

Absence of Extraocular Muscle Pathology in Duchenne's Muscular Dystrophy: Role for Calcium Homeostasis in Extraocular Muscle Sparing

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Summary

Duchenne muscular dystrophy (DMD) is characterized by clinical weakness and progressive necrosis of striated muscle as a consequence of dystrophin deficiency. While all skeletal muscle groups are thought to be affected, enigmatically, the extraocular muscles (EOM) appear clinically unaffected. Here we show that dystrophin deficiency does not result in myonecrosis or pathologically elevated levels of intracellular calcium ($[Ca^{2+}]_i$) in EOM. At variance with a previous report, we find no evidence for dystrophin-related protein/utrophin up-regulation in EOM. In vitro experiments demonstrate that extraocular muscles are inherently more resistant to necrosis caused by pharmacologically elevated $[Ca^{2+}]_i$ levels when compared with pectoral musculature. We believe that EOM are spared in DMD because of their intrinsic ability to maintain calcium homeostasis better than other striated muscle groups. Our results indicate that modulating levels of $[Ca^{2+}]_i$ in muscle may be of potential therapeutic use in DMD.

Duchenne/Becker muscular dystrophy (DMD)¹ is one of the most common inherited neuromuscular diseases. The disease is of particular importance because of its relentless course of progression and debilitating nature, for which no effective therapies currently exist (1). Over the last decade, it has emerged that the etiology of the disease is a primary defect in the DMD gene that leads to quantitative and/or qualitative abnormalities in the expression of dystrophin, the protein product of this gene (2, 3). DMD is clinically characterized by slow but progressive muscle weakness due to necrosis of skeletal muscle fibers. While all muscle groups are thought to be affected by the absence of dystrophin, enigmatically, the extraocular muscles (EOM) remain clinically unaffected during the course of the disease (1, 4). This observation has led to a great deal of interest in understanding the compensa-

tory factor(s) or mechanism(s) that allow EOM to escape the deleterious consequences of dystrophin deficiency.

Dystrophin belongs to the spectrin superfamily of proteins, the members of which share overall structural similarities (5). These proteins contain an actin-binding region at the amino terminus and a rodlike central domain consisting of tandemly repeated, spectrinlike amino acid motifs. Functionally, these proteins share properties such as morphology, calcium-binding potential, and tightly regulated cellular and subcellular distribution (5, 6). The dystrophin class of the spectrin superfamily currently consists of chromosome X-encoded dystrophin, chromosome 6-encoded dystrophin-related protein (DRP)/utrophin, and, more distantly, the 87-kD chromosome 18-encoded dystrophinlike protein (5, 7–10). The proteins share significant sequence similarity, size, evolutionary conservation, abundance, and some aspects of subcellular distribution in normal skeletal muscle, such as enrichment at neuromuscular and myotendinous junctions (2, 5, 7, 8, 11–14). Furthermore, dystrophin and DRP copurify with a complex of proteins known as the dystrophin-associated glycoprotein complex, which contributes to anchoring and localizing both proteins to the sarcolemma (15, 16). Dystrophin and DRP are critical components of the chain of cytoskeletal elements that links internal cellular structures, e.g., actin bundles, to

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¹Abbreviations used in this paper: CPA, cyclopiazonic acid; DMD, Duchenne muscular dystrophy; DRP, dystrophin-related protein; EOM, extraocular muscles; H&E, hematoxylin and eosin.

components of the extracellular matrix, e.g., laminin (17, 18). Dystrophin and its relatives are thought to help in the genesis and maintenance of subcellular membrane specializations and to protect the sarcolemma from structural damage that occurs during multiple contraction-relaxation cycles in the skeletal muscle leading to myonecrosis (17, 19).

Members of the dystrophin family have been strongly conserved over evolution. Consequently, dystrophin deficiency due to mutations in the DMD gene has been described and characterized in a number of animal species, including mice (*mdx*), dogs (*cxmd*), and cats, all of which are considered useful animal models for the disease (17, 20). These animal models share the same biochemical defect as DMD (i.e., severe dystrophin deficiency), albeit with differing functional consequences. The *mdx* mouse used in most studies (including this one) has a mutation at nucleotide position 3185 resulting in translation termination within the amino-terminal third of the molecule (21). Recently, additional allelic forms of the *mdx* mouse have been generated (*mdx*^{2-4Cv}) with mutations in the 3' end of the murine cDNA (22). Interestingly, while the *mdx* mouse demonstrates foci of myonecrosis in skeletal muscle, cardiomyopathy, and elevated creatine kinase levels similar to human DMD patients throughout most of its life, it does not exhibit extensive muscle fibrosis or clinical weakness as seen in humans, except for some muscle groups such as the diaphragm (23, 24). The feline model of DMD has a deletion in the muscle promoter region of the gene and shares features such as myonecrosis, weakness, muscle hypertrophy, and extensive fibrosis with the human disease (25). Interestingly, dystrophin-deficient cats suffer from esophageal obstruction as a consequence of muscle hypertrophy, a feature not seen in human patients (26). The dog model of DMD is remarkably similar to human muscular dystrophy, both in terms of histopathology and clinical progression due to skeletal muscle necrosis and cardiomyopathy (27). The *cxmd* mutation on a golden retriever background (used in this study) is known to be due to a messenger RNA processing error occurring because of a mutation in the consensus splice acceptor site in exon 6 of the dystrophin molecule (28). Although these animal models have species-specific differences in their clinical courses, they nevertheless occupy an important place in understanding human disease and serve as excellent models for studies concerning dystrophin deficiency.

Clinicians have long suggested that EOM dysfunction is not a cardinal feature of the otherwise widespread muscle weakness in DMD. Indeed, a recent clinical study demonstrated that saccadic eye movements in DMD patients were indistinguishable from those of controls (4). However, despite an extensive review of literature, we were unable to find any histological analyses of EOM in confirmed DMD cases. Indeed, the enigmatic clinical sparing of EOM along with the lack of histological information about EOM in DMD prompted this study. As a muscle group, EOM exhibit marked differences in their developmental, morphological, and physiological properties when compared with other skeletal muscle groups. These differences have been attributed to the unique demands made on EOM in providing the correct combination of smooth and saccadic eye movements to impart an

efficient usage of foveal vision in mammals. These differences include, but are not restricted to, a unique, well-demarcated fiber-type layering of myofibers (29); presence of motor units capable of firing at ~600 Hz compared with ~200 Hz in the extremity, and "hyper-fast" twitch fibers capable of the fastest known contractile responses (30). Additionally, EOM exhibit "en-grappe" type of neuromuscular junctions and are the only known example of multiple innervation in mammalian species (31). These features make EOM dysfunction an early and sometimes the sole presenting feature in certain diseases, e.g., myasthenia gravis, congenital fibrosis of the EOM, and some mitochondrial diseases. Conversely, clinical sparing of ocular function has been reported in DMD and motor neuron disease, where pathology is evident in a wide variety of non-EOM skeletal muscle groups (1, 4, 32).

It has been argued on theoretical grounds that, in DMD, the EOM may escape damage because they express high levels of a homologue/isoform of dystrophin, i.e., dystrophin-related protein(s), which can functionally compensate for the genetic lesion (33). Alternatively, it has been proposed that the unique anatomical and physiological properties of EOM may sufficiently explain the minimal morbidity of this muscle group in DMD (4). While these issues have not been studied in DMD patients, the first hypothesis was supported by a study that demonstrated that dystrophin-deficient *mdx* mice expressed greater amounts of DRP in skeletal, cardiac, and EOM and correlated the DRP overexpression with the lack of clinical weakness in these muscle groups (16). However, it is noteworthy that dystrophin and DRP differ in a number of properties, such as the pattern of their tissue distribution, developmental expression, and subcellular localization in both muscle and brain (7, 8, 12, 13, 34-39). To address the question of whether it is the overexpression of DRP or other physiological differences that compensate for dystrophin deficiency in the EOM of DMD patients, we used a combined approach of histological, immunohistochemical, and pharmacological methods in human, murine, and canine EOM from normal and dystrophin-deficient individuals.

Materials and Methods

Immunoblotting. The methods used for immunoblotting have been previously described (12). Briefly, weighed aliquots of tissue were solubilized in sample buffer and boiled. Protein concentrations were measured using a colorimetric assay, and samples normalized by dilution to achieve uniform loading of protein. Aliquots were fractionated on a 3.5-12.5% gradient SDS-polyacrylamide gel. Subsequently, proteins were electrotransferred onto nitrocellulose filters. Filters were stained with Ponceau S solution, which, together with the transfer of prestained protein molecular weight standards, served to control for the efficiency of transfer. Filters were incubated with the affinity-purified anti-DRP (rabbit) antibody. Antibody complexes were detected using chemiluminescence or alkaline phosphatase staining. Bands were quantified using Imagequant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Immunohistochemistry and Histopathology. Immunohistochemistry was performed using previously described methods (12). Tissue from C57 B10 *mdx* mice, *cxmd* dogs, and controls as well as from clinically and biochemically confirmed DMD patients was obtained on

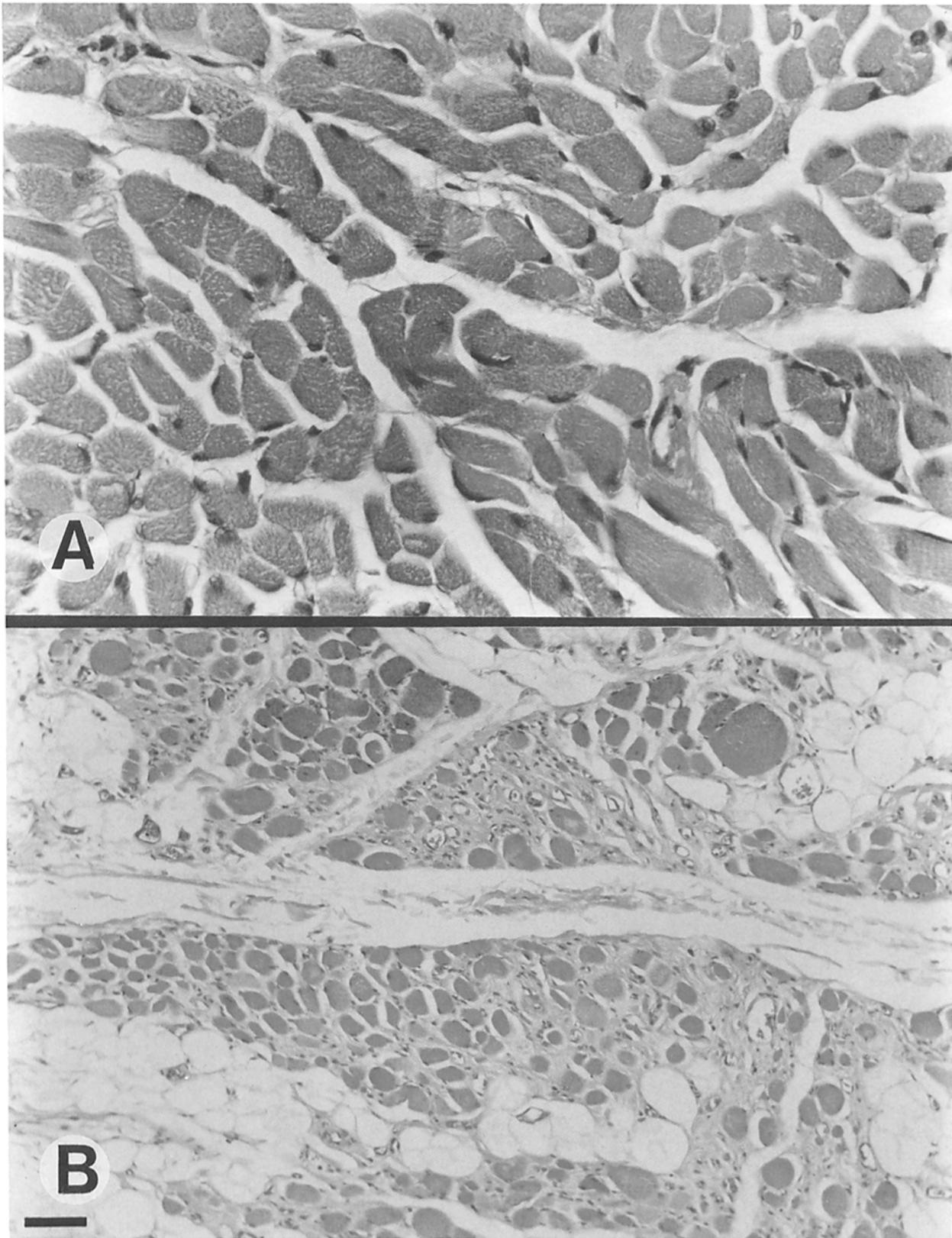


Figure 1. H&E staining of DMD muscle. Paraffin-embedded sections from 14-yr-old DMD autopsy case were prepared from (A) EOM (lateral rectus), and (B) limb muscle (quadriceps). Classical pathological changes of DMD, including fiber hypertrophy, fiber size variation, fiber splitting, central nucleation, and fatty change are clearly visible in limb muscle (B); however, the EOM from the same patient (A) are normal in appearance. Bar, 25 μm for A and 50 μm for B.

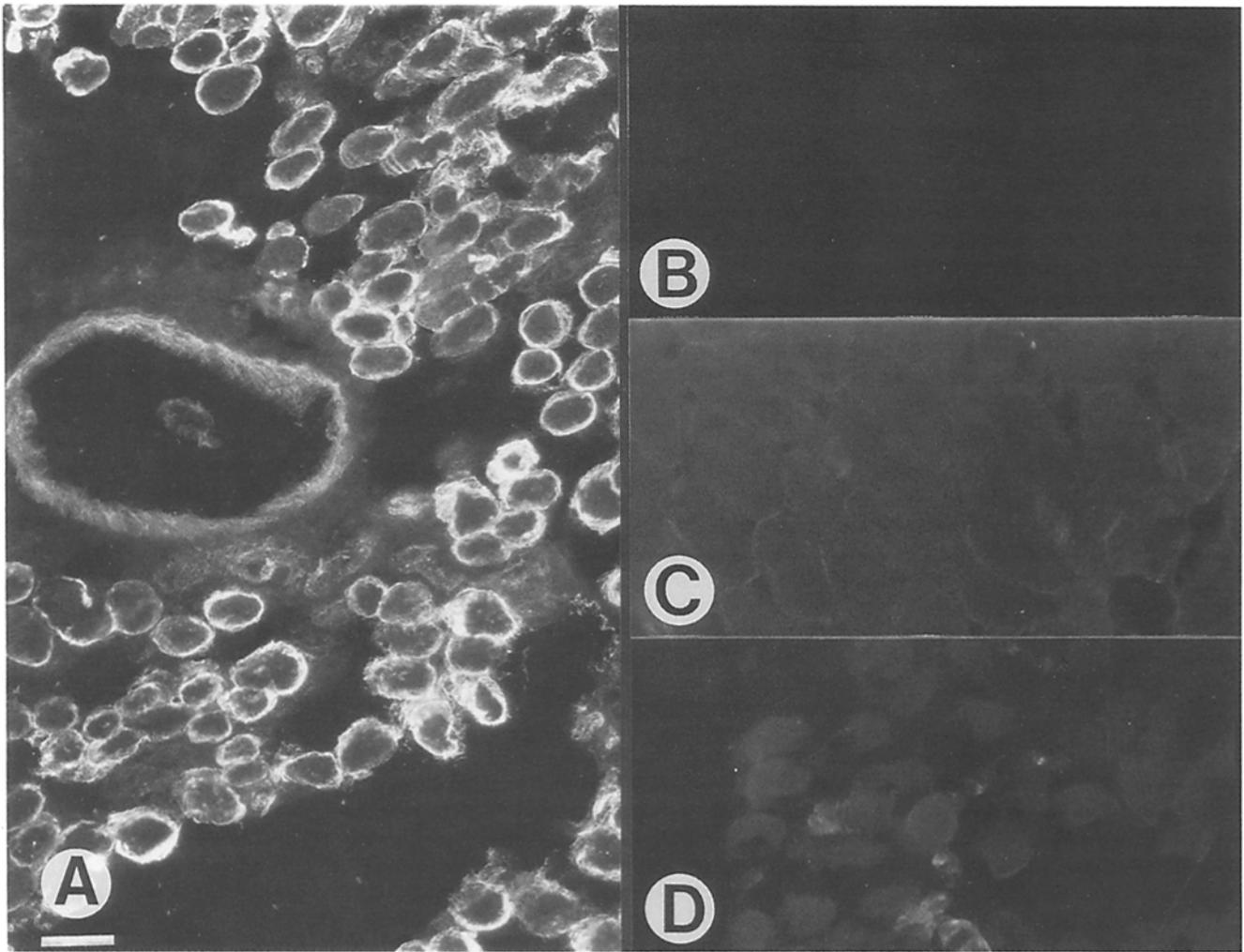


Figure 2. Dystrophin immunofluorescence of control human, DMD, *mdx*, and *cxmd* EOM. Dystrophin antibodies were used to label frozen sections of EOM obtained from (A) human control, (B) human DMD patient, (C) *cxmd* dog, and (D) *mdx* mouse. While normal sarcolemmal labeling using dystrophin antibodies is visible in control EOM (A), there is no detectable labeling in dystrophin deficient EOM from various species (B–D). Bar, 25 μ m.

autopsy, and control EOM tissue was obtained as an operation specimen. Tissue was flash frozen, and 5–10 μ m cryosections were cut and lifted onto electrostatically charged slides. Sections were fixed with cold methanol and washed with PBS before being labeled with dystrophin antibody (d6-10) in PBS with 10% calf serum at a dilution of 1:2,000 for 1 h at room temperature (35). After washing in PBS, sections were incubated with Cy3 anti-rabbit IgG antibody (Jackson Immunological Laboratories, Bethesda, MD) for 1 h. Washed sections were mounted in Aqua-mount (Lerner Laboratories, Pittsburgh, PA). Sections were examined using a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics. Hematoxylin and eosin (H&E) and alizarin red staining were performed as per standard histological protocols.

Ex Vivo Calcium Loading. Pectoral, limb, and EOM were dissected within 1 h postmortem from outbred male hounds weighing between 20 and 30 kg, used for studies of acute cardiac dysfunction at the Cardiology Research Unit (Johns Hopkins Hospital, Baltimore, MD). Small segments measuring 1 \times 0.2 \times 0.2 cm of muscle were placed on HBSS-beaded dental wax pallets and injected with HBSS, calcium ionophore A-23187 (25–100 μ g/ml), or cyclopiazonic acid (CPA; 50 μ M). 50 μ l of these reagents was

delivered by microsyringe (Hamilton Co., Reno, NV) through a 30-gauge needle centrally placed into each muscle segment. After incubation at 37°C for 30–60 min at 100% humidity, the muscle segments were washed in HBSS and submerged in 4% formalin,

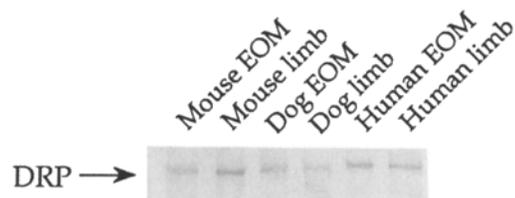


Figure 3. Immunoblot analysis of DRP content in DMD, *mdx*, and *cxmd* muscles. Aliquots of EOM and limb muscle from DMD, *mdx*, and *cxmd* were solubilized in protein sample buffer and quantitated, and equivalent amounts were separated by electrophoresis, immunoblotted with DRP antibodies, and quantified by densitometric analysis. An equivalent amount of DRP was detected in each set of lanes. Uniformity of loading and efficiency of transfer were based on Coomassie staining of posttransfer myosin as well as Ponceau S staining of the filter (not shown).

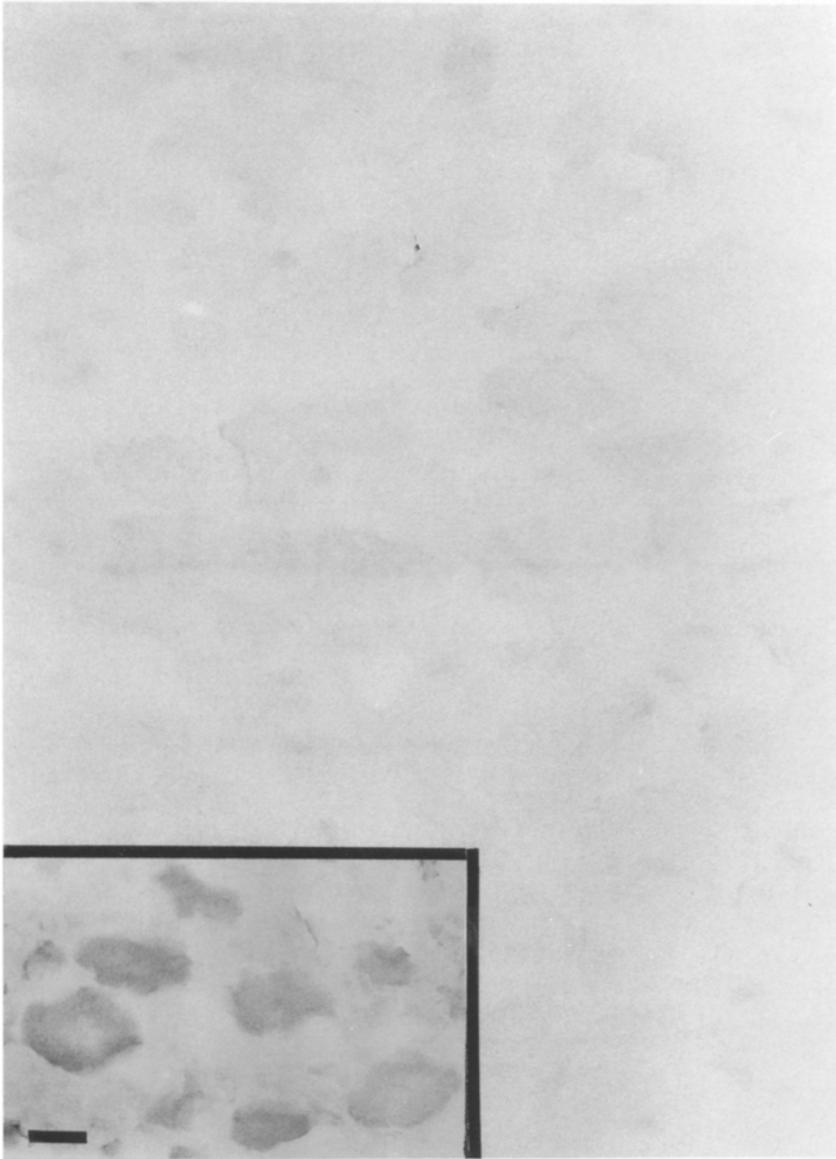


Figure 4. Alizarin red staining of DMD EOM muscle. 10- μm cryostat sections were cut from EOM from a DMD patient and stained with the calcium-sensitive dye, alizarin red. The dye failed to stain any fibers in the EOM from this patient. The inset shows a positive control of alizarin red staining in limb muscle from a different DMD patient. Bar, 30 μm for the full figure, 50 μm for the inset.

pH 7.2. After fixation, the terminal 1 mm was excised from each fragment by blue blades (The Gillette Corp., Boston, MA). Routine histologic examination of three cross-sections of each muscle segment was performed. Over 50 samples from each muscle group from 15 dogs were analyzed by this procedure.

Results and Discussion

To explore reports of clinical sparing of EOM in dystrophin deficiency, we performed histopathological analysis of EOM and limb muscles obtained from dystrophin-deficient humans (DMD), dogs (*cxmd*), and mice (*mdx*). Tissue sections stained using H&E clearly revealed that the EOM from dystrophin-deficient tissue were remarkably healthy in appearance and indistinguishable from the previously reported appearance of EOM of healthy individuals (29). Specifically, DMD EOM lacked the cardinal pathological manifestations

of dystrophin deficiency such as hypertrophy, fiber size variation, fiber splitting, central nucleation, fatty degeneration, and fibrotic scarring. In agreement with previous reports, these features were readily visible in limb musculature obtained from the same DMD individual as shown in Fig. 1. Similar histological studies in two additional DMD cases, one *cxmd* dog and two *mdx* mice, revealed sparing of the EOM despite dystrophin deficiency (data not shown).

Since EOM are known to exhibit certain physiological differences (e.g., innervation patterns, EOM-specific transcripts) when compared with other skeletal muscle, it was important to determine if the mechanism of histological sparing of EOM was associated with a lack and/or novel pattern of dystrophin expression in EOM when compared with limb musculature. Immunofluorescence analysis using the d6-10 dystrophin antibody revealed sarcolemmal labeling in control EOM in a manner indistinguishable from that previously noted in limb musculature, with strongly positive myofibers

predominating the picture (Fig. 2). EOM obtained from DMD patients, *cxmd* dogs, or *mdx* mice did not express detectable levels of dystrophin using this antibody (Fig. 2). Neither control nor DMD EOM expressed detectable levels of the non-muscle, alternative dystrophin transcripts Dp 71 or Dp 116 as determined using the carboxy-terminal specific dystrophin antibody d11 (40–42) (data not shown). Given the normal distribution of dystrophin labeling in EOM and the absence of labeling in EOM from DMD patients, *cxmd* dogs, and *mdx* mice, it is unlikely that the clinical sparing of this muscle group occurs because of EOM-specific differences in the quantity, nature, and distribution of dystrophin expression.

Despite the absence of any form of dystrophin in DMD EOM, it is possible that they could be spared of necrosis because of overexpression of DRP, which may functionally substitute for dystrophin in this muscle group. To test this hypothesis, tissue samples from EOM and limb muscle from DMD patients, *cxmd* dogs, and *mdx* mice were quantitated and immunoblotted using affinity-purified DRP antibodies that we had previously used to identify DRP (8). Fig. 3 demonstrates that similar levels of DRP are expressed in all these tissues, with no evidence of DRP overexpression as described by Matsumura et al. (16) being found in *mdx* EOM samples. To confirm these results we performed similar experiments on an additional case of DMD, *cxmd*, and *mdx* EOM and limb muscle; however, contrary to the findings of Matsumura et al., we obtained similar results in each instance. To eliminate observer bias, one set of experiments was performed “blind,” i.e., without prior knowledge of the identity of samples (data not shown). Thus, despite careful, objective analysis, we have obtained no evidence for the DRP overexpression in *mdx* EOM reported by Matsumura et al. In fact, our studies clearly demonstrate that DRP overexpression does not occur in DMD, *cxmd*, or *mdx* EOM. It is possible that the differences in DRP levels reported by these authors could be due to differences in antibody specificity, experimental error, or observer bias, since these issues were not controlled for by Matsumura et al. It should also be pointed out that the Matsumura study discussed here also reported DRP overexpression in *mdx* limb muscle, a finding for which conflicting reports currently exist in the literature (8, 14, 37). Given the number of experiments we performed and the analysis of EOM from three different species, we conclude that DRP overexpression is not responsible for the relative sparing of dystrophin-deficient EOM in either *mdx*, *cxmd*, or human DMD cases.

Since DRP overexpression was ruled out as a mechanism for sparing EOM in dystrophin deficiency, we addressed other physiological factors/processes in muscle germane to the process of myonecrosis. One such candidate process was maintenance of calcium homeostasis, which has long been suggested to play a pivotal role in DMD muscle pathophysiology (43). An elevated level of total intracellular calcium $[Ca^{2+}]_i$ and the subsequent activation of calcium-activated proteases are well-characterized steps in the final common pathway leading to muscle necrosis in dystrophin deficiency. Indeed, in DMD, ~5% of skeletal muscle fibers stain positively with the calcium sensitive dye alizarin red (44), indicating that

these fibers contain pathologically elevated levels of intracellular calcium $[Ca^{2+}]_i$. To address the role $[Ca^{2+}]_i$ plays in the lack of pathogenesis of dystrophin-deficient EOM, we stained these EOM with alizarin red to identify what fibers, if any, contained elevated levels of $[Ca^{2+}]_i$. Remarkably, the calcium-sensitive dye failed to stain any fibers in the EOM (the rectus) from a DMD patient (Fig. 4). Additionally, we tested sections cut from other EOM groups (the obliques) from this patient, from an additional DMD patient, from two *cxmd* dogs, and from three *mdx* mice, none of which showed positive staining with this dye (data not shown). The lack of fibers containing pathologically elevated, millimolar levels of $[Ca^{2+}]_i$ in dystrophin-deficient EOM is indicative of the ability of this muscle group to continue to maintain calcium homeostasis despite the lack of dystrophin.

To further analyze the calcium homeostatic mechanisms of EOM, we treated freshly dissected dog EOM and pectoral muscles with the calcium ionophore A23187. This drug causes widespread necrotic damage in the myofiber because of a marked elevation of $[Ca^{2+}]_i$. Interestingly, the pathological lesions caused by this reagent are remarkably similar to the lesions seen in DMD patients in vivo, as demonstrated by Pestronk et al. (45). Consistent with previous reports, calcium ionophore-treated pectoral muscles were markedly necrotic, and the lesions resembled the pathology usually seen in dystrophin-deficient skeletal muscle (Fig. 5). Surprisingly, administration of the same reagent in parallel did not cause any detectable muscle damage or necrosis in EOM (Fig. 5). To validate these results, we used another pharmacological agent, CPA, to perturb the calcium homeostatic mechanism in these muscles. CPA blocks the reuptake of $[Ca^{2+}]_i$ by the sarcoplasmic reticulum and hence causes an increase in the levels of $[Ca^{2+}]_i$ by using a pathway independent of A23187 (46). As shown in Fig. 5, CPA alone and in combination with A 23187 caused the expected necrotic damage in pectoral muscle but was unable to cause detectable muscle damage or necrosis in EOM taken from the same dog. These experiments are suggestive of the superior ability of EOM to maintain calcium homeostasis when challenged with elevated intracellular calcium levels; however, the precise mechanism by which they do so remains unclear. A detailed analysis of the presence/absence and the amounts of calcium-binding proteins, mitochondria, and calcium-activated proteases (e.g., calpain), as well as quantification of the amount of $[Ca^{2+}]_i$ in these muscle groups during pharmacological manipulation and in disease states using calcium-sensitive dyes (e.g., fura-2), are some obvious future experiments in this regard.

In conclusion, we find that dystrophin-deficient EOM are spared the widespread muscle necrosis that affects other striated muscle groups (Fig. 1). At variance with a previous report, we did not find any evidence for elevated DRP levels in dystrophin-deficient EOM (Fig. 3). Based on physiological and pharmacological experiments described here, we suggest that dystrophin-deficient EOM are spared the pathological consequences of dystrophin deficiency, at least in part, because the inherent ability of these muscle groups to maintain calcium homeostasis is better than that of other muscle groups (e.g., pectoral or limb muscle) (Figs. 4 and 5). The

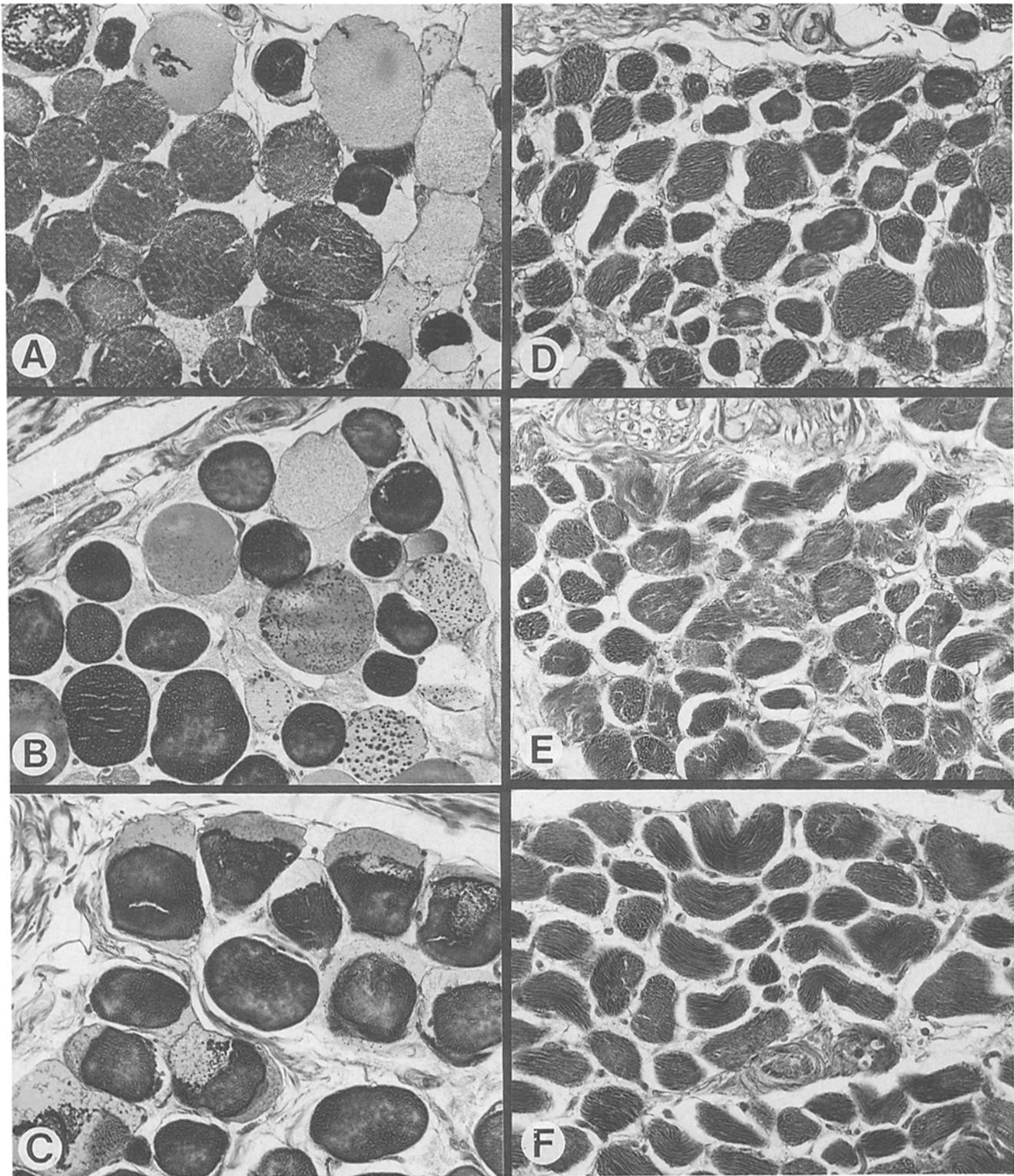


Figure 5. Ex vivo calcium loading in dog EOM and pectoral muscle. Canine pectoral muscles (A–C) or EOM (D–F) were rapidly dissected and incubated with A 23187 (A, D), CPA (B, E), or a combination of these drugs (C, F) for a period of 30 min. Specimens were embedded in paraffin and processed for routine histology. While pathological changes are visible in pectoral muscles treated with these drugs (A–C), the EOM (D–F) are resistant to pharmacological elevation of intracellular calcium levels. Original magnification: 360, reduced by the journal 26%.

central role that EOM-specific calcium homeostatic mechanisms play in compensating for the lack of dystrophin (Figs. 4 and 5) may yield important clues for developing potential therapeutic strategies for Duchenne muscular dystrophy. It may be useful to reevaluate previous drug trials using calcium channel blockers that had been undertaken in advanced cases of DMD (47) in light of our finding that efficient calcium homeostasis allows EOM to escape the onset of necrosis

rather than delaying the progression of necrosis. The ex vivo calcium-loading paradigm described in this study may be useful in identifying drugs that prevent myonecrosis and in evaluating these drugs in the prevention of clinico-pathological changes in well-studied animal models and preparations, such as dystrophin-deficient dogs, cats, and/or the diaphragm of *mdx* mice (24, 26, 27).

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