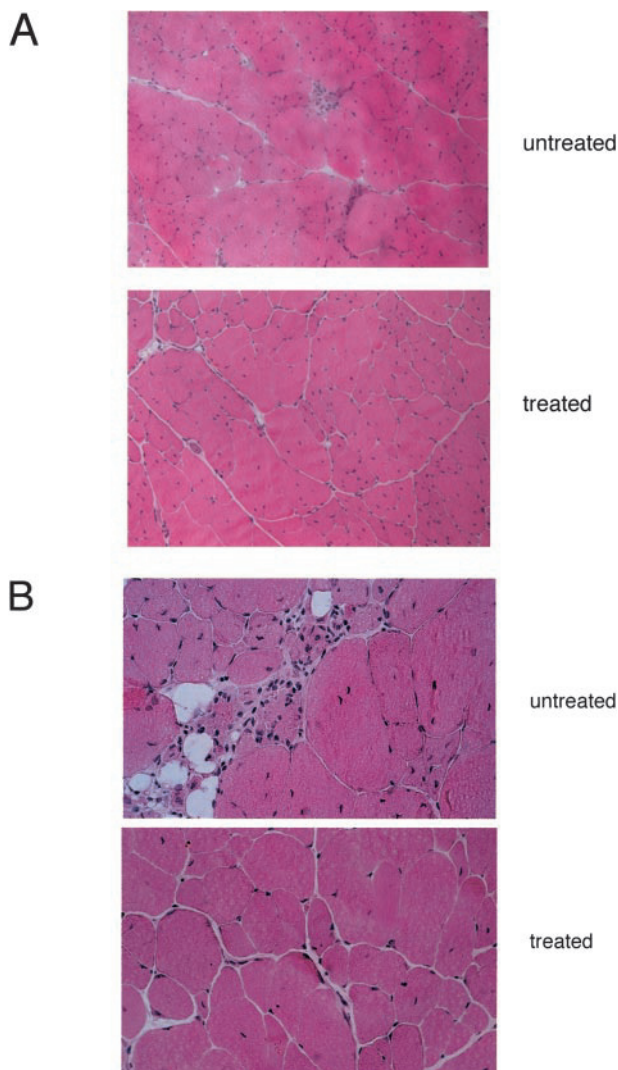


Figure 5. Histological analysis of H&E-stained skeletal muscle fibers after systemic treatment with MG-132. Mdx mice were systemically treated with MG-132 (delivered at rate of 10 $\mu\text{g/kg/24}$) for 8 days. H&E staining was performed on frozen skeletal muscle tissue sections from untreated mdx mice and MG-132-treated mdx mice. Skeletal muscle biopsies from untreated mdx mice (PBS) show signs of myopathic changes, including variability in fiber diameter, lymphocytic infiltration, lipidic droplets, and muscle fiber regeneration with central nuclei. In contrast, analysis of skeletal muscle biopsies from MG-132-treated mdx mice revealed few centrally nucleated muscle fibers and no signs of lymphocytic invasion, suggesting that the inflammatory process was reduced, and that the histopathological appearance was greatly improved. **A:** Original magnification, $\times 20$. **B:** Original magnification, $\times 40$.

from MG-132-treated mdx mice. In contrast, the expression levels of nNOS and Cav-3 were not significantly changed after treatment (Figure 6E). It is important to note that we could rescue the expression level of α -dystroglycan, α -sarcoglycan, and dystrophin by infusing mdx mice with MG-132 at the rate of 10 $\mu\text{g/kg/24}$ hours. In contrast, the rescue of the expression level of β -dystroglycan was achieved by delivering MG-132 at the rate of 1 $\mu\text{g/kg/24}$ hours.

Next, skeletal muscle lysates from untreated (PBS only) and from MG-132-treated (delivered at rates of 1 μg , 5 μg , and 10 $\mu\text{g/kg/24}$ hours) mdx mice were subjected to Western blot analysis with specific antibodies

directed against β -dystroglycan, α -dystroglycan, α -sarcoglycan, dystrophin, nNOS, and Cav-3. Figure 7 shows that the expression levels of β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin were greatly increased in skeletal muscle lysates from MG-132-treated mdx mice, as compared with untreated mdx mice. In accordance with what we observed by immunofluorescence analysis, β -dystroglycan expression levels were augmented by infusing mdx mice with MG-132 at a concentration of 1 $\mu\text{g/kg/24}$ hours, whereas α -dystroglycan, α -sarcoglycan, and dystrophin expression levels were rescued by delivering MG-132 at a concentration of 10 $\mu\text{g/kg/24}$ hours. One possible explanation could be that β -dystroglycan is more sensitive to prolonged exposure to the proteasomal inhibitor. In addition, Figure 7A shows that systemic treatment with the proteasomal inhibitor rescued the expression level of the precursor form of α -dystroglycan, in accordance with what we observed using localized treatment with MG-132 (Figure 2A). Consistent with the immunofluorescence data, we did not observe any changes in nNOS expression levels in skeletal muscle lysates from MG-132-treated mdx mice, as compared with untreated controls.

Previous studies have shown that Cav-3 expression levels are increased by ~ 2 -fold in skeletal muscle fibers from mdx mice,¹² as well in skeletal muscle fibers from DMD patients.¹³ Figure 7B reveals that Cav-3 expression levels are decreased in skeletal muscle extracts from MG-132-treated mdx mice, as compared with the untreated (PBS only) counterparts. Therefore, we speculate that treating mdx mice with the proteasomal inhibitor induces a decrease in Cav-3 expression levels. This finding highlights a discrepancy between our results obtained by immunofluorescence and Western blot analysis. One possible explanation is that immunoblotting is more sensitive than immunofluorescence and can, therefore, better reveal relatively small changes in protein expression levels.

To evaluate the possible involvement of the non-lysosomal protease calpain in protein degradation in mdx muscles, and to assess whether MG-132 efficiently and specifically blocks the ubiquitin-proteasome pathway, we analyzed calpain activity levels in skeletal muscle lysates from untreated and MG-132-treated mdx mice, using a fluorometric assay system.

Figure 8 shows that the levels of calpain activity were mildly increased in skeletal muscles from mdx mice *versus* wild-type mice. However, the levels of calpain activity were not significantly altered in skeletal muscle samples taken from MG-132-treated mdx mice, suggesting that calpain may play a role in the pathology of mdx mice, but that MG-132 treatment specifically targets the proteasomal pathway.

Taken together, these results directly support the idea that the proteasomal pathway is involved in DGC protein degradation in dystrophin-deficient muscle. The present findings demonstrate that treating mdx mice with MG-132 can efficiently block the degradation of dystrophin and of dystrophin-associated proteins, and can rescue their ex-

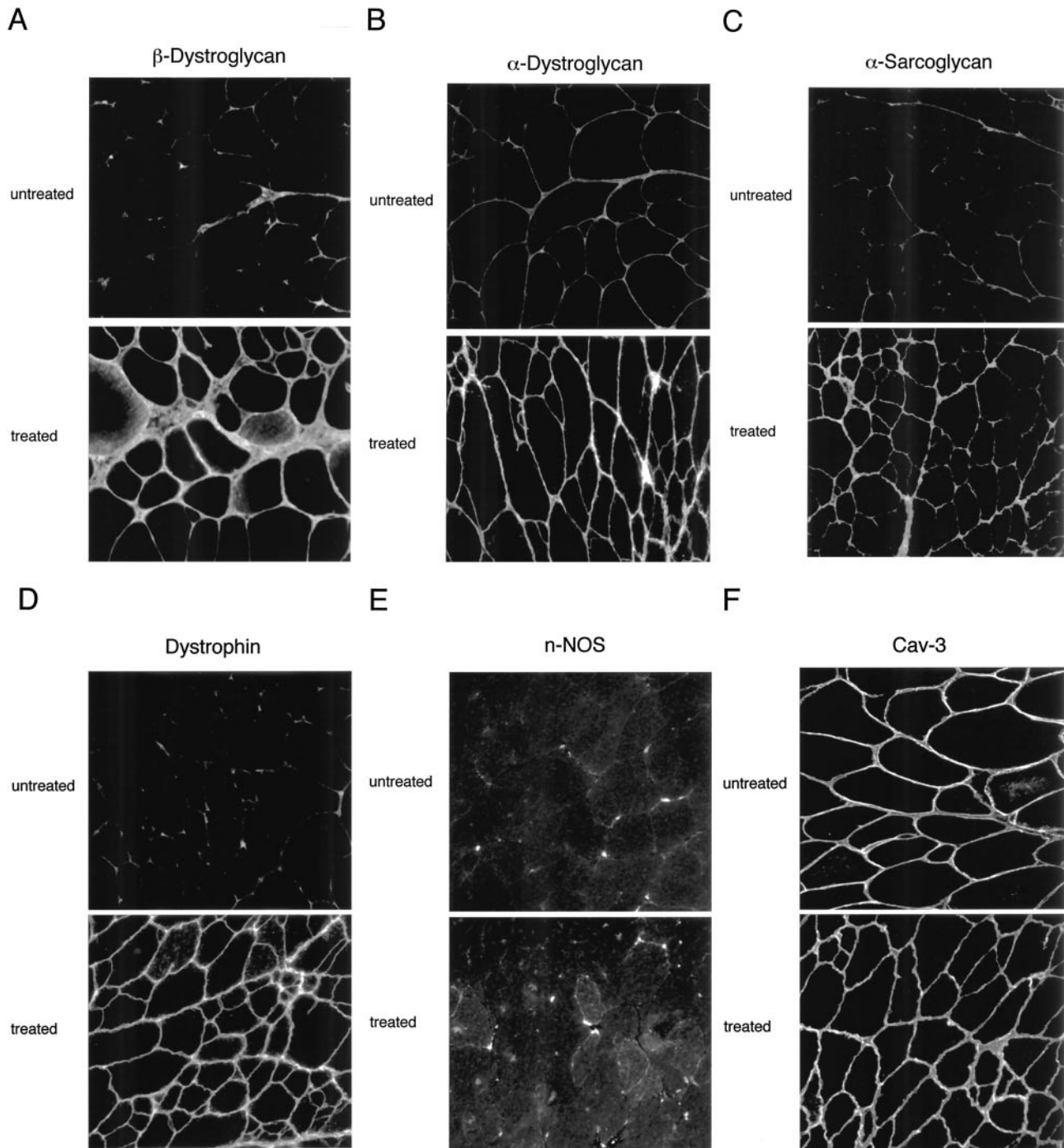


Figure 6. Systemic treatment of mdx mice with proteasomal inhibitor: immunohistochemistry. To systemically administer the proteasomal inhibitor, we subcutaneously implanted osmotic pumps in 6-month-old mdx mice. Using this approach, we were able to deliver MG-132 at a constant rate (either 1 μ g, 5 μ g, or 10 μ g/kg/24 hours) or inhibitor-diluent (PBS) for a period of 8 days. Frozen sections of gastrocnemius muscles from untreated (PBS only) mdx mice and MG-132-treated mdx mice were subjected to immunofluorescence analysis with antibodies directed against β -dystroglycan (**A**), α -dystroglycan (**B**), α -sarcoglycan (**C**), dystrophin (**D**), nNOS (**E**), and Cav-3 (**F**). As expected, β -dystroglycan, α -dystroglycan, α -sarcoglycan, dystrophin, and nNOS were absent or greatly reduced in skeletal muscle from untreated mdx mice (PBS only), whereas Cav-3 was augmented in skeletal muscles from the same mice (upper panels). Surprisingly, the expression levels of β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin were rescued in skeletal muscle from MG-132-treated mdx mice. However, after MG-132 systemic treatment, we did not detect any significant increases in the expression levels of nNOS and Cav-3 in skeletal muscles fibers. Interestingly, we achieved rescue of the expression levels of α -dystroglycan, α -sarcoglycan, and dystrophin by delivering MG-132 at the rate of 10 μ g/kg/24 hours, whereas the rescue of β -dystroglycan was accomplished by delivering MG-132 at the rate of 1 μ g/kg/24 hours. Importantly, pairs of images (treated *versus* untreated) were acquired with the same exposure time.

pression and subcellular localization at the level of the sarcolemma, as judged by immunofluorescence and Western blot analysis. Similar results were obtained by localized injection and by systemic infusion with MG-132.

In addition, histological analysis shows that systemic administration of the proteasomal inhibitor may ameliorate muscle fiber degeneration, improving skeletal muscle pathology.

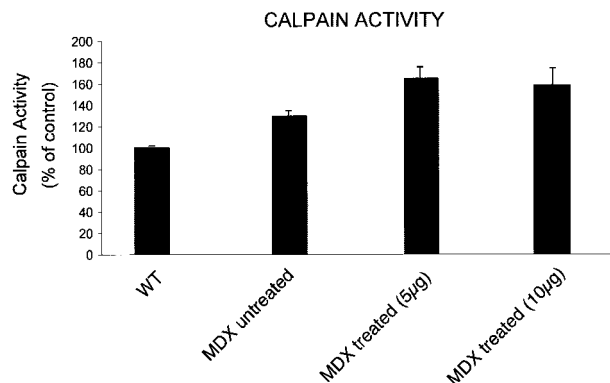
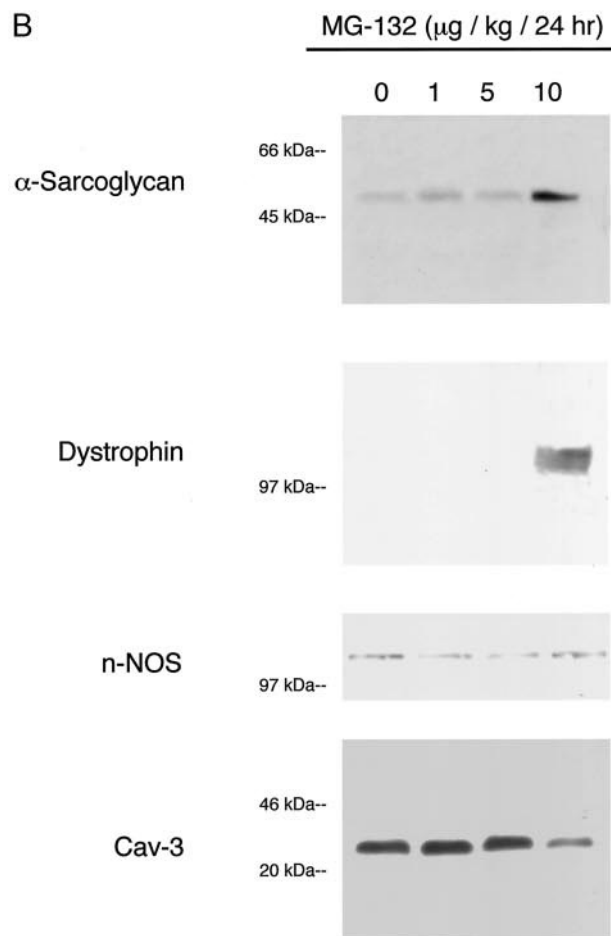
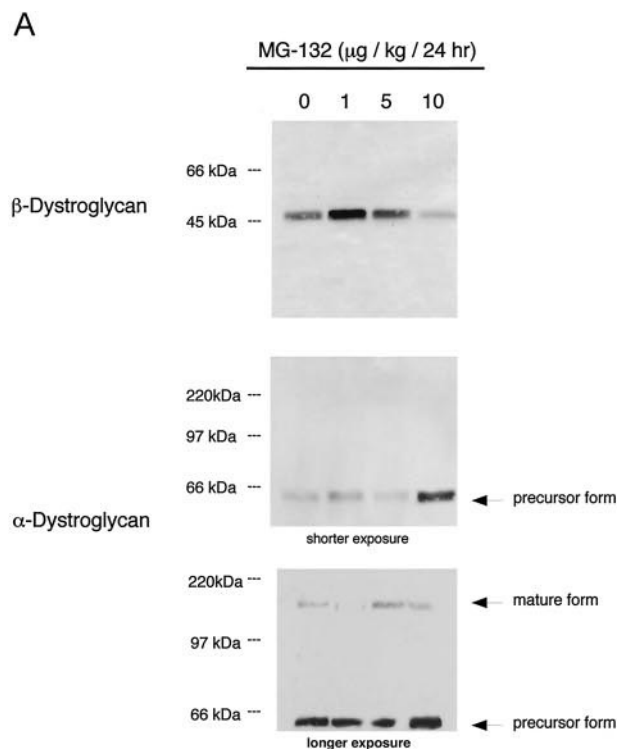


Figure 8. Treatment with MG-132 does not significantly affect calpain activity in muscle samples derived from mdx mice. Calpain activity in skeletal muscles samples was measured using a fluorimetric assay (Oncogene Research Products). Calpain activity is expressed as % of control (wild-type untreated mice). The data shown are means \pm SD of four independent experiments, each performed in duplicate. Note that calpain activity levels are increased in mdx mice *versus* wild-type mice. Importantly, there is no significant change in the levels of calpain activity in MG-132-treated mdx mice (MG-132 delivered at rate of 5 μg and 10 $\mu\text{g}/\text{kg}/24$ hours), as compared with untreated mdx mice.

Discussion

Dystrophin, the protein product of the DMD gene, is absent in the skeletal muscle fibers of DMD patients and mdx mice. At the plasma membrane of normal skeletal muscle fibers, dystrophin associates with a multimeric protein complex, termed the DGC. The complete loss of dystrophin perturbs the structural composition of the DGC, and induces the loss of expression of the components of the DGC itself. Previous studies have suggested that the loss of dystrophin in mdx and DMD muscle cells may render the DGC more susceptible to proteolytic degradation.¹⁴ However, the role of the ubiquitin-proteasome pathway in dystrophin-deficient skeletal muscle fibers remains controversial.

Previous studies have shown that the proteasomal pathway is involved in the pathogenesis of various muscle diseases, and have demonstrated that *in vitro* inhibition of the proteasomal machinery can effectively im-

Figure 7. Systemic treatment of mdx mice with proteasomal inhibitor: Western blot analysis. To systemically administer the proteasomal inhibitor, we subcutaneously implanted osmotic pumps in 6-month-old mdx mice. Using this approach, we were able to deliver MG-132 at a constant rate (either 1 μg , 5 μg , or 10 $\mu\text{g}/\text{kg}/24$ hours) or inhibitor-diluent (PBS only) for a period of 8 days. Lysates of gastrocnemius muscle biopsies were prepared from untreated mdx mice and MG-132-treated mdx mice. Equal amounts of total protein were separated by SDS-PAGE and transferred onto nitrocellulose. The expression levels of β -dystroglycan, α -dystroglycan, α -sarcoglycan, dystrophin, nNOS, and Cav-3 were assessed by Western blot analysis, using specific antibodies. Interestingly, the expression levels of β -dystroglycan, α -dystroglycan, α -sarcoglycan, and dystrophin are increased in skeletal muscles from MG-132-treated mdx mice, as compared to their untreated (PBS only) counterparts. The rescue of β -dystroglycan was achieved by delivering MG-132 at the rate of 1 $\mu\text{g}/\text{kg}/24$ hours of MG-132, while the rescue of the expression levels of α -dystroglycan, α -sarcoglycan, and dystrophin was accomplished by delivering MG-132 at a rate of 10 $\mu\text{g}/\text{kg}/24$ hours. Note that the systemic treatment rescues the precursor form of the α -dystroglycan, consistent with the results we obtained with localized treatment using MG-132 (see Figure 2). Consistent with the results we obtained by immunofluorescence analysis, nNOS expression levels are not increased in skeletal muscles from MG-132-treated mdx mice, as compared with their untreated counterparts. Interestingly, treatment of mdx mice with MG-132 actually reduces the expression levels of Cav-3.

prove the phenotype of cultured cells. In a heterologous cell system, MG-132 was shown to successfully block the protein degradation of an autosomal dominant mutant form of Cav-3 seen in LGMD-1C.²³ In addition, other evidence has suggested that muscle wasting results primarily from acceleration of the proteasomal pathway. Incubation of atrophying rat skeletal muscles with inhibitors of the proteasome (ALLN and MG-132) could suppress protein breakdown and improve overall protein balance.¹⁸

In light of this evidence, we reasoned that treatment of mdx mice with inhibitors of the proteasomal pathway could potentially rescue the expression level and localization pattern of dystrophin and of dystrophin-associated proteins. To directly test this hypothesis, we treated mdx mice with MG-132. First, we locally injected MG-132 into the gastrocnemius muscle of mdx mice, and observed the outcome after 24 hours of treatment. Second, we systemically administered MG-132, by subcutaneously implanting osmotic pumps within mdx mice. Using this approach, we were able to continuously infuse MG-132 at a constant rate over an 8-day period.

Here, we show that *in vivo* administration of MG-132 effectively restores the expression levels and the localization pattern of dystrophin and of dystrophin-associated proteins, normally absent or greatly reduced in mdx skeletal muscles. Our results clearly show that the levels of dystrophin, β -dystroglycan, α -dystroglycan, and α -sarcoglycan are elevated, and are able to properly reach the plasma membrane in skeletal muscle fibers from MG-132-treated mdx mice, as compared with untreated controls. These results were confirmed by the use of two independent methodological approaches, eg, immunofluorescence microscopy and Western blot analysis. Similar results were obtained from the analysis of skeletal muscle fibers from mdx mice in which MG-132 was locally injected, and from mdx mice in which the proteasomal inhibitor was systemically administered. The current findings directly support the idea that the proteasomal pathway plays a substantial role in protein degradation in dystrophin-deficient muscle. These results may be important in elucidating the molecular mechanisms underlying the pathogenesis of DMD.

The finding that MG-132 treatment could rescue the expression level and subcellular localization of dystrophin in mdx mice is noteworthy itself. However, it is surprising that the truncated dystrophin protein can properly reach the plasma membrane. As mentioned previously, in mdx mice, dystrophin lacks the carboxyl-terminal domain, which is thought to be necessary for binding to β -dystroglycan, and consequently targeting to the plasma membrane. Here, by immunofluorescence analysis, we show that a truncated form of dystrophin is able to reach the plasma membrane and possibly to interact with the DGC complex, through a yet uncovered mechanism. Even though expressed as a truncated protein, dystrophin could still retain residual activity with a beneficial role for muscle functioning. These findings may be of vital importance in the treatment of DMD.

Analysis of skeletal muscle fibers from mdx mice, which were systemically infused with MG-132, provides

further insight into the functional and physiological relevance of proteasomal inhibitor treatment, as judged by staining of the diaphragm and skeletal muscle tissue sections with a vital dye (Evans blue), and by histological examination of muscle biopsies. The muscle fiber integrity appears to be greatly improved by MG-132 treatment. By using the vital staining of EBD as a marker for injured muscles, we could evaluate the degeneration of muscle fibers. Diaphragms isolated from MG-132-treated mdx mice show few blue-stained fibers, as compared with diaphragms isolated from untreated mdx mice, suggesting that overall cell membrane integrity is possibly restored by treatment with the proteasomal inhibitor. Moreover, examination of skeletal muscle frozen sections by fluorescent microscopy further corroborates the idea that the integrity of muscle fibers is improved after treatment with the proteasomal inhibitor. Untreated mdx skeletal muscle fibers show a strong red autofluorescent EBD signal, whereas MG-132-treated mdx skeletal muscle fibers show little or no signal. Taken as a whole, these data suggest that treatment with MG-132 ameliorates the cell membrane lesions.

Histological examination of frozen sections from untreated and MG-132-treated mdx mice further corroborated the idea that proteasomal inhibitor administration can effectively improve the overall muscle pathology. Skeletal muscle biopsies from untreated mdx mice show all of the key histological features of muscular dystrophy, including variable fiber size, lymphocytic infiltration, lipidic vesicles, and the presence of central nuclei. However, these signs were greatly diminished in skeletal muscle biopsies from MG-132-treated mice, suggesting that proteasomal inhibitor administration can dramatically improve the histopathological appearance of dystrophin-deficient skeletal muscle.

Since it is well documented that the calpain pathway may also be involved in mediating muscle damage in the mdx model of muscular dystrophy,^{36,37} we have investigated the activity levels of calpain in skeletal muscles from untreated and MG-132-treated mdx mice. Calpain is a calcium-dependent cysteine protease, which cleaves many cytoskeletal and myelin proteins. By measuring calpain activity, we have demonstrated the specificity of MG-132 treatment. Indeed, our results show that MG-132 exerts no significant effect on the calpain activity, strongly supporting the specific effects of MG-132 in the inhibition of the ubiquitin-proteasome pathway.

Mechanical damage to the cell membrane is still considered as one of the main causes leading to the pathology in dystrophin-deficient skeletal muscle. Supporting evidence arose from the spatial and functional role that dystrophin is thought to play inside the cell. The N-terminal region of dystrophin interacts directly with the cytoskeletal protein actin, while the dystrophin C-terminal domain binds to the plasma membrane, through interaction with β -dystroglycan. As such, dystrophin is thought to provide, in contracting cells, an essential mechanical linkage between the intracellular cytoskeleton and the extracellular matrix. Loss of dystrophin leads to the disruption of this crucial link, and induces mechanical damage to the cell membrane.¹¹ In this report, we provide

evidence that correlates the rescue of expression of dystrophin and dystrophin-associated proteins with improvement of cell membrane lesions, and with amelioration of muscle damage. As such, these results directly support the idea that dystrophin plays a key role in membrane stability and that increasing the expression of dystrophin and of dystrophin-associated proteins can prevent muscle damage and allow the recovery of membrane stability. However, the fact that nNOS and β -dystroglycan expression levels are not rescued at concentrations of the proteasomal inhibitor that effectively improve muscle pathology suggests that these proteins are not necessary for the amelioration of the pathological phenotype.

Interesting insights into muscle membrane biology are gained from the finding that Cav-3 expression levels, normally elevated in dystrophin-deficient skeletal muscles, are decreased on systemic treatment with MG-132. Several lines of evidence have demonstrated the importance of muscle cell caveolae in the pathogenesis of DMD.^{12,13,38} Over two decades ago, freeze-fracture studies have demonstrated that skeletal muscle caveolae undergo particular changes in their size and distribution in patients with DMD. More recent reports have confirmed that skeletal muscle biopsies from DMD patients show an increased number of caveolae, and overexpression of the Cav-3 protein product.¹³ In addition, Cav-3 expression was shown to be elevated by ~2-fold in skeletal muscle samples from mdx mice.¹² Conversely, transgenic over-expression of Cav-3 in skeletal muscle fibers induces a Duchenne-like muscular dystrophy phenotype, and causes the down-regulation of dystrophin and of dystrophin-associated proteins.³⁹ Taken together, these results suggest that a particular ratio between Cav-3 and dystrophin may be crucial for the proper functioning of skeletal muscle. Recent studies have attempted to gain insight in the molecular basis of these observations, and have demonstrated that Cav-3 directly interacts with β -dystroglycan, at the same or an overlapping site as dystrophin. As a consequence, the interaction of Cav-3 with β -dystroglycan may competitively regulate the recruitment of dystrophin at the plasma membrane, and may control the functional assembly of the DGC.⁴⁰ Since the disruption of the delicate balance between Cav-3 and dystrophin expression levels may play a critical role in the pathogenesis of DMD, the reduction in Cav-3 expression levels that we detect in skeletal muscles after MG-132 systemic treatment may be important for the improvement of the pathological phenotype.

At the moment, no pharmacological treatment is available for DMD. Several genetic approaches have been explored for the therapeutic treatment of DMD. For example, adenovirus-mediated transfer of human minidystrophin genes was shown to effectively ameliorate muscular dystrophy in mdx mice.⁴¹ Others groups have studied the effect of transgenic over-expression of utrophin, a ubiquitous homologue of dystrophin, in mdx mice.^{42,43} In addition, a NOS transgene has been shown to reduce muscle membrane damage and inflammatory processes in mdx mice.⁴⁴ However, the development of such gene therapies is weakened by the difficulty of selectively delivering the target genes to all of the af-

fected muscle cells. An alternative approach to DMD therapy used stem cell transplantation. Intravenous injection of stem cells into lethally irradiated mdx mice resulted in the partial restoration of dystrophin expression in the affected muscle.⁴⁵ However, the complete suppression of the immune system remains a major problem.

Here, we suggest the possibility of a different route of therapy for DMD, based on a pharmacological approach. Using an inhibitor of the proteasomal pathway (MG-132), we could effectively block the degradation of dystrophin and of dystrophin-associated proteins, and we could ameliorate myopathic changes normally seen in the skeletal muscle fibers of mdx mice. As such, pharmacological administration of oral proteasomal inhibitors may provide a simpler approach to DMD therapy. Further studies on primary cultures of skeletal muscle cells isolated from DMD patients are necessary to test the effectiveness of this therapeutic approach. Also, additional analysis should be performed to evaluate the long-term toxicity of the proteasomal inhibitors to be used.

Acknowledgments

We thank Dr. R. Campos-Gonzalez (BD Transduction Laboratories) for donating mAbs directed against caveolin-3.

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