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Effects of Iron Deprivation on the Pathology and Stress Protein Expression in Murine X-Linked Muscular Dystrophy

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ABSTRACT. Duchenne muscular dystrophy (DMD) is caused by dystrophin deficiency, which results in muscle necrosis and the upregulation of heat shock/stress proteins (HSP). We hypothesized that reactive oxygen species, and in particular hydroxyl radicals ($^{\bullet}OH$), participate in muscle necrosis and HSP expression. It was assumed that iron deprivation decreases $^{\bullet}OH$ generation, restraining the disease process and reducing the oxidant-induced expression of HSP. The role of iron-catalyzed free radical reactions in the pathology of dystrophin-deficient muscle was evaluated in the murine model for Duchenne muscular dystrophy (*mdx*), by examining the effects of dietary deficiency and supplementation of iron on serum creatine kinase (CK), muscle morphology, lipid peroxidation and HSP levels in mice maintained on diets deficient in or supplemented with iron for 6 weeks. Iron-deprived *mdx* mice showed a significant decrease in the number of macrophage-invaded necrotic fibers and the expression of the 70-kDa heat shock protein (Hsp70). This suggests that the iron-dependent generation of $^{\bullet}OH$ relates to muscle necrosis in the *mdx* mouse and modulates the expression of Hsp70 *in vivo*. In contrast, iron deprivation had no influence on other HSP or on lipid peroxidation in *mdx* mice, while maintenance on either diet caused a significant decrease in serum creatine kinase activity. The potential therapeutic effects of iron deprivation in *mdx* should be considered. BIOCHEM PHARMACOL **56**;6:751–757, 1998.

KEY WORDS. Duchenne muscular dystrophy; glucose-regulated proteins; heat-shock proteins; hydroxyl radical; iron; *mdx*

DMD¶ is a severe genetic disorder affecting 1 in 3500 male births [1]. Mutations in the dystrophin gene at Xp21 prevent the expression of this cytoskeletal, membrane-associated protein in affected individuals, leading to progressive muscle wasting [2]. While the absence of dystrophin is clearly responsible for the DMD phenotype, the exact role of its deficiency in the pathobiochemistry of DMD has remained elusive [3]. One of the approaches taken in several studies to the pathogeny of DMD has been to consider free radical reactions [4–6]. There is, however, little consensus concerning the involvement of free radical reactions in the pathology of DMD, and antioxidant therapeutic trials have been inconclusive so far [for review, see 7]. In DMD muscle, the xanthine-xanthine oxidase system [5] and the respiratory burst of phagocytic cells may contribute to the generation of superoxide (O_2^-), which, in turn, is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase. The highly reactive hydroxyl radical ($^{\bullet}OH$) is produced during the metal-dependent decomposition of H_2O_2. Iron, in particular, is a major determinant in the generation of $^{\bullet}OH$ (Haber-Weiss and Fenton reactions), and its removal may provide a useful starting point for controlling free radical reactions [8].

We have shown that oxidative stress—in particular $^{\bullet}OH$ generated by the iron-catalyzed Fenton reaction—regulates the expression of the so-called HSP in human monocytes. Indeed, in the presence of exogenous iron, increased levels of the 70- and 90-kDa HSP (Hsp70, Hsp90) are expressed by human monocytes during *in vitro* infection or phagocytosis [for review, see 9]. An increased expression of HSP is also found in degenerating and regenerating myofibers of DMD [for review, see 10]. While HSP are induced in response to a variety of stresses other than HS and oxidative stress, including differentiation, development and growth, other stress proteins are unique to particular stresses, e.g. the GRP that are induced by alterations in

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¶ *Abbreviations:* CK, creatine kinase; DMD, Duchenne muscular dystrophy; Fe-Df, iron-deficient diet; Fe-S, iron-supplemented diet; GRP, glucose regulated proteins; HSP, heat shock/stress proteins; LPP, lipid peroxidation products; MDA, malondialdehyde; and *mdx,* murine X-linked muscular dystrophy.

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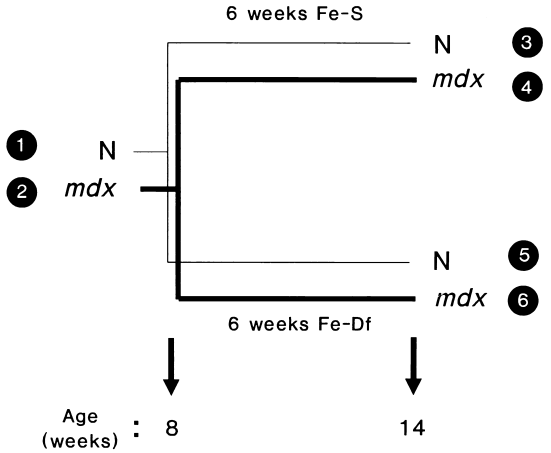

FIG. 1. Schematic presentation of the experimental protocol. Eight-week-old normal mice (N) and mutant C57BL/10ScSn inbred mice with X-linked muscular dystrophy (*mdx*) were either sacrificed at the onset of the study (groups 1 and 2) or maintained either on an iron-supplemented (Fe-S) diet containing 270 mg of iron/kg (groups 3 and 4) or an iron-deficient (Fe-Df) diet containing <5 mg of iron/kg (groups 5 and 6) for a period of 6 weeks. Blood samples were collected once every 2 weeks and when mice were sacrificed to monitor blood iron status. Western blot analyses were performed on skeletal muscle collected at termination.

calcium homeostasis and glucose deprivation [11]. All of these stresses might occur in muscle tissue in DMD.

Our approach in investigating the role of $^{\bullet}OH$ in DMD was to study the effects of dietary iron deprivation in *mdx*. *Mdx* represents a spontaneous mutation found in C57BL/10ScSn inbred mice [12]. The *mdx* model is genetically homologous to DMD: it shows a lack of dystrophin, impaired calcium metabolism, elevated serum CK and early pathological features, including hypercontraction, necrosis and regeneration [13, 14]. Chelating agents, such as desferrioxamine, may exert specific effects distinct from iron deprivation [15]. We tested dietary iron deprivation as the method of choice for decreasing iron levels. We report a significant and parallel decrease in muscle necrosis and in the expression of Hsp70 in iron-deprived *mdx* mice.

MATERIALS AND METHODS

Animals

Eighteen normal male C57BL/10ScSn mice (Harlan Olac) and eighteen mutant (*mdx*) male C57BL/10ScSn mice (Roslin Institute), 7–9 weeks old, weighing 20 to 25 g and raised on a diet containing 104 mg of iron/kg diet, were randomly assigned to three groups: one group was sacrificed at the onset of the study (N = 6), while the other two groups of animals (each N = 6) were maintained for 6 weeks on a purified mice diet containing 270 mg of iron/kg, (Fe-S) or a purified diet from which iron sulphate was omitted (<5 mg of iron/kg) (Fe-Df) (Fig. 1). The basic, semi-chemically defined diet consisted of the following ingredients (g/kg): casein (150), corn oil (80), salt mixture (50), vitamin mixture (10), cellulose (10), water (50), sugar (550), and cornstarch (100). One kilogram of salt mixture contained (g): NaCl (139.3), KI (0.79), KH_2PO_4 (389.0), $MgSO_4$ anhyd. (57.3), $CaCO_3$ (381.4), $FeSO_4 \cdot H_20$ (27.0), $MnSO_4 \cdot H_2O$ (4.01), $ZnSO_4 \cdot H_2O$ (0.548), $CuSO_4 \cdot H_2O$ (0.477), and $CoCl_2 \cdot H_20$ (0.023). The vitamin mixture consisted of vitamin A (2000 IU), vitamin D (200 IU), vitamin E (10 IU), menadione (0.5 mg), choline (200 mg), *p*-aminobenzoic acid (10 mg), inositol (10 mg), nicotinic acid (4 mg), Ca-D-pantothenate (4 mg), riboflavin (6.8 mg), thiamine · HCI (0.5 mg), pyridoxine · HCI (0.5 mg), folic acid (0.2 mg), biotin (0.4 mg), and vitamin B12 (0.003 mg). NaF (2.22 mg: 1 mg F) was added to the diet. $FeSO_4 \cdot H_2O$ was omitted from the Fe-Df diet. The diets met or exceeded the nutrient requirements of the mice for growth and development, except for iron, as specified by Special Diets Services, Essex, U.K. The Fe-S diet contained 2.6 times more iron than the diet fed up to the age of 8 weeks and 54 times more iron than the Fe-Df diet. Food and distilled, deionized water were provided *ad lib*. Iron levels in diets as well as in bedding material were verified by atomic absorption spectrophotometry. Blood samples (300–500 μL) were collected once every 2 weeks from the retro-orbital venous sinus with a heparinized microhematocrit tube to monitor blood iron status. The physical activity of mice in all experimental groups was monitored on a regular basis and found not to be significantly different between groups. All mice were sacrificed following ether inhalation, subsequent to blood sampling by cardiac puncture of anaesthetized animals. Hind limb skeletal muscle was snap-frozen in liquid-nitrogen-cooled isopentane and stored in liquid nitrogen. The principles of laboratory animal care promulgated by the South African Medical Research Council were observed.

Blood Iron Status

Hemoglobin and hematocrit were measured on a Coulter Counter, while serum iron levels were determined by automated analysis [16] using reagents from Boehringer Mannheim. These parameters were monitored at the onset of the study and at 2-week intervals up to 6 weeks of maintenance on either Fe-S or Fe-Df.

Morphology

Morphometric analysis to determine the lesion severity was carried out on hematoxylin-eosin-stained sections of *mdx* gastrocnemius muscle. Gastrocnemius muscle was used in all cases. Macrophage-infiltrated fibers, regenerating fibers and fibers with centralized nuclei were scored in two separate fields, randomly chosen in one cryosection of skeletal muscle obtained from each mouse.

Serum CK and Lipid Peroxidation

Serum CK activity was determined by automated analysis [17] using reagents from Boehringer Mannheim. MDA and

4-hydroxyalkenals, as indexes of lipid peroxidation, were determined in skeletal muscle using a colorimetric assay [18] (Bioxytech S.A.). LPP, as reported here, represent the combined values for MDA and 4-hydroxyalkenals (including 4-hydroxy-2(E)-noneal [4-HNE]), as described [18].

HSP Levels

Stress protein levels in skeletal muscle were determined by immunoblotting. Equal amounts of muscle protein in SDS sample buffer [19] were electrophoresed on SDS gels. Proteins were transferred to polyvinylidene difluoride membranes with a semi-dry transfer unit (Hoefer Scientific Instruments) at 90 mA/gel in a Kyhse-Anderson buffer [20]. Blots were subsequently blocked and probed for stress proteins. Secondary antibodies were revealed by means of chemiluminescence (Boehringer Mannheim). A scanning densitometer (Hoefer Scientific Instruments) was used to determine the relative peak areas to assess the relative amount of protein in immunoreactive bands. Mouse monoclonal antibodies specific for Hsp90 (AC88), Grp75 (30A5) and the inducible form of Hsp70 (C92F3A-5/SPA-810) were obtained from StressGen (Victoria, Canada). Secondary antibodies were anti-mouse, IgG peroxidase linked F(ab′)2 fragments obtained from Amersham Laboratories.

Statistical Analysis

All data are expressed as means (SEM). ANOVA and a comparison of means were performed using CoStat (CoHort Software). Differences in mean values were considered significant if the least-significant-difference value (LSD) was exceeded, as calculated from the pooled variance.

RESULTS

The Effects of Dietary Iron Supplementation or Deprivation on Blood Iron Status

In order to evaluate whether 6 weeks of maintenance on either Fe-S or Fe-Df diets resulted in a significant difference in iron status between these groups, a number of hematologic parameters were evaluated. At the onset of the study, hemoglobin and hematocrit levels were significantly lower in *mdx* mice (Fig. 1, Group 2) as compared to normal mice (Fig. 1, Group 1 and Table 1), while serum iron levels were not significantly different. Hemoglobin and hematocrit levels in mutant mice maintained on the Fe-Df diet (Fig. 1, Group 6) were significantly lower as compared to their Fe-S counterparts (Fig. 1, Group 4 and Table 1). Serum iron levels were not significantly different between these groups.

The Effects of Dietary Iron Supplementation or Deprivation on Muscle Morphology

Dystrophin-deficient muscle displays a number of characteristic morphological features. In order to evaluate the influence of Fe-S and Fe-Df feeding on muscle pathology, we investigated differences in muscle morphology in *mdx* muscle between the different experimental groups. The total number of fibers, including normal fibers, counted in two fields of cryosections from six mice each per treatment, averaged approximately 200 and did not differ significantly between the different treatments, indicating that fiber size was not influenced by the dietary manipulations. In contrast, the number of macrophage-invaded necrotic fibers was significantly lower in Fe-Df mice as compared to Fe-S mice ($P < 0.05$, Table 2). Unlike necrotic fibers, centralized nucleation and regeneration were not significantly different between *mdx* mice fed with either the Fe-S or Fe-Df diets (Table 2). The decrease in macrophage-invaded necrotic fibers is illustrated in Fig. 2, which shows a representative muscle histology of the three groups of *mdx* mice (A: Onset, B: Fe-S and C: Fe-Df); gastrocnemius muscle was used in all cases and the fibers shown in Fig. 2 are representative of the average pattern observed for the different groups.

TABLE 1. Effects of *mdx* and 6 weeks of either iron supplementation (Fe-S) or deprivation (Fe-Df) on hemoglobin, hematocrit, and serum iron levels

Experimental group	Hemoglobin g/dL	Hematocrit %	Serum iron mmol/L
Onset			
Normal	16.2 (0.17)	42.2 (1.37)	37.4 (2.51)
mdx	14.9* (0.18)	37.5* (1.12)	34.6 (1.63)
Fe-S			
Normal	17.1 (0.31)	43.7 (1.67)	22.0 (1.78)
mdx	17.4 (0.51)	46.4 (1.15)	23.1 (2.51)
Fe-Df			
Normal	15.8 (0.53)	42.4 (1.59)	21.1 (5.13)
mdx	15.4† (0.47)	42.1† (1.43)	18.8 (2.54)

Each point represents the mean (SEM) of six animals in each group.
*$P < 0.01$ in comparison with normal mice within the same group.
†$P < 0.01$ in comparison with Fe-S counterparts.

The Effects of Dietary Iron Supplementation or Deprivation on Serum CK and Lipid Peroxidation

Serum CK activity, measured as U/mL, was significantly different ($P < 0.01$) between 8-week-old *mdx* mice

TABLE 2. Quantification of morphological features in skeletal muscle from *mdx* mice at the onset of the study and following six weeks of maintenance on either Fe-Df or Fe-S diets

Experimental group	Macrophage-invaded necrotic fibers	Regenerating fibers	Centralized nuclei
Onset	8.67 (2.12)	35.33 (12.52)	20.25 (4.00)
Fe-S	11.67 (3.88)	29.25 (6.32)	23.00 (2.85)
Fe-Df	6.17* (3.10)	31.83 (5.81)	22.25 (5.40)

Macrophage-invaded necrotic fibers, regenerating fibers and fibers with centralized nuclei were counted in two fields of cryosections from each of six mice/treatments. Values are means (SEM).
*$P < 0.05$ in comparison with Fe-S data.

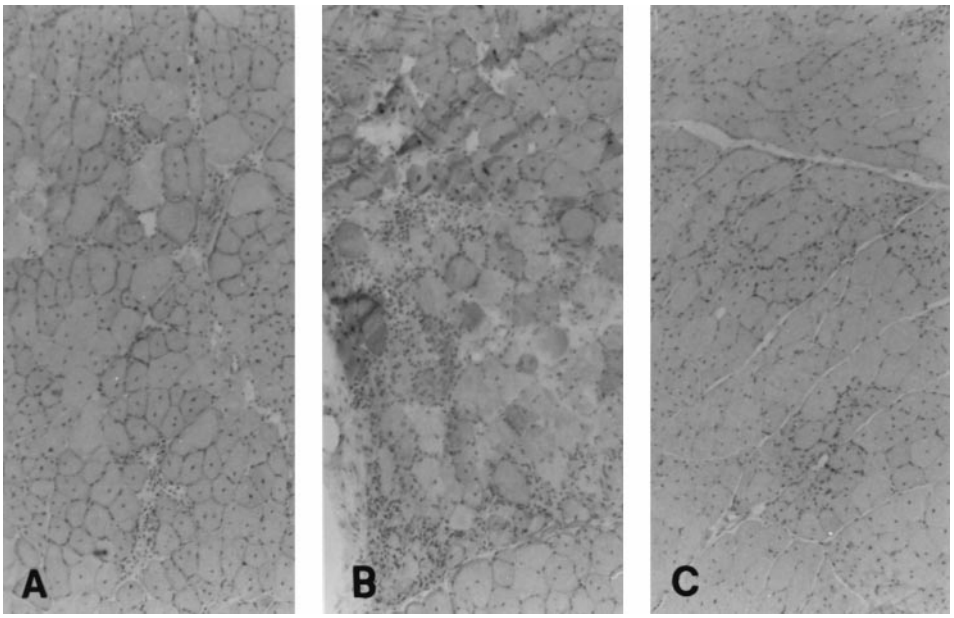

FIG. 2. The effect of iron supplementation or deprivation on the histopathology in skeletal muscle from *mdx* mice. Hematoxylin and eosin-stained cryosections from *mdx* gastrocnemius muscle collected at the onset of the study ((A) group 2, Fig. 1) and after the mice were maintained for 6 weeks on diets either supplemented (Fe-S) ((B), group 4, Fig. 1) or deficient (Fe-Df) ((C), group 6, Fig. 1) in iron. Necrotic fibers were significantly ($P < 0.05$) decreased in *mdx* mice following 6 weeks of maintenance on the Fe-Df diet compared to mice maintained on Fe-S.

(13.28 ± 2.24) and normal mice (2.77 ± 0.95). This difference was, however, abolished in both the Fe-S and Fe-Df groups by the end of the study (Fe-S: normal 1.55 ± 0.19, *mdx* 3.25 ± 0.16, Fe-Df: normal 2.43 ± 0.39, *mdx* 2.48 ± 0.39)

LPP were significantly elevated in skeletal muscle from 8-week-old *mdx* mice as compared to normal 8-week-old mice ($P < 0.01$) (Fig. 3: Onset). The levels of lipid peroxidation products in *mdx* mice compared to normal mice remained significantly higher following Fe-S or Fe-Df feeding at a 99% ($P < 0.01$) and 95% ($P < 0.05$) confidence level, respectively.

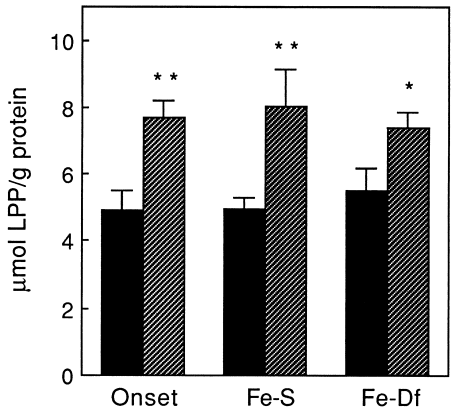

FIG. 3. Effects of iron supplementation or deprivation on LPP in skeletal muscle from normal (black bars) and *mdx* mice (hatched bars). The content of MDA and 4-hydroxyalkenals was colorimetrically determined in hind limb skeletal muscle at the onset of the study (groups 1 and 2, Fig. 1) and after the mice were maintained for 6 weeks on either Fe-S (groups 3 and 4, Fig. 1) or Fe-Df diets (groups 5 and 6, Fig. 1). Each point represents the mean (SEM) of six animals in each group; $^{**}P < 0.01$; $^{*}P < 0.05$ in comparison with normal muscle from animals receiving the same treatment. LPP was significantly higher in *mdx* muscle at the onset and following Fe-S feeding at a level of 99% significance, while it was significantly elevated at a 95% level of confidence following Fe-Df feeding.

The Effects of Dietary Iron Supplementation or Deprivation on HSP Levels

Representative immunoblot and densitometric quantification of the stress proteins analyzed in skeletal muscle from mice in the different experimental groups are illustrated in Fig. 4. In 8-week-old mice, the levels of Hsp70, Hsp90 and Grp75 were significantly increased in *mdx* muscle compared to normal muscle (Fig. 4A: a, b, and c, compare lanes 1 and 2, Fig. 4B, C, D, compare black and hatched bars for Onset). This increase in stress proteins was related to the elevated levels of HSP we previously reported in DMD [10]. No significant difference was observed in the levels of Hsp70 between normal and *mdx* mice maintained for 6 weeks on Fe-S, while a significant difference between normal and mdx mice maintained on Fe-Df was observed. In addition, the level of Hsp70 in Fe-Df *mdx* mice was significantly lower as compared to Fe-S *mdx* mice (Fig. 4B, compare hatched bars for Fe-S and Fe-Df). The decrease in Hsp70 expression thus paralleled the decrease in macrophage-invaded fibers upon iron deprivation. This was, however, not the case for Hsp90: there was no significant difference in Hsp90 levels in *mdx* muscle as compared to normal mice in both groups maintained for 6 weeks on either Fe-S or Fe-Df diets (Fig. 4C).

Neither iron supplementation nor deprivation had any influence on the elevated accumulation of Grp75 observed in 8-week-old *mdx* mice in comparison to normal mice. This implies that the upregulation of Grp75 proceeds through mechanism(s) independent of iron, e.g. the elevation of calcium. The level of confidence was 99% in the Fe-S group compared to 95% for the Fe-Df group.

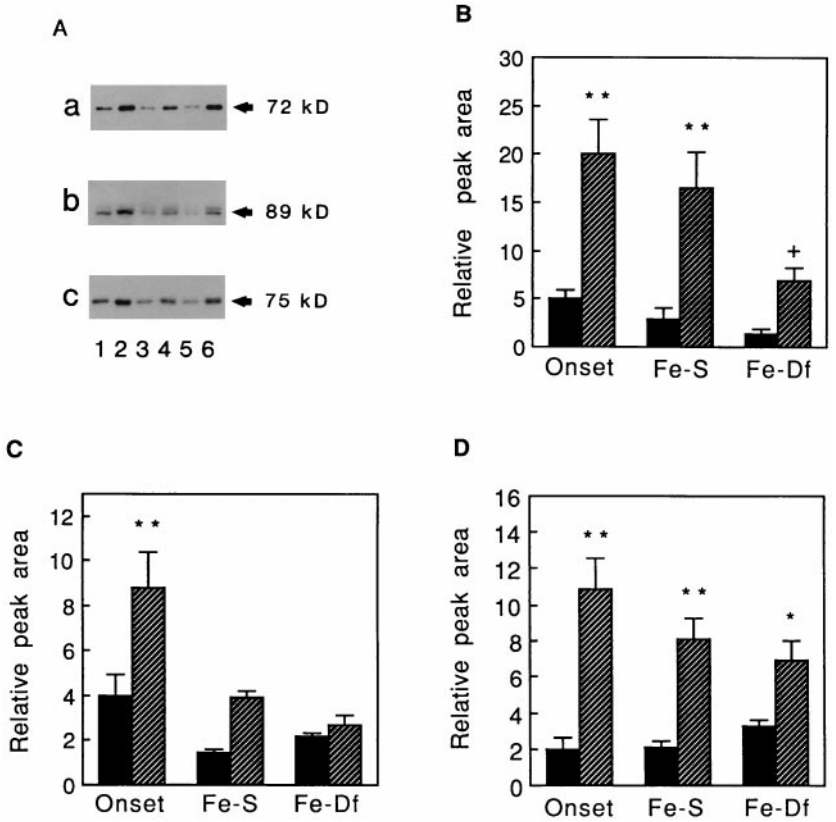

FIG. 4. Western blot analysis of stress protein levels in skeletal muscle. (A) Representative immunoblots for Hsp70 (a), Hsp90 (b) and Grp 75 (c) in skeletal muscle from normal (lanes 1, 3 and 5) and mdx mice (lanes 2, 4 and 6) at the onset of the study (lanes 1 and 2) or subsequent to 6 weeks of maintenance on an Fe-Df (lanes 3 and 4) or an Fe-S (lanes 5 and 6) diet. The relative amounts of Hsp70 (B), Hsp90 (C) and Grp75 (D) in normal (black bars) and *mdx* mice (hatched bars) was assessed from immunoblots by means of scanning densitometry. Each bar represents the mean ± SEM (N = 6); $^{**}P < 0.01$; $^{*}P < 0.05$ in comparison with normal mice similarly treated. $^{+}P < 0.05$ in comparison with the *mdx* mice maintained on the Fe-S diet.

The significance of differences observed between *mdx* mice maintained on either Fe-S or Fe-Df are summarized in Table 3. Hemoglobin, hematocrit, macrophage-invaded necrotic fibers and Hsp70 levels were among the parameters which were significantly lower in Fe-Df mice as compared to Fe-S mice.

DISCUSSION

The most important result of this study was the significant decrease in the number of macrophage-invaded necrotic fibers in Fe-Df as compared to Fe-S mice. Along with the decrease of disease severity, we also observed a selective decrease in the associated overexpression of Hsp70, while other HSP were unaffected. This may imply that the iron-driven generation of $^{\bullet}OH$ does indeed play a role in necrosis of dystrophin-deficient muscle fibers and the consequent attraction of inflammatory cells, as well as in the upregulation of Hsp70.

TABLE 3. Statistical significance of differences in parameters between mdx mice maintained on diets either deficient in or supplemented with iron for six weeks (groups 4 and 6, Fig. 1).

	Statistical significance	
Parameter	**Significant**	**Not significant**
Iron status	Hemoglobin* Hematocrit*	Serum Fe
Morphology	Macrophage-invaded necrotic fibers†	Regeneration, centralized nuclei
Biochemical		Lipid peroxidation (MDA, hydroxyalkenals) Serum CK
Stress proteins	Hsp70†	Hsp90 Grp75

*$P < 0.01$.
†$P < 0.05$.

Classic antioxidant therapies in DMD have been limited by the heterogeneity of the clinical course of the disease and by homeostatic mechanisms that complicate the specificity of tissue and subcellular targeting of antioxidants [21]. The reduction in muscle necrosis in the *mdx* mouse following iron deprivation we report here may provide the basis for new, iron chelating and/or antioxidant therapeutic approaches in the treatment of DMD patients. Use of the iron chelator desferrioxamine has been proposed for DMD [22], but has not been investigated so far. However, taking a similar approach, it was found that the severity of bleomycin-induced pulmonary fibrosis (a disease in which the pathology is also mediated by reactive oxygen species and in particular $^{\bullet}OH$) was significantly decreased in hamsters maintained on diets deficient in iron [23]. In contrast, the influence of iron deficiency on the inflammatory response in DMD was unknown up to now, while in a number of other conditions associated with acute and chronic inflammation, such as adjuvant-induced joint inflammation, nutritional iron deficiency was reported to relieve inflammation [24].

The induction of Hsp70 in *mdx* mice probably involves

the iron-catalyzed production of $^{\bullet}OH$ from O_2^- generated via the xanthine-xanthine oxidase system or NADPH oxidase during phagocytosis. Abnormal and/or degraded proteins—that represent a possible common pathway for the induction of a HS response [25]—may further enhance the induction of Hsp70 in dystrophin-deficient muscle. We have provided evidence for the interdependence of a number of parameters in the regulation of the stress response, including oxidative stress and notably $^{\bullet}OH$, in the *in vitro* regulation of HSP expression [9, 10, 26, 27]. The cytotoxic potential of iron to promote oxidative injury and influence the stress response is further supported by the observation of increased levels of Hsp70 and ubiquitin mRNA in neuroblastoma cells following iron exposure [28].

Increased levels of Hsp90 that occurred in skeletal muscle from 8-week-old *mdx* mice decreased after 6 weeks of maintenance on either Fe-S or Fe-Df diets. We previously observed the increased expression of Hsp90 in regenerating fibers of DMD muscle, and in a subsequent comparative study of mature and immature muscle established the developmental regulation of Hsp90 in skeletal muscle [reviewed in 10]. The elevated levels of Hsp90 in *mdx* mice at 8 weeks of age and the significant decrease at fourteen weeks, irrespective of iron status, may suggest a similar regulatory mechanism, influenced by age and muscle regeneration. The higher level of regeneration observed at the onset of the study may further reflect the phasic nature of the *mdx* pathology, which is characterized by a bout of myonecrosis and regeneration after 20 days that declines significantly beyond the age of 80 to 100 days [29].

In contrast to Hsp70 and Hsp90, Grp75 was significantly elevated in *mdx* as compared to normal mice irrespective of the diet they were fed. The iron-independent increase in Grp75 in *mdx* mice is likely due to increases in intracellular calcium [11]. Indeed, calcium content is elevated in *mdx* mice skeletal muscle throughout the animals' lifespan.

Thus, the expression of HSP in dystrophin-deficient muscle relates to specific events: while Hsp70 is regulated by iron-dependent mechanisms in necrotic fibers, the regulation of Hsp90 and Grp75 is iron-independent and involves myofiber regeneration and elevated calcium levels, respectively, as previously suggested [10]. The long-lasting controversy as to whether increased expression of HSP in disease is a marker for severity or for protection is also addressed by this study. At least in *mdx* mice, the former appears to hold true, since the expression of Hsp70 *decreased* when the disease *improved*.

Other significant differences were observed between normal and *mdx* mice. Hemoglobin and hematocrit levels were lower in 8-week-old *mdx* mice as compared to normal mice, a finding possibly related to altered iron metabolism, such as increased iron sequestration, as previously described in vitamin E-deficient muscular dystrophy [30, 31]. The elevated serum CK activity in *mdx* as compared to normal mice unexpectedly declined in both Fe-Df and Fe-S animals over time, which for the latter could be explained by *in vivo* inactivation of CK activity by iron loading [32]. The decrease in CK activity in both groups of *mdx* mice would accordingly be secondary to distinct mechanisms for Fe-Df mice (decrease in muscle necrosis) and for Fe-S mice (CK inactivation by iron). The lower CK activity of Fe-S normal mice as compared to Fe-Df normal mice also supports the postulate that iron supplementation might inactivate CK activity.

In contrast to the above-mentioned significant differences, lipid peroxidation was elevated in *mdx* mice maintained on either diet, and centralized nucleation and regeneration were not different in *mdx* mice fed the Fe-S or Fe-Df diets (Table 3), although for lipid peroxidation, there was a decreasing tendency in significance in the absence of dietary iron (Fig. 3). This suggests that in these mice, lipid peroxidation, regeneration and centralized nucleation are regulated by mechanisms not influenced by iron. Those may include elevation in other transition metals such as copper [33], membrane physicochemical instability, loss in calcium homeostasis, $^{\bullet}OH$ production by myeloperoxidase [34], active ferryl species produced by the reaction of H_2O_2 with myoglobin [8], or the state of membrane lipids in dystrophic muscle [35].

In conclusion, we report that iron deprivation selectively decreased muscle necrosis in *mdx* mice, along with a decrease in Hsp70 expression. The decreased generation of cytotoxic ROS, especially $^{\bullet}OH$, may restrain the progressive nature of the disease and improve the prognosis of affected individuals. The close correlation between Hsp70 expression and the pathology of the disease suggests that Hsp70 could be employed as a marker during therapy in DMD patients. However, whether the observed decrease in muscle necrosis, in the absence of effects on centralized nucleation and regeneration and on lipid peroxidation, will, or not, be associated with an overall benefit to the disease in mice and in humans will have to be established by other studies.

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