Lipid Peroxidation Inhibition Blunts Nuclear Factor- κ B Activation, Reduces Skeletal Muscle Degeneration, and Enhances Muscle Function in *mdx* Mice

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Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease resulting from lack of the sarcolemmal protein dystrophin. However, the mechanism leading to the final disease status is not fully understood. Several lines of evidence suggest a role for nuclear factor (NF)- κ B in muscle degeneration as well as regeneration in DMD patients and *mdx* mice. We investigated the effects of blocking NF- κ B by inhibition of oxidative stress/lipid peroxidation on the dystrophic process in *mdx* mice. Five-week-old *mdx* mice received three times a week for 5 weeks either IRFI-042 (20 mg/kg), a strong antioxidant and lipid peroxidation inhibitor, or its vehicle. IRFI-042 treatment increased forelimb strength (+22%, $P < 0.05$) and strength normalized to weight (+23%, $P < 0.05$) and decreased fatigue (−45%, $P < 0.05$). It also reduced serum creatine kinase levels ($P < 0.01$) and reduced muscle-conjugated diene content and augmented muscle-reduced glutathione ($P < 0.01$). IRFI-042 blunted NF- κ B DNA-binding activity and tumor necrosis factor- α expression in the dystrophic muscles ($P < 0.01$), reducing muscle necrosis ($P < 0.01$) and enhancing regeneration ($P < 0.05$). Our data suggest that oxidative stress/lipid peroxidation represents one of the mechanisms activating NF- κ B and the consequent pathogenetic cascade in *mdx* muscles. Most importantly, these new findings may have clinical implications for the pharmacological treatment of patients with DMD. (Am J Pathol 2006; 168:918–926; DOI: 10.2353/ajpath.2006.050673)

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease leading to loss of ambulation by the 13th year and to death, usually in early adulthood.¹ The disease results from absence of the protein dystrophin, which is an essential component of the dystrophin-glycoprotein complex that maintains membrane integrity of muscle fibers by linking cytoskeleton to extracellular matrix.^{2–5} Although the primary genetic defect is known, how this mutation gives rise to the final disease status is not fully understood. The mechanisms responsible for the pathological hallmarks of the dystrophic process, such as necrosis, phagocytosis, infiltration of inflammatory cells, initial efficient regeneration followed by a decline and secondary fibrosis, have not been definitively identified. DMD pathogenesis is frequently studied in the genetically homologous animal, the *mdx* mouse, despite relevant clinical and pathological differences. The murine model exhibits late muscle weakness, a slow disease progression, similar extensive degeneration and regeneration occurring between 2 and 12 weeks of age, but no proliferation of connective tissue in limb muscles.^{6,7}

Several lines of evidence suggest that oxidative stress might be involved in the dystrophic process. Free radical injury may contribute to loss of membrane integrity in muscular dystrophies⁸ and dystrophic muscle cells have an increased susceptibility to reactive oxygen intermediates.^{9–11} Markers of oxidative stress have been detected in muscles of either DMD patients or *mdx* mice.^{9,12,13} An involvement of reactive oxygen intermediates is also supported by observations of increased biological by-products of oxidative stress,¹⁴ reduced cellular antioxidants

Supported by a Research Program of Relevant National Interest grant from the Italian Ministry of Education, University, and Research (to G.V.) and by other departmental funding.

Accepted for publication December 8, 2005.

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(glutathione and vitamin E), and altered concentrations of antioxidant enzymes.^{12,15}

Recently, the role of nuclear factor- κ B (NF- κ B) in the skeletal muscle-wasting process is gaining increasing attention, mainly because NF- κ B is activated in response to several inflammatory molecules that cause muscle loss.^{16,17} NF- κ B is an ubiquitous transcription factor regulating the expression of a plethora of genes involved in inflammatory, immune, and acute stress responses.¹⁸ In fact NF- κ B, after proteasomal degradation of the inhibitory protein I- κ B (I- κ B), translocates to the nucleus and binds target DNA elements in the promoter of different genes expressing cytokines, chemokines, cell adhesion molecules, immunoreceptors, and inflammatory enzymes such as nitric oxide synthase, matrix metalloproteinases, and phospholipase A2.^{19–21} On the other hand, NF- κ B is activated in response to several inflammatory molecules, such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), metalloproteinases, whose circulating levels have been found elevated in DMD and other types of muscular dystrophies.^{22–25} Moreover oxidative stress strongly activates NF- κ B,^{26–28} which is involved in the up-regulation of antioxidant enzymes such as glutathione peroxidase and catalase.²⁸ An involvement of NF- κ B in myogenesis has been suggested because its activity was shown to be required by human and rat myoblasts to fuse into myotubes and to express muscle-specific proteins such as myosin heavy chain and caveolin 3.²⁹ In addition, it has also been demonstrated that systemic administration of the NF- κ B inhibitor curcumin stimulates muscle regeneration after traumatic injury, suggesting that modulation of NF- κ B activity within muscle tissue could be beneficial for muscle repair.³⁰

Very recently, NF- κ B activity has been demonstrated to be increased in muscles of either DMD patients²¹ or *mdx* mice,^{16,17} but its effective role in DMD pathogenesis is not clear to date. Interestingly, we have reported the novel observation of increased immunoreactivity for NF- κ B in the cytoplasm of all regenerating fibers and in 20 to 40% of necrotic fibers in DMD as well as in inflammatory myopathies.²¹

Taken together, 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 the subsequent release of inflammatory mediators. This work hypothesis would also indicate that the interruption of this cascade might have a therapeutic potential. To confirm and clarify this issue, we used as a pharmacological tool ($\pm$)-5-emissuccinoyl-2-[2-(acetylthio)ethyl]-2,3-dihydro-4,6,7-trimethylbenzofuran (IRFI-042), a synthetic, vitamin E analogue. Vitamin E has been suggested to act as potential inhibitor of NF- κ B activation³¹; nevertheless the marked lipophilicity of this vitamin limits its therapeutic potential with low circulating levels and poor tissue distribution after somministration. IRFI-042 is a less lipophilic compound with powerful antioxidant properties due to the combination in the same molecule of a chain-breaking moiety (characteristic of phenols related to α -tocopherol) with the reducing ability of a thiol group (dual antioxidant). Moreover this compound shows no systemic toxicity even after high dosage

(up to 1 g/kg).³¹ IRFI-042 possesses a strong inhibitory activity on both oxidative stress/lipid peroxidation and NF- κ B activation demonstrated in different experimental models, such as endotoxin-induced shock,³² organ ischemia/reperfusion injury,³³ neurotoxicity,³⁴ and impaired wound healing process.³⁵ The aim of our study was to test the novel hypothesis that the modulation of 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 patterns.

Materials and Methods

Animals

Male *mdx* and wild-type C57BJ/10 (WT) mice were obtained from The 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 with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no.85-23, revised 1996). Five-week-old *mdx* and WT mice have been treated for 5 weeks with intraperitoneal injections with either IRFI 042 (n:8; 20 mg/kg three times a week) or vehicle (n:8; dimethyl sulfoxide/NaCl 0.9%; 0.1:1 v/v; 0.2 mg/kg three times a week). At the end of the experiments, animals were anesthetized with an intraperitoneal administration of sodium pentobarbital (80 mg/kg). Then, blood, collected by intracardiac puncture, was drawn to analyze creatine kinase (CK) levels and the biceps, quadriceps, and extensor digitorum longus (EDL) muscles were removed bilaterally and immediately frozen in liquid nitrogen-cooled isopentane and stored at -80°C for morphological and biochemical evaluations.

Animal Examinations

Mice were weighed and examined for forelimb strength at baseline and after 5 weeks of treatment. Strength testing consisted of five separate measurements using a grip meter attached to a force transducer that measures peak force generated (Stoelting Co., Wooddale, IL). The mouse grabs the trapeze bar as it is pulled backward and the peak pull force in grams is recorded on a display. The three highest measurements for each animal were averaged to give the strength score. We calculated also the degree of fatigue by comparing the first two pulls to the last two pulls. The decrement between pulls one and two and pulls four and five gives a measure of fatigue.⁶

Serum CK Evaluation

Blood samples were centrifuged at 6000 rpm and the serum was stored at -80°C until the day of analysis. Serum CK was evaluated at 37°C using a commercially available kit (Randox Laboratories Ltd., Antrim, UK). The results were expressed as U/L.

Histological Studies

Ten- μm -thick transverse cryostat sections were obtained from the midpoint of the biceps and EDL muscle body. The whole muscle cross-sections (corresponding to a mean area of 2.15 mm² in biceps and 1.69 mm² in EDL), stained with hematoxylin and eosin (H&E), were examined by a blinded observer, using the AxioVision 2.05 image analysis system equipped with Axiocam camera scanner (Zeiss, Munchen, Germany). The following four areas were recognized with patchy distribution: 1) normal fibers, identified by the presence of peripheral nuclei; 2) centrally nucleated fibers, identified by normal size but with central nuclei; 3) regenerating fibers, identified by small size, basophilic cytoplasm, and central nuclei; 4) necrotic fibers, identified by pale cytoplasm and phagocytosis. The results were expressed as the ratio of the area occupied by normal fibers, centrally nucleated fibers, regenerating fibers, or necrotic fibers divided by the total surface area as a percentage.

Immunocytochemistry

Seven- μm -thick transverse cryostat sections from biceps and EDL muscles were incubated for 120 minutes at 37°C in rabbit polyclonal antibody against phospho-NF- κB p65 subunit (Ser276) (1:50; Cell Signaling Technology, Beverly, MA). It selectively binds to the NF- κB p65 only when phosphorylated at serine 276, ie, it is activated and can then undergo nuclear translocation. Nonspecific binding of immunoglobulin was blocked with 5% normal horse serum. Immunodetection was performed using a biotin-avidin system (DAKO, Milan, Italy) followed by horseradish peroxidase staining with 3,3'-diaminobenzidine tetrahydrochloride.

Evaluation of Conjugated Dienes (CDs) Content

Estimation of the tissue content of CDs was performed to evaluate the extent of lipid peroxidation in tissue as previously shown.³¹ Samples of biceps muscle were collected in polyethylene tubes and then washed with 1 ml of butylated hydroxytoluene (BHT) (1 mg/ml in phosphate buffer). The samples, after drying in absorbent paper, were frozen at 4°C until the analysis. The biochemical assay of CDs required previous lipid extraction from the tissue samples by chloroform/methanol (2:1). The lipid layer was dried under nitrogen atmosphere and then dissolved in cyclohexane. Muscle contents of CDs was measured at 232 nm by using a spectrophotometric technique. The amount of muscle CDs was expressed as $\Delta\text{ABS}/\text{mg}$ protein.

Evaluation of Reduced Glutathione (GSH) Levels

GSH activity was evaluated to estimate endogenous defenses against oxidative stress. The levels in biceps muscles were determined as previously described.³¹ Briefly, tissue samples were homogenized with a Ultra-turrax (IKA, Staufen, Germany) homogenizer in a solution con-

taining 5% trichloroacetic acid and 5 mmol/L ethylenediamine tetraacetic acid at 4°C. Then each sample was centrifuged at 15,000 $\times g$ for 10 minutes at 4°C. Homogenate supernatant (0.4 ml) was added in polyethylene dark tubes containing 1.6 ml of Tris-ethylenediamine tetraacetic acid buffer 0.4 mol/L, pH 8.9. After vortexing, 40 μl of 10 mmol/L dithiobisnitrobenzoic acid were added. The samples were vortexed again and the absorbance was read after 5 minutes at 412 nm. The values of unknown samples were drawn from a standard curve plotted by assaying different known concentrations of GSH. The amount of muscle GSH was expressed as $\mu\text{mol}/\text{g}$ protein.

Electrophoretic Mobility Shift Assay

NF- κB binding activity in quadriceps muscle specimens was performed in a 15- μl binding reaction mixture containing 1% binding buffer [50 $\mu\text{g}/\text{ml}$ of double-stranded poly (dl-dC), 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.5 mmol/L ethylenediamine tetraacetic acid, 0.5 mmol/L dithiothreitol, 1 mmol/L MgCl₂, and 10% glycerol], 15 μg of nuclear proteins, and 35 fmol (50,000 cpm, Cherenkov counting) of double-stranded NF- κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Promega, Madison, WI) that was end-labeled with [γ -³²P] ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Life Sciences, Arlington Heights, IL) using T4 polynucleotide kinase. The binding reaction mixture was incubated at room temperature for 20 minutes and analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. After electrophoresis, the gels were dried using a gel-drier and exposed to Kodak X-ray films at -70°C. The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profile; Celbio, Milan, Italy). The results were expressed as relative integrated intensity compared with normal controls, considering exposure time, background levels, and known protein concentration of an Epstein-Barr virus nuclear antigen-1 extract, which was used as electrophoretic mobility shift assay control.

Western Blot Analysis

Samples from quadriceps muscles were homogenized in lysis buffer (1% Triton X-100, 20 mmol/L Tris/HCl, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 5 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L phenylmethyl sulfonyl fluoride, 1% aprotinin, 15 $\mu\text{g}/\text{ml}$ leupeptin). Protein samples (40 μg) were denatured in reducing buffer (62 mmol/L Tris, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 5% β -mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis on sodium dodecyl sulfate (12%) polyacrylamide gel with prestained standard proteins (Bio-Rad, Milan, Italy) to achieve a more accurate molecular weight determination. The separated proteins were transferred onto a nitrocellulose membrane using the transfer buffer (39 mmol/L glycine, 48 mm Tris, pH 8.3, 20% methanol) at 200 mA for 1 hour. The membranes were stained with Ponceau S (0.005% in

1% acetic acid) to confirm equal amounts of protein and were blocked with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween for 1 hour at room temperature, washed three times for 10 minutes each in Tris-buffered saline-0.015% Tween, and incubated with rabbit monoclonal antibody against TNF- α (Chemicon, Temecula, CA) in Tris-buffered saline-0.1% Tween overnight at 4°C. After washing three times for 10 minutes each in Tris-buffered saline-0.15% Tween, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (Pierce, Milan, Italy) for 1 hour at room temperature. 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 a bio-image analysis system (Bio-Profil, Celbio). The results from each experimental group were expressed as relative integrated intensity compared with control muscle measured with the same batch. Equal loading of protein was assessed on stripped blots by immunodetection of β -actin with a rabbit monoclonal antibody (Cell Signaling, Celbio) diluted 1:500 and peroxidase-conjugated goat anti-rabbit IgG (Pierce) diluted 1:15,000. All antibodies are purified by protein A and peptide affinity chromatography.

Drug

IRFI 042 was supplied by Biomedica Foscama Research Centre, Ferentino, Italy. All substances were prepared fresh daily and administered in a volume of 1 ml/kg.

Statistical Analysis

Results are expressed as mean $\pm$ SD. Statistical evaluation was performed by using one-way analysis of variance followed by Dunnett's post hoc tests and paired Student's *t*-test with the use of the InPlotPrism software version 3.0 (GraphPad Software, San Diego, CA). *P* values <0.05 were considered significant.

Results

Body Weight, Forelimb Strength, and Fatigue

Body weight was not significantly different among the animal groups at baseline as well as after treatment. Comparing the values longitudinally, at the end of the experiment all groups had an increased body weight ($P < 0.01$) (Figure 1A). At baseline, strength and strength normalized to weight were significantly lower in both *mdx* mice groups (assigned to IRFI 042 or to vehicle treatment) compared to WT groups (allocated to IRFI 042 or to vehicle treatment) ($P < 0.05$). At the end of treatment, IRFI 042-treated *mdx* mice had higher forelimb strength (+22%, $P < 0.05$) and strength normalized to weight (+23%, $P < 0.05$) compared to vehicle-treated *mdx* mice (Figure 1, B and C). In all groups the somatic growth paralleled with an increment of strength if compared with baseline values ($P < 0.01$ in *mdx* + IRFI 042, $P < 0.05$ in

Figure 1. Effects of IRFI 042 and vehicle treatment on body weight (A), forelimb strength (B), forelimb strength normalized to weight (C), and fatigue (D) in WT and *mdx* mice ($n = 8$ in each group). * $P < 0.05$ and $^{\S}P < 0.01$ versus baseline value; ** $P < 0.05$ IRFI 042-treated versus vehicle-treated *mdx* mice. For statistical analysis between *mdx* and WT mice at baseline and at the end of treatment, see text.

the other groups) (Figure 1B), but when normalized to weight only the IRFI 042-treated *mdx* mice showed a significant amelioration in strength ($P < 0.05$) (Figure 1C).

At baseline, the percentage of fatigue was significantly higher in both *mdx* mice groups compared to WT groups ($P < 0.01$); furthermore, there was not any significant difference between the two *mdx* groups (Figure 1D). After treatment we found in both *mdx* groups a higher level of fatigue compared to WT groups ($P < 0.001$ in *mdx* + vehicle, $P < 0.01$ in *mdx* + IRFI 042), but the value was significantly lower in IRFI 042-treated compared to vehicle-treated *mdx* (−45%, $P < 0.05$). Comparing the data longitudinally, the percentage of fatigue increased in the vehicle-treated ($P < 0.05$) and remained stable in the IRFI 042-treated *mdx* mice (Figure 1D). IRFI 042 did not cause any significant change in the forelimb strength, strength normalized to body weight, and fatigue of WT animals.

CK Level Evaluation

Low CK levels were observed in WT animals treated either with vehicle or IRFI 042 (WT + vehicle = 221 ± 33 U/L, WT + IRFI 042 = 145 ± 28 U/L). *Mdx* mice showed a significant increase in serum CK levels (*mdx* + vehicle = 2662 ± 79 U/L, $P < 0.01$ versus WT + vehicle). IRFI 042 administration resulted in a marked reduction of the enzyme levels (*mdx* + IRFI 042 = 681 ± 173 U/L, $P < 0.01$ versus *mdx* + vehicle) (Figure 2).

Histological Studies

Wild-type animals showed a normal architecture of the biceps and EDL muscles that was not modified by treatment with IRFI 042 (Figure 3). Both biceps and EDL muscles from vehicle-treated *mdx* mice showed necrosis and regeneration (Figures 3, 4, and 5). Quantitative morphological evaluation of biceps muscle from IRFI 042-

Figure 2. Effects of IRFI 042 and vehicle treatment on serum CK levels ($n = 8$ in each group). * $P < 0.01$ versus vehicle-treated WT mice; § $P < 0.01$ versus vehicle-treated *mdx* mice.

treated *mdx* mice revealed a significant increase in regenerating area ($P < 0.05$) and a decrease in necrotic area ($P < 0.01$) (Figures 3 and 5). Similarly, EDL muscle from IRFI 042-treated *mdx* mice showed an increase in regenerating area ($P < 0.05$) and a decrease in necrotic area ($P < 0.01$) (Figures 4 and 5).

Immunocytochemistry

In vehicle-treated *mdx* mice muscles, a strong nuclear NF- κ B immunoreactivity was seen in 3 to 5% of normal fibers, 70 to 80% of regenerating fibers, and 90 to 95% of centrally nucleated fibers. NF- κ B immunoreactivity was absent in necrotic fibers with or without myophagia. In IRFI 042-treated *mdx* mice, NF- κ B immunoreactivity was markedly reduced (Figure 6).

Figure 3. Surface area of biceps muscles occupied by normal area (A), necrotic area (B), regenerating area (C), and centrally nucleated fiber area (D) in the different animal groups ($n = 8$ in each group). * $P < 0.05$ and § $P < 0.01$ versus vehicle-treated *mdx* mice.

Figure 4. Surface area of EDL muscles occupied by normal area (A), necrotic area (B), regenerating area (C), and centrally nucleated fiber area (D) in the different animal groups ($n = 8$ in each group). * $P < 0.05$ and § $P < 0.01$ versus vehicle-treated *mdx* mice.

CD and GSH Level Evaluations

Low CD content and a normal basal amount of GSH levels were observed in WT animals treated either with vehicle or IRFI 042 (Figure 7). *Mdx* mice showed markers of oxidative stress damage, characterized by a significant increase in the tissue content of CD, accompanied by a concomitant decrease in the muscle levels of GSH, when compared to wild-type animals ($P < 0.01$) (Figure 7). IRFI 042 administration in *mdx* mice resulted in a reduction of CD level and an increase in GSH value ($P < 0.01$) (Figure 7).

Figure 5. Histological appearance of biceps (A) and EDL (B) muscles in vehicle-treated (left column) and IRFI 042-treated (right column) *mdx* mice. IRFI 042-treated *mdx* mice showed decreased necrosis and enhanced muscle fiber regeneration in both muscles. H&E staining. Original magnifications, $\times 55$.

H&E

NF- κ B

Vehicle

IRFI 042

Figure 6. H&E staining (**A, B**) and NF- κ B immunoreactivity (**C, D**) on serial sections of biceps muscles in vehicle-treated (**left column**) and IRFI 042-treated (**right column**) *mdx* mice. A strong nuclear immunoreactivity for the activated form of NF- κ B was found in fibers with central nuclei in vehicle-treated *mdx* mice (**A, C**); necrotic fibers and macrophages (**top right corner**) were negative. IRFI 042 treatment drastically reduced NF- κ B immunoreactivity (**B, D**). Original magnifications, $\times 55$.

NF- κ B Binding Activity

NF- κ B DNA binding activity revealed by electrophoretic mobility shift assay was markedly increased in *mdx* mice administered with vehicle, when compared with WT mice ($P < 0.01$). Treatment with IRFI 042 drastically reduced NF- κ B binding activity in dystrophic mice ($P < 0.01$) (Figure 8A).

TNF- α Expression

TNF- α expression was very low in WT animals treated either with vehicle or IRFI 042. By contrast a marked increase in the expression of TNF- α was observed in vehicle-treated *mdx* mice ($P < 0.01$). The administration of IRFI 042 significantly reduced TNF- α expression in *mdx* mice ($P < 0.01$) (Figure 8B).

Discussion

In this study we contributed to clarify the relationship among oxidative stress/lipid peroxidation, NF- κ B activation, and dystrophic process in dystrophin-deficient *mdx* mouse. The ameliorated functional parameters and the reduced dystrophic pathology in IRFI 042-treated animals support the hypothesis that NF- κ B contributes to the progression of the dystrophic damage.

Recently, Nakae and colleagues³⁶ further supported the role of oxidative stress in DMD pathogenesis. They demonstrated an accumulation of lipofuscin, a product of oxidative degradation of cellular macromolecules caused by free radicals and redox-active metal ions, in muscles of DMD patients and *mdx* mice. In our study, the elevated

Conjugated dienes levels

Glutathione levels

Figure 7. Levels of muscular CD (**A**) and GSH (**B**) ($n = 8$ in each group). * $P < 0.01$ versus WT mice; § $P < 0.01$ versus vehicle-treated *mdx* mice.

CD levels in vehicle-treated *mdx* mice demonstrate an increased lipid peroxidation during the development of skeletal muscle damage. Moreover the low levels of reduced glutathione, an essential tripeptide that reacts with

Figure 8. **A:** Electrophoretic mobility shift assay of muscular NF- κ B binding activity. **B:** Western blot analysis of muscular TNF- α . On the **left** of each figure are graphs with quantitative data and on the **right**, representative autoradiograms ($n = 8$ in each group). * $P < 0.01$ versus WT mice; § $P < 0.01$ versus vehicle-treated *mdx* mice.

free radicals, indicate that the antioxidant defense mechanism is most likely unable to blunt the increased oxygen radical formation. These findings are in keeping with the reported increase of glutathione cycling components in DMD muscle fibers¹⁵ and of other lipid peroxidation products (thiobarbituric acid-reactive substances) in *mdx* mice⁹ associated with an up-regulation of several antioxidant enzymes activity, such as superoxide dismutase, catalase, and glutathione peroxidase.¹⁰ Interestingly, IRFI 042 treatment resulted in a significant reduction of CD levels and an increase in GSH content, accompanied by enhanced muscle function, blunted serum CK levels, and reduced myofiber degeneration. This suggests that lipid peroxidation inhibition by IRFI 042 could have induced a significant attenuation of membrane injury, restoring the defense mechanism in the muscle cells. In previous studies, cell oxidative state has been shown to influence the induction of NF- κ B activation, and in fact reactive oxygen intermediates can induce I- κ B phosphorylation by influencing the activity of tyrosine kinases.³⁵ Moreover, treatment of muscle cells with *N*-acetyl-L-cysteine, a free radical scavenger, completely inhibits stress-induced activation of NF- κ B.²⁷ In our experiment we confirmed that the augmented oxidative stress parallels an increased activation of NF- κ B in vehicle-administered *mdx* mice and consistent with these findings, we demonstrated that IRFI 042 treatment was able to strongly reduce this pathological cascade. Furthermore, this strong activation of NF- κ B in *mdx* mice muscles mostly occurs in regenerating fibers, since we reported the novel observation of a strong NF- κ B immunoreactivity in the nuclei of muscle cells at different levels of differentiation. IRFI 042 treatment almost completely blunted the NF- κ B immunoreactivity.

Several evidences demonstrated that the activation of NF- κ B can lead to an augmented expression of several inflammatory molecules such as IL-1 β , IL-6, TNF- α , cell adhesion molecules, and matrix metalloproteinase-9,^{19,16,37} furthermore increased levels of some of them have been observed in inflammatory myopathies, DMD, and *mdx* mice.^{16,22,25} The abnormal increase of IL-1 β and TNF- α has been suggested as a mechanism that promotes muscle wasting also in other cachexia-associated diseases.^{38,39} TNF- α is also one of the most important NF- κ B inducers, contributing to a positive feedback loop. Therefore it might be postulated that a positive feedback perpetuates the effects of the activation of NF- κ B signaling pathway in the context of the dystrophic process. In our study we found a marked enhancement of TNF- α expression in *mdx* muscle, strongly reduced by IRFI 042 treatment. These data are in agreement with the recently reported delay and reduction of the breakdown of dystrophic muscle in young *mdx* mice after pharmacological blockade of TNF- α activity with Remicade.⁴⁰ Moreover, our data would suggest that the protective effect of Remicade against muscle damage might be induced, at least in part, by inhibition of NF- κ B activity.

In *mdx* mice, muscle weakness and necrosis are present at ~4 to 5 weeks of age, and then a morphological recovery begins with an apparent stabilization of myopathy.^{6,41–43} For this reason we chose to start the

Figure 9. Synthetic scheme of the dystrophic process pathological cascade, showing hypothetical interactions between oxidative stress/lipid peroxidation, and NF- κ B activation in *mdx* mice.

study at the 5th week of age and to end it by the 10th week to better verify the effect of treatment on both functional and morphological patterns. In our study, we confirmed in *mdx* mice the presence of weakness and fatigue already at 5 weeks of age. At 10 weeks of age, after somatic growth the strength was increased, but it was evident a trend toward a decline in strength normalized to weight and a significant increment of fatigue; these data were accompanied by the presence of muscle necrosis and regeneration. On the contrary, IRFI 042-treated *mdx* mice showed an increment of strength, strength normalized to weight, and a stabilization of the levels of fatigue throughout the 5-week period. This beneficial effect was also supported by the histological findings, consisting in a significant decrease in the area occupied by necrosis and an increase in the area occupied by regenerating fibers in IRFI 042-treated compared to vehicle-treated *mdx* mice. The IRFI 042 effects on blunting NF- κ B immunoreactivity in regenerating cells and on promoting regeneration are also consistent with the results obtained by Thaloer and colleagues³⁰ of an enhanced muscle

repair after NF- κ B inhibition in a different model of muscle damage. Other NF- κ B inhibitors, such as corticosteroids, have been shown to reduce necrosis and to enhance regeneration.^{44,45} It can be hypothesized that the well known positive effect of corticosteroids in the treatment of DMD might be partially mediated through a NF- κ B activity inhibition, which in turn down-regulates expression of cytokines and adhesion molecules. Moreover other drugs with demonstrated positive effects in *mdx* mice, such as creatine, which improves intracellular Ca^{++} handling⁴⁶ and green tea, with antioxidant effects,⁴⁷ could inhibit at different steps the NF- κ B signaling pathway.

In several chronic inflammatory diseases, such as rheumatoid arthritis⁴⁸ and asthma,⁴⁹ the role of NF- κ B has been demonstrated in the amplification and perpetuation of the inflammatory process. In the *mdx* mouse model, Kumar and colleagues demonstrated a skeletal muscle-specific activation of NF- κ B even before the onset of muscular dystrophy and postulated that this might lead to an augmented level of TNF- α and IL-1 β .¹⁶ Herein we have obtained data showing that a cross-talk between oxidative stress/lipid peroxidation and NF- κ B activation is likely to occur in *mdx* mice, in turn triggering an inflammatory cascade contributing to muscle damage (Figure 9). Finally modulation of this cascade, obtained through IRFI 042 treatment, might represent a rational pharmacological approach to limit muscle damage in dystrophinopathies. However this hypothesis deserves further experiments to delineate possible therapeutic implications in DMD.

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