

Modulation of p38 MAPK Cascade and Metalloproteinase Activity in Diaphragm Muscle in Response to Free Radical Scavengers administration in Dystrophin-Deficient *mdx* mice

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[‡]Abbreviations: DGC, dystrophin glycoprotein complex; DAPC, dystrophin associated protein complex; β -DG, Beta-dystroglycan; ALA, Alpha-lipoic acid; L-Car, L-carnitine; CK, creatine kinase; TBARS, thiobarbituric acid reactions; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MyHC, myosin heavy chain; HO-1, Heme oxygenase-1; NF- κ B, nuclear factor- κ B; MMP, Matrix Metalloproteinase; MAPK, Mitogen-activated protein Kinase; ERK, Extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; Wt, Wild-type; *mdx*, dystrophin-deficient mice.

Duchenne muscular dystrophy muscles undergo increased oxidative stress and altered calcium homeostasis, which contribute to myofibre loss by triggering both necrosis and apoptosis. Here we asked if treatment with free radical scavengers' could improve the dystrophic pattern of *mdx* muscles. 5 weeks old *mdx* mice were treated for 2 weeks with lipoic acid (ALA)/L-carnitin (L-Car). This treatment decreases the plasmatic creatine kinase level, the antioxidant enzyme activity, and lipid peroxidation products in *mdx* diaphragm. Free radical scavengers also modulate the phosphorylation/activity of some component of the MAP kinase cascades: p38 MAPK, the extracellular signal-related kinase (ERK), and the Jun kinase (JNK). The β -dystroglycan a multifunctional adaptor or scaffold capable of interacting with components of the ERK-MAP kinase cascade was also affected after treatment. In the *mdx* muscles β -dystroglycan (43kDa) was cleaved by the matrix metalloproteinase (MMPs) into a 30 kDa form (β -DG₃₀). We showed that the pro-inflammatory protein, nuclear factor- κ B activator (NF- κ B), decreased after the treatment which leads to a significant reduction of MMPs activity in the *mdx* diaphragm. Our data highlights the implication of oxidative stress and cell signalling defects in dystrophin deficient muscle via the MAP kinase cascade- β -dystroglycan interaction and NF- κ B mediated inflammation process.

Keywords: *mdx*, oxidative stress, NF- κ B, MMP, β -dystroglycan, MAP kinase cascades

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder, caused by mutations in the dystrophin gene¹. Dystrophin links intracellular actin and extracellular laminin via its interaction with the dystrophin-glycoprotein complex (DGC) composed of dystroglycans and sarcoglycans^{2,3}. In the cytoplasmic region, dystrophin interacts with the dystrophin associated protein complex (DAPC) i.e. dystrobrevin, syntrophins and the neuronal isoform of nitric synthase (nNOS)⁴⁻⁶. Besides providing mechanical stability⁷, several proteins of the DGC play a role in cell signalling^{8,9}. The dystroglycan complex is an important adhesion receptor and has a vital role in maintaining muscle integrity, its loss leads to muscular dystrophy¹⁰, and it has recently been implicated in the maintenance of cell polarity^{11,12}. Previous studies have also shown that β -dystroglycan, the transmembrane component of the dystroglycan complex which links the C-terminal region of dystrophin, is associated with a number of adaptor proteins involved in a variety of signalling cascades^{13,14}. It has also been demonstrated that the interaction of dystroglycan with laminin has an inhibitory effect on the activation of the ERK-MAP (ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein) kinase cascade in response to the binding of integrins to laminin⁸. The ability of dystroglycan to interact with several components of ERK-MAP kinase cascade may be part of the mechanism involved in dystroglycan modulating ERK activity in response to several cell stimuli^{8,15}.

Altered cell signalling is thought to increase the susceptibility of muscle fibres to secondary triggers, such as functional ischemia and oxidative stress¹⁶. Several lines of evidence suggest that free radical injury to the membrane may contribute to the loss of membrane integrity in muscular dystrophies¹⁷. The increase action of oxidative stress in DMD muscle is indicated in part by an increased changes in proteins¹⁶, enhanced lipid peroxydation and induction of antioxidant enzymes¹⁷. 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 diseases¹⁸. HO-1 induction inhibits cytokine production in macrophages and monocyte adherence¹⁹. Moreover, it has been shown that HO-1 can be upregulated under inflammatory condition in several tissues and induction of HO-1 in vivo occurs as a response to cytokine released locally at the site of inflammation²⁰. The dystrophic muscle displays inflammatory cell infiltration reflecting the immune response to tissues damage²¹. Despite these finding, no study has yet investigated the regulation of HO-1 in dystrophic muscles. The nuclear factor- κ B (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 responses²⁴. After proteosomal 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 cytokine, chemokines, cell adhesion molecules, immunoreceptors, and inflammatory enzymes such as nitric Oxide synthase (nNOS) and metalloproteinase (MMPs)^{25,26}. Recently, NF- κ B activity has been demonstrated to be increased in the muscle of both DMD patients and mdx mice²². Novel observations have also reported an increased immunoreactivity of NF- κ B in the cytoplasm of all regenerating fibres and in 20 to 40% of necrotic fibres in DMD²⁷. 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 peroxydation inhibition may influence the skeletal muscle pathology in mdx mice with respect to the functional, morphological, and biochemical pattern²⁸. Taking together these finding 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 signalling 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 damage²⁹. It has been shown to scavenge superoxide anion, to inhibit lipoperoxidation of linoleic acid and to protect against damage induced by H_2O_2 ³⁰. Similarly, 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 glutathione³¹. This study demonstrated that *in vivo* ALA/L-Car treatment re-establishes antioxidant enzymes activity (SOD, CAT, and GPx), decreases the index of lipid peroxidation (TBARS), plasmatic 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 MMPs 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.

Material and Methods

Animals

Five-week-old Male wild-type C57BL/10 (Wt) and dystrophin-deficient (mdx) mice were purchased from Jackson Laboratory (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 Institute of Health (NIH). The animals were killed by rapid cervical dislocation and experiments were carefully designed to minimize the number of animals and their suffering. Animal (Wt; n = 7 and mdx; n = 8,) were treated for 2 weeks with intraperitoneal injections with (α -lipoic acid/L-carnitine, Sigma, 250 μ l vol. at a Cc of 250 mg/kg) of dissolved in physiological saline solution (Ss). Control groups were injected with saline solution (mdx-Ss; n = 7 and wild-type-Ss; n = 8). At the end of the experiment, animals were weighed and killed by cervical dislocation. After beheading, blood was collected to analyse creatine kinase (CK) levels and the diaphragm was dissected into 2 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 ethylenediamine- tetraacetic acid (EDTA) 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 peroxydation 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 (TBARS) according to Draper et al.³². The superoxide dismutase (SOD) activity was measured based on epinephrine auto-oxidation according to the previous protocol³³. The activity of catalase (CAT) was measured as the amount of H₂O₂ consumed per minute per mg of protein assayed by the protocol of Aebi et al.³⁴. GPx was analysed by measuring the amount of reduced glutathione (GSH)³⁵. Protein concentrations were determined by the BCA protein assay (Pierce). Heme oxygenase-1 (HO-1) expression was measured by western blot and bands were quantified by NIH image analysis program.

Morphometrical analysis

Ten- μ m transverse cryostat sections of mdx and wild-type muscles were stained by hematoxylin and eosine (H&E). Morphometric analysis was performed on 5 cross-sections from each muscle using the Histolab program (version 5-13-1, Microvision Instrument). The histological parameters: % normal fibres, % centrally nucleated fibres, non muscle area (cm²), and Variance coefficient "*Feret's diameter*" was determined as described previously³⁶⁻³⁸.

Antibodies

Rabbit anti-Heme-Oxygenase-1 was from Stressgen Bioreagents. For the proinflammatory and apoptotic cascade respectively polyclonal antibodies against NF- κ B, and Bcl2 protein family (BAX and Bcl2) were purchased from assay designs. Rabbit polyclonal antibody to macrophage inflammatory protein 1 alpha/CCL3 was purchased from abcam. Polyclonal antibodies against β -dystroglycan (LG5), utrophin C-terminal residues (3423-3433), and α 7B-integrin were produced and characterized, as previously described³⁸. The polyclonal anti- β -actin antibody was from Rockland. Caveolin-3 and nNOS commercial antibodies were purchased respectively from SantaCruz Biotechnologies and BD Transduction Laboratories. Pan- and phospho-monoclonal antibodies which recognize respectively the non phosphorylated and phosphorylated forms of p38 MAPK, ERK1/2, and JNK1/2 were purchased from R&D system. All the polyclonal antibodies were tested in competition with their corresponding peptides. Monoclonal antibody was tested according to the manufactures recommendation using the protein positive controls.

Immunofluorescence

10- μ m unfixed cryostat sections were incubated with the primary antibody used at the appropriate dilution for 1h at RT. After washing with phosphate-buffered saline (PBS) solution, sections were incubated with a secondary antibody (Cy3-goat anti-rabbit IgG, Chemicon International). For negative controls only the second

antibody was applied. Sections were visualized under a Nikon optiphot-2 microscope 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% SDS buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 5% SDS) supplemented with 1% trypsin inhibitor, 1% saponin, and 15 μ g/ml leupeptin. After centrifugation (10 min at 13,000 \times g), protein concentration was estimated in the supernatant using the BCA protein assay kit (Pierce). Protein homogenates (40mg) recovered from the obtained supernatant of each sample were denatured for 5 min 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 submitted, in duplicate, to SDS-PAGE (3-10% or 5-15%) with prestained standard proteins (Bio-Rad) to achieve a more accurate molecular weight determination. The resulting gel, containing the separated protein, was transferred onto 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 (RT). All membranes were incubated with primary antibodies overnight at 4°C. After washing 3 times for 10 minutes in Tris-buffer, 0.1% Tween 20 (TBST), the membranes were incubated with peroxidase-conjugated antibodies (Chemicon International) for 1 hour. After washing the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham). The protein signals were quantified by scanning densitometry using the NIH program package. The results from each experimental group were expressed as relative integrate intensity compared to the control samples. Equal loading of proteins was assessed on stripped blots by immunodetection using a β -actin antibody.

Zymography

Zymography was carried out according to Heussen and Dowdle³⁹. Zymogram gel (5-15% polyacrylamide) was impregnated with gelatin at 1mg/ml. After electrophoresis, the gel was washed in 2.5% Triton X100 solution at room temperature and incubated for 24 h in a substrate buffer (50mM Tris-HCl, pH 8.0, 5mM CaCl₂, 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 h 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 colour and the MMP bands were quantified using the NIH image analysis program.

Statistics

Results are expressed as mean \pm SD. Statistical analysis was performed by unpaired Student's test and multiple statistical comparisons between groups was performed by one-way analysis variance followed by Bonferroni's t-test posthoc correction for allowing a better evaluation of intra- and inter-group variability and avoiding false positives using Statview program (version 5.0, SAS Institute Inc). Statistical significance was set at $^{\S, \text{¶}}$. * $P < 0.01$.

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 ($^{\text{¶}}$ $P < 0.01$, Table 1). Before treatment mdx mice were 7% heavier than wild-type. After two weeks, no significant change was observed in either the untreated groups. ALA/L-Car treatment significantly increased body weights by two fold in wild-type-T group and by three fold in the mdx-T group. In the mdx group, among the muscles analysed, only the weight of the diaphragm was increased by more than 10% (* $P < 0.01$, Table 1). As shown in Fig. 1A in the left panel the dystrophin deficient diaphragms (c and d) presents extensive areas of degeneration, with small centro-nucleated and clustered necrotic fibres with several inflammatory cells³⁷ as compared to wild-type muscle (image a and b). The treatment improves the histological pattern of the mdx-T diaphragms (Fig. 1A, image d). The morphometrical analysis is summarized in table 2, there is 10% increase in the number of a normal size fibres, with 35% decrease in centrally nucleated fibres. 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 non-muscle areas are also reduced by 52% after treatment (* $P < 0.01$).

Creatine kinase (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). 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). Data are expressed \pm SD and significance is taken at * $P < 0.01$ (Fig. 2).

SOD, GPx, CAT and lipid peroxydation 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 following ALA/L-Car treatment. The Diaphragm is considered in the mdx mice, as the best model to study dystrophin-deficiency. Thus to clarify our finding, all the following data will be presented in detail only in the diaphragm. Fig.3 shows the fold increase of SOD, GPx and CAT activities in the experimental groups (panel A, B, 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. Similarly, TBARS was also

higher in mdx than in wild-type muscles and was significantly decreased in the mdx-T group After ALA/L-Car treatment ($*P<0.01$) (Fig. 3, panel D).

HO-1 expression evaluation

HO-1 was either absent or gave a very weak staining pattern in normal muscle tissue (Fig.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 fibres were HO-1 negative (Fig 4 panel A). 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 (Fig 4 panel B). The decreased level of HO-1 could be attributed to a decreased number of infiltrating macrophage in mdx diaphragms which leads to a reduced diffusion of the free radical species in the micro environment of the dystrophic muscle fibres.

Inflammation and apoptotic cascade

The inflammatory process is activated in the mdx muscles essentially by infiltrating cells (such as macrophages) which produce NF- κ B and other molecules involved in inflammatory, immune, and acute stress response. Our experiments showed as expected increased levels of NF- κ B in mdx diaphragms compared to Wt samples (Fig. 5 panel A and B). Interestingly, we observed a decreased level of NF- κ B in mdx diaphragms samples from the mdx-T group compared to -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 pro-apoptotic 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 also had not affected their respective levels in Wt-T group (Fig 5 panel A and B). This experiment is an agreement with a recent study which suggested that Bcl2-mediated apoptosis appears 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 -dystroglycan level and processing in mdx-diaphragms

In the mdx-T group, a significant change concerning the β -dystroglycan level was found. In mdx muscles β -DG was cleaved by the matrix degrading metalloproteinase (MMPs) into a C-terminal 30 kDa β -DG fragment. Using a polyclonal C-terminal antibody which recognizes the two forms of β -DG, we analyzed the level of the two forms of β -DG by western blot detection. β -DG full form (β -DG₄₃) was increased after treatment in the mdx-T groups whereas the β -DG₃₀ form was decreased which suggest that our treatment inhibited the processing mediated by the MMPs in mdx-T diaphragms compared to mdx-Ss one (Fig 6). The β -DG₃₀ was weakly or absent in Wt samples and the treatment did not affect the level of β -DG₄₃ in Wt group (Fig. 6 panel A and B).

Free radical scavenger inhibit MMPs activity in mdx diaphragms

MMPs especially MMP-2 and MMP-9 gelatinases were involved in tissue remodelling which occurs during skeletal muscle degeneration and regeneration^{41, 42}. When compared to Wt diaphragms, our zymography results demonstrate that mdx diaphragm samples, exhibited an increased activity of both MMP-9 and MMP-2 (respectively detected at approximately 100 kDa and a major 60 kDa form, Fig. 7, panel A). 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 present 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) (Fig. 7, histograms panel B). 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 kinases 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 signalling 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 (non phosphorylated forms, pan-) of these proteins (Fig. 8, panel A), whereas the treatment affects the activity/phosphorylation (p-) status of p38, ERK1/2, and JNK1/2 in the mdx diaphragms (Fig. 8, panel B). As expected the level of phosphorylated p38 MAPK (p-p38) was decrease in mdx samples compared to Wt groups. p-p38 MAPK level was significantly decreased in mdx-T compared to mdx-Ss whereas no significant difference was observed between Wt-Ss and Wt-T groups. The p-ERK1 and p-ERK2 dually phosphorylated at T202/Y204 and T185/Y187, respectively and were detected as double bands at 44 kDa (p-ERK1) and 42 kDa (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 was observed in Wt-T groups compared to Wt-Ss one. The p-JNK1 and p-JNK2 proteins were analyzed separately. Our experiment was in agreement with previous reports which showed that p-JNK1 was overexpressed in mdx muscle compared to Wt samples. Furthermore,

the p-JNK1 phosphorylation level was significantly decreased in treated mdx group (mdx-T) compared to 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 to control group (Wt-Ss). All the data obtained after western blot revelation are summarized in the histograms shown in Fig 8, panel C.

Discussion

In this study we investigated the relationship between oxidative stress, NF- κ B/MMPs activation, apoptotic status, and the dystrophic process in dystrophin-deficient diaphragm muscle from 5 week-old mdx mice after a 2 week 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 peroxydation level after ALA/L-Car treatment in mdx diaphragm clearly demonstrate that the oxidative damage and activation of the proinflammatory cascade NF-KB/MMPs contribute to the progression of dystrophic pattern in the mdx muscles.

The over activation of the MMPs in mdx muscle accentuates the processing of β -DG which leads to disintegration of the dystroglycan complex and enhances DGC disorganization⁴⁵. In addition, ROS 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 defence mechanism is most likely unable to blunt the increased oxygen radical formation²⁸. This work showed that several antioxidant enzymes such SOD, GPx, and CAT, as well as the lipid peroxydation levels were significantly decreased after a free radical scavenger (ALA/L-Car) administration in mdx mice. This suggests that lipid peroxydation inhibition could induce a significant attenuation of membrane injury, restoring the defence 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 which suggests 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 to 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 analysed TA, VL and cardiac muscle. This could be explained by the fact that mdx mouse diaphragm showed a severe degenerative and dystrophic pattern due to perpetual contraction/relaxation cycles⁴⁶. Moreover, Nakae et al. have reported that oxidative stress may contribute to necrosis in mdx diaphragm muscle: most necrotic myofibres, myosatellite and interstitial inflammatory cells in mdx diaphragm muscles containing accumulated lipofuscin^{47, 48}. In this context recently Messina et al. have demonstrated that the NF- κ B inhibitor IRF1 042 was able to strongly reduce the pathological cascade in mdx muscles²⁸. In fact, evidences demonstrated that the

activation of NF- κ B can lead an increased expression of inflammatory molecules such as IL-6, TNF- α , cell adhesion molecules, and matrix metalloproteinase-9²⁶. Chen et al. have also shown a strong induction of NF- κ B soon after birth in DMD muscle. They have observed a stage-specific remodelling of DMD muscle with inflammatory pathways predominating in the presymptomatic stage and acute activation of transforming growth factors (TGF)- 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 mdx treated 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 interaction⁵⁰. 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 disorganisation⁴⁵. Over-activation of MMP-9 was also observed in injured and mdx muscle in response to a chronic treadmill exercise⁴¹ which suggests that this metalloproteinase could be associated with either degeneration or regeneration cycles in muscle⁴¹. In agreement with these reports, our data suggests that MMPs activity could be also associated with the pathological pattern observed in mdx muscles and also reflects the degree of membrane injury caused by ROS 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 types¹⁸. In mdx diaphragm muscle, p38 MAPK and ERK1/2 phosphorylation was different from the other muscles⁴⁴. In cardiac muscle phosphorylation of p38 MAPK was also dramatically increased in exercise trained mdx mice⁵¹ and utrophin-dystrophin-deficient mice⁵². Our treatment decreases the phosphorylation level of p38 in mdx diaphragms and could be associated to the efficiency of our treatment on cell survival, slowing degeneration and inflammatory pattern. JNK is also thought to be associated with cell death and apoptosis⁵³. We demonstrated that JNK1 and JNK2 phosphorylation was decreased in mdx diaphragm following 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 features⁵⁴, JNK2 activation was also implicated in the progression of dystrophic features after exercise in mdx muscle⁵¹. 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 signalling molecules of the ERK-MAP kinase cascade⁸.

The situation regarding ERK activation is unclear in the literature. Kolodziejczyk et al. 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 mouse⁵⁴. Furthermore, significantly elevated levels of active ERK were found in the hearts of non-exercised and exercised mdx mice compared to controls⁵¹.

ERK activity was also found to be increased in the hearts of caveolin-3 null mice probably because caveoline-3 is able to interact with ERK and suppress its activation⁵⁵. 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 DGC signalling to ERK might be mediated⁸.

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 -DG⁵⁶. 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 suggested 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 -dystroglycan/ α -dystroglycan interaction by MMP processing leads to an imbalance in ERK-MAP kinase signalling that could affect cell cycle progression and gene expression thus contributing to the muscular dystrophy phenotype (see hypothetical schema, Fig 9).

In conclusion our understanding of the role of damage by oxygen species and the associated signalling cascades in dystrophin-deficient muscles is always at a relatively early stage. But 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 open new strategies of treatment that could modify the dystrophic process and slow down its progression.

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Figure Legends

Figure 1. A, Hematoxylin and eosine (H&E) staining on 10- μ m 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 centro-nucleated and less connective tissue with less areas of mononuclear cell infiltrate. Scale bar: 25 μ m.

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 material 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 = grey light bar, mdx-Ss = grey dark bar, and mdx-T = black bar). Values are expressed \pm 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 enzymes activity of the wild-type group injected with the saline solution (Wt-Ss). Panel TBARS (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 \pm 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, images A3 and A4, respectively) showed that this protein is expressed by inflammatory cells such as macrophages (image A5 and A6). ALA/L-Car treatment decreases the level of these infiltrating cells in mdx-T diaphragm (image 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 diaphragms extracts.

Figure 5. A, Effect of ALA/L-Car on NF- κ B, BAX, and Bcl2 protein level. 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) means not significant.

Figure 6. A. Effect of ALA/L-Car treatment on β -dystroglycan level. Western blot detection of β -dystroglycan (β -DG) in diaphragm extracts of each experimental groups showed that this protein was expressed as two forms the full length form with a Mr of 43 kDa (β -DG₄₃) and a cleaved form with Mr of about 30 kDa (β -DG₃₀). **B.** Histograms under the blots showed quantification of protein bands (arbitrary unit). 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 were expressed as means \pm SD and statistical significance was set at $*P < 0.01$.

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 to mdx-Ss. **B.** Gelatin zymography gels were treated with black/white colour and the MMP bands were quantified using the NIH image analysis program. Histogram representation confirmed that MMP-2 and MMP-9 activity were decreased in mdx-T group (n = 8) compared to 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 $^{\$}P < 0.01$ for MMP-9.

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 analysed via western blot using each

phosphorylated (p) (**A**) and non-phosphorylated (pan) (**B**) antibody raised against these proteins. Lane 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. Quantitative analysis of p-p38 MAPK, p-ERK1/2, p-JNK1, and p-JNK2 is showed under histogram representation after analysis of each experimental groups (Wt-Ss: n = 7, Wt-T: n = 7, mdx-Ss: n = 8, and mdx-T: n = 8). Data were expressed as means \pm SD and statistical significance was set at $*P < 0.01$.

Figure 9. A. Synthetic representation of muscle fibre membrane (sarcolemma) showing hypothetical cascade involving NF-kB/MMPs activity and several MAP kinase in dystrophic process. Activated MMPs in dystrophic myofibres, leads to disintegrate the dystroglycan complex by cleaving the N-terminal part of β -DG into β -DG₃₀. ALA/L-Car treatment can decreased ROS and proinflammatory molecules level and attenuates dystrophic pattern by stabilizing the β -DG₄₃ form in sarcolemma. **B** represents normal dystroglycan complex through the sarcolemma.

Table 1: Effect of ALA/L-Car treatment on body and muscle weight. n: number of animal per experimental group; body weight at the beginning (time 0) and after 2 weeks of treatment. Muscle weight in (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 $^{\text{¶}}P < 0.01$ (wild type versus mdx mice) and at $*P < 0.01$ (ALA/L-Car versus Saline solution injected mice). Data are expressed as mean \pm SD.

Table

| | n | 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 \pm 1.2 | 19.4 \pm 0.64 | 0.055 \pm 0.002 | 0.112 \pm 0.009 | 0.079 \pm 0.008 | 0.107 \pm 0.011 |
| Wild type T | 7 | 16.5 \pm 0.56 | 20.8 \pm 0.75 | 0.061 \pm 0.004 | 0.118 \pm 0.011 | 0.081 \pm 0.006 | 0.106 \pm 0.010 |
| mdx Ss | 8 | 18.9 \pm 0.96 [¶] | 20.5 \pm 0.34 [¶] | 0.072 \pm 0.007 [¶] | 0.141 \pm 0.010 [¶] | 0.101 \pm 0.007 [¶] | 0.113 \pm 0.019 [¶] |
| mdx T | 8 | 18.0 \pm 0.88 | 22.3 \pm 0.83 | 0.078 \pm 0.003 | 0.142 \pm 0.013 | 0.112 \pm 0.002 [*] | 0.114 \pm 0.016 |

Table 2: Morphometrical analysis of diaphragm sections. 5 sections from each muscle of all experimental groups were analyzed by Histolab program. Each section contains a number of muscle cell ranging 100 to 150 fibres. The parameters evaluated were: the percentage of normal fibres, the percentage of centrally nucleated fibres, the non-muscle area (the amount of space between fibres or between groups of muscle fibres that is not stained as muscular tissue), and the variance coefficient (showed the regularity of fibres dimensions). Data are expressed \pm SD and significantly difference was set at $*P < 0.01$.

Table

| Diaphragm | | | | |
|---------------------|----------------------|------------------------------|-------------------|-----------------------------|
| | Normal fibers | % Centrally nucleated | Non muscle | Variance coefficient |
| 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 * |