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Evidence of oxidative stress in *mdx* mouse muscle: Studies of the pre-necrotic state

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Abstract

Considerable evidence indicates that free radical injury may underlie the pathologic changes in muscular dystrophies from mammalian and avian species. We have investigated the role of oxidative injury in muscle necrosis in mice with a muscular dystrophy due to a defect in the dystrophin gene (the *mdx* strain). In order to avoid secondary consequences of muscle necrosis, all experiments were done on muscle prior to the onset of the degenerative process (i.e. during the 'pre-necrotic' phase) which lasted up to 20 days of age in the muscles examined. In pre-necrotic *mdx* muscle, there was an induction of expression of genes encoding antioxidant enzymes, indicative of a cellular response to oxidative stress. In addition, the levels of lipid peroxidation were greater in *mdx* muscle than in the control. Since the free radical nitric oxide ($NO^{\bullet}$) has been shown to mediate oxidative injury in various disease states, and because dystrophin has been shown to form a complex with the enzyme nitric oxide synthase, we examined pre-necrotic *mdx* muscle for evidence of $NO^{\bullet}$-mediated injury by measuring cellular nitrotyrosine formation. By both immunohistochemical and electrochemical analyses, no evidence of increased nitrotyrosine levels in *mdx* muscle was detected. Therefore, although no relationship with $NO^{\bullet}$-mediated toxicity was found, we found evidence of increased oxidative stress preceding the onset of muscle cell death in dystrophin-deficient mice. These results lend support to the hypothesis that free radical-mediated injury may contribute to the pathogenesis of muscular dystrophies.

Keywords: Muscular dystrophy; *mdx* mouse; Antioxidant enzyme; Free radicals; Oxidative stress

1. Introduction

The most common forms of muscular dystrophy are due to defects in the dystrophin gene [15]. In the absence of dystrophin, muscle undergoes characteristic necrotic degeneration in both humans [Duchenne muscular dystrophy (DMD)] and mice (the *mdx* strain), yet the pathogenetic mechanisms leading to the death of muscle cells are unknown. Conversely, the mechanism by which dystrophin protects muscle from this degenerative process remains unexplained [35]. Because dystrophin localizes to the plasma membrane [5], and because membrane abnormalities have long been viewed as the primary site of cellular dysfunction in muscular dystrophies, there has been considerable interest in changes in membrane integrity in dystrophin-deficient muscle [35]. Indeed, measurements both in vivo and in vitro suggest that the membrane of dystrophin-deficient muscle cells is fragile or 'leaky' [26,38,48], although this appears to be a property that develops in these muscles rather than being an intrinsic, obligatory characteristic [34].

Loss of membrane integrity may be due to direct damage, cellular energy depletion, or both [33]. Several lines of evidence suggest that free radical injury to membrane may contribute to loss of membrane integrity in

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muscular dystrophies [39]. First, there are similarities between the pathologic changes that occur in the dystrophies and changes that occur in muscle under different conditions of oxidative stress such as ischemia, exhaustive exercise, and vitamin E deficiency [27,37,39]. Second, numerous studies have documented biochemical changes in dystrophic muscle that are characteristic of oxidative injury [21,22,39,43]. Third, we have shown that dystrophin deficient muscle cells are specifically more sensitive to injury from oxidative stress than are control muscle cells [44]. Fourth, we have recently described a form of muscular dystrophy in mice with genetically altered free radical metabolism that leads to increased oxidative stress in muscle [45]. Finally, recent evidence indicates an association between the dystrophin complex of proteins and neuronal nitric oxide synthase (nNOS) [6,7,9], an enzyme involved in the metabolism of the free radical $NO^{\bullet}$. This association is disrupted in dystrophin-deficient muscle, and there is a significant reduction in nNOS activity. How this reduction in nNOS activity affects levels of cellular $NO^{\bullet}$, which is known to have both protective and destructive actions [13], is as yet undetermined. Nevertheless, it is intriguing in that it is the first demonstration of a direct connection between dystrophin and pathways of cell injury or cell protection, and those pathways involve free radicals.

One of the difficulties with the interpretation of previous biochemical studies of oxidative injury in dystrophic muscle is that nearly all studies have used tissue during the active phase of the disease. Since both cell death and inflammation can themselves induce free radical generation [19], the discrimination between cause and effect is not possible. The *mdx* strain provides an excellent model in which to study the pathogenetic process without this confounding feature. Since muscle necrosis does not begin in this strain until the mice are several weeks of age [1,12,49], it is possible to study the 'pre-necrotic' muscle for evidence of pathogenetic mechanisms. During this period, none of the secondary consequences from cell death or inflammation are occurring, thus allowing a study of the primary pathogenetic processes.

Here we report the results of studies to test the hypothesis that oxidative stress and free radical injury cause muscle cell death in the *mdx* mouse [39]. We examined muscle during the pre-necrotic phase of the disease for evidence of oxidative stress using measures of the oxidative state of the muscle and direct measures of oxidative injury. We show that, preceding the onset of muscle degeneration, there is evidence of increased oxidative stress in *mdx* muscle compared to control. In studies of the role of $NO^{\bullet}$ in this process, we found no evidence of increased $NO^{\bullet}$-mediated injury in *mdx* muscle. The role of $NO^{\bullet}$ in muscle cell survival and muscle cell death in the muscular dystrophies remains to be determined. Nevertheless, our results support the hypothesis that free radical injury may be the cause of cell necrosis in dystrophin-deficient muscle.

2. Material and methods

2.1. Animals and animal care

Control (C57BL/10SnJ) and *mdx* (CS7BL/10ScSn-*mdx*) mice were maintained in breeding colonies in the Veterinary Medical Unit at the Palo Alto VA Medical Center from founders originally obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were handled in accordance with guidelines of the Administrative Panel on Laboratory Animal Care of Stanford University.

2.2. Tissue collection and preparation

For biochemical or molecular studies, hindlimb muscles were dissected and immediately frozen in liquid nitrogen. For histological studies, the muscles were placed in cryomolds containing embedding medium (O.C.T. compound, Miles Laboratories, Elkhart, IN), and frozen in isopentane cooled to −160°C in liquid nitrogen. Eight micron cross-sections were obtained with a Leica cryostat at −18°C. The sections were collected onto gelatin-coated slides and stained with hematoxylin and eosin (H&E) or used for immunohistological detection of dystrophin expression or nitrotyrosine (NT) formation (see below).

2.3. Dystrophin immunohistochemistry

Frozen sections were stained with a mouse monoclonal antibody to dystrophin (Mandys 8; Sigma Chemical, St. Louis, MO) at a 1:400 dilution for 1 h, followed by Texas Red-coupled secondary antibody (Cappel Research Products, Durham, NC) for 30 min at a dilution of 1:250. The sections were mounted with an aqueous mounting medium, and the slides were examined and photographed with a Zeiss Axioskop microscope.

2.4. Lipid peroxidation assay

Lipid peroxidation was measured using the thiobarbituric acid (TBA) assay [14]. This assay measures the level of cellular malondialdehyde (MDA) which is proportional to the degree of lipid peroxidation. Tissues were homogenized in 50 mM Tris-HCl, pH 7.6 at 4°C, and incubated at 37°C for 30 min. To 300 μl of each sample was added 900 μl H_2O and 600 μl 28% trichloroacetic acid (w/v). The samples were centrifuged at 3000 *g* for 15 min at 4°C. To 1.38 ml of supernatant was added 0.23 ml 1% TBA (w/v) in 50 mM NaOH. The absorbance was measured at 532 nm. The amount of MDA was determined using a standard curve, and expressed as nmoles MDA/mg protein. Protein concentrations were determined using a Bradford protein assay kit (BioRad, Hercules, CA).

2.5. Northern blot analysis

Total RNA from C57 or *mdx* limb muscle was

homogenized in TRI-reagent (Sigma) as described in the manufacturer's protocol. The homogenates were mixed with 1-bromo-3 chloropropane (BCP) (Molecular Research Center, Cincinnati, OH) then centrifuged at 9000 rpm. RNA was precipitated from the aqueous phase by the addition of isopropanol, washed in ethanol and dissolved in water. RNA (20 μg/lane) was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto a nylon membrane in 10×SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0). The membrane was hybridized to ^{32}P-labeled riboprobes for Cu,Zn-superoxide dismutase (SOD-1) or Mn-superoxide dismutase (SOD-2), or to ^{32}P-labeled DNA probes for catalase (CAT), glutathione peroxidase (GPx), and glyceraldehyde phosphate dehydrogenase (GAPDH). Antisense probes were synthesized using ^{32}P-UTP, linearized plasmid containing SOD-1 or SOD-2, and appropriate T7 or T3 RNA polymerase following the method supplied by the Stratagene in vitro transcription kit (La Jolla, CA). DNA probes were obtained by appropriate digestion to release the sequences of catalase (268 bp fragment), GPx (a pool of 650, 580, 300 bp fragments) or GAPDH (1.2 kb fragment). The ^{32}P-labeled DNA probes were generated following Amersham (Arlington Heights, IL) random priming kit protocol using ^{32}P-dCTP. The hybridizations were performed for 1 h in ExpressHyb solution (Clontech, Palo Alto, CA), then washed consecutively in 2×SSC/0.1% SDS, 1×SSC/0.1% SDS, and 0.2×SSC/0.1% SDS for 20 min each. The membranes were exposed for 24 h, and the signal intensities were recorded using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The RNA levels for SOD-1, SOD-2, GPx, and CAT were normalized to the level of GAPDH message for each sample.

2.6. Nitrotyrosine detection: Immunohistochemistry

Frozen sections were fixed in methanol (−20°C for 5 min) and stained with a rabbit polyclonal antibody to NT (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:10 for 30 min at room temperature. After rinsing, the specific binding of the primary antibody was detected using a biotin-streptavidin detection kit (Vector Laboratories, Burlingame, CA) with fluorescein coupled to streptavidin. No staining was detected when the primary antibody was left out of the reaction, or when the primary antibody was pre-incubated with 10 mM NT for 30 min before application to the tissue.

2.7. Nitrotyrosine quantitation: HPLC and electrochemical detection

Muscle tissue was dissected and rapidly frozen in liquid nitrogen. The tissue was thawed, homogenized in 0.1 M $HClO_4$, and centrifuged at 10 000 *g* for 10 min at 4°C. The supernatants were stored at −80°C. The samples were thawed and analysis was performed using HPLC with 16-electrode electrochemical detection as previously described [2]. 3-NT levels are expressed as the ratio of 3-NT to total cellular tyrosine to normalize for any variations in sample size.

2.8. Statistical methods

Data are expressed as mean±S.D. Comparisons between samples were made using ANOVA, and differences were considered to be statistically significant at the $P<0.05$ level.

3. Results

3.1. The pre-necrotic state: Histologic determination

To investigate the possibility that free radical induced injury causes muscle cell necrosis, we focused all experiments on the period of time preceding the onset of muscle cell death, i.e. the pre-necrotic state. The age of onset of muscle necrosis has been reported to be between 2 and 4 weeks of age in previous studies [1,12,23,49], and appears to vary somewhat among breeding colonies. Thus, we sought to define the ages of the pre-necrotic state in our colony of *mdx* mice by histological examination of muscle. In our colony, there were no signs of muscle necrosis before 20 days of age in any of the hindlimb muscles examined (quadriceps, tibialis anterior, gastrocnemius and hamstrings) (Fig. 1). By 25 days of age, there was

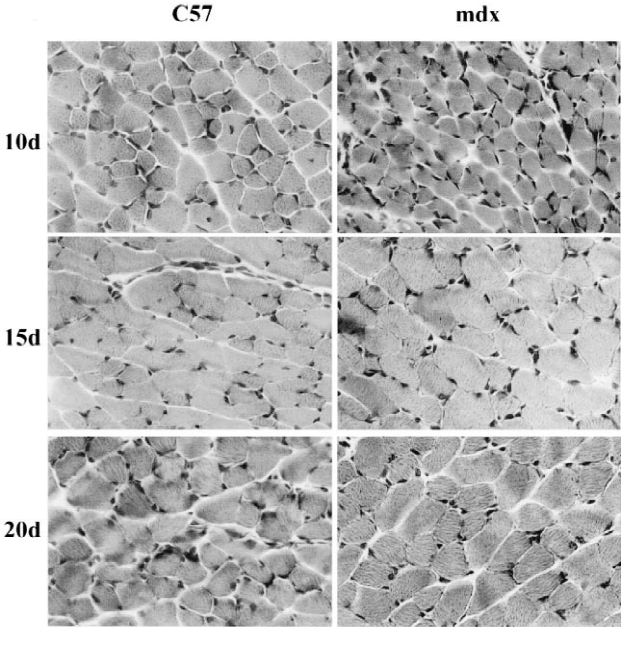

Fig. 1. Histologic determination of the pre-necrotic state. Representative photomicrograph of quadriceps muscle from 10, 15 and 20 day-old C57 and *mdx* mice. H&E staining was performed as described in Section 2. No evidence of muscle necrosis was seen in any of the muscles examined from *mdx* mice at these ages. The pre-necrotic phase of the disease was thus determined to last up to 20 days of age (magnification: 200×).

evidence of active necrosis, including fiber degeneration and macrophage infiltration, in all hindlimb muscles examined. We thus concluded that the pre-necrotic phase of the disease extended from birth to at least 20 days of age, and all biochemical and molecular analyses focused exclusively on this age range.

3.2. Increase of antioxidant gene expression: Marker of oxidative stress during the pre-necrotic state

Oxidative stress of a tissue may lead to induction of one or more antioxidant enzymes [20]. To examine the oxidative state of *mdx* muscle during the pre-necrotic phase of the disease, we first analyzed *mdx* and control muscle for expression of genes encoding SOD-1, SOD-2, GPx and CAT. Northern blot analysis revealed that there was an induction of the expression of each of these genes in muscle of 15 and 20 day-old *mdx* mice (Fig. 2A). The level of expression of each gene was normalized to the expression of GAPDH from each muscle. Statistical analysis of four separate experiments demonstrated that the level of antioxidant gene expression in *mdx* muscle was significantly greater than in control muscle (Fig. 2B).

3.3. Oxidative injury to muscle precedes muscle necrosis

Induction of antioxidant enzyme gene expression, while indicative of oxidative stress, is a measure of a cellular response to that stress. To test directly for evidence of oxidative injury during the pre-necrotic state, we measured the level of lipid peroxidation in *mdx* and in control muscle. We used the TBARS assay to assess oxidative injury, measuring the level of MDA which is proportional to the extent of lipid peroxidation in the tissue [14]. Fig. 3 shows the level of MDA is greater in *mdx* muscle than in control muscle during the pre-necrotic state. The level of MDA was also greater in *mdx* muscle after the onset of necrosis (data not shown), but these results do not necessarily reflect the primary pathogenetic process since necrotic cell death itself can increase lipid peroxidation in a tissue [19]. Our results indicate oxidative injury to muscle membrane is elevated in dystrophin-deficient muscle prior to the onset of the degenerative process.

3.4. Nitric oxide mediated injury

Although dystrophin is not known to have any antioxidant properties itself, the demonstration of an association between the dystrophin complex and nNOS is intriguing [6,7,9]. Since $NO^{\bullet}$ is a product of nNOS activity and has been demonstrated to mediate oxidative injury in certain disease states, we considered the possibility that the oxidative injury of dystrophin deficient muscle might be mediated by $NO^{\bullet}$. To test this idea, we examined pre-necrotic muscle for the formation of nitrotyrosine (NT), a product of $NO^{\bullet}$-mediated injury [3].

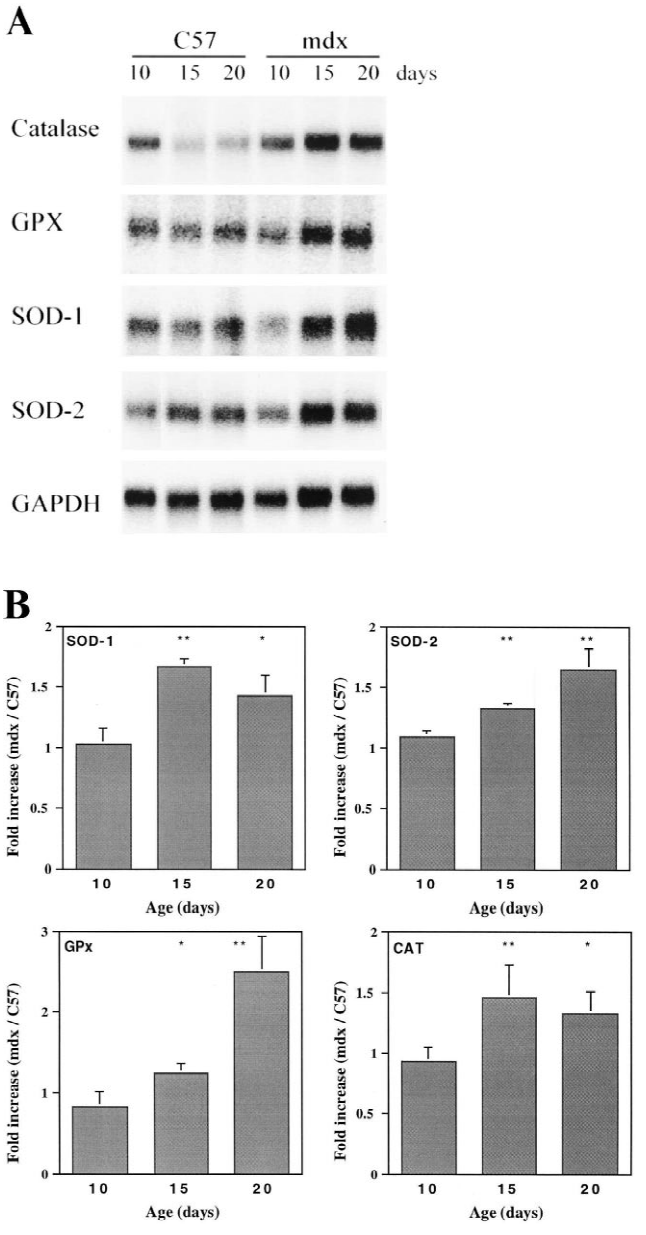

Fig. 2. Oxidative stress in *mdx* muscle during the pre-necrotic state. (A) Representative Northern blots showing expression of genes encoding the antioxidant enzymes SOD1, SOD2, GPx and CAT in C57 and *mdx* muscle. Northern analysis was performed on hindlimb muscle at the ages indicated. (B) Quantitative densitometry of Northern blots. The levels of expression for each antioxidant gene were normalized to the level of GAPDH expression in each sample. Results are expressed as fold-increase in *mdx* muscle compared with muscle of age-matched C57 mice. Data are presented as mean±S.D. ($n=4$; $^{*}P<0.05$; $^{**}P<0.005$).

We examined pre-necrotic *mdx* and control muscle for NT immunohistochemically. Frozen sections of muscle were incubated with a polyclonal antibody to NT, and muscles were analyzed for the distribution and relative intensity of the specific staining (Fig. 4). NT staining appeared most intense around the cell membrane, although patchy areas of staining were seen scattered throughout the cytoplasm. The staining was completely abolished when the antibody was pre-incubated with NT. There was

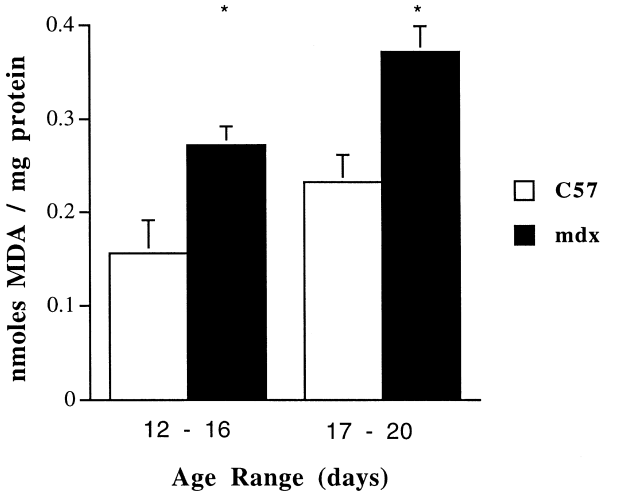

Fig. 3. Oxidative injury in pre-necrotic *mdx* muscle. The extent of oxidative injury was determined in C57 and *mdx* muscle at different age ranges during the pre-necrotic state of the disease. Oxidative injury was determined by the amount of lipid peroxidation using the TBA assay (see Section 2) which 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±S.D. ($n=5$; *$P<0.05$).

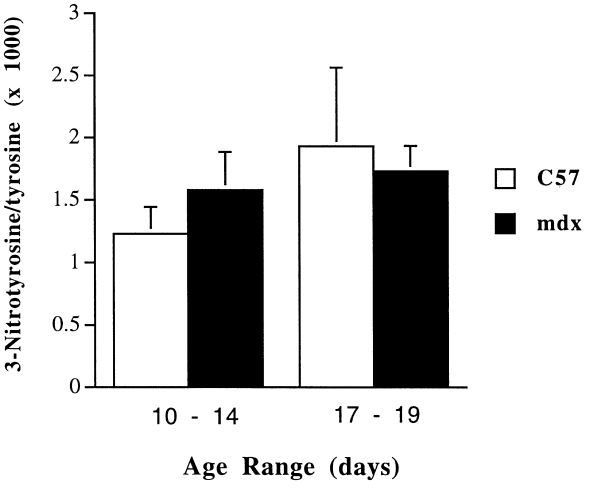

Fig. 5. Nitrotyrosine quantitation in control and *mdx* muscle. To obtain more quantitative data on the production of NT in muscle of *mdx* mice compared to controls, muscle homogenates were subjected to HPLC and total NT levels were quantified using 16-electrode electrochemical detection [2]. Cellular NT levels were normalized to total cellular tyrosine levels in the same sample. No significant difference was detected between NT levels in *mdx* and control muscle at the two age ranges indicated. Data are presented as mean±S.D. ($n=5$).

considerable variability among samples, even when comparing muscles from mice of the same strain and same age. However, there was no consistent difference in intensity or distribution when comparing C57 and *mdx* muscle, nor did the staining pattern change with age during the pre-necrotic state. After the onset of necrosis in the *mdx* strain, we saw increases in NT-staining in the muscle which was not seen in C57 muscle of the same age, but these increases could be secondary to the necrotic process itself since there is an associated inflammatory cell infiltration [4].

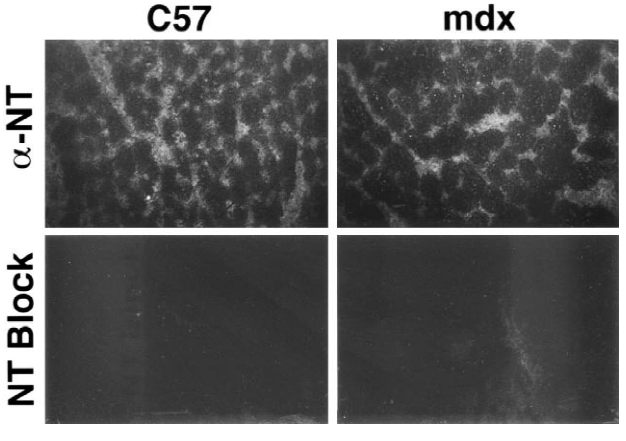

Fig. 4. Nitrotyrosine formation in control and *mdx* muscle. To assess $NO^{\bullet}$-mediated injury, we stained frozen sections of control and *mdx* muscle with an antibody to NT. Antibody binding was assessed using a three-step biotin-streptavidin detection system (see Section 2) with fluorescein as the fluorochrome (top panels: 'α-NT'). NT was seen primarily at the periphery of the muscle fibers for both C57 and *mdx* muscle, and no significant difference was seen in the intensity or distribution of the stain between the samples. The specificity of this staining for cellular NT is shown by the abolition of all staining when the antibody was preincubated with NT (bottom panels: 'NT block') showing the specificity of the staining for cellular nitrotyrosines.

Because the immunohistochemical analysis provided only a qualitative assessment of NT levels, we tested *mdx* muscle for NT levels quantitatively. Control and dystrophin-deficient muscles were homogenized, and total NT levels were determined using HPLC and 16-electrode electrochemical detection [2]. During the pre-necrotic state, when there was evidence of increased oxidative injury in *mdx* muscle (see Figs. 2 and 3), there were no significant differences in NT levels between *mdx* and C57 muscle (Fig. 5). Thus, although we find evidence of increased oxidative stress in *mdx* muscle during the pre-necrotic state, we find no evidence that injury is mediated by the toxic effects of $NO^{\bullet}$.

4. Discussion

The results presented here are consistent with the hypothesis that free radical mediated injury contributes to the pathogenesis of muscle necrosis in the muscular dystrophies [39]. This hypothesis was initially based on the similarities between the pathology in the dystrophies and the pathology of muscle exposed to oxidative stress in vitamin E deficiency [31]. Subsequently, other conditions of oxidative stress to muscle such as ischemia/reperfusion injury [16,37] and exhaustive exercise [27,41,50] have also been shown to result in similar pathologic changes as those seen in the muscular dystrophies [39].

Lipid peroxidation is a common index of free radical mediated injury [19], and induction of antioxidant enzymes is a common cellular response [20]. Previous measurements of lipid peroxidation in dystrophin-deficient muscle have indicated elevated levels in both humans and mice [28,30,36,43]. Further evidence of lipid peroxidation in humans with DMD has been shown by the increase in expired pentane and the increased levels of fluorescent pigments and conjugated dienes in muscle tissue [18,25]. Oxidation of cellular proteins has also been shown to be increased in dystrophin-deficient muscle from mouse and humans [21,22]. Previous studies have provided conflicting results of the levels of antioxidant enzyme activities in dystrophin-deficient muscle. Burr et al. [8] reported that both SOD-1 and SOD-2 activities were reduced in muscle from patients with DMD, but that GPx activity was increased. Kar and Pearson [30] and Mechler et al. [36] found no change in the levels of SOD-1 activity. In the *mdx* mouse, Ragusa et al. [42] found no clear correlation between the pattern of antioxidant enzyme activity and disease severity among different muscles.

However, these studies have all been done using muscle during the active phase of muscle degeneration. This is problematic because cellular degeneration itself, as well as the secondary process of inflammatory cell infiltration that occurs in response to cell necrosis, may be associated with increases in oxidative stress [19]. All biochemical measurements of oxidative injury obtained during the active phase of the disease may thus be a consequence of the degenerative process rather than a cause. Discrepancies between results could be due to variations in the amount of active necrosis, in the extent of tissue degeneration with replacement of muscle with adipose and connective tissue, in the amount of active regeneration, and in the extent of the inflammatory response.

To avoid these complications, we have focused all of our studies on the pre-necrotic phase of the disease. The age range of the pre-necrotic state is defined by the absence of evidence of active necrosis and extended up to 20 days of age in our colony. During this time, there was both direct evidence of oxidative injury (lipid peroxidation) and evidence of oxidative stress (induction of antioxidant genes) in dystrophin-deficient muscle compared to control. Because there was no active cell necrosis over this age range, these changes could not be secondary to the degenerative process. As further support for this concept, we have recently shown that dystrophin-deficient muscle cells are specifically more sensitive to oxidative injury than are normal muscle cells [44]. We would thus suggest that dystrophin-deficiency is a condition of increased susceptibility to free radical mediated injury (similar to vitamin E deficiency) rather than a condition of increased generation of free radicals (such as in ischemia/reperfusion).

There have not been any previous studies of gene expression of the antioxidant enzymes in pre-necrotic *mdx* mice. We found that expression of SOD-1, SOD-2, GPx and CAT were all induced, albeit at low levels. It is likely that the level of gene induction in the tissue is not uniform, but instead represents a greater induction in a subset of fibers, averaged across the whole tissue. One of the hallmarks of the degenerative process is that fibers within a muscle do not undergo simultaneous necrosis, but rather there are focal areas of necrosis with other areas unaffected at any given time [15]. Thus, during the pre-necrotic phase of the disease, molecular or biochemical changes measured in the tissue as a whole may represent changes occurring only in the subset of fibers that is destined to undergo necrosis most imminently. The actual changes in those individual fibers would be much greater than predicted by assuming that the changes measured in the tissue homogenate were evenly distributed among all cells.

Clearly, the expression of dystrophin in muscle conveys protection against the degenerative process that occurs in its absence. Still, the functional role of dystrophin in the cell remains a mystery [35]. There is substantial evidence of membrane fragility in dystrophin-deficient muscle cells. When tested in vitro in assays that challenge membrane integrity, dystrophin-deficient muscle cells have been found to be more susceptible to injury [26,38]. Uptake of Evans blue, injected intravenously, into muscle fibers of *mdx* mice and mice with other (although not all) forms of muscular dystrophy suggests that leaky membrane is a characteristic feature of dystrophin deficient muscle [48]. Interestingly, muscle fibers of 40 day-old *mdx* mice demonstrated uptake of procion orange, whereas pre-necrotic *mdx* muscles (14 days old) did not [34]. This result suggests that membrane fragility is not an intrinsic feature of dystrophin-deficient muscle, but rather that membrane fragility develops as a stage during the degenerative process. We suggest that membrane fragility may develop during the pre-necrotic stage by cumulative oxidative injury to membrane lipids (Fig. 3).

If our hypothesis is correct, then it would be necessary to postulate a role for dystrophin in protecting membrane against oxidative injury. The demonstration of the association between dystrophin and nNOS [6,7,9] may provide an important clue as to what that role might be. In normal muscle, nNOS is localized to the plasmalemma by virtue of its binding to the dystrophin complex. In dystrophin-deficient muscle, that association is disrupted and nNOS is displaced to the cytoplasmic compartment and is less abundant [6,7,9]. The free radical $NO^{\bullet}$ is generated via the action of nNOS, and the levels of $NO^{\bullet}$ may thus be quite different in normal and dystrophin-deficient muscle. There is also a correlation between the reduction of nNOS localization and/or activity and the severity of the dystrophy in humans and mice with different mutations in the dystrophin gene [10,11]. By contrast, *mdx* mice that lack nNOS do not develop a more severe dystrophy [11]. Our results suggest that the nNOS delocalization in dystrophin-deficient muscle is not associated with an increase in $NO^{\bullet}$-mediated injury by both qualitative and quantitative assessments (Figs. 4 and 5). Interestingly, $NO^{\bullet}$ has been shown to have both destructive and protective

properties as a free radical [13]. Thus, the role of nNOS (and $NO^{\bullet}$) in the pathogenesis of muscular dystrophies remains to be determined, but one possibility is that $NO^{\bullet}$ protects muscle against oxidative injury [52,53] and that protection is reduced in dystrophin-deficient muscle.

Extraocular muscles are spared the degenerative process in dystrophin-deficient animals [15], and there has been an interest in whether this sparing is due to unique properties of those fibers. Ragusa et al. [43] did not find any specific pattern of antioxidant enzyme activity that might explain the relative resistance, and Wehling et al. [51] did not find any difference of nNOS activity or localization in these muscles compared to limb muscles. Khurana et al. [32] showed that extraocular muscles are able to maintain calcium homeostasis better than limb muscles. This could explain the resistance to any process in which loss of membrane integrity led to elevated intracellular calcium concentrations. It is also unknown why the degenerative process appears to decline in older *mdx* mice, as if there is an increased resistance among the older, regenerated fibers [1]. In rat muscle, age-related increases in antioxidant enzyme activity have been reported [29]. It is not known whether older *mdx* muscles likewise have enhanced antioxidant capacity. However, biochemical studies of muscles of older *mdx* mice would be complicated by the ongoing degeneration in the tissue [40], and any results would be subject to the caveats described above.

The beneficial effect of antioxidant therapy would be compelling support of the hypothesis that oxidative stress contributes to the initiation of muscle cell necrosis in the muscular dystrophies. Antioxidants have been tested in different animal models of muscular dystrophy and in humans with Duchenne muscular dystrophy [17,39,46,47]. The results have been mostly discouraging, but all human studies have involved boys with advanced disease. The recent report of Hubner et al. [24] suggests that dietary supplementation with components of wheat kernel is beneficial to the *mdx* mouse when administered soon after the onset of degeneration, but supplementation with α-tocopherol did not show the same benefit. Therapeutic studies in *mdx* mice begun during the pre-necrotic phase of the disease, during which antioxidant therapy might be most effective, should prove useful for considering the possible application of antioxidant therapy for humans with DMD.

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