Musculoskeletal Pathology

Modulation of p38 Mitogen-Activated Protein Kinase Cascade and Metalloproteinase Activity in Diaphragm Muscle in Response to Free Radical Scavenger Administration in Dystrophin-Deficient Mdx Mice

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Duchenne muscular dystrophy muscles undergo increased oxidative stress and altered calcium homeostasis, which contribute to myofiber loss by triggering both necrosis and apoptosis. Here, we asked whether treatment with free radical scavengers could improve the dystrophic pattern of mdx muscles. Five-week-old mdx mice were treated for 2 weeks with α -lipoic acid/L-carnitine. This treatment decreased the plasmatic creatine kinase level, the antioxidant enzyme activity, and lipid peroxidation products in mdx diaphragm. Free radical scavengers also modulated the phosphorylation/activity of some component of the mitogen-activated protein kinase (MAPK) cascades: p38 MAPK, the extracellular signal-related kinase, and the Jun kinase. β -Dystroglycan (β -DG), a multifunctional adaptor or scaffold capable of interacting with components of the extracellular signal-related kinase-MAP kinase cascade, was also affected after treatment. In the mdx muscles, β -DG (43 kD) was cleaved by matrix metalloproteinases into a 30-kD form (β -DG₃₀). We show that the proinflammatory protein nuclear factor- κ B activator decreased after the treatment, leading to a significant reduction of matrix metalloproteinase activity in the mdx diaphragm. Our data highlight the implication of oxidative stress and cell signaling defects in dystrophin-deficient muscle via the MAP kinase cascade- β -DG interaction and nu-

clear factor- κ B-mediated inflammation process. (Am J Pathol 2007; 170:633–643; DOI: 10.2353/ajpath.2007.060344)

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder caused by mutations in the dystrophin gene.¹ Dystrophin links intracellular actin and extracellular laminin via its interaction with the dystrophin-glycoprotein complex composed of dystroglycans and sarcoglycans.^{2,3} In the cytoplasmic region, dystrophin interacts with the dystrophin-associated protein complex, ie, dystrobrevin, syntrophins, and the neuronal isoform of nitric synthase (nNOS).^{4–6} Besides providing mechanical stability,⁷ several proteins of the dystrophin-glycoprotein complex play a role in cell signaling.^{8,9} The dystroglycan complex is an important adhesion receptor and has a vital role in maintaining muscle integrity: its loss leads to muscular dystrophy,¹⁰ and it has recently been implicated in the maintenance of cell polarity.^{11,12} Previous studies have also shown that β -dystroglycan (β -DG), the transmembrane component of the dystroglycan complex that links the C-terminal region of dystrophin, is associated with a number of adaptor proteins involved in a variety of signaling cascades.^{13,14} It has also been demonstrated that the interaction of dystroglycan with laminin has an inhibitory effect on the activation of the extracellular signal-regulated kinase (ERK)-mitogen-activated protein (MAP) kinase cascade in response to the binding of integrins to laminin.⁸ The ability of dystroglycan to interact with several components of ERK-MAP kinase

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cascade may be part of the mechanism involved in dystroglycan-modulating ERK activity in response to several cell stimuli.^{8,15}

Altered cell signaling is thought to increase the susceptibility of muscle fibers to secondary triggers, such as functional ischemia and oxidative stress.¹⁶ Several lines of evidence suggest that free radical injury to the membrane may contribute to the loss of membrane integrity in muscular dystrophies.¹⁷ The increased action of oxidative stress in DMD muscle is indicated in part by increased changes in proteins,¹⁶ enhanced lipid peroxidation, and induction of antioxidant enzymes.¹⁷ Heme oxygenase-1 (HO-1) and the metabolites produced from its action on heme play a key role in protection against the oxidative stress and inflammation associated with several diseases.¹⁸ HO-1 induction inhibits cytokine production in macrophages and monocyte adherence.¹⁹ Moreover, it has been shown that HO-1 can be up-regulated under inflammatory conditions in several tissues, and induction of HO-1 *in vivo* occurs as a response to cytokine released locally at the site of inflammation.²⁰ The dystrophic muscle displays inflammatory cell infiltration reflecting the immune response to tissue damage.²¹ Despite these findings, no study has yet investigated the regulation of HO-1 in dystrophic muscles. The nuclear factor (NF)- κ B is also activated in response to several inflammatory molecules that cause muscle loss.^{22,23} NF- κ B is a ubiquitous transcription factor regulating the expression of a plethora of genes involved in inflammatory, immune, and acute stress responses.²⁴ After proteasomal degradation of the inhibitory protein (I- κ B), NF- κ B translocates to the nucleus and binds target DNA elements in the promoter of different genes resulting in the expression of cytokines, chemokines, cell adhesion molecules, immunoreceptors, and inflammatory enzymes such as neuronal nitric-oxide synthase (nNOS) and matrix metalloproteinases (MMPs).^{25,26} Recently, NF- κ B activity has been demonstrated to be increased in the muscle of both DMD patients and mdx mice.²² Novel observations have also reported an increased immunoreactivity of NF- κ B in the cytoplasm of all regenerating fibers and in 20 to 40% of necrotic fibers in DMD.²⁷ This evidence suggests that reactive oxygen intermediates might be involved in the dystrophic process, triggering an inflammatory cascade that leads to NF- κ B activation and to subsequent release of inflammatory mediators. The modulation of the NF- κ B activity by oxidative stress/lipid peroxidation inhibition may influence the skeletal muscle pathology in mdx mice with respect to the functional, morphological, and biochemical pattern.²⁸ Taken together, these findings indicate that interruption of oxidative stress and the inflammatory mediator cascade might have a therapeutic potential. As yet, no study has investigated the impact of such inhibition on the dystrophin-associated protein complex and the associated signaling pathways interacting with some of the DAG components. To clarify this issue we have used a pharmacological tool, free radical scavengers such as α -lipoic acid (ALA) and L-carnitine (L-Car). L-Car has a beneficial effect on pathological cells, in which it is possible to recognize free radicals as a potential mediator of

cellular damage.²⁹ It has been shown to scavenge superoxide anion, to inhibit lipoperoxidation of linoleic acid, and to protect against damage induced by H₂O₂.³⁰ Likewise, the reduced form of ALA reacts with oxidants such as superoxide radicals, hydroxyl radicals, peroxy radicals, and singlet oxygen. It also protects membranes by reducing oxidized vitamin C and glutathione.³¹ This study demonstrated that *in vivo* ALA/L-Car treatment re-establishes antioxidant enzymes activity [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)], decreases the index of lipid peroxidation (thiobarbituric acid reactive substances) and plasmatic creatine kinase (CK) level, and improves the dystrophic pattern in the mdx diaphragm muscle. We showed that the oxidative status can control β -DG processing by inhibiting NF- κ B and MMP activity. The second important point is that free radical scavengers can directly have an impact on activity/phosphorylation of some component of the MAP kinase cascade in the mdx diaphragm. This study highlights the use of free radical scavengers to counterbalance the progression of muscle injury in dystrophin-deficient muscles.

Materials and Methods

Animals

Five-week-old male wild-type C57BL/10 (Wt) and dystrophin-deficient (mdx) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in our animal facilities. Mice were housed in plastic cages in a temperature-controlled environment with a 12-hour light/dark cycle and free access to food and water. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, Bethesda, MD. The animals were sacrificed by rapid cervical dislocation, and experiments were carefully designed to minimize the number of animals and their suffering. Animals (Wt, $n = 7$; mdx, $n = 8$) were treated for 2 weeks with intraperitoneal injections of α -lipoic acid/L-carnitine (250- μ l vol at a cc of 250 mg/kg; Sigma, St. Louis, MO) dissolved in physiological saline solution (Ss). Control groups were injected with saline solution (mdx-Ss, $n = 7$; wild-type-Ss, $n = 8$). At the end of the experiment, animals were weighed and sacrificed by cervical dislocation. After decapitation, blood was collected to analyze CK levels, and the diaphragm was dissected into two parts. One part was immediately frozen in liquid nitrogen-cooled isopentane and stored at -80°C , and the other part was used to determine antioxidant enzymatic activities and lipid peroxidation level.

Serum CK Evaluation

After animal decapitation, blood samples were collected in ethylenediaminetetraacetic acid and centrifuged at 6000 rpm for 10 minutes. The serum was stored at -80°C until analysis. Serum CK was evaluated at 37°C by standard spectrophotometric analysis using a commercially

available kit (Randox Laboratory Ltd., Antrim, UK). The results were expressed as U/l.

Evaluation of Antioxidant Enzymatic Activity and Lipid Peroxidation Level

Freshly dissected muscles were rapidly placed in 10% (w/v) potassium phosphate buffer (pH 7.8) and homogenized on ice. Lipid peroxidation was determined by the extent of thiobarbituric acid reactive substances according to Draper and colleagues.³² The SOD activity was measured based on epinephrine auto-oxidation according to the previous protocol.³³ The activity of CAT was measured as the amount of H₂O₂ consumed per minute per mg of protein assayed by the protocol of Aebi.³⁴ GPx was analyzed by measuring the amount of reduced glutathione (GSH).³⁵ Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL). HO-1 expression was measured by Western blot, and bands were quantified by the National Institutes of Health Image analysis program (Bethesda, MD).

Morphometrical Analysis

Ten- μ m transverse cryostat sections of mdx and wild-type muscles were stained by hematoxylin and eosin (H&E). Morphometric analysis was performed on five cross-sections from each muscle using the Histolab program (version 5-13-1; Microvision Instrument, Evry, France). The histological parameters: percent normal fibers, percent centrally nucleated fibers, nonmuscle area (cm²), and variance coefficient Feret's diameter were determined as described previously.^{36–38}

Antibodies

Rabbit anti-HO-1 was from Stressgen Bioreagents (Ann Arbor, MI). For the proinflammatory and apoptotic cascade, respectively, polyclonal antibodies against NF- κ B and Bcl2 protein family (BAX and Bcl2) proteins were purchased from Assay Designs (Ann Arbor, MI). Rabbit polyclonal antibody to macrophage inflammatory protein 1 α /CCL3 was purchased from Abcam (Cambridge, MA). Polyclonal antibodies against β -DG (LG5), utrophin C-terminal residues (3423 to 3433), and α 7B-integrin were produced and characterized, as previously described.³⁸ The polyclonal anti- β -actin antibody was from Rockland (Gilbertsville, PA). Caveolin-3 and nNOS commercial antibodies were purchased, respectively from Santa Cruz Biotechnologies (Santa Cruz, CA) and BD Transduction Laboratories (Lexington, KY). Pan- and phospho-monoclonal antibodies, which recognize the nonphosphorylated and phosphorylated forms, respectively, of p38 MAPK, ERK1/2, and JNK1/2, were purchased from R&D Systems (Minneapolis, MN). All of the polyclonal antibodies were tested in competition with their corresponding peptides. Monoclonal antibody was tested according to the manufacturer's recommendation using the protein-positive controls.

Immunofluorescence

Ten- μ m unfixed cryostat sections were incubated with the primary antibody used at the appropriate dilution for 1 hour at room temperature. After washing with phosphate-buffered saline solution, sections were incubated with a secondary antibody (Cy3-goat anti-rabbit IgG; Chemicon International, Temecula, CA). For negative controls, only the second antibody was applied. Sections were visualized under a Nikon optiphot-2 microscope (Tokyo, Japan), and fluorescence intensity was analyzed using the Histolab program (version 5-13-1; Microvision Instrument).

Total Protein Extract and Western Blotting

Muscles (0.01 g) were homogenized in 150 μ l of 5% sodium dodecyl sulfate (SDS) buffer (50 mmol/L Tris/HCl, pH 8.0, 10 mmol/L ethylenediaminetetraacetic acid, and 5% SDS) supplemented with 1% trypsin inhibitor, 1% saponin, and 15 μ g/ml leupeptin. After centrifugation (10 minutes at 13,000 $\times$ g), protein concentration was estimated in the supernatant using the BCA protein assay kit (Pierce). Protein homogenates (40 mg) recovered from the obtained supernatant of each sample were denatured for 5 minutes at 100°C in reducing buffer (50 μ l of SDS buffer containing 5% SDS, 0.01% bromophenol blue, 10% glycerol, and 5% β -mercaptoethanol). Protein extracts were analyzed in duplicate by SDS-polyacrylamide gel electrophoresis (3 to 10% or 5 to 15%) with prestained standard proteins (Bio-Rad, Hercules, CA) to achieve a more accurate molecular weight determination. The resulting gel, containing the separated protein, was transferred onto a 0.2- μ m nitrocellulose membrane using the transfer buffer (25 mmol Tris-HCl, pH 8.3, 192 mmol glycine, and 20% methanol). The membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm that equal amounts of protein had been loaded and were blocked with Tris buffer, 0.1% Tween 20 (TBST) containing 3% bovine serum albumin (w/v), for 1 hour at room temperature. All membranes were incubated with primary antibodies overnight at 4°C. After washing three times for 10 minutes in TBST, the membranes were incubated with peroxidase-conjugated antibodies (Chemicon International) for 1 hour. After washing membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham, Little Chalfont, Buckinghamshire, UK). The protein signals were quantified by scanning densitometry using the National Institutes of Health program package. The results from each experimental group were expressed as relative integrate intensity compared with the control samples. Equal loading of proteins was assessed on stripped blots by immunodetection using a β -actin antibody.

Zymography

Zymography was performed according to Heussen and Dowdle.³⁹ Zymogram gel (5 to 15% polyacrylamide) was

Table 1. Effect of ALA/L-Car Treatment on Body and Muscle Weight

	<i>n</i>	Body weight (g), time 0	Body weight (g) after treatment	Tibialis anterior (g)	Vastus lateralis (g)	Diaphragm (g)	Cardiac muscle (g)
Wild-type Ss	7	16.1 ± 1.2	19.4 ± 0.64	0.055 ± 0.002	0.112 ± 0.009	0.079 ± 0.008	0.107 ± 0.011
Wild-type T	7	16.5 ± 0.56	20.8 ± 0.75	0.061 ± 0.004	0.118 ± 0.011	0.081 ± 0.006	0.106 ± 0.010
mdx Ss	8	18.9 ± 0.96 [†]	20.5 ± 0.34 [†]	0.072 ± 0.007 [†]	0.141 ± 0.010 [†]	0.101 ± 0.007 [†]	0.113 ± 0.019 [†]
mdx T	8	18.0 ± 0.88	22.3 ± 0.83	0.078 ± 0.003	0.142 ± 0.013	0.112 ± 0.002*	0.114 ± 0.016

n, number of animals per experimental group; body weight at the beginning (time 0) and after 2 weeks of treatment. Muscle weight (g) of the different groups: wild-type Ss (C57BL/10 injected with the saline solution), wild-type-T (C57BL/10 mice injected with ALA/L-Car solution mixture), mdx-Ss (injected with saline solution) and mdx-T (injected with ALA/L-Car). Statistical significance was set at [†]*P* < 0.01 (wild-type versus mdx mice) and at **P* < 0.01 (ALA/L-Car versus saline solution injected mice). Data are expressed as mean ± SD.

impregnated with gelatin at 1 mg/ml. After electrophoresis, the gel was washed in 2.5% Triton X-100 solution at room temperature and incubated for 24 hours in a substrate buffer (50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L CaCl₂, and 0.02% NaN₃) at 37°C. MMPs are secreted in a latent form and require cleavage of a peptide from their NH₂ terminus for activation. However, exposure of proenzymes of the tissue extracts to SDS during the gel separation procedure leads to activation without proteolytic cleavage. The gel was stained in Coomassie Blue R250 for 1 hour and destained in water overnight. Gelatin-degrading enzymes were visualized as clear bands, indicating proteolysis of the substrate protein. The gel was treated in black/white color, and the MMP bands were quantified using the National Institutes of Health image analysis program.

Statistics

Results are expressed as mean ± SD. Statistical analysis was performed by unpaired Student's test, and multiple statistical comparisons between groups were performed by one-way analysis variance followed by Bonferroni's *t*-test post hoc correction for allowing a better evaluation of intra- and intergroup variability and avoiding false-positives using Statview program (version 5.0; SAS Institute Inc., Cary, NC). Statistical significance was set at [§], **P* < 0.01.

Results

Body/Muscle Weight and Morphometrical Analysis

As expected the body weights were significantly higher in the dystrophin-deficient group (mdx) than in wild-type group as shown ([§]*P* < 0.01, Table 1). Before treatment, mdx mice were 7% heavier than wild-type mice. After 2 weeks, no significant change was observed in either of the untreated groups. ALA/L-Car treatment significantly increased body weights by twofold in wild-type-T group and by threefold in the mdx-T group. In the mdx group, among the muscles analyzed, only the weight of the diaphragm was increased by more than 10% (**P* < 0.01, Table 1). As shown in Figure 1 in the left panel, the dystrophin-deficient diaphragms (Figure 1, c and d) presents extensive areas of degeneration, with small centro-

nucleated and clustered necrotic fibers with several inflammatory cells³⁷ compared with wild-type muscle (Figure 1, a and b). The treatment improves the histological pattern of the mdx-T diaphragms (Figure 1d). The morphometrical analysis is summarized in Table 2; there is 10% increase in the number of a normal size fibers, with 35% decrease in centrally nucleated fibers. The variance coefficient of the Feret's diameter was 165% higher in the mdx diaphragm before treatment and reduced to 146% in mdx-T. The nonmuscle areas are also reduced by 52% after treatment (**P* < 0.01).

CK Level Evaluation

Low CK levels were observed in wild-type animals treated either with Ss (Wt-Ss) or with ALA/L-Car (Wt-T) (Wt-Ss = 293 ± 12 U/l, Wt-T = 287 ± 18 U/l). As expected, mdx-Ss mice showed a significant increase in serum CK levels (mdx-Ss = 3026 ± 63 U/l). Free radical scavenger administration resulted in a marked reduction of the enzyme level (mdx-T = 872 ± 51 U/l). Data are

Figure 1. H&E staining on 10-μm cryostat sections showed morphological features of mdx diaphragms from wild-type-Ss (a), wild-type-T (b), mdx-Ss (c), and mdx-T (d). Mdx-T diaphragm section (d) showed more regular-sized centronucleated and less connective tissue with less areas of mononuclear cell infiltrate. Scale bar = 25 μm.

Table 2. Morphometrical Analysis of Diaphragm Sections

	Diaphragm			
	Normal fibers (%)	% Centrally nucleated fibers	Nonmuscle area (cm ²)	Variance coefficient Feret's diameter
Wild-Type Ss	100	0	1 ± 0.8	255.3 ± 9.4
Wild-type T	100	0	1 ± 0.6	264.2 ± 11
mdx Ss	46 ± 8	57 ± 11	8.1 ± 3	423 ± 17
mdx T	51 ± 6	42 ± 4*	5.3*	387 ± 13*

Five sections from each muscle of all experimental groups were analyzed by Histolab program. Each section contains a number of muscle cells ranging 100 to 150 fibers. The parameters evaluated were the percentage of normal fibers, the percentage of centrally nucleated fibers, the nonmuscle area (the amount of space between fibers or between groups of muscle fibers that is not stained as muscular tissue), and the variance coefficient (showed the regularity of fibers dimensions). Data are expressed ± SD and significant difference was set at **P* < 0.01.

expressed ± SD, and significance is taken at **P* < 0.01 (Figure 2).

SOD, GPx, CAT, and Lipid Peroxidation Evaluation

The antioxidant enzyme activities of SOD, GPx, and CAT were compared between control (Wt-Ss and mdx-Ss) and treated animals (Wt-T and mdx-T). No significant change was observed in tibialis anterior, vastus lateralis, and cardiac muscles (not shown). However, enzyme activities were significantly modified in the mdx diaphragms after ALA/L-Car treatment. The diaphragm in the mdx mice is considered the best model to study dystrophin deficiency. Thus to clarify our finding, all of the following data will be presented in detail only in the diaphragm. Figure 3 shows the fold increase of SOD, GPx, and CAT activities in the experimental groups (Figure 3, A–C, respectively). No change was observed with wild-type samples after ALA/L-Car treatment, but the respective activities of all these enzymes were increased in the mdx-Ss group. Likewise, thiobarbituric acid reactive substance was also higher in mdx

than in wild-type muscles and was significantly decreased in the mdx-T group after ALA/L-Car treatment (**P* < 0.01) (Figure 3D).

HO-1 Expression Evaluation

HO-1 either was absent or gave a very weak staining pattern in normal muscle tissue (Figure 4). However, in mdx samples, HO-1 gave a strong staining pattern. In fact, it has been reported that in inflammatory conditions, such those observed in the mdx diaphragms or in injured muscle, HO-1 is expressed in some small, elongated, irregularly shaped cells infiltrating injured muscle such as macrophages, whereas muscle fibers were HO-1-negative (Figure 4A). The administration of the free radical scavenger to the mdx group has an impact on HO-1 levels in the mdx diaphragms. The HO-1 level was decreased significantly in the mdx-T group, whereas it remained unchanged in the Wt-T diaphragms (Figure 4B). The decreased level of HO-1 could be attributed to a

Figure 2. Effect of ALA/L-Car and Ss administration on serum CK level. Blood samples from Wt (*n* = 7 for each treated and control group) and mdx mice (*n* = 8 for each treated and control group) were collected as described in Materials and Methods. The serum CK level was measured by standard spectrophotometric analysis, and the corresponding values were recorded under histogram representation (Wt-Ss, white bar; Wt-T, gray light bar; mdx-Ss, gray dark bar; and mdx-T, black bar). Values are expressed ± SD, and **P* < 0.01.

Figure 3. Quantitative analysis of the antioxidant enzyme activities, SOD (A), GPx (B), and CAT (C) in mdx diaphragms of the experimental groups (Wt-Ss, *n* = 7; Wt-T, *n* = 7; mdx-Ss, *n* = 8; and mdx-T, *n* = 8). The level of activity of each enzyme in experimental groups was normalized to the level of enzyme activity of the wild-type group injected with the saline solution (Wt-Ss). Panel TBARS (thiobarbituric acid reactive substances) (D) represents the oxidative injury determined by the amount of lipid peroxidation using the TBA assay, which measures MDA formation as an index of lipid peroxidation. MDA formation is normalized to protein levels from the muscle extract for each sample. Data are presented as mean ± SD. Statistical significance was set at **P* < 0.01.

Figure 4. A: Immunofluorescence revelation of HO-1 in 10- μ m cryostat sections of Wt and mdx diaphragms. No immunoreactivity was observed in either Wt-Ss (A1) or Wt-T (A2) sections, whereas in mdx sections, HO-1 staining (white arrow, A3 and A4, respectively) showed that this protein is expressed by inflammatory cells such as macrophages (A5 and A6). ALA/L-Car treatment decreases the level of these infiltrating cells in mdx-T diaphragm (A4 and A6). **B:** Western blot analysis of HO-1 in Wt and mdx diaphragm extracts. Under the blot histogram represents quantitative data of HO-1 level in each experimental group (Wt-Ss, *n* = 7; Wt-T, *n* = 7; mdx-Ss, *n* = 8; and mdx-T, *n* = 8). Values (arbitrary units) are presented as mean $\pm$ SD. Statistical significance was set at **P* < 0.01 versus mdx-Ss-mdx-T diaphragm extracts.

Figure 5. A: Effect of ALA/L-Car on NF- κ B, BAX, and Bcl2 protein levels. Western blot analysis of NF- κ B, BAX, and Bcl2 level was performed in Wt and mdx diaphragm (Wt-Ss, *n* = 7; Wt-T, *n* = 7; mdx-Ss, *n* = 8; and mdx-T, *n* = 8). All samples were equilibrated according to β -actin immunodetection. **B:** Histogram represents quantitative data of Western blots. Values (arbitrary units) are presented as mean $\pm$ SD. Statistical significance was set at **P* < 0.01; ns, not significant.

decreased number of infiltrating macrophages in mdx diaphragms, which leads to a reduced diffusion of the free radical species in the microenvironment of the dystrophic muscle fibers.

Inflammation and Apoptotic Cascade

The inflammatory process is activated in the mdx muscles essentially by infiltrating cells (such as macrophages) that produce NF- κ B and other molecules involved in inflammatory, immune, and acute stress response. As expected, our experiments showed increased levels of NF- κ B in mdx diaphragms compared with Wt samples (Figure 5, A and B). Interestingly, we observed a decreased level of NF- κ B in mdx diaphragm samples from the mdx-T group compared with mdx-Ss-injected group. NF- κ B was either weakly or not expressed in the Wt diaphragms, and the free radical scavenger administration did not affect its expression in the Wt-T group. We examined two members of the apoptotic cascade (Bcl-2 and BAX), which have antagonist functions during the muscle cell death cascade. ALA/L-Car administration showed that both the anti-apoptotic Bcl2 and the proapoptotic BAX proteins remain unchanged between mdx-T and mdx-Ss diaphragms. BAX and Bcl2 are not present at a significant level in Wt diaphragms, and treatment had not affected their respective levels in

Figure 6. A: Effect of ALA/L-Car treatment on β -DG level. Western blot detection of β -DG in diaphragm extracts of each experimental group showed that this protein was expressed as two forms, the full-length form with a M_r of 43 kD (β -DG₄₃) and a cleaved form with M_r of ~30 kD (β -DG₃₀). B: Histograms under the blots showed quantification of protein bands (arbitrary units). Experiment was performed in duplicate in each experimental group (Wt-Ss, $n = 7$; Wt-T, $n = 7$; mdx-Ss, $n = 8$; and mdx-T, $n = 8$). Data are expressed as means $\pm$ SD, and statistical significance was set at $*P < 0.01$.

Wt-T group (Figure 5, A and B). This experiment is in agreement with a recent study suggesting that Bcl2-mediated apoptosis seems to play a significant role in congenital muscular dystrophies such as laminin α 2 deficiency but not in dystrophin-deficient muscle (DMD and mdx).⁴⁰

Utrophin and Associated Protein

Utrophin, nNOS, α 7B-integrin, and caveolin-3 were analyzed by Western blotting in diaphragms from treated and untreated animal groups. No significant difference in utrophin, α 7B-integrin, and caveolin-3 levels were observed in wild-type groups (Wt-Ss and Wt-T) or between mdx groups (mdx-Ss and mdx-T) (not shown).

Administration of ALA/L-Car Affects β -DG Level and Processing in Mdx Diaphragms

In the mdx-T group, a significant change concerning the β -DG level was found. In mdx muscles, β -DG was cleaved by MMPs into a C-terminal 30-kD β -DG fragment. Using a polyclonal C-terminal antibody that recognizes the two forms of β -DG, we analyzed the levels of the two forms of β -DG by Western blot detection. Full-length β -DG (β -DG₄₃) was increased after treatment in the mdx-T groups, whereas the β -DG₃₀ form was decreased, suggesting that our treatment inhibited the processing mediated by the MMPs in mdx-T diaphragms compared with mdx-Ss one (Figure 6). The β -DG₃₀ was weakly produced or absent in Wt samples, and the treatment

Figure 7. A: Gelatin zymography analysis of diaphragm muscle in each experimental group. In Wt-Ss and Wt-T samples, only the MMP-2 was active under these conditions. In mdx diaphragm, samples both MMP-2 and MMP-9 are up-regulated. ALA/L-Car administration showed a decreased MMP-2 and MMP-9 activity in mdx-T diaphragm samples compared with mdx-Ss. B: Gelatin zymography gels were treated with black/white color, and the MMP bands were quantified using the National Institutes of Health Image analysis program. Histogram representation confirmed that MMP-2 and MMP-9 activity were decreased in mdx-T group ($n = 8$) compared with mdx-Ss ($n = 8$), whereas no significant difference was observed between Wt-T ($n = 7$) and Wt-Ss ($n = 7$) groups. Data were expressed as means $\pm$ SD, and statistical significance was set at $*P < 0.01$ for MMP-2 and at $^{\S}P < 0.01$ for MMP-9.

did not affect the level of β -DG₄₃ in Wt group (Figure 6, A and B).

Free Radical Scavengers Inhibit MMP's Activity in Mdx Diaphragms

MMPs, especially MMP-2 and MMP-9, and gelatinases were involved in tissue remodeling that occurs during skeletal muscle degeneration and regeneration.^{41,42} When compared with Wt diaphragms, our zymography results demonstrate that mdx diaphragm samples, exhibited an increased activity of both MMP-9 and MMP-2 (detected an ~100-kD and a major 60-kD form, respectively; Figure 7A). In Wt diaphragm samples, only the MMP-2 form was active. MMP-9 is produced essentially by both inflammatory and activated satellite cells. Numerous histological studies have identified the presence of macrophages and mast cells in mdx muscles. These cell types are known to store and produce MMP-9 in response to different stimuli such as oxidative stress from necrotic tissue and represent one important source of MMP-9. Our experiments showed that ALA/L-Car treatment had a significant effect on MMP-9 and MMP-2 activities in the mdx diaphragms (MMP-9: mdx-Ss = 112.3 ± 17 versus mdx-T = 62 ± 9 , MMP-2: mdx-Ss = 190.4 ± 32 versus mdx-T = 136.7 ± 21) (Figure 7B, histograms). The native activity of MMP-2 in Wt diaphragms was unchanged between Wt-T and Wt-Ss groups, whereas MMP-9 expression was almost absent in Wt groups.

Free Radical Scavengers Modulate MAP Kinase Activity in Mdx Diaphragm

Several component of the MAP kinase cascade were differentially expressed in the mdx diaphragm, which is in agreement with the idea that different signaling pathways were distinctly activated depending on the severity of the dystrophic phenotype.^{43,44} We analyzed three components involved in the MAP kinase cascade after the treatment (p38 MAP kinase, ERK1/2, and JNK1/2). No changes were observed in the core protein expression (nonphosphorylated forms, pan-antibody) of these proteins (Figure 8A), whereas the treatment affects the activity/phosphorylation (p-) status of p38, ERK1/2, and JNK1/2 in the mdx diaphragms (Figure 8B). As expected, the level of phosphorylated p38 MAPK (p-p38) was decreased in mdx samples compared with Wt groups. p-p38 MAPK level was significantly decreased in mdx-T compared with mdx-Ss, whereas no significant difference was observed between Wt-Ss and Wt-T groups. The p-ERK1 and p-ERK2 were dually phosphorylated at T202/Y204 and T185/Y187, respectively, and were detected as double bands at 44 kd (p-ERK1) and 42 kd (p-ERK2). Western blot detection showed that the level of p-ERK1/2 was higher in mdx samples (mdx-Ss and mdx-T) than in Wt samples (Wt-Ss and Wt-T). Administration of ALA/L-Car leads to a decreased level of this protein in mdx-T diaphragms, whereas no effects were observed in Wt-T groups compared with the Wt-Ss one. The p-JNK1 and p-JNK2 proteins were analyzed separately. Our experiment was in agreement with previous reports showing that p-JNK1 was overexpressed in mdx muscle compared with Wt samples. Furthermore, the p-JNK1 phosphorylation level was significantly decreased in the treated mdx group (mdx-T) compared with the mdx-Ss one. No change in phosphorylated p-JNK1 was observed between Wt-Ss and Wt-T diaphragm samples. In contrast, the treatment leads to a decreased p-JNK2 level in mdx-T samples, whereas we have noted no changes in phosphorylation level of this protein in Wt treated diaphragms (Wt-T) compared with control group (Wt-Ss). All of the data obtained after Western blot revelation are summarized in the histograms shown in Figure 8C.

Discussion

In this study, we investigated the relationship between oxidative stress, NF- κ B/MMP activation, apoptotic status, and the dystrophic process in dystrophin-deficient diaphragm muscles from 5-week-old mdx mice after 2 weeks' treatment with ALA/L-Car. The improvement, which was observed in histological parameters, antioxidant enzymes (SOD, GPx, and CAT), as well as in the lipid peroxidation level after ALA/L-Car treatment in mdx diaphragm clearly demonstrate that the oxidative damage and activation of the proinflammatory cascade NF- κ B/MMP contribute to the progression of dystrophic pattern in the mdx muscles. The overactivation of the MMPs in mdx muscle accentuates the process-

Figure 8. Effect of ALA/L-Car on p38 MAPK, ERK1/2, JNK1, and JNK2 phosphorylation in diaphragm muscle of Wt and mdx mice. Muscle lysates were analyzed via Western blot using each phosphorylated (p) (A) and nonphosphorylated (pan) (B) antibody raised against these proteins. Wt-Ss: wild-type mice treated with a vehicle (saline solution); Wt-T: wild-type mice treated with ALA/L-Car solution mixture; and mdx-Ss and mdx-T indicates, respectively, mdx mice vehicle (saline solution)- and ALA/L-Car-injected mice. C: Quantitative analysis of p-p38 MAPK, p-ERK1/2, p-JNK1, and p-JNK2 is shown under histogram representation after analysis of each experimental group (Wt-Ss, $n = 7$; Wt-T, $n = 7$; mdx-Ss, $n = 8$; and mdx-T, $n = 8$). Data are expressed as means $\pm$ SD, and statistical significance was set at $*P < 0.01$.

ing of β -DG, which leads to disintegration of the dystroglycan complex and enhances dystrophin-glycoprotein complex disorganization.⁴⁵ In addition, reactive oxygen species damage and inflammation in dystrophin-deficient muscle leads to changes in the phosphorylation/activity of several MAP kinase components (ERK1/2, JNK1/2, and p38 MAPK).

ALA/L-Car Treatment Re-Establishes the Oxidative Balance and Decreases Inflammation in the Mdx Diaphragms

Recently, several reports have supported the role of oxidative stress in DMD pathogenesis. They have shown that elevated levels of free radicals indicate that the antioxidant defense mechanism is most likely unable to blunt the increased oxygen radical formation.²⁸ This work showed that several antioxidant enzymes, such as SOD, GPx, and CAT, as well as the lipid peroxidation levels were significantly decreased after free radical scavenger (ALA/L-Car) administration in mdx mice. This suggests that lipid peroxidation inhibition could induce a significant attenuation of membrane injury, restoring the defense mechanism in the diaphragm muscle cells. Moreover, we showed that expression of HO-1 by infiltrating macrophages could be considered as a novel marker of the oxidative status, suggesting that these cells were the most efficient in inducing injury mediated by these inflammatory cells in the dystrophin-deficient mdx diaphragm.

Our treatment seems more efficient in the mdx diaphragm compared with the other muscles (TA, VL, and cardiac muscle). Despite little difference between treated and control muscles, no significant changes in morphometrical parameters or in antioxidant enzyme status were observed when we have analyzed TA, VL, and cardiac muscle. This could be explained by the fact that mdx mouse diaphragm showed a severe degenerative and dystrophic pattern attributable to perpetual contraction/relaxation cycles.⁴⁶ Moreover, Nakae and colleagues^{47,48} have reported that oxidative stress may contribute to necrosis in mdx diaphragm muscle: most necrotic myofibers, myosatellite, and interstitial inflammatory cells in mdx diaphragm muscles contained accumulated lipofuscin. In this context, recently Messina and colleagues²⁸ have demonstrated that the NF- κ B inhibitor IRFI 042 was able to strongly reduce the pathological cascade in mdx muscles. In fact, evidence demonstrated that the activation of NF- κ B can lead an increased expression of inflammatory molecules such as interleukin-6, tumor necrosis factor- α , cell adhesion molecules, and MMP-9.²⁶ Chen and colleagues⁴⁹ have also shown a strong induction of NF- κ B soon after birth in DMD muscle. They have observed a stage-specific remodeling of DMD muscle with inflammatory pathways predominating in the pre-symptomatic stage and acute activation of transforming growth factor- β .⁴⁹ Our data demonstrated that activation of the NF- κ B cascade was inhibited by the free radical scavenger mixture (ALA/L-Car) and leads to decreased activity of the MMP-2 and MMP-9 in treated mdx diaphragms. These molecules are responsible for the processing of the β -DG₄₃ into β -DG₃₀, which leads to the disruption of the α -DG/ β -DG interaction.⁵⁰ β -DG processing was observed in skeletal muscles from DMD patients and sarcoglycan-deficient muscle, which are muscular dystrophies characterized by membrane fragility and protein membrane disorgani-

Figure 9. **A:** Synthetic representation of muscle fiber membrane (sarcolemma) showing hypothetical cascade involving NF- κ B/MMP activity and several MAP kinases in dystrophic process. Activated MMPs in dystrophic myofibers lead to disintegration of the dystroglycan complex by cleaving the N-terminal part of β -DG into β -DG₃₀. ALA/L-Car treatment can decrease reactive oxygen species and proinflammatory molecule levels and attenuate dystrophic pattern by stabilizing the β -DG₄₃ form in sarcolemma. **B:** Normal dystroglycan complex through the sarcolemma.

zation.⁴⁵ Overactivation of MMP-9 was also observed in injured and mdx muscle in response to a chronic treadmill exercise,⁴¹ which suggests that this metalloproteinase could be associated with either degeneration or regeneration cycles in muscle.⁴¹ In agreement with these reports, our data suggest that MMP activity could also be associated with the pathological pattern observed in mdx muscles and may reflect the degree of membrane injury caused by reactive oxygen species damage.

ALA/L-Car Treatment Decreases the Phosphorylation/Activity of MAP Kinase Components in the Mdx Diaphragms

Several studies have shown that MMPs are a possible downstream substrate of p38 MAPK and ERK1/2 in several cell types.¹⁸ In mdx diaphragm muscle, p38 MAPK and ERK1/2 phosphorylation were different from the other muscles.⁴⁴ In cardiac muscle, phosphorylation of p38 MAPK was also dramatically increased in exercise-trained mdx mice⁵¹ and utrophin-dystrophin-deficient mice.⁵² Our treatment decreases the phosphorylation level of p38 in mdx diaphragms and could be associated with the efficiency of our treatment on cell survival, slowing degeneration and inflammatory pattern. JNK is also thought to be associated with cell death and apoptosis.⁵³ We demonstrated that JNK1 and JNK2 phosphorylation

was decreased in mdx diaphragm after ALA/L-Car treatment. A recent report stated that JNK1 is activated in mdx muscle and that JNK1 inhibitory JIP1 dramatically attenuated the progression of dystrophic features,⁵⁴ JNK2 activation was also implicated in the progression of dystrophic features after exercise in mdx muscle.⁵¹ The reason why the decreased phosphorylation of the JNK after treatment is not known and the fact that this protein is important in controlling programmed cell death or apoptosis leads us to speculate its implication in the dystrophic pattern of mdx diaphragm. The activation of all these kinases in dystrophic muscle may result from an intrinsic loss of the dystrophin protein and/or the cognate binding partner. In particular, several components of the dystrophin protein complex have been shown to retain signaling molecules of the ERK-MAP kinase cascade.⁸

The situation regarding ERK activation is unclear in the literature. Kolodziejczyk and colleagues⁵⁴ claim no difference in ERK activity in either cardiac or skeletal muscle of mdx or mdx:MyoD^{-/-} mice, despite the clear presence of a slower migrating p-ERK1 band in the skeletal mdx mouse. Furthermore, significantly elevated levels of active ERK were found in the hearts of nonexercised and exercised mdx mice compared with controls.⁵¹ ERK activity was also found to be increased in the hearts of caveolin-3-null mice, probably because caveolin-3 is able to interact with ERK and suppress its activation.⁵⁵ The interaction of caveoline-3 with β -DG and the observed ability of β -DG to interact with MEK and ERK provide potential clues as to how dystrophin-glycoprotein complex signaling to ERK might be mediated.⁸

ALA/L-Car Treatment Inhibits β -DG Processing in the Mdx Diaphragms

Similar results have been observed in DMD and mdx muscle concerning the ability of MMPs to cleave the extracellular domain of β -DG.⁵⁶ In fact, in our work we confirmed that β -DG has two forms in mdx diaphragm, full-length β -DG (β -DG₄₃) and its cleaved form (β -DG₃₀). We suggest that, in contrast to β -DG₄₃, β -DG₃₀ is unable to sequester and inactivate or prevent the activation of MEK and therefore reduce the activity of its downstream kinase ERK. Taken together, the association of β -DG with components of ERK-MAP kinase cascade may play an important role in normal muscle homeostasis. Perturbation of β -DG/ α -DG interaction by MMP processing leads to an imbalance in ERK-MAP kinase signaling that could affect cell cycle progression and gene expression, thus contributing to the muscular dystrophy phenotype (hypothetical schema in Figure 9).

In conclusion, our understanding of the role of damage by oxygen species and the associated signaling cascades in dystrophin-deficient muscles is always at a relatively early stage. However, given the enormous advances that have been made in recent years in understanding the implication of these cascades in muscular dystrophies, it is likely that in the near future the use of specific antioxidants could provide new strategies of

treatment to modify the dystrophic process and slow down its progression.

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References

1. Koenig M, Monaco AP, Kunkel LM: The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 1988, 53:219–226
2. Zubrzycka-Gaarn EE, Bulman DE, Karpati G, Burghes AH, Belfall B, Klamut HJ, Talbot J, Hodges RS, Ray PN, Worton RG: The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. *Nature* 1988, 333:466–469
3. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP: Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 1992, 355:696–702
4. Nawroztzki R, Loh NY, Ruegg MA, Davies KE, Blake DJ: Characterisation of alpha-dystrobrevin in muscle. *J Cell Sci* 1998, 111:2595–2605
5. Adams ME, Butler MH, Dwyer TM, Peters MF, Murnane AA, Froehner SC: Two forms of mouse syntrophin, a 58 kd dystrophin-associated protein, differ in primary structure and tissue distribution. *Neuron* 1993, 11:531–540
6. Brenman JE, Chao DS, Xia H, Aldape K, Brecht DS: Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 1995, 82:743–752
7. Matsumura K, Campbell KP: Deficiency of dystrophin-associated proteins: a common mechanism leading to muscle cell necrosis in severe childhood muscular dystrophies. *Neuromuscul Disord* 1993, 3:109–118
8. Spence HJ, Dhillon AS, James M, Winder SJ: Dystroglycan, a scaffold for the ERK-MAP kinase cascade. *EMBO Rep* 2004, 5:484–489
9. Lapidus KA, Kakkar R, McNally EM: The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ Res* 2004, 94:1023–1031
10. Côté PD, Moukles H, Lindenbaum M, Carbonetto S: Chimeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nat Genet* 1999, 23:338–342
11. Muschler J, Levy D, Boudreau R, Henry M, Campbell K, Bissell MJ: A role for dystroglycan in epithelial polarization: loss of function in breast tumor cells. *Cancer Res* 2002, 62:7102–7109
12. Deng WM, Schneider M, Frock R, Castillejo-Lopez C, Gaman EA, Baumgartner S, Ruohola-Baker H: Dystroglycan is required for polarizing the epithelial cells and the oocyte in *Drosophila*. *Development* 2003, 130:173–184
13. James M, Nuttall A, Ilsley JL, Ottersbach K, Tinsley JM, Sudol M, Winder SJ: Adhesion-dependent tyrosine phosphorylation of (beta)-dystroglycan regulates its interaction with utrophin. *J Cell Sci* 2000, 113:1717–1726
14. Buday L, Downward J: Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell* 1993, 73:611–620
15. Winder SJ: The complexities of dystroglycan. *Trends Biochem Sci* 2001, 26:118–124
16. Haycock JW, MacNeil S, Jones P, Harris JB, Mantle D: Oxidative damage to muscle protein in Duchenne muscular dystrophy. *Neuroreport* 1996, 8:357–361
17. Ragusa RJ, Chow CK, Porter JD: Oxidative stress as a potential pathogenic mechanism in an animal model of Duchenne muscular dystrophy. *Neuromuscul Disord* 1997, 7:379–386
18. Alcaraz MJ, Fernandez P, Guillen MI: Anti-inflammatory actions of the heme oxygenase-1 pathway. *Curr Pharm Des* 2003, 9:2541–2551
19. Petit-Bertron AF, Fitting C, Cavallion JM, Adib-Conquy M: Adherence influences monocyte responsiveness to interleukin-10. *J Leukoc Biol* 2003, 73:145–154

20. Vicente AM, Guillin MI, Alcaraz MJ: Participation of heme oxygenase-1 in a model of acute inflammation. *Exp Biol Med* (Maywood) 2003, 228:514–516
21. 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
22. 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
23. Kumar A, Takada Y, Boriek AM, Aggarwal BB: Nuclear factor-kappaB: its role in health and disease. *J Mol Med* 2004, 82:434–448
24. Karin M: The beginning of the end: ikappaB kinase (IKK) and NF-kappaB activation. *J Biol Chem* 1999, 274:27339–27342
25. Karin M, Delhase M: The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin Immunol* 2000, 12:85–98
26. Vellaichamy E, Khurana ML, Fink J, Pandey KN: Involvement of the NF-kappa B/matrix metalloproteinase pathway in cardiac fibrosis of mice lacking guanylyl cyclase/natriuretic peptide receptor A. *J Biol Chem* 2005, 280:19230–19242
27. Monici MC, Aguenouz M, Mazzeo A, Messina C, Vita G: Activation of nuclear factor-kappaB in inflammatory myopathies and Duchenne muscular dystrophy. *Neurology* 2003, 60:993–997
28. Messina S, Altavilla D, Aguenouz M, Seminara P, Minutoli L, Monici MC, Bitto A, Mazzeo A, Marini H, Squadrito F, Vita G: Lipid peroxidation inhibition blunts nuclear factor-kappaB activation, reduces skeletal muscle degeneration, and enhances muscle function in mdx mice. *Am J Pathol* 2006, 168:918–926
29. Jacob C, Belleville F: L-Carnitine: metabolism, functions and value in pathology. *Pathol Biol (Paris)* 1992, 40:910–919
30. Vanella A, Russo A, Acquaviva R, Campisi A, Di Giacomo C, Sorrenti V, Barcellona ML: L-Propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector. *Cell Biol Toxicol* 2000, 16:99–104
31. Arivazhagan P, Juliet P, Panneerselvam C: Effect of dl-alpha-lipoic acid on the status of lipid peroxidation and antioxidants in aged rats. *Pharmacol Res* 2000, 41:299–303
32. Draper HH, Polensek L, Hadley M, McGirr LG: Urinary malondialdehyde as an indicator of lipid peroxidation in the diet and in the tissues. *Lipids* 1984, 19:836–843
33. Wahlund G, Marklund SL, Sjoquist PO: Extracellular-superoxide dismutase type C (EC-SOD C) reduces myocardial damage in rats subjected to coronary occlusion and 24 hours of reperfusion. *Free Radic Res Commun* 1992, 17:41–47
34. Aebi H: Catalase in vitro. *Methods Enzymol* 1984, 105:121–126
35. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG: Selenium: biochemical role as a component of glutathione peroxidase. *Science* 1973, 179:588–590
36. De Luca A, Nico B, Liantonio A, Didonna MP, Frayssé B, Pierno S, Burdi R, Mangieri D, Rolland JF, Camerino C, Zallone A, Confalonieri P, Andreetta F, Arnoldi E, Courdier-Fruh I, Magyar JP, Frigeri A, Pisoni M, Svelto M, Conte Camerino D: A multidisciplinary evaluation of the effectiveness of cyclosporine a in dystrophic mdx mice. *Am J Pathol* 2005, 166:477–489
37. Brigueat A, Courdier-Fruh I, Foster M, Meier T, Magyar JP: Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscul Disord* 2004, 14:675–682
38. Chazallet D, Hnia K, Rivier F, Hugon G, Mornet D: Alpha7B integrin changes in mdx mouse muscles after L-arginine administration. *FEBS Lett* 2005, 579:1079–1084
39. Heussen C, Dowdle EB: Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* 1980, 102:196–202
40. Dominov JA, Kravetz AJ, Ardelt M, Kostek CA, Beermann ML, Miller JB: Muscle-specific BCL2 expression ameliorates muscle disease in laminin [alpha]2-deficient, but not in dystrophin-deficient, mice. *Hum Mol Genet* 2005, 14:1029–1040
41. Kherif S, Lafuma C, Dehaupas M, Lachkar S, Fournier JG, Verdier-Sahuque M, Fardeau M, Alameddine HS: Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: a study in experimentally injured and mdx muscles. *Dev Biol* 1999, 205:158–170
42. Roma J, Munell F, Fargas A, Roig M: Evolution of pathological changes in the gastrocnemius of the mdx mice correlate with utrophin and beta-dystroglycan expression. *Acta Neuropathol (Berl)* 2004, 108:443–452
43. 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
44. Lang JM, Esser KA, Dupont-Versteegden EE: Altered activity of signaling pathways in diaphragm and tibialis anterior muscle of dystrophic mice. *Exp Biol Med* (Maywood) 2004, 229:503–511
45. Matsumura K, Zhong D, Saito F, Arai K, Adachi K, Kawai H, Higuchi I, Nishino I, Shimizu T: Proteolysis of beta-dystroglycan in muscular diseases. *Neuromuscul Disord* 2005, 15:336–341
46. Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B, Narusawa M, Leferovich JM, Sladky JT, Kelly AM: The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 1991, 352:536–539
47. Nakae Y, Stoward PJ, Kashiwama T, Shono M, Akagi A, Matsuzaki T, Nonaka I: Early onset of lipofuscin accumulation in dystrophin-deficient skeletal muscles of DMD patients and mdx mice. *J Mol Histol* 2004, 35:489–499
48. Nakae Y, Stoward PJ, Shono M, Matsuzaki T: Most apoptotic cells in mdx diaphragm muscle contain accumulated lipofuscin. *Histochem Cell Biol* 2001, 115:205–214
49. Chen YW, Nagaraju K, Bakay M, McIntyre O, Rawat R, Shi R, Hoffman EP: Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. *Neurology* 2005, 65:826–834
50. Yamada H, Saito F, Fukuta-Ohi H, Zhong D, Hase A, Arai K, Okuyama A, Maekawa R, Shimizu T, Matsumura K: Processing of beta-dystroglycan by matrix metalloproteinase disrupts the link between the extracellular matrix and cell membrane via the dystroglycan complex. *Hum Mol Genet* 2001, 10:1563–1569
51. Nakamura A, Yoshida K, Ueda H, Takeda S, Ikeda S: Up-regulation of mitogen activated protein kinases in mdx skeletal muscle following chronic treadmill exercise. *Biochim Biophys Acta* 2005, 1740:326–331
52. Nakamura A, Harrod GV, Davies KE: Activation of calcineurin and stress activated protein kinase/p38-mitogen activated protein kinase in hearts of utrophin-dystrophin knockout mice. *Neuromuscul Disord* 2001, 11:251–259
53. Johnson GL, Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002, 298:1911–1912
54. Kolodziejczyk SM, Walsh GS, Balazsi K, Seale P, Sandoz J, Hierlihy AM, Rudnicki MA, Chamberlain JS, Miller FD, Megeney LA: Activation of JNK1 contributes to dystrophic muscle pathogenesis. *Curr Biol* 2001, 11:1278–1282
55. Woodman SE, Park DS, Cohen AW, Cheung MW, Chandra M, Shirani J, Tang B, Jelicks LA, Kitsis RN, Christ GJ, Factor SM, Tanowitz HB, Lisanti MP: Caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAPK cascade. *J Biol Chem* 2002, 277:38988–38997
56. Blake DJ, Esapa CT, Martin-Rendon E, McIlhinney RA: Glycosylation defects and muscular dystrophy. *Adv Exp Med Biol* 2005, 564:97–98