Elevated Levels of Albumin in Soleus and Diaphragm Muscles of *mdx* Mice (44061)

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Abstract. **Muscle damage is often associated with an influx of extracellular fluid containing albumin into the muscle. Muscles affected by muscular dystrophy undergo severe muscle damage; therefore, the hypothesis was tested that muscles of dystrophic (*mdx*) mice contain elevated levels of albumin. Albumin levels in diaphragm (DIA) and soleus (SOL) muscles of control and *mdx* mice were measured at 3 months and 1 year of age. Albumin in *mdx* DIA at 1 year of age was twice that of control. In *mdx* SOL at 1 year of age albumin was increased 25% compared with control. The increase in albumin correlates well with the decline in function in *mdx* DIA and SOL muscles. Electron microscopy of muscles suggests that albumin is co-localized with transverse tubules of muscle fibers and thus may be mainly located in extracellular fluid.**

We conclude that albumin is elevated in muscles affected by muscular dystrophy and suggest that this may be of clinical importance in view of substances bound to albumin under physiological conditions. [P.S.E.B.M. 1996, Vol 213]

Muscle damage is often associated with leakage of enzymes (e.g., creatine kinase) out and entry of ions (e.g., Ca^{2+}) into the muscle fiber. It is thought that these processes take place because of damage to the muscle cell membrane (sarcolemma) causing it to become more permeable to biological molecules. Some reports have indicated that this damage causes plasma proteins to leak into the muscle cell (1, 2) and albumin has even been used as a marker for muscle cell damage (3–5).

Muscles of patients with Duchenne muscular dystrophy (DMD) exhibit severe muscle damage and leakage of muscle enzymes. Cornelio and Dones (6) reported increased levels of albumin in muscles from DMD patients and suggested that albumin accumulates in muscle fibers as a result of sarcolemmal lesions. Increased levels of albumin in muscles can be of physiological importance. Heilig and Pette (7) showed that with increased contractile activity the amount of albumin in muscles increased. They suggested a role for albumin in aiding the enhanced transport demands related to increased metabolic activity. In a pathological state like muscular dystrophy, however, increased levels of albumin may influence the concentration of albumin-bound substances such as hormones, drugs, or fatty acids in muscles that do not exhibit increased metabolic demands.

The *mdx* mouse model described by Bulfield *et al.* (8) has a genetic defect homologous to patients with DMD. The defective gene is located on the Xp21 region of the X chromosome (9, 10), and its normal protein product is called dystrophin (11). Dystrophin is a 427-kDa protein located at the intracellular side of the sarcolemma (12–14). Because of its resemblance to structural proteins and its anchorage to the cell membrane, it is thought to function in preserving the integrity and flexibility of the sarcolemma (14–16). However, its precise physiological role is still unknown.

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Dystrophin is absent in muscles from DMD patients and *mdx* mice (11, 17) and is present as a protein with altered molecular weight in muscles from Becker muscular dystrophy (BMD) patients (18). The absence of dystrophin in *mdx* mice and DMD patients could lead to structural deficits in the muscle membrane causing it to become more permeable. However, the absence of dystrophin in *mdx* mice does not lead to extensive necrosis and early death as in DMD patients. Hindlimb muscles in *mdx* mice go through a phase of rapid degeneration and regeneration and almost fully regain function (19–21). In contrast, diaphragm muscle progressively degenerates with time (22–24). Indeed, *mdx* diaphragm most closely resembles DMD muscles (22, 23, 25). Also, recently it has been shown that all muscles of *mdx* mice older than 18 months of age exhibit DMD-like symptoms of muscular dystrophy (26).

This study was conducted to determine if albumin content is increased in muscles affected by muscular dystrophy in the *mdx* mouse. The goal was to determine albumin levels in diaphragm and soleus muscles in control and dystrophic mice, to correlate levels of albumin with functional decline of dystrophic muscles as measured previously (23, 27) and to investigate the possible location of albumin in muscles.

Materials and Methods

Animals and Tissue Collection. C57BL/SNJ10 (control) and dystrophic (*mdx*) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in accredited facilities at UTHSCSA. All procedures were performed in accordance with institutional guidelines for the care and use of animals. Male and female mice of 3 months and 1 year of age were used for this study. It has been shown previously that there are no differences between males and females (23). Mice of these ages were used because hindlimb muscles of *mdx* mice at 3 months of age have just gone through an accelerated stage of degeneration and regeneration, but their diaphragm muscle has not yet degenerated to a large extent; at 1 year of age hindlimb muscles of *mdx* mice have regained most of their function while the diaphragm is markedly degenerated (23).

Animals were anesthetized with methoxyflurane and blood was collected *via* heart puncture. Animals were sacrificed by cervical dislocation and diaphragm (DIA) and soleus (SOL) muscles were dissected. SOL was chosen as a representative hindlimb muscle since it has been shown that fast and slow muscles go through a similar disease process in *mdx* mice (23). Muscles were frozen in liquid nitrogen and stored at −80°C for SDS polyacrylamide gel electrophoresis (PAGE) ($n = 8$). Soleus and diaphragm muscles of one control and one *mdx* mouse were fixed in phosphate buffered/1% glutaraldehyde/4% formaldehyde for electron microscopic immunocytochemistry.

Protein Sequencing. Albumin was tentatively identified by its molecular weight, but to ensure its identity a sequence analysis was performed. The protein was sequenced by Edman degradation of Coomassie blue-identified bands on Pro-Blott membranes (polyvinylidene difluoride, Applied Biosystems, Foster City, CA) electroblotted from SDS polyacrylamide gels (28). The membrane was loaded into an Applied Biosystems 477A protein sequencer equipped with a 120A on-line PTH analyzer located at the UTHSC Institutional Protein Core Facility in the Department of Medicine.

Quantitation of Muscle Albumin. Muscles were homogenized in buffer (330 m*M* sucrose, 0.1 m*M* EGTA, 5 m*M* $MgCl_2$, 2 m*M* β-mercaptoethanol, 10 m*M* Tris HCl, pH 7.5). Protein concentrations of the muscle samples were measured according to the method of Bradford (29). Samples were boiled in SDS sample buffer for 5 min and proteins were separated on a 10% SDS-polyacrylamide gel (28). Gels were stained with Coomassie brilliant blue (R250), destained, and dried onto filter paper. The gels were then scanned with a ScanJet IIcx (Hewlett Packard) and quantitated using the image analysis program Image 1.44 (NIH). Intensity of the albumin band was expressed as a ratio compared with the intensity of the myosin band. Myosin is the most abundant muscle protein and has been suggested as a standard for normalization of other muscle constituents (30).

Serum Albumin Determination. Whole blood was centrifuged and serum was collected to measure albumin concentrations. Serum albumin was measured in blood samples from *mdx* and control mice using a colorimetric method based on the fact that bromcresol purple (BCP) binds quantitatively to albumin to form a stable complex with an absorbance maximum at 600 nm. The albumin reagent (BCP) was purchased from Sigma Chemical Co. (St. Louis, MO).

Electron Microscopic Immunocytochemistry. DIAand SOL muscles from control and *mdx* mice were dissected and placed in phosphate buffered 4% glutaraldehyde, 1% formaldehyde for 1–2 hr and washed in buffer. The muscle tissues were embedded in LR Gold (LRG) according to Nir *et al.* (31). Briefly, muscles were sequentially dehydrated with 50% (10 min), 70% (20 min), and 90% (20 min) ethanol. Muscles were then infiltrated with 50% LRG monomer/50% ethanol for 15 min; 70% LRG monomer/30% ethanol for 30 min; 100% LRG monomer for 60 min; 100% LRG monomer and initiator (benzoin methyl ether) for 30 min; and then in 100% LRG monomer and initiator over night at 4°C. Polymerization was carried out under ultraviolet light for 20 hr. Thin sections (70–80 nm) were cut at different regions of the muscle with a diamond knife. Sections were positioned on carbon coated Formvar grids and immunolabeling was per-

formed using the three stage labeling protocol described by Schneider and Papermaster (32). In all solutions bovine serum albumin (BSA) was replaced by ovalbumin, because the protein of interest was albumin. Rabbit anti-mouse albumin IgG-fraction (Accurate, Westbury, NY) was used as the primary antibody. Sections were viewed and photographed using a Philips 301 microscope. Photographs were taken randomly from at least 20 sites in the muscles. These procedures were performed by the Pathology Core Facilities at UTHSC SA.

Statistics. Values are reported as means ± SEM. Student's *t* tests were used to determine statistically significant differences between *mdx* and control values at 1 year and 3 months of age (33). Statistical significance was assumed at $P < 0.05$.

Results

Sequencing. The band that migrates with the standard bovine serum albumin band was assumed to be mouse albumin, but to ensure that this was indeed the case a sequence analysis was performed on the first 20 amino acids of the electroblotted protein. The sequence of the first 20 amino acids is as follows:

EAHKSEIAHRYNDLGEQHFK

The Genbank database was searched and this above printed sequence is identical to rat albumin except for amino acids 11 and 12 which are F and K, respectively, in rat. This protein was positively identified as albumin.

Quantitation of Albumin. Figure 1 shows a representative sample of gels of SOL and DIA muscles of 1-year-old control and *mdx* mice. More intense staining was observed in muscle samples from *mdx* mice compared with control. Figure 2 shows the results of analysis of intensity of albumin bands compared with myosin. Albumin was increased significantly at 1 year of age in both SOL and DIA muscles. Albumin content of *mdx* DIA was more than twice as high compared with control; however, albumin in *mdx* SOL was elevated only by about 25%. At 3 months of age, there was a tendency for albumin to be increased, but this did not reach statistical significance.

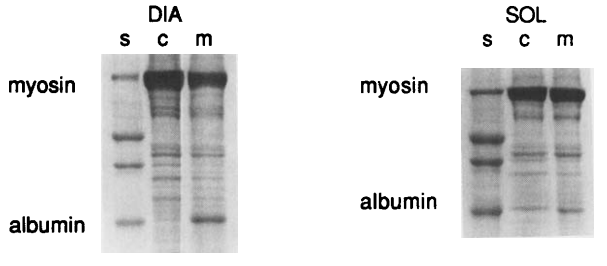

Figure 1. SDS-PAGE gels of diaphragm (DIA) and soleus (SOL) muscles at 1 year of age. Lanes: s, molecular weight standards; c, control; m, *mdx*.

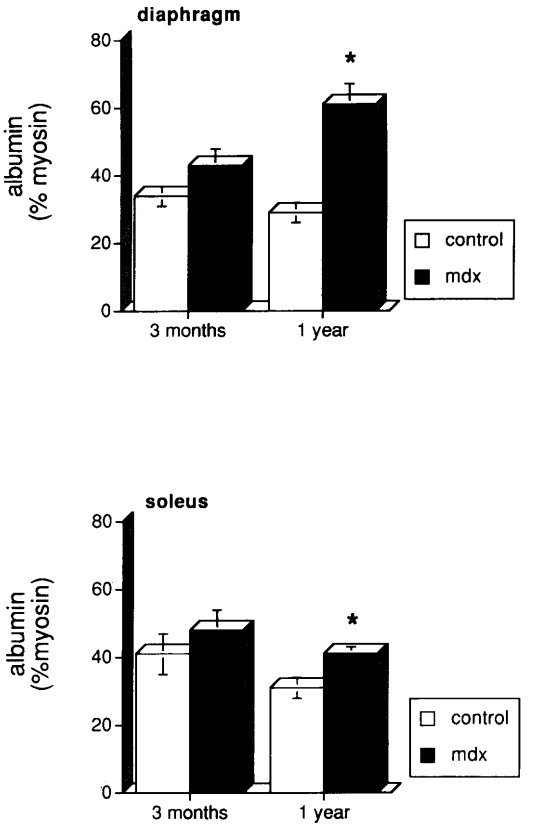

Figure 2. Albumin levels as a percentage of myosin in diaphragm (top) and soleus (bottom) muscles of control (open bars) and *mdx* (closed bars) mice at 3 months and 1 year of age. $n = 8$ for each group. Values are means ± SEM. *Significantly different from control ($P < 0.05$).

Serum Albumin Determination. Serum albumin was measured to investigate if contamination of muscle samples by blood and different levels of albumin in the blood of *mdx* and control mice could account for the differences seen in whole muscles. No significant differences were observed in serum albumin levels between control (3 months: 2.74 ± 0.14 g/dl; 1 year: 2.82 ± 0.34 g/dl) and *mdx* (3 months: 2.58 ± 0.17 g/dl; 1 year: 3.03 ± 0.18 g/dl) at both ages.

Electron Microscopic Immunocytochemistry. Figure 3 shows electron micrographs of control and *mdx* diaphragm muscles at 1 year of age. These pictures are representative of the pictures that were taken from both soleus and diaphragm muscles. Albumin as indicated by dark grains is localized in the regions where transverse tubules(t-tubules) are also located. Co-localization of albumin with t-tubular structures suggests that albumin is contained in the extracellular fluid in thet-tubules.

Discussion

The data presented in this study show that muscles affected by muscular dystrophy contain elevated levels of albumin. Interestingly, the diaphragm, which is the muscle most affected by the disease in *mdx* mice

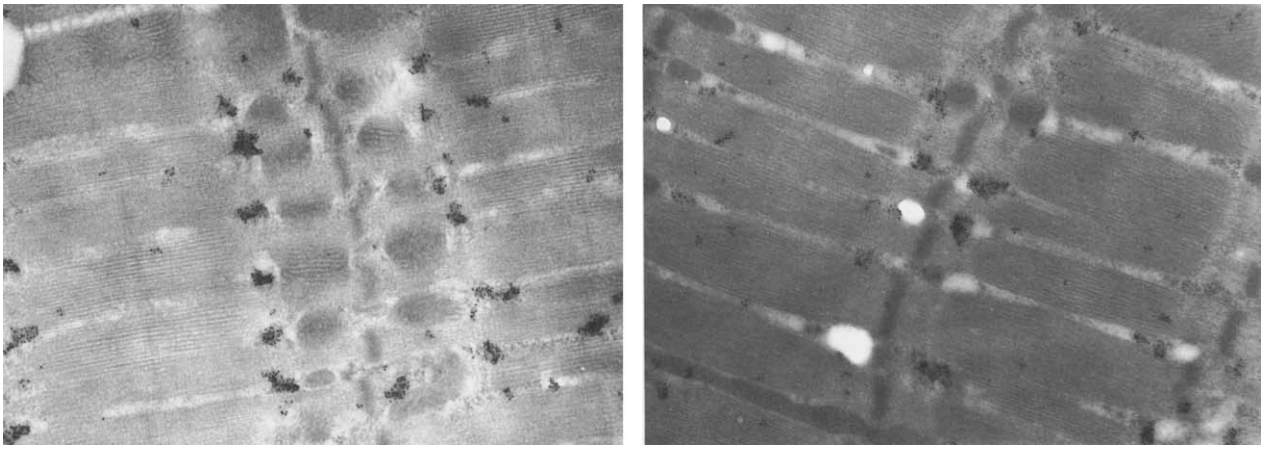

Figure 3. Electron micrographs of control (A) and *mdx* (B) diaphragm muscles at 1 year of age. Black spots indicate position of albumin antibody. Magnification: ×58,000.

and which mimics DMD muscles most closely (22, 23), contains more than twice as much albumin as control diaphragm. In contrast, albumin levels in *mdx* soleus are only increased by about 25%. This correlates well with functional data measured on control and *mdx* mice. In previous studies (23, 27), it was shown that at 3 months of age both soleus and diaphragm muscles of *mdx* mice exhibited a 35%–40% decrease in muscle strength but at 1 year of age muscle strength in diaphragm of *mdx* mice was decreased by 65% compared with control and soleus muscle strength was decreased by only 25%. In this study albumin level in *mdx* diaphragm at 1 year of age is more than twice the level in control diaphragm correlating well with a more than 50% decrease in muscle strength at that same age. In soleus muscles at 1 year of age the 25% decrease in muscle strength in *mdx* mice correlates well with a 25% increase in albumin.

The increase in albumin in muscles of *mdx* mice could not be attributed to an increase in albumin concentration in serum, because no differences in serum albumin between control and *mdx* mice were observed at either age. Heilig and Pette (7) also showed that the increase in albumin in muscles subjected to contractile activity was not due to an increase in intravascular albumin, because after perfusion of the muscles with physiological saline, elevated albumin levels persisted. We therefore conclude that albumin is elevated in muscles of *mdx* mice compared with control.

Some studies have suggested that different forms of injury to skeletal muscle cells cause an increase of plasma proteins in muscles (1, 2). In this respect, Greaves *et al.* (34) reported interesting findings in their studies on the muscle protein parvalbumin. These authors found an increase in a 65-kDa protein in muscles of dystrophic mice compared with control muscles but did not further investigate the identity of this protein. It is possible that the elevated protein is albumin and that the increase in albumin in dystrophic muscle can be considered as a marker for muscle damage in muscular dystrophy. Morandi *et al.* (35) indeed used albumin as an endogenous marker of extracellular fluid penetration. This was based on earlier data by Cornelio and Dones (6) which showed that necrotic fibers in muscles of patients with muscular dystrophies exhibited penetration by albumin-rich extracellular fluid. Other investigators (3–5) have used albumin as a marker for muscle fiber wounding due to exercise-induced injury or mechanical damage. Clarke *et al.* (36) showed that albumin containing muscle fibers were more frequently observed in *mdx* mice than in control mice.

Controversy exists regarding the exact location of albumin in muscle. While Müller and Heizmann (37) localized albumin to the region where thick and thin filaments overlap, implying an intracellular location, Yokota (38) and Heilig and Pette (7) localized albumin only in the interstitial fluid, t-tubular fluid and some sarcolemmal vesicles, suggesting confinement to extracellular space. Albumin can be expected to be in the extracellular space only, because it is not produced in muscle (7) and there is no known mechanism for albumin to be transported into the muscle cell. However, in muscular dystrophy, where membrane lesions are common, it is possible that albumin leaks into muscle cells. Therefore, we studied the localization of albumin in muscle cells by electron microscopy. In the muscles investigated in this study, no significant amounts of albumin were found in the intracellular space of intact muscle fibers. Albumin appeared to be specifically located at regions where t-tubules invade muscle fibers and was abundant in the extracellular space (data not shown). It is possible that we did not observe intracellular location of albumin because of the limited area of muscle fibers being studied when using an electron microscope, but our data suggest that albumin may be localized mainly in the extracellular space.

The increase in albumin levels in dystrophic muscles of *mdx* mice could be due to an increase in extracellular space in these muscles. Indeed, it has been shown that there is increased spacing between muscle fibers in *mdx* soleus and diaphragm muscles compared with control muscles at 3 months and 1 year of age and the increase in extracellular space is more severe in diaphragm than in soleus muscles of *mdx* mice (25). This correlates well with our observation that albumin content is increased to a higher degree in *mdx* diaphragm than soleus muscles. Heilig and Pette (7) also found a good correlation between the increase in albumin concentration and an increase in extracellular (chloride) space (39) in muscles after chronic stimulation. Moreover, we have shown that myosin concentration is significantly decreased in *mdx* diaphragm at 1 year of age compared with control (27), suggesting a decrease in contractile protein concentration. This observation, as well as the fact that *mdx* muscles are generally larger but lower in total protein concentration (27), suggests that there is an increased extracellular space. Elevated albumin levels in *mdx* diaphragm muscles is not just a reflection of a decrease in myosin, because albumin is increased by more than 100% and myosin is decreased by only 30% (27). Thus, these findings suggest that the increase in albumin in muscles affected by muscular dystrophy is at least partly due to an increase in extracellular space. McArdle *et al.* (40) have shown that there is an increased influx of extracellular fluid into *mdx* muscles and have shown that this is secondary to muscle degeneration and not due to an increase in permeability as a primary effect of dystrophin deficiency. Therefore, we believe that there is an influx of albumin into muscle fibers of *mdx* mice, but the largest part of the measured elevated levels in whole muscle can be attributed to an increase mainly in extracellular space.

Even though multiple physiological functions for albumin in plasma have been identified (41), its function in muscle is controversial. Heizmann *et al.* (42) suggested that albumin in muscle was indistinguishable from β-actinin, a regulatory protein of fine muscle structure. However, Maruyama and Kimura (43) disputed this and showed that albumin did not have the same physiological actions as β-actinin and concluded that β-actinin and albumin are two different proteins. Heilig and Pette (7) suggested that albumin may aid in the enhanced transport demands related to increased metabolic activity in contracting muscles. However, dystrophic muscles in *mdx* mice do not exhibit enhanced metabolic activity (44). The increased levels of albumin in dystrophic muscles without an increase in metabolic demand may become important when considering albumin-bound substances such as hormones, drugs, and fatty acids and their increased levels in muscles. It may be that some of these substances are present in higher concentrations in dystrophic muscles than in normal muscles. The importance of this possibility needs further investigation.

In summary, albumin levels are elevated in dystrophic muscles of *mdx* mice and the extent of albumin increase is correlated with disease severity. One possible explanation for the increase in albumin is an increase in extracellular space in dystrophic muscles, but an increased leakage into damaged muscle fibers cannot be excluded. Elevated levels of albumin in muscles may be of physiological and clinical importance because albumin binds and transports substances such as hormones, drugs, and fatty acids.

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