Increased structure-bound proteolytic activity in maturing dystrophic skeletal muscles

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Abstract

Duchenne Muscular Dystrophy (DMD) is a severe X-linked progressive muscle wasting disease resulting from the absence of the membrane-associated protein dystrophin and the secondary components of the dystrophin-glycoprotein complex. Although the genetic basis of the disease has been known for over 15 years, the onset mechanism of the disease is not yet known and no treatment is yet available to significantly increase the lifespan of DMD patients.

Increased levels of intracellular calcium have been noted in dystrophic muscle (Turner et al., 1991) and increased intracellular levels of calcium in skeletal muscle lead to increased levels of calcium-dependent proteolysis (Zeman et al., 1985). Increased levels of calpain, a calcium-dependent protease have been reported as early as age 4 weeks in mdx (dystrophin-deficient) mice (Spencer et al., 1995). Increased calpain activity has been demonstrated in mdx myotubes (Alderton et al., 2000a). There is also evidence of a role for calpain in DMD, but the contribution of calpain activity to the onset of DMD has not yet been determined.

The purpose of this study was to test the hypothesis that increased calpain activity contributes to the onset of DMD in maturing (birth to weaning) dystrophic skeletal muscles and to determine if increased calpain activity was due to the relative distribution of calpain and calpastatin, calpain's endogenous inhibitor. Calpain activity was assessed in quadriceps and diaphragm muscle homogenate supernatant and pellet fractions from C57BL/6 control and mdx mice at ages 7, 14, and 21 days. Total calpain and calpastatin content were determined by Western analysis.

In both the quadriceps and diaphragm samples, calpain activity in the supernatant increased with age. There was a significant increase (47.7%; $p<0.05$) in calcium-dependent calpain activity in mdx quadriceps pellet compared to control at age 7 days. In the quadriceps at age 7 days, calpain activity in the pellet in the presence of calcium was significantly greater than at age 14 (61.2%) and 21 days (52.6%; $p<0.05$). In the diaphragm, there were no significant differences in pellet activity in either the presence or absence of calcium at any age between control and mdx samples. In both control and

mdx diaphragms, pellet calpain activity in the absence compared with the presence of calcium was significantly greater at both age 7 (control, 46.4%; mdx, 45.4%) and 14 days (control, 42.4%; mdx, 43.6%; $p<0.05$). At age 21 days, both control and mdx calpain activities in the diaphragm supernatants in the presence of calcium were significantly greater than those at ages 7 (control, 66.7%; mdx, 72.1%) and 14 days (control, 39.9%; mdx 49.5%; $p<0.05$). In general, there were no differences in total calpain and calpastatin content that would account for the differences in calpain activity. There were similar patterns of calpain activity and total calpain and calpastatin content in both control and mdx samples, suggesting a similar pattern of development in control and mdx muscle from ages 7-21 days. The increase in calcium-dependent calpain activity in mdx quadriceps pellet compared to control at age 7 days may be due to differences in regulation and/or distribution of the calpain system early in mdx maturation compared to control. From the present study, the role of calpain in the onset of DMD appears to be minor if global calcium-dependent activity is evaluated.

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List of Important Definitions

1. DMD: Duchenne muscular dystrophy
2. DGC: Dystrophin-glycoprotein complex
3. Ca^{2+}: Calcium
4. $[Ca^{2+}]_i$: Intracellular calcium concentration
5. AU: Arbitrary units
6. EGTA: ethylenebis (oxy-ethlenenitrilo) tetraacetic acid
7. EDTA: ethylenediaminetetracetic acid tetrasodium salt dihydrate
8. DTT: Dithiothreitol
9. PMSF: Phenylmethylsulfonyl fluoride
10. SDS: Sodium dodecyl sulfate

Chapter 1
Introduction

Introduction

Duchenne Muscular Dystrophy (DMD) is a severe X-linked progressive muscle wasting disease resulting from the absence of the membrane-associated protein dystrophin. The absence of dystrophin results in the secondary loss of the components of the dystrophin-glycoprotein complex (DGC) (Roberts, 2001). There are two proposed functions of the DGC, structural and/or signaling (Blake et al., 2002a), both of which may affect calcium homeostasis and calcium-dependent signaling.

The exact mechanisms leading to the onset of DMD are not yet known, but changes in intracellular calcium may be involved. Increased levels of intracellular calcium have been reported in dystrophic muscle (Blake et al., 2002a). Protein degradation rates in normal muscles are affected by increases in intracellular calcium (Zeman et al., 1985) and this is also true in dystrophic muscle. Increased intracellular calcium in dystrophic muscle has been correlated with necrosis (Reeve et al., 1997).

The calcium-dependent calpains (calcium-activated neutral proteinase) (Molinari et al., 1997) are good candidate protease(s) for a role in DMD pathophysiology. These cysteine/thiol proteases cleave the carboxyl side of a tyrosine (Tyr), methionine (Met), or arginine (Arg) residue in the P_1 position (position where cleavage takes place) if the P_2 position (on the amino side of the P_1 position) is occupied by a hydrophobic amino acid. They have a wide variety of substrates, such as myofibrillar proteins and the ryanodine receptor (Spencer et al., 1996, Ruegg et al., 2002). One important characteristic of calpain action is that it modifies rather than degrades its substrates, sometimes modulating their function (perrin, 2002). There are three calpain isoforms in skeletal muscle: ì-calpain, which is activated at calcium concentrations from 1-70 ìM; m-calpain activated from 10-800 ìM (Spencer et al. 1995, 1996); and a monomeric muscle-specific isoform p94 activated by nanomolar calcium concentrations (Shevchenko et al., 1998, Molinari et al., 1997). Both ì- and m-calpain consist of a small regulatory subunit (30kD) and a large catalytic subunit (80kD) (Spencer et al., 1996) and both ì- and m-calpain are regulated by their endogenous inhibitor, calpastatin. P94 or calpain 3, is not regulated by calpastatin.

Turner et al. (1993) measured protein-degradation rates by the release of tyrosine in mdx and normal muscle that had been incubated with leupeptin, a nonspecific thiol

protease inhibitor, which has been shown to be effective at preventing calcium induced protein degradation. This group found that mdx muscles from mice aged 3-6 weeks not treated with leupeptin had a higher tyrosine-release rate than normal untreated muscle, indicating that mdx muscle had an increased protein-degradation rate (Turner et al., 1993).

Higher levels of intracellular free calcium have been shown to increase rates of leupeptin-sensitive proteolysis in intact soleus muscle (Alderton et al., 2000b). A fluorogenic calpain substrate was used to monitor the calcium-dependent proteolysis in mdx and normal myotubes. Over time, mdx myotubes showed increased hydrolysis in parallel with increased intracellular calcium.

Sultan et al. (2000) stated that increased calpain activity, without changes in total content, could result from a decreased interaction with the endogenous calpain inhibitor, calpastatin. The calpain:calpastatin ratio may represent an important indicator of net calpain breakdown potential (Enns et al., 2002, Spencer et al., 2002). In mdx mice that overexpressed calpastatin, reductions in muscle necrosis were evident as early as age 4 weeks, suggesting that suppressing calpain activity may reduce the histological features of dystrophin deficiency, such as tissue degradation.

Collectively, these studies demonstrate a possible role for calcium activation of calpains in the pathogenesis of DMD. The purpose of this study, therefore, was to evaluate the calcium-dependent calpain activity in the dystrophin-deficient (mdx) mouse model of DMD during early maturation (age 7-21 days), prior to onset of the overt signs of disease, using a calpain activity assay and Western analysis. The overall aim was to determine if the onset of muscle degeneration was due to increased calpain activity and if this activity was modulated by the distribution of calpain and calpastatin.

Statement of Problem

Although the relationship between the absence of dystrophin and the DGC and the onset of DMD has not yet been clearly defined, there are two main hypotheses. The first is the mechanical or structural hypothesis, which is based on the DGC being a link between the subsarcolemmal cytoskeleton and the extracellular matrix. The disruption in the DGC, due to the absence of dystrophin, may lead to sarcolemmal instability, membrane tears, and eventually muscle cell necrosis (Mendell et al., 1995, Campbell,

1995). The suggestion is that the DGC offers structural and functional integrity to the sarcolemma (Blake et al., 2002b) by stabilizing it against stresses imposed during muscle contraction or stretch (Rybakova et al., 2000). The second hypothesis involves signaling and is based on the involvement of dystrophin and the DGC in signaling mechanisms involving calcium and/or nitric oxide (NO) and/or unknown pathways.

In maturing muscles (i.e., birth to weaning, age 21 days), the mechanical hypothesis may not accurately reflect the onset mechanism of the disease (Grange et al., 2002, Pasternak et al., 1995). The role of neuronal nitric oxide synthase (nNOS) or nitric oxide (NO) in DMD is controversial with some investigators claiming either no role (Crosbie et al., 1998) or a potential role in immune response (Wehling et al., 2001). Conversely, the role of calcium and its abnormal homeostasis in dystrophic muscle has been widely studied. In DMD, the increase in intracellular calcium concentration ($[Ca^{2+}]_I$) has been linked with an increase in proteolysis; thus, calcium-activated calpains may be responsible in part for this increased protein degradation. The increased proteolysis may cause additional influx of extracellular calcium creating a positive feedback loop that leads to muscle necrosis and loss of muscle function.

Even though evidence suggests a role for calpain in DMD, the age at which calpain activity increases has not yet been established. This age may be important in determining calpain activity as having a primary or secondary role in the onset of DMD. Several studies have been conducted in myotubes (Alderton et al., 2000a, Fong et al., 1990, Franco et al., 1990), but most of the work in either whole muscle or fibers has been at age 3 weeks or older, though at least one group worked with mdx mice at 14 days (Badalamente et al., 2000).

The purpose of this study, therefore, was to evaluate the calcium-dependent calpain activity in the dystrophin-deficient (mdx mouse) model of DMD during early maturation (age 7-21 days) using a calpain activity assay and Western analysis. The overall aim was to determine if the onset of muscle degeneration was due to an increase in calpain activity.

Significance of Study

Duchenne Muscular Dystrophy is a severe and fatal muscle-wasting disease affecting 1 in 3500 boys. Although the genetic basis of the disease has been known for over 15 years, the onset mechanism of the disease is not yet known and no treatment is yet available to significantly increase the lifespan of DMD patients.

Increased levels of intracellular calcium have been noted in dystrophic muscle (Turner et al., 1991) and increased intracellular levels of calcium in skeletal muscle lead to increased levels of calcium-dependent proteolysis (Zeman et al., 1985). Increased levels of calpain, a calcium-dependent protease have been reported as early as age 4 weeks in mdx mice (Spencer et al., 1995). Increased calpain activity has been demonstrated in mdx myotubes (Alderton et al., 2000a). There is also evidence of a role for calpain in DMD, but the contribution of calpain activity to the onset of DMD has not yet been determined. The goal of the present study was to determine if increased calpain activity may play a role in the onset of DMD. If calpain is defined as part of the onset mechanism, therapeutic treatments could be designed to intervene in the onset of DMD.

Specific Aims

1. To test the hypothesis that increased calpain activity contributes to the onset of DMD in maturing dystrophic skeletal muscles. Calpain activity refers to proteolytic activity by cleavage of a fluorogenic substrate specific for calpain.
2. To test the hypothesis that increased calpain activity is due to the relative distribution of calpain and calpastatin.

Research Hypotheses

Main Hypotheses:

1. Increased calpain activity contributes to onset of DMD in maturing dystrophic skeletal muscles.
2. The increased calpain activity is due to an increased calpain:calpastatin ratio.

Specific Hypotheses:

H_{01}: Calpain activity will not be different between the quadriceps supernatant of control and mdx mice at any age.

H_{02}: Calpain activity will not be different between the quadriceps pellet of control and mdx mice at any age.

H_{03}: Calpain activity will not be different between the diaphragm supernatant of control and mdx mice at any age.

H_{04}: Calpain activity will not be different between the diaphragm pellet of control and mdx mice at any age.

H_{05}: Calpain activity will not be different between supernatant and pellet fractions of control and mdx mice at any age.

H_{06}: Total calpain and calpastatin content will not be different between quadriceps supernatant of control and mdx mice at any age.

H_{07}: Total calpain and calpastatin content will not be different between quadriceps pellet of control and mdx mice at any age.

H_{08}: Total calpain and calpastatin content will not be different between diaphragm supernatant of control and mdx mice at any age.

H_{09}: Total calpain and calpastatin content will not be different between diaphragm pellet of control and mdx mice at any age.

H_{10}: Total calpain and calpastatin content will not be different between supernatant and pellet fractions of control and mdx mice at any age.

Basic Assumptions

The following are basic assumptions made by the investigator:

1. All mice were well fed and hydrated.
2. Control animals were free from disease.
3. The mdx mouse is a suitable model for the study of dystrophy in murine skeletal muscle.
4. The muscle homogenates retained calpain activity.
5. The supernatant fraction contained only soluble cytosolic proteins, and the pellet fraction contained membrane structures and its associated proteins, and myofibrillar proteins.
6. The antibodies used to detect total calpain and calpastatin were specific; the band intensities on Western blots determined by densitometry reflected the content of the protein in each sample; and the protein content could be normalized to the intensity of a control band on the same blot.

Limitations

The following were limitations of the current study:

1. The current study was limited to the quadriceps and diaphragm muscles of mice.
2. The calpain activity measurements include p94 activity. The total calpain antibody recognized the 3 isoforms of calpain in muscle, including p94, which is not inhibited by calpastatin. Therefore, some of the calpain activity cannot be predicted by relative distribution of calpain and calpastatin.

Chapter 2
Review of Literature

Review of Literature

Brief History and Clinical Characteristics of Duchenne Muscular Dystrophy

Between 1830-1879, Duchenne Muscular Dystrophy (DMD) was recognized as a clinical disorder, but it was first described in detail by Edward Meryon in 1852. He portrayed the disease as one that primarily affected muscle tissue, had a tendency to affect males, and tended to occur within families (Emery, 1993). Duchenne de Boulogne, for whom the disease is named, characterized the disease in 1861. He called it pseudohypertrophic muscular dystrophy. He noted a childhood onset, a prevalence of the disease in boys, its occurrence in children within the same family, a progressive muscle weakness, a gradual increase in size of many affected muscles, and based on muscle biopsies, abundant fibrosis and adipose tissue at later stages of the disease (Emery, 1993).

In 90% of patients, the onset of DMD is at age 5 years. A delay in speech and motor development in early childhood are most noticeable. Walking is delayed until 18 months compared to a normal 13 months. A fundamental feature in early stages is enlargement of calf muscles, some of which is due to adipose and connective tissue and is, therefore, called pseudohypertrophy. The muscle involvement is bilateral and symmetrical, first affecting the lower limbs. The proximal muscles are more affected than the distal muscles. Additional features are a waddling gait due to weakness in hip abductors, lumbar lordosis because of weak gluteals, and the Gower's maneuver, which is difficulty rising from the floor or a chair, owing to weakened knee and hip extensors. One third of DMD patients also show intellectual impairment. By age 12, 95% of patients are confined to a wheelchair. Contractures arise at the elbows, knees, and hips and a severe kyphoscoliosis can develop. There is steady deterioration of pulmonary function. By age 20, 90% of DMD patients die, most from cardiac failure and respiratory insufficiency (Emery, 1993).

By 1959, it was recognized that serum creatine kinase levels were raised in patients and female carriers (Ebashi et al., 1959). Between 1978-1979, DMD had been mapped to locus Xp21 (Verellen et al., 1978, Lindebaum et al., 1979). The protein dystrophin was identified in 1987 as the protein product from this gene locus (Hoffman et al., 1987). Today, DMD is recognized as a severe X-linked muscle-wasting disease that affects 1 in 3500 boys. One third of all DMD cases arise from a sporadic mutation in the

dystrophin gene. This is about 105 x 10^{-6} genes/generation, which is one of the highest mutation rates reported and most likely a result of the large size of the locus (Vogel, 1990).

Dystrophin and Dystrophin-Glycoprotein Complex

The gene for the protein dystrophin, located on chromosome X at locus p21 (Blake et al., 2002a), is the largest gene (2.4 Mb) characterized to date. The dystrophin gene has 79 coding exons and 7 tissue-specific promoters for 7 protein isoforms. Three promoters produce "full-length" transcripts whose 427kDa proteins only differ in their amino-terminal sequences. These isoforms are brain (B), muscle (M) which is expressed in skeletal and cardiac muscle, and Purkinje (P), which is expressed in both cerebellar Purkinje cells and skeletal muscle (Blake et al., 2002a). The other promoters produce smaller isoforms of dystrophin (Figure 1).

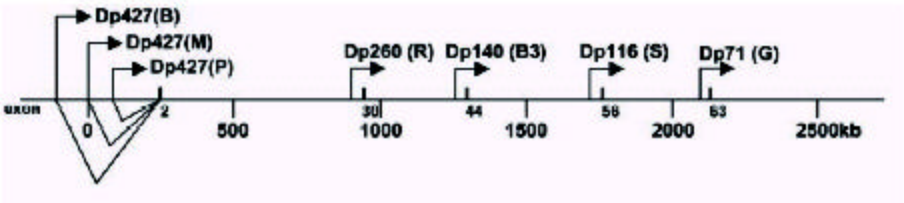

Figure 1. Schematic of human dystrophin gene. Dp-dystrophin promoter, B-brain, M-muscle, P-Purkinje, R-retina, B3-brain, **S**-Schwann cells, G-general (ubiquitously expressed) (Blake et al., 2000a)

The dystrophin protein is expressed in both invertebrates and vertebrates. Vertebrate dystrophin is located at the cytoplasmic membrane of skeletal, cardiac and smooth muscle and at synapses in the central nervous system (Roberts, 2001). The 427kDa cytoskeletal protein dystrophin is a member of the â-spectrin/á-actinin protein family, which is characterized by an amino-terminal actin-binding domain. Dystrophin has four separate regions: (1) amino-terminal actin-binding domain; (2) a central rod domain; (3) a cysteine-rich domain; and (4) a carboxyl-terminal domain (Blake et al., 2002a). The cysteine-rich domain is the most critical because it interacts with the intracellular tail of â-dystroglycan, which links dystrophin to the membrane and

preserves the entire dystrophin-glycoprotein complex (DGC) (Roberts, 2001, Straub et al., 1997, Ervasti et al., 1990).

The absence of dystrophin results in the secondary loss of the components of the DGC (Roberts, 2001). The DGC (Figure 2) acts as a scaffold to connect the cytoskeleton to the basal lamina of the muscle fiber. It is made up of several subcomplexes, including the dystroglycan complex and sarcoglycan:sarcospan complex, and the peripheral proteins of the cytoplasmic dystrophin-containing domain. The dystroglycan complex is composed of á- and â-dystroglycan. â-dystroglycan, an integral membrane protein, interacts with the amino-terminus of dystrophin in the cytosol and with á-dystroglycan in the extracellular matrix, which in turn binds to laminin-2. The sarcoglycan:sarcospan complex, composed of á, â, ã, and ä sarcoglycans and sarcospan, is important for stabilizing the dystroglycan complex in the sarcolemma (Blake et al., 2002a, Roberts, 2001, Straub et al., 1997). In muscle, the cytoplasmic dystrophin-containing domain contains á-dystrobrevin that binds directly to dystrophin, but its exact role is unknown. â-dystrobrevin is found in the DGC of nonmuscle tissues. Another component of this domain are the syntrophins, adapter proteins that link membrane-associated proteins, dystrophin and dystrobrevin to the DGC (Blake et al. a, 2002 Roberts, 2001). Several other proteins with unknown functions are associated with the complex, including syncoilin, biglycan, and filamin-2 (Roberts, 2001).

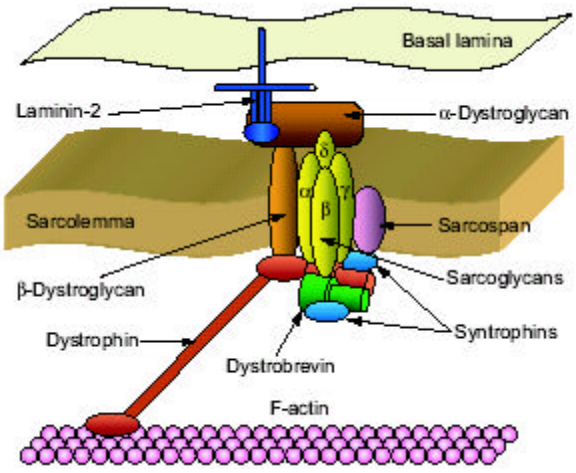

Figure 2. Schematic of DGC with currently understood relationship of the components. (Roberts, 2001)

There are two proposed functions of the DGC, structural and/or signaling. The structural role is thought to stabilize the sarcolemma from shear stresses imposed during eccentric muscle contractions (Rybakova et al., 2000, Blake et al., 2002b). However, there is also increasing evidence that the DGC plays an important signaling role (Blake et al., 2002b, Hack et al., 2000), such as in the interaction between nNOS and á-syntrophin. For example, there are decreased levels of sarcolemmal associated nNOS in patients with DMD (Blake et al., 2002a) that may affect inflammation in dystrophic muscle (Wehling et al., 2001). These functions will be discussed further in the section on mechanisms leading to DMD.

Animal Models of DMD

There are several animal models of DMD, three of which are cat, dog and mouse. In the cat, hypertrophic feline muscular dystrophy (HFMD) is the result of deletions in the Muscle and Purkinje promoters of the dystrophin gene. The reduced level of dystrophin results in abnormal gait, marked hypertrophy, and histological necrosis. However, fibrosis is limited, which in part makes this a poor model for DMD (Blake et al., 2002a).

Canine X-linked muscular dystrophy (CXMD), best characterized by the golden retriever, is the animal model most similar to DMD because it is genetically homologous and shares the severe myopathy, clinical development, and significantly reduced lifespan of human DMD (Cozzi et al., 2001, Blake et al., 2002a). Connective tissue proliferation is significant in 15-day old puppies indicating prior loss of muscle fibers (Cozzi et al., 2001). At 2 months, muscle weakness is progressive and apparent and the muscles show evidence of necrosis, fibrosis, and regeneration (Blake et al., 2002a). In older dystrophic CXMD dogs, the percentage of necrotic fibers declines from ~5% to ~2% and the extent of fibrosis drops and plateaus from ~60% to ~30%. In adult dogs, there is a decline in muscle fiber regeneration, which may be due to the depletion of regeneration potential (Cozzi et al., 2001). One problem with this model is the phenotypic variation between litters that makes maintaining a kennel difficult (Allamand et al., 2000).

There are two mouse models for DMD, mdx and dystrophin-deficient/utrophin-deficient. The mdx mouse has an X-linked (Bulfield et al., 1984) point mutation in the dystrophin gene that results in a truncated protein (Sicinski et al., 1989). The point

mutation occurs in exon 23 where a thymine is replaced by a cytosine, which results in a termination codon instead of the normal codon for glutamine. The protein product is 27% of the normal length. The truncated protein might still be able to bind cytoskeletal elements, add stability to the membrane, and it could play a positive residual role if not degraded, however the researchers did not test these hypotheses (Sicinski et al., 1989).

Mdx mice have a near normal lifespan (Blake et al., 2002a) and show little weakness after age 6 weeks (Durbeej et al., 2002). At age 4 days, there are no histological lesions evident. At age 3 weeks, the muscles demonstrate excessive atrophy (Bulfield et al., 1984), variation in fiber size, and degeneration of some fibers. In addition, the plasma demonstrates elevated levels of pyruvate kinase and creatine kinase. There is also the presence of phagocytic cells in place of the lost fibers, but there is no replacement of lost muscle by adipose cells (Bulfield et al., 1984). By age 3 weeks in the mdx mice, there is a vigorous regeneration response based on the presence of many centrally nucleated fibers (Grady et al., 1997). In the first 6 weeks, there is an expansion of satellite cell population and hypertrophy (Durbeej et al., 2002). By age 9 weeks, muscle fibers appear normal other than the presence of some centralized nuclei. In older adult mice, atrophy and fibrosis are found in limb muscles (Blake et al., 2002a). Functionally, force production and power output of the skeletal muscles are significantly reduced between 10-12 days (Grange et al., 2002) and 6-28 months (Blake et al., 2002a). At 12 months, phagocytosis of necrotic muscle tissue is evident (Bulfield et al., 1984).

The most affected muscle in the mdx mouse is the diaphragm (Blake et al., 2002a, Durbeej et al., 2002, Stedman et al., 1991). The pattern of degeneration, fibrosis, and functional deficit, which includes reductions in strength, elasticity, and contractile speed, is similar to that of DMD limb muscles, though before age 25 days, changes are rare (Stedman et al., 1991). At age 6 months, degeneration resumes with continued necrosis and connective tissue degeneration, but regenerative activity persists. This situation causes no respiratory compromise. By age 16-22 months, there is a drop in diaphragm isometric strength. At age 18 months, there is a loss of tissue compliance (increased stiffness). The difference in mdx limb muscle and diaphragm may reflect the intrinsic differences in regenerative capacity (Stedman et al., 1991). About 90% of the fibers in the diaphragm (at ages 2, 8, 10 weeks) stained for fast myosin heavy chain (MHC),

slightly more than the percentage in wild-type fibers. This increase in glycolytic fibers in a muscle needed for oxidative activity may strain the energy output of the muscleand lead to respiratory failure (Deconinck et al., 1997).

The second DMD mouse model is the dystrophin-deficient/utrophin-deficient (mdx/utr$^{-/-}$) model that was produced by breeding dystrophin-deficient mdx stock with utrophin-deficient stock (Grady et al., 1997, Deconinck et al., 1997). This model is characterized by a reduced lifespan (<20 weeks), weight loss, severe muscle weakness with joint contractures, obvious growth retardation, kyphosis, diminished mobility, and cardiomyopathy similar to DMD (Durbeej et al., 2002, Grady et al., 1997, Deconinck et al., 1997). The onset of degeneration and regeneration cycles is evident at age 2 weeks, earlier than in the mdx mouse. Their pathology is similar to that of the mdx mouse at age 4-5 weeks, including variation in muscle fiber size, presence of connective tissue, and extensive regeneration. By age 10 weeks, they demonstrate prominent interstitial fibrosis. Adipose cells replace the muscle cells and there is an invasion of macrophages (Deconinck et al., 1997, Grady et al., 1997). There are also fewer folds in the neuromuscular junction than in the mdx model, but the importance of the folding remains unknown (Grady et al., 1997).

In the diaphragm at age 6 days, there are necrotic muscle fibers and connective tissue already present. At 2 weeks, there is increased fiber regeneration evident. The diaphragm stains for 99% fast MHC, compared to the ~90% in mdx mice. Developmental MHC is also present. These mdx/utr$^{-/-}$ mice have difficulty breathing (Deconinck et al., 1997).

Functionally, the muscles of the mdx/utr$^{-/-}$ model generate only 40-60% as much tension as controls but, when this is normalized to cross-sectional area, the tension is reduced to 25% (Grady et al., 1997), suggesting that some loss of force is due to the smaller muscle size. There is also an increased relaxation time following contraction that indicates a disturbance in calcium homeostasis (Grady et al., 1997). In 9-12 day mdx/utr$^{-/-}$ mice, there is a 70% depression of stress generation in the extensor digitorum longus (EDL), compared to mdx, which had a depression in maximal stress compared to control (Grange et al., 2002). This model more closely follows the severe pathology of DMD.

Dystrophin deficiency: Potential mechanisms leading to DMD

The relationship between dystrophin, the DGC, and the onset of DMD remains undefined. However, there are several hypotheses to explain how the absence of dystrophin and the DGC may play a role in DMD. The first is the mechanical or structural hypothesis, which is based on the DGC being a link between the subsarcolemmal cytoskeleton and the extracellular matrix. The disruption of the DGC, due to the absence of dystrophin, may lead to sarcolemmal instability, membrane tears, and eventually muscle-cell necrosis (Mendell et al., 1995, Campbell, 1995). The suggestion is that the DGC offers both structural and functional integrity to the sarcolemma (Blake et al., 2002b) by stabilizing it against stresses imposed during muscle contraction or stretch (Rybakova et al., 2000). The second hypothesis is based on the involvement of dystrophin and the DGC in signaling mechanisms that may include calcium and/or nitric oxide (NO) and/or unknown pathways.

Dystrophin deficiency: Mechanical hypothesis

In normal tissues that are subjected to mechanical stress, cells have transient disruptions in the plasma membrane, but in dystrophic membranes from mice aged 90-110 days, there is a higher frequency of these transient disruptions (Petrof et al., 1993).

The lack of dystrophin and loss of the DGC is considered both to make the membrane less stiff (ΔForce/ΔLength) and to change the threshold for work-induced injury, which causes a disturbance in the balance between injury and repair in muscle fibers (Stedman et al., 1991, Grange et al., 2002). Muscle enzymes, such as creatine kinase, and extracellular fluids including calcium could be exchanged through these membrane ruptures, could alter intracellular ionic concentrations, and eventually lead to cell death (Carlson, 1998). Some evidence for the mechanical hypothesis is that the DGC had been found to be a strong physical link between the sarcolemmal membrane and ã-actin of the costameric cytoskeleton (Rybakova et al., 2000). Costameres are cytoskeletal protein assemblies that link the sarcomere to the sarcolemma and transmit contractile forces laterally through the sarcolemma to the basal lamina. Without dystrophin, there could be a disruption of the costameres because dystrophin may directly bind actin filaments (Rybakova et al., 2000). By disrupting this costameric structure and thereby

altering the transmission of contractile forces, it is conceivable that membrane tears can occur. Muscle fibers in the mdx mouse at age 90-110 days have been shown to be more susceptible to contraction-induced sarcolemmal rupture (Petrof et al., 1993). This structural stability of the sarcolemma in DMD could be compromised early in development and progressively decline over time (Mendell et al., 1994).

Mdx diaphragm and EDL muscle from mice aged 90-110 days, which were subjected to three contraction protocols *in vitro* including eccentric, isometric, and passive lengthening, demonstrated greater sarcolemma breakage compared to control muscles in all instances (Petrof et al., 1993). Damage was detected using procion orange, which is unable to permeate an undamaged sarcolemma, but can pass through disrupted cellular membranes, therefore allowing detection of damaged fibers. The eccentric-stretch protocol (~9%) resulted in the most dye uptake compared to either the isometric protocol (~7%) or passive stretching (~3.5%). Mdx fibers also had a higher percentage of damaged fibers at peak force (indicating mechanical stress) than control fibers (Petrof et al., 1993).

There is evidence, however, that this mechanical hypothesis may not be responsible for the onset of DMD. Grange et al. (2002) subjected the EDL of 9-12 day old control, mdx and mdx/utr$^{-/-}$ mice to an acute stretch-injury protocol similar to that of Petrof et al. (1993). Before the stretch-protocol, muscle fibers from both dystrophic genotypes had greater dye uptake (mdx, ~9% and mdx/utr$^{-/-}$, ~6%) compared to control (~2%), indicating sarcolemmal damage. Following the stretch-protocol, the sarcolemmal membrane did not display additional damage in any genotype (Grange et al., 2002). Additional evidence against the mechanical hypothesis is that dystrophic compared to non-dystrophic membranes were 4-times less stiff (mdyne/ìm) when suction was applied over individual *mdx* membrane patches (Pasternak et al., 1995). This lack of stiffness may indicate that the membranes have a greater capacity to stretch and yield to transmembrane forces (Carlson, 1998). The mechanical hypothesis suggests that contractile forces produced during exercise could increase damage to the fragile membrane. Surprisingly, mdx mice that have been placed on long-term exercise regimens have shown that the exercise may actually have a small beneficial effect instead of the expected enhancement of the disease progress (Carlson, 1998).

The mechanical hypothesis suggests that dystrophin and the DGC stabilize the sarcolemmal membrane by acting as a scaffold to connect the cytoskeleton to the basal lamina. Evidence is not conclusive to support this hypothesis during early maturation. In maturing mice, stretch did not appear to impose additional tears in the sarcolemma based on the uptake of procion orange (M_r 631), a molecule much larger than calcium (M_r 40). However given the size of calcium, it is possible that calcium could leak across a damaged membrane.

Dystrophin deficiency: Signaling hypothesis

The second major hypothesis to explain how the absence of dystrophin and the DGC may lead to the onset of DMD is the signaling hypothesis. At present, there is little known about this mechanism in the pathogenesis of DMD. Signaling molecules are known to be associated with the DGC and they are important to muscle cell survival. This association is the basis of the hypothesis that dystrophin and the DGC play a signaling role and is not just a molecular scaffold (Blake et al., 2002b). Two signaling cascades that may be affected by the DGC involve nitric oxide (NO) and calcium.

Nitric oxide is produced by the Ca^{2+}-calmodulin dependent enzyme neuronal nitric oxide synthase (nNOS). nNOS is associated with the DGC through the adaptor proteins syntrophins. In á-dystrobrevin-deficient mice, nNOS is displaced from the sarcolemmal membrane and results in impaired nitric oxide (NO) signaling (Grady et al., 1999, Ruegg et al., 2002). With the impaired NO signaling, as measured by a decrease in cGMP, the vascular regulation in dystrophic mouse muscle may be compromised (Thomas et al., 1998). The decrease in cGMP may cause enhanced vasoconstriction in arterioles of skeletal muscle, limiting blood supply to contracting muscles. For example, muscle ischemia during hand-grip exercise has been demonstrated in DMD boys (Sander et al., 2000).

NO may act as an anti-inflammatory molecule in skeletal muscle (Wehling et al., 2001). A reduction in NO may cause an increase in reactive oxygen species (ROS) and inflammation because of impaired superoxide scavenging by NO. Increased formation of ROS has been found in muscles of both DMD patients and mdx mice (Ruegg et al., 2002).

A strong potential signaling molecule is calcium, for several reasons. One is that calcium binding is selective which is based on its charge-to-size ratio. Divalent calcium (0.95 Å) is closer in size to monovalent cations sodium (1.00Å) and potassium (1.33 Å) than divalent magnesium (0.65Å) (Da Silva, 1991). Calcium also has a high coordination number (±7), similar to sodium (±6) and potassium (±8). This high coordination number allows calcium to bind to many ligands at once (Silberberg et al, 2000), contributing to its selective binding. It is also able to interact with neutral oxygen donors, such as carbonyls and ethers. Magnesium does not share these last two characteristics (Da Silva, 1991).

Another important property in calcium signaling is calcium kinetics. Calcium exchanges water 10^9/sec, which is much faster than magnesium 10^6/sec. Calcium is a good messenger because of its fast response (Da Silva, 1991, Carafoli and Klee, 1999). The fast exchange of water for other binding groups is due to bond angles and bond distances, which contribute to a flexible geometry for the molecule (Carafoli and Klee, 1999).

According to Carafoli and Klee (1999), the best messengers are substances that are not metabolized, exchange quickly, but also bond well. Calcium is not metabolized, it has fast kinetics, 1000 times faster than magnesium. Its high coordination numbers and flexible geometry allow it to bind selectively. Therefore, calcium is an excellent signaling molecule that may be involved in the onset of DMD.

Dystrophin deficiency: The calcium hypothesis

The calcium hypothesis states that, in the absence of dystrophin, there would be an influx of calcium through the sarcolemma due to either abnormally functioning mechanosensitive channels or cation-leak channels. The increased calcium entry would cause a rise in cytosolic free calcium, activate proteases, and eventually lead to muscle necrosis (Mallouk et al., 2000, Alderton et al., 2000a, Alderton et al., 2000b). The small but constant elevation in intracellular calcium that causes activation of calcium-dependent proteases would result in destruction of the cytoskeleton and consequently breakdown of the sarcolemma. The sarcolemmal breakdown would allow larger influxes of calcium and a higher intracellular calcium concentration in a positive feedback loop that ultimately results in muscle fiber necrosis (Carlson, 1998). A chronic elevation in $[Ca^{2+}]_i$, which is produced by an increase in calcium channel activity in human DMD and

mdx myotubes (Fong et al., 1990, Franco and Lansman, 1990) and enhanced calcium influx has been reported in mdx dystrophic muscle fibers from mice aged 3-6 week (Turner et al., 1991). Six to fifteen days after myoblast fusion, DMD human myotubes and mdx myotubes had resting free intracellular calcium levels that were elevated compared to control; in human, 76±5nM vs. 55±5nM and in mouse, 110±7nM vs. 82±7nM (Fong et al., 1990). In dystrophic fibers from mice aged 3-6 week, resting $[Ca^{2+}]_i$ was elevated from 2- to 4-fold over normal fibers when measured using fura-2, a calcium-binding dye (Turner et al., 1991, Alderton et al., 2000b).

Turner et al. (1988) demonstrated that mdx dystrophic fibers had a reduced ability to regulate $[Ca^{2+}]_i$ because $[Ca^{2+}]_i$ varied more dramatically with a change in the external calcium ion concentration ($[Ca^{2+}]_o$). When $[Ca^{2+}]_o$ was reduced 10-fold, $[Ca^{2+}]_i$ was reduced in the mdx fibers by 40% but only by 10% in normal mouse fibers. Furthermore, protein degradation, measured by tyrosine release, in the mdx fibers was lowered to normal levels, suggesting a defect in the sarcolemma involving calcium regulation (Turner et al., 1988).

After an increase in $[Ca^{2+}]_o$, adult dystrophic *mdx* muscle fibers have a reduced ability to regulate calcium, especially near the sarcolemma (Turner et al., 1991). Using calcium-activated K^+ channels in the sarcolemma as a calcium sensor, a significant increase in subsarcolemmal free calcium was found in mdx fibers (115nM) compared to control (40nM) (Mallouk et al., 2000). This group also indicated by inside-out patch-clamp experiments that the calcium overload (~3 fold) present in mdx muscle was restricted to the subsarcolemmal compartment and suggested that the influx of calcium may overcome the local calcium sequestering mechanisms in mdx muscle (Mallouk et al., 2000).

Abnormal calcium regulation in DMD has been reported in several studies. In 1991, Turner et al. hypothesized that the increased calcium influx in dystrophic muscles leading to elevated $[Ca^{2+}]_i$ and increased protein degradation was due to calcium leak channels, assuming that the number of leak channels in both dystrophic and normal cells was the same. Dystrophic cells that were exposed to nifedipine, a drug that increases leak-channel activity, had a significantly increased $[Ca^{2+}]_i$, suggesting that it is leak channel activity which can regulate $[Ca^{2+}]_i$ levels (Turner et al., 1991). Using the patch-

clamp technique, the occurrence of channels, which was determined by the number of patches in which a calcium current was detected per number of patches sampled, was 1.6-times greater in mdx (85%) compared to control C57 (54%) mice (Vandebrouck et al., 2002). However, voltage-gated calcium channels (Vandebrouck et al., 2002) and mechanosensitive leak channels, which could contribute to the higher resting [Ca^{2+}] probably are not a significant pathway for calcium entry into dystrophic myotubes because blockers of voltage-gated channels did not return $[Ca^{2+}]_i$ to normal levels and the change in Ca^{2+} permeability in the mechanosensitive channels was not enough to account for the increased $[Ca^{2+}]_i$ (Turner et al., 1991).

Franco and Lansman (1990) demonstrated that mdx myotubes had a larger percentage of stretch-inactivated channels than normal myotubes. They recorded single channel activity using cell-attached patches. They found that these channels were open for extended periods of time and could contribute to the increased levels of $[Ca^{2+}]_{i.}$

In cultured myotubes and adult muscle fibers from mdx mice aged 3-5 weeks, both leak and stretch-regulated calcium channels had a significantly higher open probability (Mallouk et al., 2000). Mdx muscle fibers had small increases in the open state probability for membrane stretch-sensitive channel activity (Carlson, 1998). Evidence for increased calcium entry includes elevated $[Ca^{2+}]_i$ in dystrophic myotubes. This elevated $[Ca^{2+}]_i$ has been associated with a persistent activation of a calcium-specific leak channel that normally opens in response to a depletion of endoplasmic recticulum calcium stores. The normal function of this channel is to allow additional calcium influx to refill the endoplasmic recticulum during a sustained period of contractions. Vandebrouck et al. (2002) demonstrated that the normal function of the voltage-independent calcium channels that are abnormally activated in mdx muscle fibers from mice aged 2-3 months, is sensitive to calcium store depletion by using thapsigargin to inhibit Ca^{2+} ATPase and caffeine to stimulate ryanodine receptors. However, in dystrophic human Duchenne and mdx mouse muscle cells, this open probability is greater than in normal muscle cells (Fong et al., 1990, Turner et al., 1988). In non-contracting mdx myotubes, most of the elevated proteolysis is stimulated by increased calcium influx through abnormally active calcium leak channels. This was demonstrated when mdx mytotubes were exposed to short-term treatment with AN1043, a dihydropyridine analog

that blocks calcium leak channel activity. This treatment reduced fluorogenic substrate hydrolysis in mdx myotubes to nearly normal levels (Alderton et al., 2000a).

Transient receptor potential channel (TRPC) proteins were identified as proteins that may compose part of the store-dependent calcium leak channels by repression of their expression with antisense nucleotides. There are several isoforms of the proteins and 1, 4, and 6, are all located in the plasma membrane. The TRPC 1 channel is known to have a dystrophin homology domain (Vandebrouck et al., 2002).

Membrane delta lesions are characteristic of dystrophic muscles (Carlson, 1998) as early as infancy in human DMD patients (Wakayama et al.,1983). Delta lesions are focal defects in the plasma membrane of non-necrotic fibers (Cozzi et al., 2001). In the channel aggregation model, the delta lesions occur due to the activity-independent calcium-activated cytoskeletal breakdown (e.g., gelsolin) and not activity-induced membrane rupture (Carlson, 1998). However, Alderton et al. (2000b) propose that there are "hot spots" of persistent calcium entry that result from sarcolemmal wounds. During the resealing process of the plasma membrane, vesicles containing calcium leak channels may be incorporated near the wound. When investigators ruptured specific areas of the sarcolemma, they observed calcium leak channel activity in the membrane within a few ìm of the resealed rupture (Alderton et al., 2000b). In dystrophic muscle, which can have an increased frequency of transient wounds, the local calcium influx may lead to local calcium-activated proteolysis altering the activity of calcium-specific leak channels close to the wound site (Alderton et al., 2000a).

Within the calcium hypothesis, abnormal calcium homeostasis may not be a direct effect of dystrophin deficiency. The cause of the abnormal homeostasis may be either a result of the muscle degeneration already in progress or be due to transient sarcolemmal wounds as a result of contraction. McArdle et al. (1994) suggested that the abnormal membrane permeability to calcium is a secondary effect rather than a direct consequence of the absence of dystrophin; the changes in permeability reflect the stage of the degenerative process. ^{45}Ca was used to measure the muscle membrane permeability of the EDL in age 14 and 40 day mdx and control mice. The ^{45}Ca accumulation in the muscle from the extracellular fluid was normal in the age 14 day mdx mice, but in age 40 day mdx mice, the ^{45}Ca levels were higher than in age-matched controls. Membrane

permeability was also assessed through both the release of cytoplasmic proteins and the uptake of the vital stain, procion orange. In the age 14 day mice, there was no evidence of abnormal muscle membrane permeability, supporting the hypothesis that the alteration of membrane permeability was a secondary effect (McArdle et al., 1994, Grange et al., 2002). Additional support for the secondary effect of abnormal permeability was that, in mdx muscle, total calcium content rose acutely in limb muscles at an age that correlated with a period of necrosis and then returned to normal values during an ensuing regenerative phase (Reeve et al., 1997).

It may be possible that altered membrane permeability in the age 14 day mice may not be evident because mice at this age are not actively moving around the cage and, therefore, contraction of muscles, such as the EDL, may be limited. Alderton et al. (2000a) found that abnormal calcium homeostasis correlated with the onset of contraction in mdx myotubes, suggesting that contraction is necessary before changes in $[Ca^{2+}]_i$ occur.

The increased frequency of transient sarcolemmal wounds, sustained during contraction in both dystrophic muscle cells and Duchenne and mdx myotubes, may be exacerbated by abnormal calcium homeostasis (Alderton et al., 2000a). This abnormal calcium homeostasis has been correlated to a history of contraction. When contraction in mdx myotubes was inhibited with the paralytic agent tetrodoxin for 5-7 days, the abnormal rise in resting intracellular free calcium levels was mostly prevented. However, when the myotubes were treated briefly with tetrodoxin, there was no change in the abnormal calcium handling. From these studies, it was concluded that the damage from long-term contractile activity and/or increased calcium leak channel activity are the likely causes of differences in calcium homeostasis, not the calcium influx from individual contractions (Alderton et al., 2000a). Hypo-osmotic shock, used to keep cells in a stretched configuration for an extended period of time, induced a 2-fold increase in calcium influx and elevation of global intracellular calcium in mdx myotubes (Ruegg et al., 2002). Mdx fibers from mice aged 3-6 weeks also show an inability to maintain low calcium concentrations at rest and also have a prolonged elevation of $[Ca^{2+}]_i$ during stimulation (Turner et al., 1988).

Increased levels of $[Ca^{2+}]_i$ have been reported by several investigators in mdx mice as early as age 3 weeks. More specifically, increased calcium has been shown to localize at the sarcolemma, which may be due to calcium leak channels. It is not yet clear if the increase in $[Ca^{2+}]_i$ is a primary or secondary effect of dystrophin-deficiency, however, the increased $[Ca^{2+}]_i$ has been correlated with the onset of contraction in myotubes, suggesting it is a secondary effect (Alderton et al., 2000a).

Calcium and proteolysis

Abnormal calcium homeostasis may lead to increased levels of calcium-dependent proteolysis. Non-dystrophic rat soleus muscles incubated with the calcium ionophore A23187 demonstrated a similar increase both in protein degradation and in tension, suggesting that the proteolysis occurred in the same compartment that contained the contractile proteins (Zeman et al., 1985). Elevated levels of K^+ were used to increase the intracellular free calcium level and produced a 55% increase in proteolysis in soleus and EDL. Both of these treatments suggest that the increased level of intracellular calcium contributed to the increased proteolysis (Zeman et al., 1985). To confirm that increased calcium was initiating the increased proteolysis, the muscles were treated with different solutions and protein degradation was measured. A hypertonic sucrose solution that increased $[Ca^{2+}]_i$ caused a 24% increase in protein degradation. A 64% increase in proteolysis was measured following treatment with TMB-8 (8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride), which antagonizes calcium release from the sarcoplasmic recticulum. Dantrolene and tetracaine both inhibit depolarization induced calcium release from the sarcoplasmic reticulum. Soleus muscles in these solutions showed reduced levels of proteolysis, of 10% and 31%, respectively (Zeman et al., 1985). Because dantrolene and tetracaine block sarcoplasmic reticulum calcium release, these results suggest that the calcium that stimulates proteolysis is in part coming from the sarcoplasmic reticulum.

Calpain

A number of studies support the idea that intracellular calcium concentrations are increased in dystrophic muscle, although the age at which this occurs is not clearly defined. Abnormal calcium homeostasis has been shown to be increased by calcium-dependent proteolysis. Calpains, calcium-dependent proteases are good candidate protease(s) responsible for degradation in dystrophic muscle.

Calpains are ubiquitous calcium-dependent proteases thought to be necessary for changes in cell architecture that occur with myoblast alignment and fusion (Alderton et al., 2000b). In addition, these proteases cleave a wide variety of substrates, such as myofibrillar proteins, the ryanodine receptor (Spencer et al., 1996, Ruegg et al. 2002), p53, protein kinase C, Ca^{2+}ATPase, and talin (Hosfield et al., 1999). One important characteristic of calpain action is that it modifies rather than degrades its substrates. These protein fragments may affect signaling pathways (Perrin, 2002). Johnson (1990) suggested that calpain proteolytic activity is specific, by modifying such events as intracellular metabolism and cellular structure, rather than a general turnover of proteins.

Calpain isoforms

There are three calpain isoforms in muscle: ì-calpain, which is activated at calcium concentrations from 1-70 ìM; m-calpain activated from 10-800 ìM of calcium (Spencer et al. 1995, 1996); and a monomeric muscle-specific isoform p94 activated by nanomolar calcium concentrations (Shevchenko et al., 1998, Molinari et al., 1997). P94 is associated with connectin, a myofibrillar protein (Sorimachi et al., 1995), and is believed to specifically cleave one site on the ryanodine receptor/calcium release channel (Shevchenko et al., 1998). This isoform does not require calcium for activation and is not inhibited by calpastatin (Sorimachi et al., 1997).

Both ì- and m-calpain consist of an identical small regulatory subunit (30kD) and a large catalytic subunit (80kD) that differs between the isoforms because they are the products of separate genes. The large subunit contains the catalytic site and is made up of domains I-IV. The small subunit contains domains V and VI (Hosfield et al., 1999). An N-terminal anchor in domain I (DI) is thought to play a role in calpain activation. Domain II (DII) is divided into 2 subdomains IIa and IIb and contains the catalytic triad (m-, Cys_{105}, His_{262}, Asn_{286},; ì-, Cys_{115}, His_{272}, and Asn_{295}) (Reverter et al.,

2001). In the absence of calcium, the active site is not assembled. DIII is a C2 domain, which is a Ca^{2+}-dependent lipid-binding domain (Hosfield et al., 1999). These C2 domains have been found in both intracellular signaling and membrane trafficking proteins (Reverter et al., 2001). DIII also contains a loop of acidic amino acids (Reverter et al., 2001) that may play a role in calpain activation. DIV and DVI are the calcium-binding domains. They each contain 5 EF-hand domains (Hosfield et al., 1999), which have a helix-loop-helix motif that has several acidic residues that help to coordinate calcium binding (Todd et al., 2003). DV, along with DI and DIII, connects the calcium-binding domains with the catalytic domains (Reverter et al., 2001).

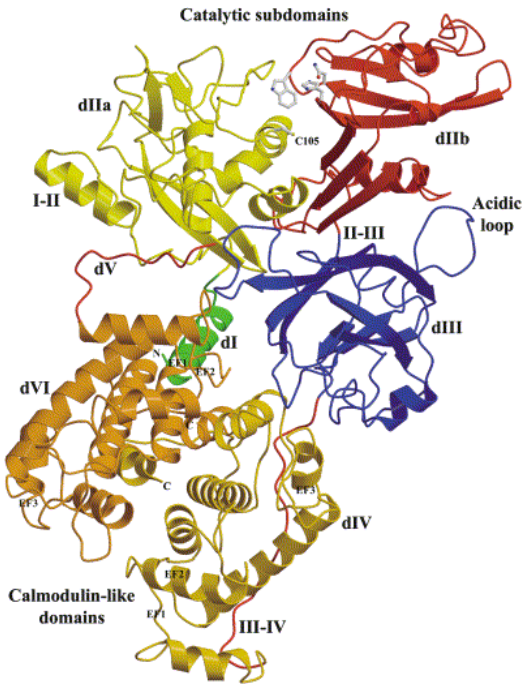

Figure 3. Ribbon structure of m-calpain. Reprinted from *Trends Cardiovasc Med*,Vol. 11, Reverter et al., The structure of calcium-free human m-calpain: implications for calcium activation and function., 222-229, Copyright 2001, with permission from Elsevier.

Calpain activation requires the calcium-dependent assembly of the catalytic triad. Calcium binds to DIV causing a conformational change that is transmitted through a 15 residue linker between DIV and DIII (Hosfield et al., 1999). The acidic loop in DIII may electrostatically interact with an amphipathic helix in DII, so that the conformational change transmitted to DIII would move DIIb as well (Hosfield et al., 1999). Autolysis of

the N-terminal anchor lowers the calcium requirement for activation, but is not required for activity. Release of this anchor may alleviate tension between DIV and DIIa, allowing DIIa freedom to move toward DIIb to form the catalytic domain (Reverter et al., 2001).

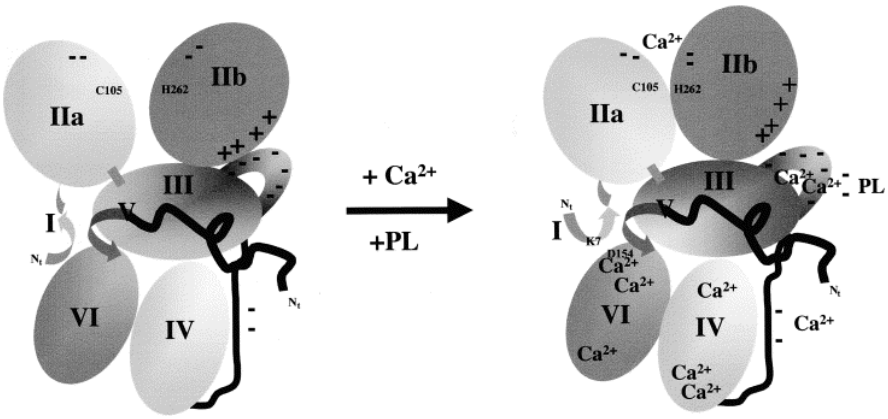

Figure 4. Schematic representation of hypothetical calpain activation process on exposure to calcium and in the presence of phospolipids. . Reprinted from *Trends Cardiovasc Med*,Vol. 11, Reverter et al., The structure of calcium-free human m-calpain: implications for calcium activation and function., 222-229, Copyright 2001, with permission from Elsevier.

Calcium can also bind to DIII and disrupt the electrostatic interactions between DIIb and DIII assisting the movement of DIIb toward DIIa to form the catalytic triad. This may explain the difference in calcium requirement for activation between m- and ì-calpain because m-calpain has 10 acidic residues in the DIII loop compared to the 8 residues spread over a longer loop in ì-calpain (Reverter et al., 2001). When calcium binds to the acidic loop, its coordination spheres may be incomplete, initiating attraction to other ligands including oxygen from nearby phospholipids of membranes (Strobl et al., 2000). Acidic phospholipids have also been shown to reduce the amount of calcium required for activation (Saido et al., 1992). This observation suggests that when calpain is located near phospholipids of the membrane, the calpain calcium requirement for activation may be reduced.

Calpain activity in DMD

Calpains are good candidates for the protease(s) responsible for degradation in dystrophic muscle. Turner et al. (1993) measured protein-degradation rates by the release of tyrosine in mdx and normal muscle that had been treated with leupeptin, a nonspecific

thiol protease inhibitor that has been shown to be effective at preventing calcium-induced protein degradation. They found that mdx muscle from mice aged 3-6 weeks not treated with leupeptin had a higher tyrosine-release rate (~0.26nM Tyr/mg/hr) than control untreated muscle (~0.175nM Tyr/mg/hr), indicating that mdx muscle had an increased protein degradation rate (Turner et al. 1993). Treatment with leupeptin prevented both an increase in mdx voltage-independent leak channel open probability (P_o) and a rise in free calcium levels compared to untreated muscles, 100nM vs.160nM, respectively (Turner et al., 1993). In the same study, with the addition of 1 ìM free calcium to normal and mdx cell free homogenates, a significant increase in protein degradation occurred. This effect was blocked by leupeptin and calpain inhibitors. Higher levels of intracellular free calcium have been shown to increase rates of leupeptin-sensitive proteolysis in intact soleus muscle (Alderton et al., 2000a).

Badalamente et al. (2000) injected leupeptin intramuscularly for 30 days starting at age 14 days and measured calpain activity in muscle homogenates using a ^{14}C-casein substrate. Increased myofiber diameters were consistent with decreased calpain activities. It was also suggested that the treated muscles went through fewer degeneration/regeneration cycles because a greater number of peripheral nuclei and fewer centralized nuclei were observed following the leupeptin treatment (Badalamente et al., 2000).

Calpain activation in muscle from mice aged 4 weeks was determined by immunoblot analysis of amino-terminal propeptides of autoproteolytic active forms of calpain. When the activity was averaged over the whole muscle, it increased 2-3 fold in mdx over control, so it was proposed that, in degenerating fibers, calpain activation could be as high as 20-30 fold (Spencer et al., 1995).

Calpain activity was measured by casein cleavage in homogenates of soleus and gastrocnemius from both mdx and C57 mice aged 5 months to 1 year (Spencer et al., 1992). In mdx mice, calpain activity was scarcely measurable and was continually lower than the activity values of control (Spencer et al., 1992).

Calcium-dependent proteolysis is increased in dystrophic compared to control muscle cells (Alderton et al., 2000b). A fluorogenic calpain substrate was used to monitor the calcium-dependent proteolysis in both mdx and normal myotubes. When the

substrate was hydrolyzed, fluorescence increased. Over time, mdx myotubes showed increased hydrolysis in parallel with increased intracellular calcium. Also, higher rates of fluorogenic substrate hydrolysis were correlated with the development of contraction in mdx but not in control myotubes (Alderton et al., 2000a). This result supported the hypothesis that calcium entry through transient sarcolemmal wounds activated calpains. To further confirm the majority of protein degradation was due to calpains and not other proteolytic processes, control and mdx myotubes were pretreated with 280nM calpeptin, an endogenous calpain inhibitor of ì- and m-calpain, and 20mM ammonium chloride. Ammonium chloride specifically inhibits lysosomal protein degradation in skeletal muscle. After pretreatment, both control and mdx myotubes showed the same minimal level of substrate hydrolysis, supporting the role of calpain as a primary contributor to proteolysis in dystrophic mdx muscle (Alderton et al., 2000a).

Calpain content in DMD

Immunoblots were used to determine the content of calpains in whole muscle extracts of mdx muscle (Spencer et al., 1995). At age 2 weeks, there was no difference in calpain content between mdx and control C57 muscles (Spencer et al., 1995). However at age 4 weeks, m-calpain levels were increased (Spencer et al., 1995, Alderton et al., 2000a). By age 14 weeks, both ì- and m-calpain levels were increased. Because there was no increase in mRNA levels, it appears that an increased half-life of the proteins and not an increased transcription rate was responsible for the increased calpain concentration in the mdx tissues (Spencer et al., 1995). In soleus and gastrocnemius from mice aged 5 months to 1 year, mdx mice had increased calpain content compared to control as measured by immunoblot analysis (Spencer et al., 1992).

Calpastatin

Calpastatin is the specific endogenous inhibitor of calpain. Calpastatin is a competitive inhibitor of calpain (Maki et al., 1988) and has 4 repeated inhibitory domains (Todd et al., 2003). Each of these domains has 3 conserved regions, A, B, and C. These conserved regions each bind to a separate domain of calpain. Domain A of calpastatin binds to DIV of calpain, while domain C binds to DVI. Domain A and C allow calpastatin to bind calpain tightly, wheras domain B causes inhibition by binding to the active site cysteine in DII of calpain (Todd et al., 2003). Calpastatin inhibition of calpain

is calcium dependent; inhibition occurs at *in vitro* [Ca^{2+}] from 42-80nM for ì-calpain and at 150-1000nM for m-calpain (Kapprell et al., 1989). Calpastatin is activated by phosphorylation of a serine residue near the N-terminus by protein kinase C (Averna et al., 1999). Calpastatin is a calpain substrate, but when cleaved, the fragments still retain their inhibitory activity (Melloni et al., 1989).

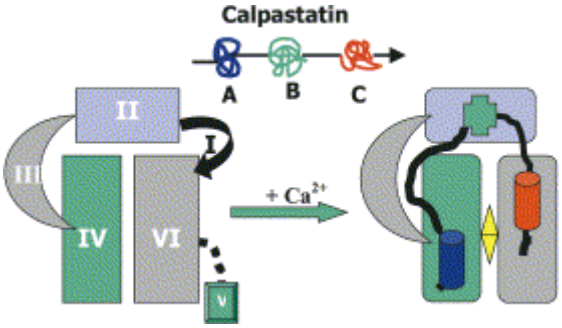

Figure 5: Cartoon representation of the model for the proposed calpastatin inhibition of calpain. Reprinted from *J Mol Bio*, Vol. 328Todd et al., A Structural Model for the Inhibition of Calpain by Calpastatin: Crystal Structures of the Native Domain VI of Calpain and its Complexes with Calpastatin Peptide and a Small Molecule Inhibitor, 31-146, Copyright 2003, with permission from Elsevier

Calpain:calpastatin ratio

Sultan et al. (2000) stated that increased calpain activity, without changes in total content could result from: (1) a decreased interaction with the calpastatin; (2) translocation that favors increased activity; and/or (3) increased modulation by specific activators. The calpain:calpastatin ratio may represent an important indicator of net calpain breakdown potential (Enns et al., 2002, Spencer et al., 2002). The calpain:calpastatin ratio has been assessed as a ratio of purified calpain activity to purified calpastatin activity (Enns et al., 2002), but the actual content of the proteins has not been assessed as a ratio. In mdx mice that overexpressed calpastatin, reductions in muscle necrosis were evident as early as age 4 weeks, suggesting that suppressing calpain activity may reduce tissue degradation associated with dystrophin deficiency. Mice overexpressing calpastatin were also used in a hindlimb suspension model of muscle

atrophy. The transgenic mice showed 33% less atrophy than non-transgenic controls (Tidball et al., 2002).

Phospholipids

Specific activators of calpain also likely modulate calpain activity, particularly because the calcium concentrations needed to activate calpains 2-20ìM and 200-700ìM for ì- and m-, respectively, are higher than normal physiological concentrations of calcium (0.2-0.8ìM). Calcium concentration requirements for calpain activation must be decreased in some way for calpains to be active (Argiles et al., 1998). Coolican and Hathaway (1984) found that phospholipids, particularly phosphatidylinositol, were able to activate calpains at a lower [Ca^{2+}]. Phosphatidylinositol stimulates the autolysis of calpain isoforms, which makes the enzyme more sensitive to calcium. Another reason calpain activity must be induced at a lower calcium concentration is that calpastatin will bind and inhibit calpain at the same or lower concentrations of calcium (Kaprell et al., 1989).

Calpain localization

Translocation of calpain to the membrane upon calcium activation may play a regulatory role in calpain inhibition or activation. In the earliest morphologically detectable stages of mdx dystrophy, changes in calpain distribution were consistent with enzyme activation (Spencer et al., 1996). In control C57 muscle, calpain was diffuse throughout the cytoplasm and slightly enhanced at the cell membrane at all ages. In mdx fibers, calpain distribution was different between areas of necrosis, regeneration, or absence of morphological signs of the disease as detected by immunostaining for calpain, acid phosphatase, and developmental myosin heavy chain. For example, in prenecrotic (age 2 weeks) mdx muscle, calpains were both membrane-associated and found in the cytosol, but in the early degenerative fibers (age 4 weeks), calpain was diffuse and not membrane-associated. Therefore, during mdx muscular dystrophy, changes in calpain distribution relate to the state of the disease and not the age of the animal (Spencer et al., 1996), suggesting that increased calpain activity may be a secondary effect of the disease process.

Mallouk et al. (2000) proposed that calpain activation required association with the cell membrane. Alderton et al. (2000a) reported that abnormal calpain translocation during muscle fiber necrosis in mdx mice was associated with increased calpain activity. In platelet activation, calpain is autolyzed in the cytosol, translocated near the plasma membrane, and then it proteolyzes its substrates; therefore, in skeletal muscle, translocation may be an early event associated with activation (Spencer et al., 1996).

Most intracellular calpain is cytosolic, while 7-30% is associated with membrane structures (Johnson, 1990). Fluorescent localization of m- and ì-calpain and calpastatin in Spraque-Dawley rat soleus showed all 3 proteins to be localized to the myofibril with most intensity at the Z-disc (Kumamoto et al., 1992). m-calpain was also more concentrated in a band around the plasma membrane as well as being found throughout the cell. The same group used immunogold labeling to test the localization of the calpains and calpastatin. These researchers detected more calpain in the nuclei than in the mitochondria, while there was more calpastatin in the mitochondria than in the nuclei (Kumamoto et al., 1992).

In mdx and C57 mice aged 5 months to 1 year, immunolocalization of calpain was performed on longitudinal sections. In the control mice, calpain was found in sharp periodic striations that corresponded with the Z-disc. However, in mdx mice, the calpain was more diffuse, only appearing slightly more concentrated at the Z-disc than throughout the fiber (Spencer et al., 1992).

The highest levels of uncleaved ì-calpain were found at the plasma membrane in mdx regenerated fibers from mice aged 14 weeks. There was no significant change in the concentration of ì-calpain at peak necrosis, but the ratio of autoproteolyzed active ì-calpain increased considerably (Spencer et al., 1995).

Calpastatin localization

In rabbit skeletal muscle, calpastatin was found localized at the sarcolemma (DeSantis et al., 1992). Immunogold labeling of rabbit vastus lateralis revealed calpastatin not just at the sarcolemma, but also in a band 1ìm into the subsarcolemmal sarcoplasm (Nori et al., 1993). Calpastatin labeling was also detected at the myofibrils with almost twice the amount at the Z-disc compared to the I and A bands. A similar

amount of labeling was found in the mitochondria. Labeling was also detected in nuclear structures, with most of it chromatin-associated (Nori et al., 1993).

Collectively, the results of these studies suggest calpains may be responsible for the increased proteolysis in dystrophic muscle. They are calcium-activated and $[Ca^{2+}]_i$ levels are increased in dystrophic muscle. In studies in which calpain activity was blocked with leupeptin or its endogenous inhibitor calpastatin, protein degradation was decreased. This evidence suggests that calpain is important in the pathogenesis of DMD. However, the age in which calpain activity is first increased in not clear and increased calpain activity has not yet been established as a primary or secondary effect of DMD.

Summary

The role of dystrophin in the pathogenesis of DMD is not presently known. Dystrophin and the DGC may function mechanically to stabilize the sarcolemma and/or play a signaling role. In maturing muscles, the mechanical hypothesis may not accurately reflect the onset mechanism of the disease. The role of calcium and its abnormal homeostasis in dystrophic muscle has been widely studied. The increase in $[Ca^{2+}]_i$ has been linked with an increase in proteolysis. Calcium-activated calpains may be responsible in part for this increased level of proteolysis. The increased proteolysis may cause additional influx of extracellular calcium that results in a positive feedback loop that further exacerbates proteolysis leading to muscle necrosis and loss of function in the muscle.

Chapter 3
Methods

Methods

Mice

Control (C57BL/6) and dystrophin-deficient (mdx) mice, a model of DMD, were used. The overt signs of dystrophy begin around age 3 weeks in mdx mice. The mechanism of onset of DMD is unknown. Calpain activity and both calpain and calpastatin content were evaluated in control and mdx muscle to determine if calpain proteolysis plays a role in the onset of DMD.

The Animal Care and Use Committee of Virginia Tech approved all procedures used in this study. Mice were housed at the Virginia Tech Laboratory Animal Resource facility. Animals were allowed to eat (Purina Rodent Laboratory Chow) and drink *ad libitum* and were exposed to a 12 hour light/dark cycle. Tissue was obtained from mice at 3 ages, 7, 14, and 21 days. Six mice of each genotype at 14 and 21 days were assessed. At 7 days, 3 animals were pooled as 1 sample, so 18 animals of each genotype were assessed.

Calpain Activity Assay

A global calpain activity assay in the presence of endogenous calpastatin (Sultan et al., 2000) was used to detect changes in calpain activity in homogenates of maturing dystrophic quadriceps and diaphragm muscles. Cytosolic and membrane fractions were assessed separately to determine if calpain association with the membrane affected its activity.

Muscle preparation

Control C57BL/6 and mdx mice were anesthetized with an intraperitoneal injection of 2mg xylazine and 20mg ketamine per 100g body mass. Quadriceps and diaphragm muscles were quickly excised, powdered under liquid nitrogen, and stored at -80ºC for later use. Powdered muscles were further homogenized on ice in a 10-fold volume of buffer (20mM Tris pH 7.4, 5mM EDTA, 5mM EGTA, 1mM DTT, 10µg/mL PMSF, and 10µg/mL pepstatin A), using a motor driven glass pestle for 3 rounds of 60s at 3 min intervals in 1ml ground glass homogenizer tubes. The speed of the motor was set by rheostat at 40V on a 140V scale.

Each homogenate was centrifuged at 1000g for 10 min at 4ºC to remove debris. The supernatant was separated into soluble (S-100 supernatant, e.g., cytosolic) and total

particulate (P-100 pellet, e.g., mitochondrial, microsomal, and myofibrillar) fractions by centrifugation at 100,000g for 60 min (TL-100 Beckman ultracentrifuge). The pellet was removed to a clean tube and the pellet was resuspended in homogenization buffer.

Protein concentration

The soluble and particulate protein concentrations were determined by the Bradford method. One ml Bradford reagent (Sigma) was pipetted into 2ml cuvettes and allowed to come to room temperature. Ten µL of either standard or sample was added to each cuvette. The cuvette was inverted to mix and then incubated for ~10 min at room temperature. Absorbance at 595 nm was determined on a Hewlett-Packard 8453 UV-vis spectrophotometer. A standard curve was constructed using bovine serum albumin as the standard (0.05mg/mL-5.00mg/mL).

Calpain Assay

Thirty µL of muscle extract (supernatant or pellet) was incubated for 10 min at 37ºC in either control or assay buffer. The control buffer contained: 20mM Tris (pH 7.4), 1mM DTT, 10µg/mL PMSF, 10µg/mL pepstatin A, 10mM EDTA, and 10mM EGTA. The assay buffer contained: control buffer plus 5mM $CaCl_2$ and excluding EDTA and EGTA. After incubation in either control or assay buffer with stirring, 5µL of the fluorogenic substrate SLY-AMC (Suc-Leu-Tyr-7-Amido-4-methylcoumarin) was added. The fluorescence was monitored for 15 min at 37ºC at an excitation wavelength of 380nm and an emission wavelength of 460nm (Perkin Elmer LS50B fluorometer). Calpain activity was determined as the change in absorbance units (AU; represents cleavage of SLY-AMC) over a minimum of 50s. Activity was expressed as AU (AU per milligram per minute of protein).

Succinate-Leucine-Tyrosine-7-amido-4-methylcoumarin (SLY-AMC) as indicator of calpain activity

In general, calpain selectively cleaves the carboxyl side of a tyrosine (Tyr), methionine (Met), or arginine (Arg) residue in the P_1 position (position where cleavage takes place) if the P_2 position (on the amino side of the P_1 position) is occupied by a hydrophobic amino acid. Substrates that are particularly susceptible to cleavage by calpain contain Leucine (Leu)-Tyr, Leu-Met, or Leu-Lysine (Lys) in the P_2-P_1 positions

respectively (Sasaki et al., 1984). This makes Suc-Leu-Tyr-AMC a good substrate for determining the activity of calpains. When the AMC is cleaved by calpain, electrons in the conjugated diene of AMC fluoresce at an emission wavelength of 440-460nm when excited at a wavelength of 360-380nm.

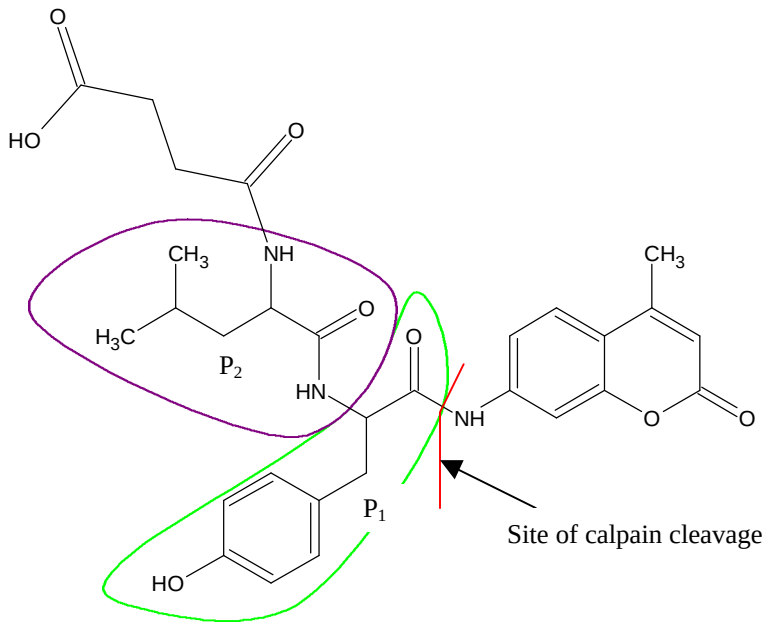

Figure 6. SLY-AMC fluorogenic calpain substrate.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Analysis

The purpose of SDS-PAGE is to separate sample proteins by relative molecular weight and to determine the relative abundance of proteins in a sample. In Western analysis, an antibody is used to detect a specific protein of interest in the presence of other proteins on a membrane, such as nitrocellulose. Briefly, the supernatant and pellet fractions of each sample were run on a polyacrylamide gel, then transferred to nitrocellulose to determine the content of ì- and m-calpains, and the endogenous calpain inhibitor, calpastatin, by Western analysis. To quantify the content of these proteins, a reference sample, a quadriceps supernatant from an age 36 day control mouse, was run on each gel and transferred. Sample protein content relative to the protein content in the reference sample was determined by densitometry. Both calpain and calpastatin had the

potential for the presence of 2 bands following transfer. In the case of calpain it was fragments of ~80 and/or 58 kd. In the case of calpastatin, the two bands represented isoforms of ~40 and /or ~36 kd. For both calpain and calpastatin, both bands were not present in all samples. In those Western blots where two bands were evident for either calpain or calpastatin, the densities of the two bands for each respective protein were summed and expressed relative to the summed density of the appropriate bands from the reference sample. Similarly, the density of a single band for a sample was expressed relative to the sum of the reference densities.

SDS-PAGE

The Laemmli (1970) or discontinuous gel system was used to separate sample proteins. Precast gels with a 4% stacking gel, 7.5% separating gel, and 50µL wells were purchased from Bio-Rad.

Samples were mixed with 2X sample buffer (240mM â-mercaptoethanol, 1M Tris (pH 6.8), 20% glycerol, 0.1% bromophenol blue, and 10% SDS). Samples were boiled for two minutes to denature the proteins. Remaining samples were frozen at -80ºC until further use.

The mini-PROTEAN® III cell apparatus was assembled. The gel sandwich was properly positioned and the inner and outer chambers of the apparatus were filled with 1X running/electrode buffer (3g/L Tris, 14.4g/L glycine, and 1.0g/L SDS) covering both the bottom and top of the gel so the current could run only through the gel. Ten µL of broad range molecular markers (7kD-206kD, Bio-Rad) were loaded into well 1; loaded into wells 2 and 3 were 0.6ìg of m- and ì-calpain, respectively, as positive controls; loaded into well 4 was a reference control sample used for protein normalization during densitometry; and the remaining wells were loaded with different samples (30ìg of total protein). The band density of the reference control sample was used to normalize the content per well of m- and ì-calpain and calpastatin in each sample during Western analysis. Gels were run at a constant 100V for 1 hour. To prevent overheating, the apparatus was placed in ice.

Some of the samples were too dilute to load 30ìg of total protein in wells with a volume of 50µL. These samples were evaporated in the Labconco Centrivap until dry.

Samples were resuspended in double deionized water and protein concentrations were reevaluated. If the sample was still too dilute, the evaporation was done a second time and then the samples were resuspended in 1X sample buffer (120mM â-mercaptoethanol, 0.5M Tris (pH 6.8), 10% glycerol, 0.05% bromophenol blue, and 5% SDS).

Western Analysis

Western analysis was used to detect the presence of a specific protein in a sample by use of specific antibodies. Once the sample proteins were separated on an SDS-PAGE gel, they were transferred to a nitrocellulose membrane.

Before transfer in a Bio-Rad Trans-blot® cell, a piece of nitrocellulose membrane, two sheets of filter paper (cut to the size of the gel), and the gel were soaked in transfer/blotting buffer (3g/L Tris, 14g/LmM glycine, and 300ml/L methanol) for 15 min before transfer. The transfer sandwich was assembled as follows. Starting from the anode side: sponge, filter paper, nitrocellulose membrane, gel, filter paper, sponge, and ending on the cathode side. The transfer sandwich was positioned in the transfer chamber so that the negatively charged proteins would migrate from the gel toward the anode onto the nitrocellulose membrane. An ice pack was inserted into the transfer cell and the transfer/blotting buffer was added. The transfer was run at a constant 15mA for approximately 2 hours.

Following transfer, the nitrocellulose was washed in Tris buffered saline (TBS) (pH 7.5) for 15 min at room temperature. The gel was stained with Coomasie Blue overnight and then destained to check for completeness of transfer. The membrane was incubated in blocking buffer (3-5% non-fat dry milk in TBST (TBS + 0.05%Tween 20)) for an hour with shaking. The membrane was washed 3 times, each for 15 min in TBST. The membrane was incubated with primary antibody (Table 1) in blocking buffer overnight at 4ºC, and then was washed in TBST 3 times, each for 15 min. The membrane was incubated with secondary antibody (Table 2) in blocking buffer for 1.5 hours. After incubation, the membrane was washed in TBST 3 times, each for 15 min.

Table 1. Antibodies and Dilutions used for Primary Incubations in Western Analysis

Protein	1º Antibody	Host	Manufacturer	Dilution
Calpain	NCL-CALP-11B3	Mouse	Novo Castra	1:50
Calpastatin	C 1683	Rabbit	Sigma	1:2500

Table 2. Antibodies and Dilutions used for Secondary Incubations in Western Analysis

2º Antibody	Host	Manufacturer	Dilution
Peroxidase-conjugated AffiniPure Anti-Mouse IgG (H+L)	Goat	Jackson Immuno Research Laboratories, Inc.	1:2500
Peroxidase-conjugated AffiniPure Anti-Rabbit IgG (H+L)	Goat	Jackson Immuno Research Laboratories, Inc.	1:2500

The presence or absence of the protein of interest was visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Pierce). Horseradish peroxidase (HRP) was conjugated to the secondary antibody to catalyze the oxidation of luminol by hydrogen peroxide. The nitrocellulose membrane was incubated in 2mL of peroxide solution and 2mL of the luminol solution for 2 min. The membrane was taken out of solution, blotted, placed on a glass plate, and covered with plastic wrap (e.g., Saran wrap). Ten min following incubation, the membrane was exposed to Kodak X-Omat film placed over the membrane for varying lengths of time (30s-10min) in a dark room. The film was then processed in a Konica SRX-101A Medical Film Processor.

Protein band density was determined with an Epson Expression 1680 Pro Scanner and GeneTools Analysis Software Version 3.0200 from SynGene. The densities were measured over the full-length of each band. To normalize the content of the calpain and calpastatin proteins in each sample, a reference/control sample was run on each gel and transferred. The control sample was an age 36 day control supernatant that expressed both total calpain and calpastatin. In the reference sample, total calpain or calpastatin content was determined from the sum of the individual band densities representing the calpain fragments or the calpastatin isoforms. The mean density for total calpain in the reference sample when determined at an exposure time of 20s on each blot was 177,809 ± 24,601 arbitrary density units (mean ± SEM; n=12). The mean density for calpastatin when determined at an exposure time between 1-3min was 48,615 ± 10,101 arbitrary density units (mean ± SEM; n=10). Relative densities for the calpain and calpastatin

bands for each sample were normalized to the densities of the respective reference bands. It should be noted that optimal exposure time was dependent on the appearance of the calpain and calpastatin bands from the samples. Thus, the exposure time used for determination of calpain on each blot was not always 20s (range: 20s-2min), and for calpastatin varied between 20s-10min.

Relative calpain activity

Relative calpain activity was determined to resolve whether increased calpain activity was due to either increased total calpain content or more activity per unit of calpain. Relative calpain activity was determined by dividing each sample's total calpain activity by its total calpain content as established by Western Analysis.

Total Calpain:Calpastatin Ratio

The total calpain:calpastatin ratio was determined to resolve whether total calpain activity correlated with the calpain:calpastatin ratio as suggested in the literature. This ratio represented the relative total calpain to calpastatin content. The average total calpain content as determined by Western Analysis for each age, genotype, and either supernatant or pellet fraction was divided by the average calpastatin content as determined by Western Analysis for the same data set. Average values of calpain and calpastatin content were used because some samples, based on the absence of antibody immunoreactivity, had zero calpain and zero calpastatin content.

Statistics

Descriptive statistics, mean and standard error of the mean (SEM), were determined for morphological data including body and muscle mass, calpain activity, and total calpain and calpastatin content.

Data were analyzed by a two-way ANOVA with two between factors, age and genotype. Significant main effects and/or interactions were further assessed by one-way ANOVA, and the differences between means determined by Student-Neuman Keuls post hoc analysis ($p<0.05$).

Correlation plots of calpain activity dependent on total calpain:calpastatin ratio were used to assess the possible relationship between activity and content. Descriptive plots of calpain activity and total calpain:calpastatin ratio across age were performed to visualize possible trends in total calpain:calpastatin ratio and calpain activity.

Chapter 4
Results

Results

Control (C57BL/6) and mdx mice quadriceps and diaphragm muscles were analyzed for calpain activity and calpain and calpastatin content to determine a possible role for increased calpain activity in the onset of DMD.

Mice

Body and quadriceps and diaphragm muscle masses from ages 7, 14, and 21 day control (C57BL/6) and mdx mice are shown in Table 3. Quadriceps and diaphragm muscle came from the same animal for all samples except control aged 7 days and mdx aged 21 days.

Table 3. Average body mass and muscle mass from mice used in analyses.

	Quadriceps Samples		Diaphragm Samples	
Age/Genotype	Body Mass (g)	Muscle Mass (g)	Body Mass (g)	Muscle Mass (g)
7d control	5.3	0.0159	5.1	0.0184
7d mdx	5.0	0.0136	5.0	0.0194
14d control	7.5	0.0558	7.5	0.0246
14d mdx	7.9	0.0471	7.9	0.0335
21d control	10.4	0.1058	10.4	0.0434
21d mdx	12.6	0.0831	12.6	0.0444

Calpain Activity Assay

Calpain activity was determined in homogenates from quadriceps and diaphragm muscles from age 7, 14, and 21 days control (C57BL/6) and dystrophic (mdx) mice. Briefly, 30µL of muscle extract (supernatant or pellet) was incubated for 10 min at 37ºC in either control ($-Ca^{2+}$) or assay ($+Ca^{2+}$) buffer. After incubation in either control or assay buffer with stirring, 5µL of the fluorogenic substrate SLY-AMC (Suc-Leu-Tyr-7-amido-4-methylcoumarin) was added. Fluorescence was monitored for 15 min at 37ºC. Calpain activity was determined as the change in AU (represents cleavage of SLY-AMC) for a minimum of 50s on that portion of the curve where slope was maximal. Figure 7 represents sample traces from the assay.

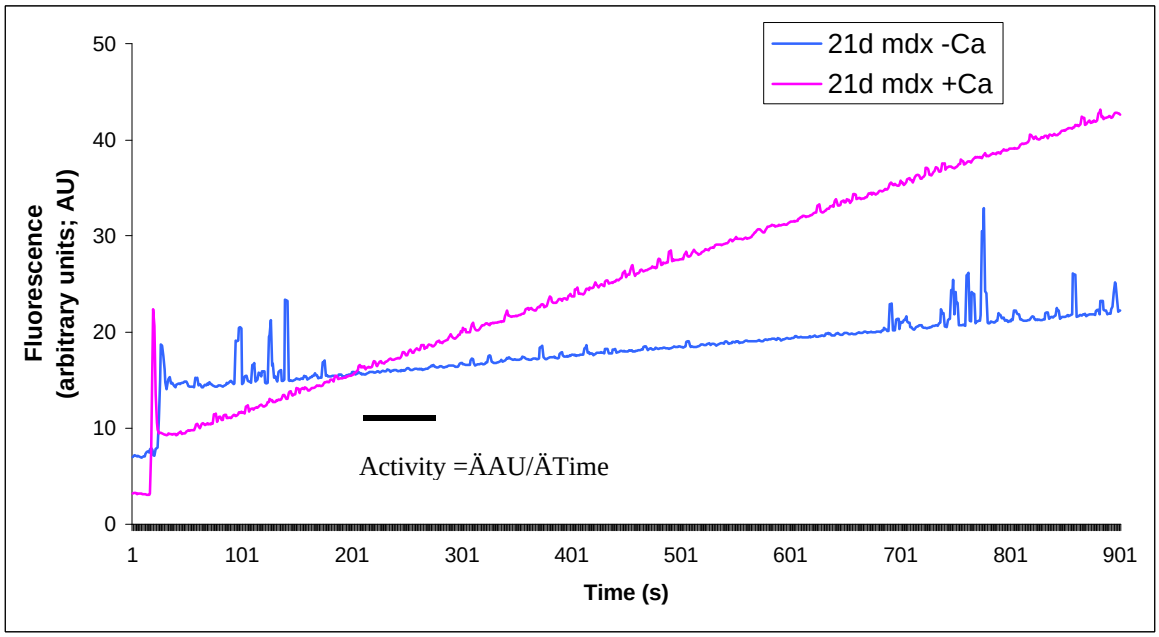

Figure 7. Raw tracings from calpain activity assay. Calpain activity determined as change in AU (represents cleavage of SLY-AMC) over minimum of 50s.

Quadriceps

General trends across age and within genotype

In general, supernatant calpain activity increased and pellet activity decreased between 7 and 21 days in both the control and mdx samples (Figure 8). Calpain activity in the pellet fraction was significantly greater at age 7 days compared to age 14 and 21 days for both control and mdx samples respectively ($p<0.05$).

Within age and genotype

At age 7 days, control pellet calpain activity in the presence (all values arre means; 23.5AU) and absence (29.6AU) of calcium was significantly greater than supernatant activity in the presence (9.6AU) or absence (6.4AU; $p<0.05$) of calcium. This was also true in the mdx at age 7 days as pellet calpain activities in the presence (44.9AU) and absence (23.4AU) were significantly greater than both supernatant activity in the presence (9.3AU) and absence (5.4AU; $p<0.05$) of calcium.

At age 14 days, control supernatant calpain activity in the absence (12.6 AU) of calcium was significantly less than supernatant activity in the presence (16.6AU) of

calcium or the pellet activity in the presence (19.2AU) or absence (18.1AU; $p<0.05$) of calcium. The mdx pellet calpain activity in the absence (27.9AU) but not the presence (17.4AU) of calcium at age 14 days was significantly greater than mdx supernatant activity in the presence (10.7AU) and absence (11.2AU; $p<0.05$) of calcium.

The control supernatant calpain activity at age 21 days in the presence (20.5AU) of calcium was significantly greater than supernatant activity in the absence (12.3AU) of calcium or the pellet activity in the presence (13.6AU) or absence (12.4AU; $p<0.05$) of calcium. In mdx muscles at age 21 days, there were no significant differences between supernatant and pellet calpain activities in either the presence or absence of calcium.

Between genotypes within age

Significant differences between control and mdx calpain activities were evident at age 7 and 21 days. At age 7 days, the calpain activity in the mdx compared to the control pellet in the presence of calcium was 47.7% greater (44.9 vs. 23.5AU, respectively, $p<0.05$; Figure 8A). At age 21 days, the calpain activity in the control compared to the mdx supernatant in the presence of calcium was significantly greater (20.5 vs.17.3AU, respectively, $p<0.05$; Figure 8C).

Diaphragm

General trends across age and within genotype

In general, supernatant calpain activity tended to increase with maturation. Calpain activity in the control supernatant in the presence of calcium at age 21 days (31.8AU) was significantly greater than at 7 (7.33AU) and 14 (18.7AU) days ($p<0.05$; Figure 9). Calpain activity at age 21 days in the mdx supernatant in both the presence (30.3AU) and absence (25.6AU) of calcium was significantly greater than at 7 days (8.5 and 8.9AU) and 14 days (15.3 and 14.8AU; $p<0.05$). There were no significant differences between the calpain pellet activities in the presence or absence of calcium in control and mdx samples.

Within age and genotype

At age 7 days, control pellet calpain activity in the presence (20.5AU) and absence (38.2AU) of calcium was significantly greater than the supernatant activity in the presence (7.3AU) or absence (10.3AU; $p<0.05$) of calcium. This was also true in the mdx at age 7 days as the pellet calpain activity in the presence (19.6AU) and absence

(35.9AU) of calcium was significantly greater than the supernatant activity in the presence (8.5AU) or absence (8.9AU; $p<0.05$) of calcium.

At age 14 days, control pellet calpain activity in the absence (31.3AU) of calcium was significantly greater than the pellet activity in the presence of calcium (18.2AU) and the supernatant activity in the presence (18.7AU) or absence (16.8AU; $p<0.05$) of calcium. The mdx pellet activity in the absence of calcium at age 14 days was significantly greater than the pellet activity in both the presence of calcium (20.2AU) and the supernatant activity in either the presence (15.3AU) or absence (14.8AU; $p<0.05$) of calcium. At age 21 days, there were no significant differences between supernatant and pellet activities in the presence or absence of calcium in the control or mdx samples.

In an attempt to identify the possible cause of increased calcium-independent proteolytic activity in the diaphragm, several protease inhibitors were used to decrease the activity (Appendix D). These were inhibitors of serine, aparatic, thiol, and aminopeptidases. Although several inhibitors were used, there was no resulting decrease of calpain activity.

Between genotypes within age

There were no significant differences between control and mdx calpain activities in the supernatant or pellet in the presence or absence of calcium.

Quadriceps vs. Diaphragm

The most apparent difference between quadriceps and diaphragm is in the pellet fraction. In the quadriceps, activity in the pellet is greatest at age 7 days in the presence of calcium. However, in the diaphragm, the activity in the pellet is greatest in the absence of calcium and does not change with maturation. The supernatant activities in both quadriceps and diaphragm increase with age.

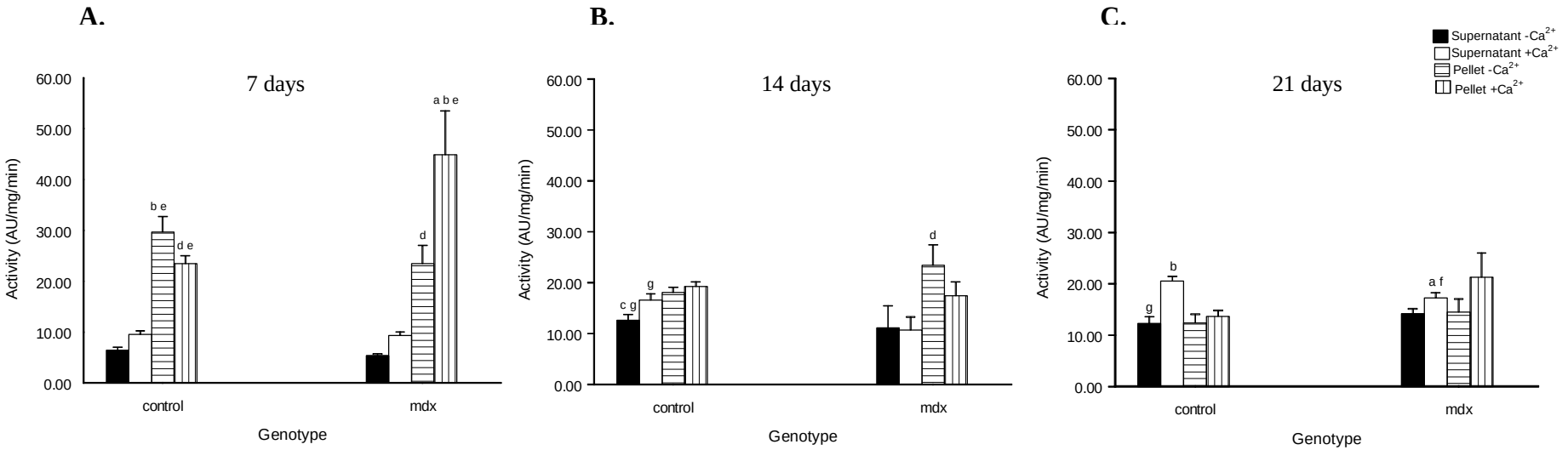

Figure 8. Total calpain activity in quadriceps (normalized to total protein content) [a]Significant difference between control and mdx. [b] Significantly greater than other conditions at the same age and genotype. [c] Significantly less than other conditions at the same age and genotype. [d]Significantly greater than both supernatant activities. [e] Calpain activity was significantly greater than at 14 and 21d. [f]Significantly greater than at 7 and 14d. [g] Significantly greater than 7d. (n=6; $p<0.05$) mean±SEM

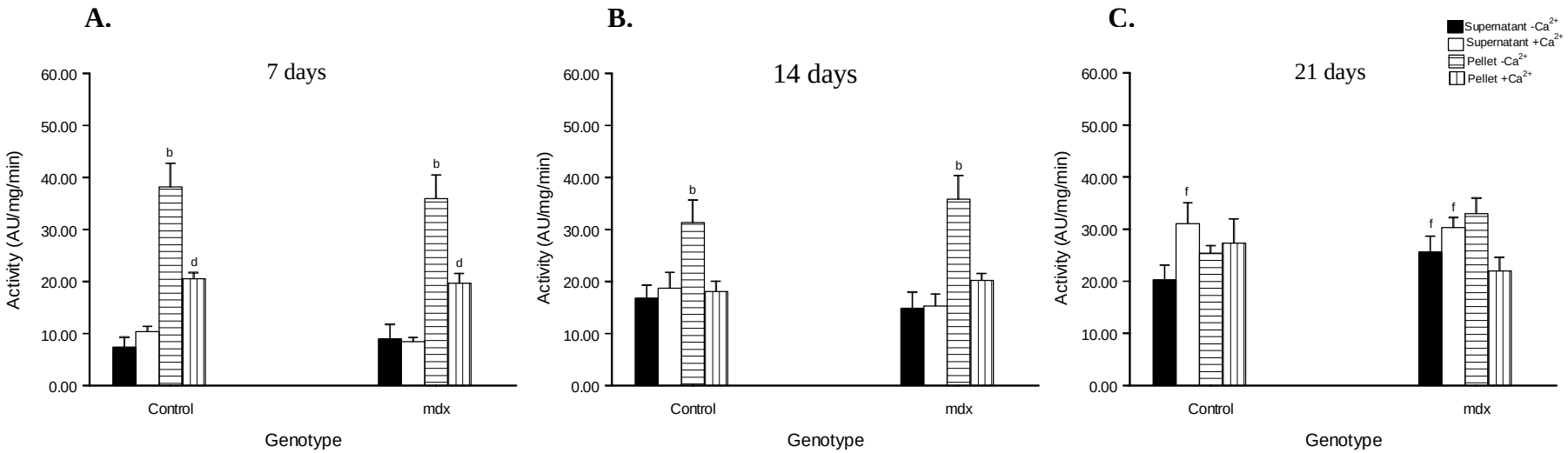

Figure 9. Total calpain activity in diaphragm (normalized to total protein content) [a]Significant difference between control and mdx. [b] Significantly greater than other conditions at the same age and genotype. [c] Significantly less than other conditions at the same age and genotype. [d]Significantly greater than both supernatant activities. [e] Calpain activity was significantly greater than at 14 and 21d. [f] Significantly greater than at 7 and 14d. [g] Significantly greater than 7d. (n=6; $p<0.05$) mean±SEM

Western Analysis

Western Analysis was performed to determine both the total calpain and calpastatin content in the quadriceps and diaphragm samples and if the relative amounts of these proteins influenced calpain activity. Total calpain and calpastatin content were determined in the same samples that were evaluated for calpain activity. Total calpain and calpastatin content were normalized to a 36d control supernatant sample loaded onto each gel. Total calpain and calpastatin contents of zero based on antibody immunoreactivity are responsible for large error in the content. Some blots with zero detectable total calpain and calpastatin often had bands at longer exposure time, but these were difficult to analyze with densitometry due to the intense reactivity of other bands distorting the image.

Quadriceps

Total calpain

Figure 10 is a representative blot of total calpain and calpastatin from quadriceps samples. There were no significant differences in total calpain content between ages within the same genotype (Figure 11). At age 21 days, the control supernatant had significantly more total calpain (2.67) than the pellet (0.37; $p<0.05$). There were no significant differences in total calpain content between control and mdx samples at any age in either the supernatant or pellet.

Calpastatin

At age 7 days, the control pellet (2.83) had significantly greater calpastatin content compared to the control pellet at age 14 (0.31) and 21 (0.08; $p<0.05$) days. At age 21 days, the control supernatant (3.91) had significantly greater calpastatin content compared to the pellet (0.08; $p<0.05$). There were no significant differences in calpastatin content between control and mdx samples at any age in either the supernatant or pellet.

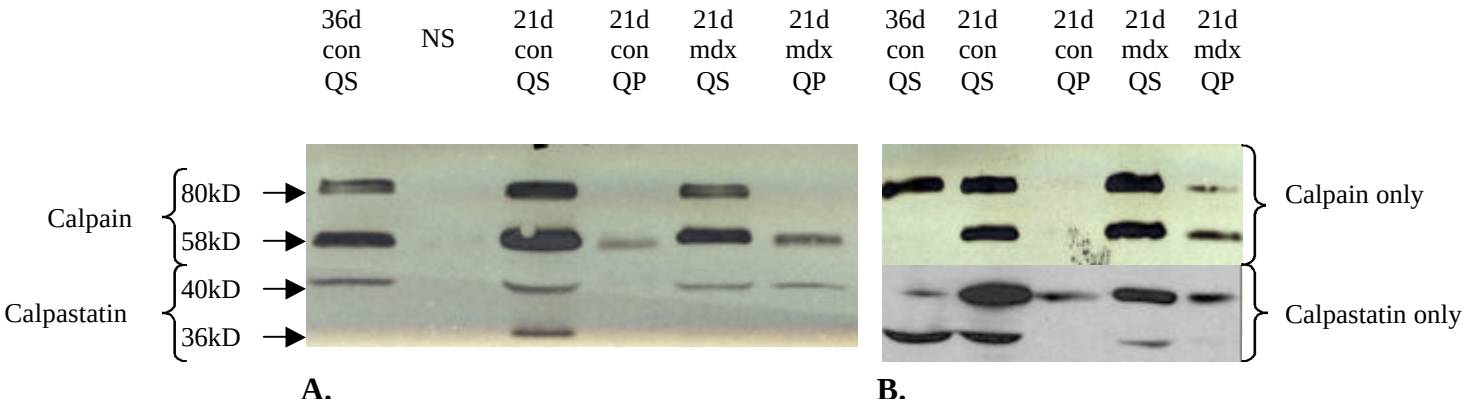

Figure 10. Immunoblots of total calpain and calpastatin. (*A)* Double labeling of calpain and calpastatin in 21d quadriceps. Both calpain and calpastatin are more prevalent in the supernatant than in the pellet. *(B)* Separate blots of calpain (top) and calpastatin (bottom) demonstrating two bands for calpain and two for calpastatin. Con: control, QS: quadriceps supernatant, QP: quadriceps pellet, NS: no sample loaded.

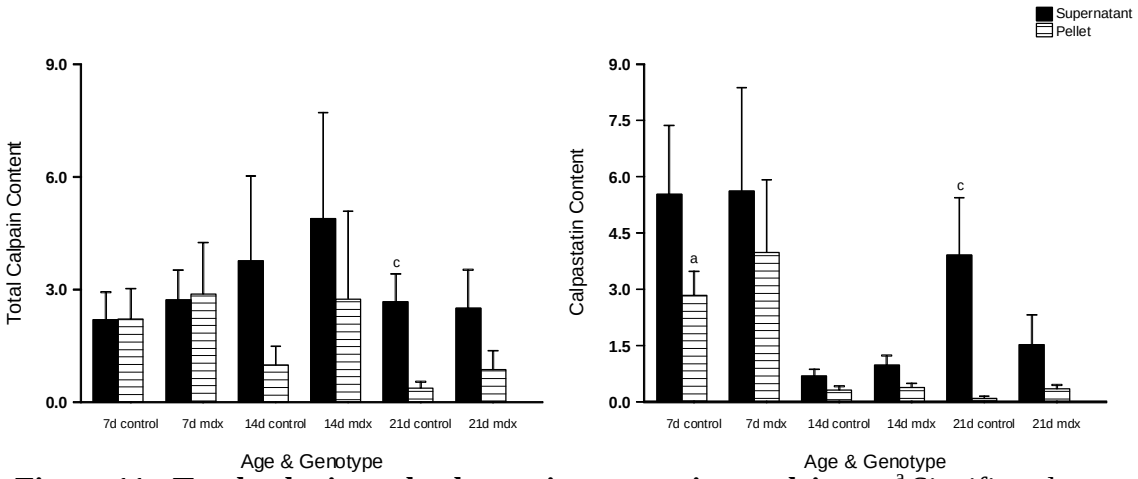

Figure 11. Total calpain and calpastatin content in quadriceps. [a] Significantly greater than other ages within the same genotype. [b] Significantly less than other ages with the same genotype. [c] Supernatant is significantly greater than the pellet fraction within the same age and genotype. (n=6, except 14d control supernatant and calpastatin both 21d control and mdx fractions n=5, $p<0.05$) mean±SEM

Diaphragm

Total calpain

Figure 12 is a representative blot of total calpain and calpastatin from diaphragm samples. At age 21 days, the mdx pellet (0.29) had significantly less total calpain content than the pellets at both age 7 (1.29) and 14 days (1.82; $p<0.05$; Figure 13). At age 7 days, the mdx supernatant (2.60) had a significantly more total calpain content than the pellet (1.29). The same was also true in the mdx supernatant (2.43) at age 21 days, with significantly more total calpain content than the pellet (0.29; $p<0.05$). There were no significant differences in total calpain content between control and mdx at any age in either the supernatant or pellet.

Calpastatin

At age 14 days, the mdx pellet (2.63) had significantly more calpastatin than the pellet at age 7 (0.15) and 21 days (2.34; $p<0.05$). There were no significant differences between supernatant and pellet calpastatin content at any age. Similar to the quadriceps results, there was no difference in calpastatin content between control and mdx samples in either the supernatant or pellet.

Relative Calpain Activity

Calpain activity was expressed with respect to total calpain content as determined by Western analysis. Some samples were excluded because immunoreactivity for calpain was not detectable. Upon longer exposure time, a band was visible in most samples, but was not quantified because longer exposure resulted in indistinct bands from the other samples and made densitometry difficult.

Quadriceps

General trends across age and within genotype

Calpain activity in the control pellet in the presence of calcium at age 21 days (75.4) was significantly greater than the control pellet activity at age 7 (11.6) and 14 days (26.3; $p<0.05$; Figure 14). Also at age 21 days in control, the pellet activity in the absence of calcium was significantly greater than at age 7 days. There were no significant differences in relative calpain activity across age in the mdx samples.

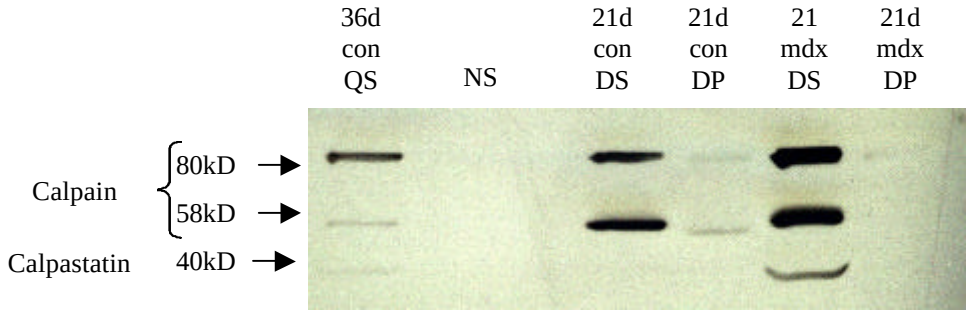

Figure 12. Immunoblot of total calpain and calpastatin in 21d diaphragm. More calpain is found in the supernatant than in the pellet in the mdx diaphragm.

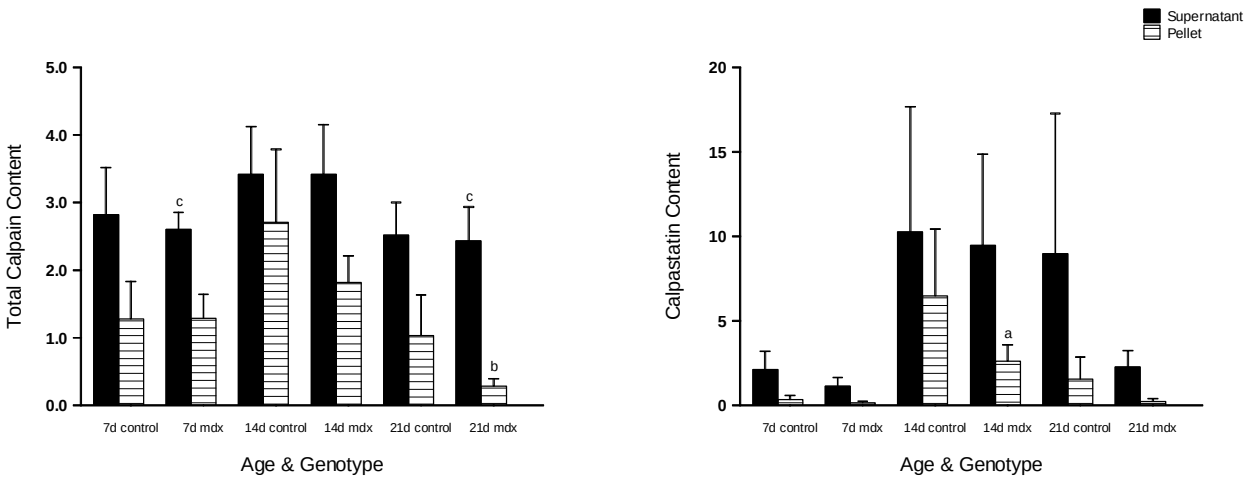

Figure 13. Total calpain and calpastatin content in diaphragm. [a] Significantly greater than other ages within the same genotype. [b] Significantly less than other ages with the same genotype. [c] Supernatant is significantly greater than the pellet fraction within the same age and genotype. (n=6, $p<0.05$) mean±SEM

Within age and genotype

At age 7 days, the control pellet calpain activity in the absence (14.9) of calcium was significantly different from the supernatant activity in both the presence (7.7) and absence (4.9; $p<0.05$) of calcium, but not different from the pellet activity in the presence of calcium. At age 7 days, the mdx pellet calpain activity in the presence (30.1) of calcium was significantly greater than the supernatant activity in both the presence (4.6) and absence (2.5) of calcium, but not different from the pellet activity in the absence of calcium. There were no significant differences in either control or mdx calpain activities between supernatant and pellet in either the presence or absence of calcium at age 14 days. At age 21 days, the control pellet calpain activity in the presence (75.4) of calcium was significantly greater than both the supernatant activity in the presence (11.3) and absence (6.8) of calcium, but not different from the pellet activity in the absence of calcium. At age 21 days, there were no significant differences between mdx supernatant and pellet calpain activity in the presence or absence of calcium.

Between genotypes within age

There were no significant differences in relative calpain activity between control and mdx samples at any age in either the supernatant or pellet in the presence or absence of calcium.

Diaphragm

General trends across age and within genotype

There were no significant differences in relative calpain activity in control samples across age. Relative calpain activity in mdx supernatant in the presence of calcium at age 21 days (14.9) was significantly greater than at age 7 (3.5) and 14 days (7.1; $p<0.05$; Figure 15). Also, at age 21 days (63.9 and 83.7), the pellet calpain activity in presence and absence of calcium was significantly greater than at 7 (21.4 and 37.6) and 14 days (9.4 and 17.7) respectively.

Within age and genotype

At age 7 days, control pellet activity in the absence (43.4) of calcium was significantly greater than supernatant activity in the presence (4.5) and absence (3.1) of calcium, but not different from pellet activity in the presence of calcium. This was also true in the mdx at age 7 days when the pellet activity in the presence (37.6) of calcium

was significantly greater than the supernatant activity in the presence (3.5) and absence (3.6) of calcium, but not different from the pellet activity in the presence of calcium. There were no significant differences in control supernatant and pellet activity in the presence or absence of calcium at age 14 days. In the mdx, pellet activity in the absence (17.7) of calcium at age 14 days was significantly greater than the pellet activity in the presence (9.6) of calcium and the supernatant activity in the presence (7.1) and absence (7.1; $p<0.05$) of calcium. At age 21 days, the control pellet activity in the presence (57.0) of calcium was significantly greater than the supernatant activity in the presence (15.1) and absence (9.8; $p<0.05$) of calcium, but not different from the pellet activity in the absence of calcium. There were no significant differences between supernatant and pellet activities in the presence or absence of calcium in mdx samples at age 21 days.

Total Calpain:Calpastatin Ratio

It has been suggested that calpain activity depends not only on calpain content but also calpastatin content. The average calpain:calpastatin ratio, which is the relative total calpain and calpastatin content, was resolved to evaluate its relationship to calpain activity. The protein contents were determined by densitometry relative to the same reference sample. A true ratio of total calpain to calpastatin could not be determined because our calpastatin antibody did not react with the calpastatin standard. A relative comparison of total calpain content to calpastatin content was used as our total calpain:calpastatin ratio. The average ratio was used because some samples, as noted previously, had no detectable calpain or calpastatin.

Quadriceps

In quadriceps, both control and mdx supernatant and pellet average calpain:calpastatin ratios were lowest at age 7 days, increased at age 14 days, and then decreased at age 21 days. The ratio in control pellet which increased to its highest level at age 21 days (Figure 16). The total calpain activity in the presence of calcium does not follow the same trend. At age 7 days, the mdx supernatant has the greatest activity that then decreases to its lowest level at age 14 days and then did not change at age 21 days. The control pellet calpain activity seems to have an inverse relationship to the calpain:calpastatin ratio because as the ratio increases, the calcium-dependent activity

decreases. Both control and mdx supernatant calcium-dependent activities did not change with age, but the calpain:calpastatin ratio peaked at age 14 days.

Diaphragm

In the diaphragm, the average calpain:calpastatin ratio is greatest at age 7 days in both mdx and control pellets and control supernatants, decreases at age 14 days, and then increases slightly at age 21days. The mdx supernatant which barely increases at age 14 days and then decreases at age 21 days. Calcium-dependent mdx and control supernatant activity increase over time, but pellet activities do not change. There does not appear to be any relationship between the calpain:calpastatin ratio and calpain activity in the diaphragm.

Correlations were performed between either quadriceps or diaphragm calpain activity and the relative total calpain:calpastatin ratio. There did not appear to be a relationship between calpain activity and the total calpain:calpastatin ratio as the largest correlation was 0.53 for the quadriceps and 0.67 for the diaphragm (data not shown).

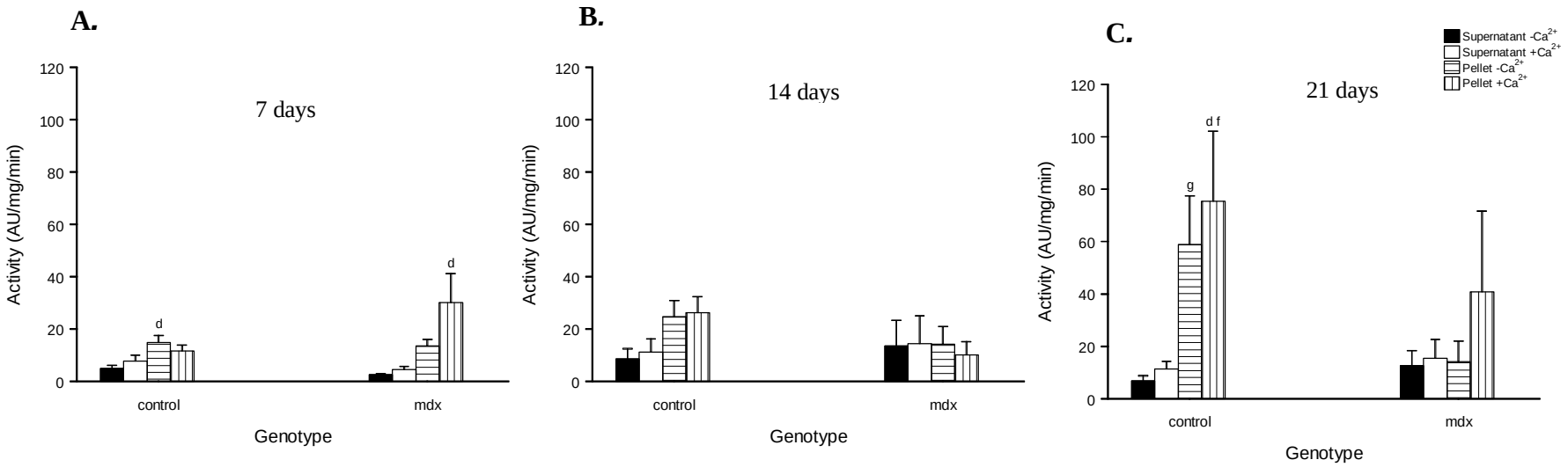

Figure 14. Relative calpain activity in quadriceps (normalized to total calpain content) [d]Significantly greater than both supernatant activities[f] Significantly greater than at 7 and 14d. [g] Significantly greater than 7d. (n=6 except 7d control pellet, 14 control supernatant and pellet, 21d control and mdx pellet n=5, 14d mdx pellet n=3 ; $p<0.05$) mean±SEM. Note: letters indicating significance are consistent with all figures reporting calpain activity.

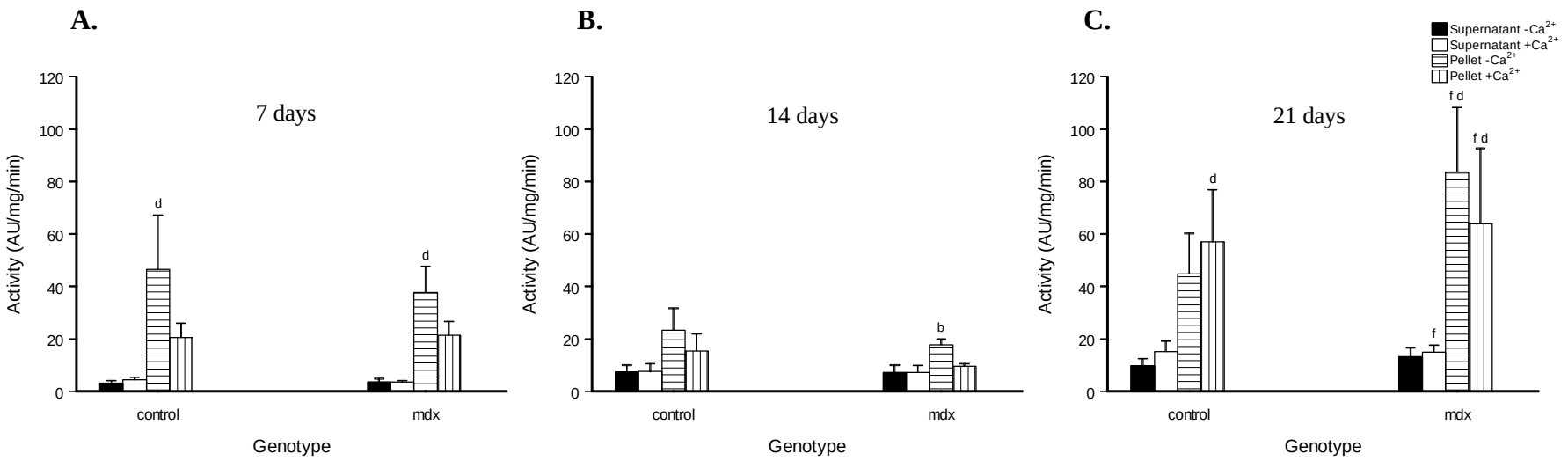

Figure 15. Relative calpain activity in diaphragm (normalized to total calpain content) [b] Significantly greater than other conditions at the same age and genotype. [d] Significantly greater than both supernatant activities. [f] Significantly greater than at 7 and 14d (n=6 except 7d control pellet, 14d control supernatant, 14d mdx pellet, 21d control pellet n=5, 21d mdx pellet n=3; $p<0.05$) mean±SEM. Significance symbols are consistent with total calpain activity, not all letters may be present. Note: letters indicating significance are consistent with all figures reporting calpain activity.

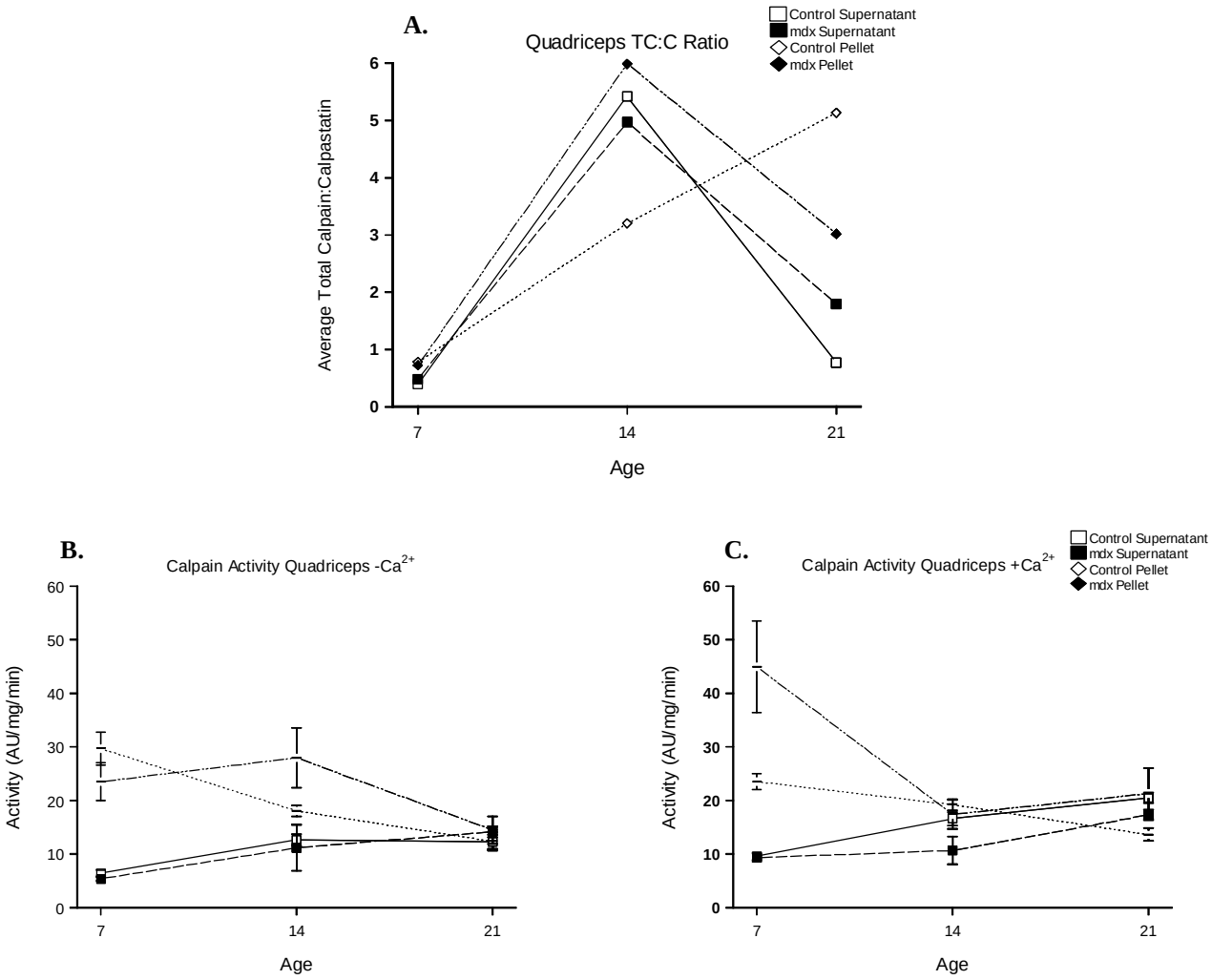

Figure 16. Average calpain:calpastatin ratio and total calpain activity in quadriceps. *(A)* Average calpain:calpastatin ratio in quadriceps ages 7, 14, 21d. *(B-C)* Total activity in the quadriceps in the absence *(B)* and presence *(C)* of calcium from 7, 14, 21d.TC=total calpain C=calpastatin

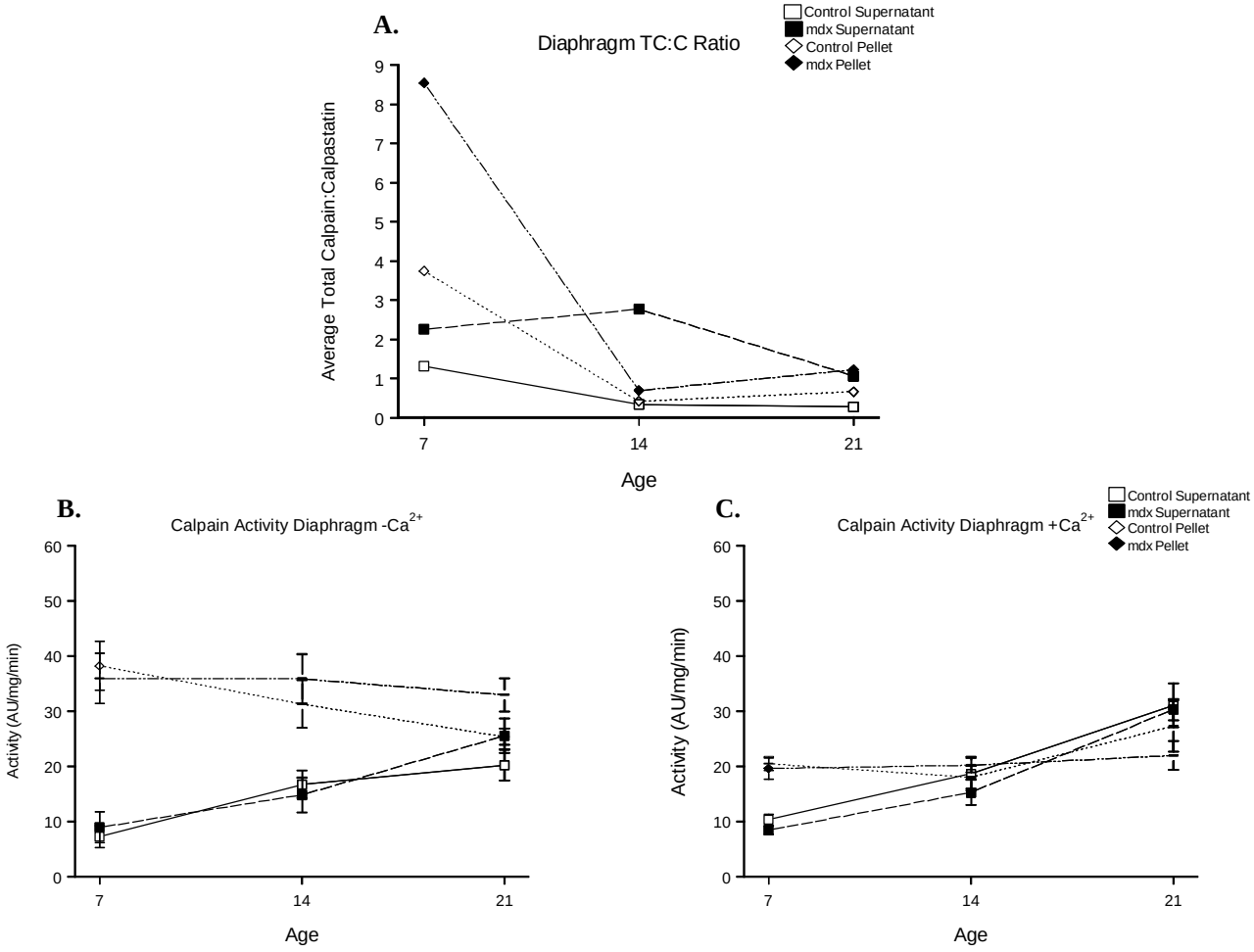

Figure 17. Average calpain:calpastatin ratio and total calpain activity in diaphragm. *(A)* Average calpain:calpastatin ratio in diaphragm at ages 7, 14, 21d. *(B-C)* Total activity in the diaphragm in the absence *(B)* and presence *(C)* of calcium from 7, 14, 21d.TC=total calpain, C=calpastatin

Chapter 5
Discussion

Discussion

Increased $[Ca^{2+}]_i$ has been observed in dystrophic skeletal muscle (Turner et al., 1988, Fong et al., 1990) in parallel with increased calcium-dependent proteolysis (Turner et al., 1991). Because calpains are calcium-dependent proteases active in skeletal muscle, they may play a role in the onset of DMD. The purpose of the present study, therefore, was to evaluate calpain activity in both the supernatant and pellet fractions of homogenized dystrophic quadriceps and diaphragm muscles obtained from maturing mice aged 7, 14, and 21 days to determine if increased calpain activity plays a role in the onset of DMD. This was done using a calpain activity assay in the presence of calpastatin, the specific endogenous inhibitor of calpain. Western analysis was also performed to examine total calpain and calpastatin content in each sample. The protein content data were used to assess if changes in calpain activity were due to differences in the amount of calpain relative to calpastatin (i.e., the calpain:calpastatin ratio) both in the supernatant and pellet fractions because this ratio has been suggested to be a potentially critical determinant of calpain activity.

Major findings

In both the quadriceps and diaphragm samples, calpain activity in the supernatant fraction increased with age. In the quadriceps at age 7 days, calpain activity in the pellet in the presence of calcium was significantly greater than at age 14 and 21 days. In the diaphragm, there were no significant differences in pellet activity in the presence or absence of calcium at any age in both control and mdx samples. In general, there were no differences in total calpain and calpastatin content that would account for the differences in calpain activity. Overall, in either the control or mdx muscles, with the exception of calcium-dependent pellet activity in the dystrophic quadriceps condition at age 7 days, the distribution of total calpain between supernatant and pellet, and their associated proteolytic activities at each age, and across age, were very similar. These findings suggest the similar pattern of total calpain content and activity in the quadriceps and diaphragm of control and mdx mice reflect a similar maturation pattern between ages 7 and 21 days. However, it should be noted that the substrate targets in control vs. mdx muscle were not determined and therefore potential changes in regulation of calpain

and/or discrete localization of the different isoforms of calpain could discriminate the dystrophic vs. control phenotype. For example, these differences may explain the increased calcium-dependent activity in the dystrophic quadriceps pellet condition at age 7 days.

Calpain Activity

Quadriceps

In the present study, at age 7 days, the mdx pellet from quadriceps demonstrated a significant increase (47.7%) in calcium-dependent calpain activity compared to control. This is consistent with a significant increase in calpain activity in mdx compared to control myotubes based on the cleavage of a fluorogenic calpain substrate (Alderton et al., 2000b). Calpain activity in the pellet fraction may be increased because the pellet contains structure-bound calpain. These structures include phospholipid membranes of the sarcolemma and sarcoplasmic recticulum. Phospholipids have been shown *in vitro* to decrease the calcium concentration required to activate calpains (Coolican and Hathaway, 1984). *In vivo,* the increased calcium sensitivity of calpain at the membrane may be important if there is increased leakage of calcium across dystrophic membranes. Furthermore, Alderton et al. (2000b) suggest that following a sarcolemmal wound, calpain would be activated locally, possibly altering the kinetics of calcium leak channels in the area of the wound, creating "hot spots" of continual calcium entry near the wound. If this is true, phospholipids and localized increases in calcium would contribute to increased calpain activity, further altering leak channel kinetics and a positive feedback loop would occur.

At age 21 days, however, in contrast to the increased rate of tyrosine release an indicator of calcium-dependent proteolysis in mdx compared to control muscle fibers from mice aged 3-6 weeks (Turner et al., 1993), the present study showed that calcium-dependent calpain activity was not different between mdx and control mice. Net tyrosine release rate is a measure of the average rate of protein turnover (Fulks et al., 1975), but calpain modifies rather than degrades its substrates, therefore, it may not contribute to the general turnover of protein (Johnson, 1990). For this reason, tyrosine-release rate may not be the most appropriate measure of calcium-dependent calpain activity.

In quadriceps samples, both control and mdx pellet activities were greatest at age 7 days and decreased at age 14 and 21 days. This may be a result of normal muscle development. Calpain is involved is myoblast fusion (Schollmeyer et al., 1986) and cleaves membrane proteins, such as â1-integrin (Barnoy et al., 1998). In the L8 rat myoblast cell line, western analysis of extracts from differentiating and fusing cells demonstrated cleavage of several calpain substrates, including talin and â1-integrin, that was then inhibited by addition of calpastatin (Barnoy et al., 1998). Inhibition of myoblast fusion by microinjection of calpastatin has also been demonstrated in C2C12 cells (Temm-Grove et al., 1999). Under the assumption that at age 7 days, muscle fibers are still developing, the increased calpain activity in the pellet, which contains microsomal membranes, may reflect continued myotube formation at this age that is not present at age 14 and 21 days. However, since fiber type characteristics (e.g., fast vs. slow) are determined largely by innervation pattern (stimulus rate and duration; Lieber, 2002), it is likely that fibers are adapting as the animal becomes more mobile at this early stage of maturation, and this adaptation requires remodeling of fiber structures by calpains.

Although calpain activity in the pellet decreased with maturation, the supernatant activity increased with age in both control and mdx quadriceps. This increase in supernatant activity may also reflect a developmental change in the muscle. The possibility of myotube formation/fiber adaptation activity at age 7 days may support the observed reduced calpain activity in the supernatant, whereas the increased calpain activity at age 14 and 21 days in the supernatant may suggest that remodeling at the membrane is declining. The increased activity in the supernatant may reflect the need for remodeling of cytosolic proteins during maturation. Calpain *in vitro* can cleave a number of soluble substrates (e.g., protein kinase A, protein kinase C; Belcastro et al., 1998). Although the substrates targeted by this shift in activity are not clear, it is likely the increased activity in the supernatant fraction reflects increased modification of soluble proteins to support the maturation process. Specific differences in calpain substrate targets in control vs. mdx fibers may explain the initiation of the dystrophic phenotype, but based on data herein, these differences cannot be discriminated.

Diaphragm

The diaphragm proteolytic activity was greatest in the pellet in the absence of calcium in both control and mdx samples. In fact, the activity in the pellet decreased with the addition of calcium. This result suggests the presence of another protease that may cleave the same substrate, but is inhibited by calcium. It also suggests the presence of another calcium-activated inhibitor of calpain. Spencer et al. (1992) also reported that calcium-dependent proteolysis of casein in soleus and gastrocnemius homogenates was less than the activity that occurred in the absence of calcium. In the present study, the calpain assay measured total calpain activity, including m-, ì-, and p94. A possible explanation for increased pellet activity in the absence of calcium could be increased activity of p94. p94 is associated with connectin (Sorimachi et al., 1995) a myofibrillar protein, which under the centrifugation conditions employed in this study, would likely be found in the pellet fraction. p94 is not inhibited by calpastatin and does not require calcium for activation (Sorimachi et al., 1997), so these characteristics may explain the increased calpain activity in the absence of calcium.

In the diaphragm, the activity in the pellet fraction remains high from age 7-21 days. However, both control and mdx supernatant activity increased significantly over this period. This change in distribution of activity may again reflect a developmental change in the muscle because of cytosolic protein remodeling.

Total Calpain and Calpastatin Content and Localization

There were no significant differences in total calpain or calpastatin content between mdx and control in either quadriceps or diaphragm samples at any age. Previous studies examining the role of calpain in DMD have used mdx mice as young as age 14 days, but also found no difference in the calpain concentrations at this age (Spencer et al., 1995).

There were several differences in calpain and calpastatin distribution between supernatant and pellet fractions in the present study. At age 21 days, control quadriceps samples had significantly greater content of total calpain than the pellet. As stated previously, this may reflect a developmental pattern. In the mdx diaphragm, a significantly greater content of total calpain was found in the supernatants compared to

pellets at age 7 and 21 days. Spencer et al. (1996) suggested that calpain distribution was related to the disease state of mdx dystrophy, but not to age. In control and pre-necrotic mdx fibers, calpain was diffuse throughout the cytosol and enriched at the membrane, but in necrotic mdx fibers, calpain was found in high concentrations and diffusely distributed (Spencer et al., 1996). In the present study there was also no difference in total calpain distribution between the supernatant and pellet across age in either control or mdx samples.

Conversely, based on fluorescent localization and immunogold labeling, calpain is localized at the myofibrils in rat skeletal muscle (Kumamoto et al., 1992), but in the present study, less total calpain was detected in the pellet that would contain myofibrillar proteins. Fluorescent localization and immunogold labeling are much more discrete methods for detecting localization of calpain than differential centrifugation into supernatant and pellet fractions followed by Western analysis, possibly contributing to the different results in distribution. Methodological differences (i.e., rat vs. mouse, immunogold labeling vs. centrifugation) may explain the greater calpain concentration in the supernatant.

At age 21 days, control quadriceps supernatant samples had significantly greater content of calpastatin than the pellet. However, in rabbit skeletal muscle, immunogold labeling detected calpastatin near the membrane and associated with myofibrils. These results may differ because of the methods used, immunogold labeling vs. Western analysis and also differences between mouse and rabbit skeletal muscle. Calpain and calpastatin content did not differ across age, but there were some differences in calpain and calpastatin distribution between the supernatant and pellet that could possibly reflect a developmental pattern.

Relative Calpain Activity and Calpain:Calpastatin Ratio

When calpain activity was expressed relative to total calpain content as determined by Western analysis, there were no significant differences in either quadriceps or diaphragm between control and mdx at any age. This result may suggest a difference in regulation of calpain and/or calpastatin between control and mdx at age 7 days in quadriceps. The total calpain:calpastatin ratio was determined to resolve whether changes in calpain activity could be associated with changes in the ratio. For example,

many groups have stated that the calpain:calpastatin ratio is the best indicator of net calpain activity potential (Sultan et al., 2000, Enns et al., 2002, Spencer et al., 2002). However, in a rat muscle ischemia-reperfusion model, the ratio was determined by measures of calpain and calpastatin activity (Enns et al., 2002), but not by protein content. Mdx mice overexpressing calpastatin had decreased levels of necrosis that was attributed to reduced calpain activity, but the content of calpain relative to calpastatin was not assessed (Spencer et al., 2002). It seems as though the calpain:calpastatin ratio is inferred rather than known. In the present study, there was no apparent relationship between total calpain:calpastatin ratio and calpain activity. However, p94 could play a role here as well. The calpain antibody should recognize all 3 muscle isoforms of calpain, m-, ì-, and p94 and the calpain activity assay is not specific for only m- and ì-calpain. The presence of p94 in the content and activity could misrepresent the relationship between the calpain:calpastatin ratio and calpain activity. For example, p94 is associated with connectin (Sorimachi et al., 1995), a myofibrillar protein, and could be found in the pellet fraction, thus contributing to pellet activity in the absence of calcium and also to the total calpain content.

Summary

Calpain activity was increased significantly at age 7 days in mdx quadriceps pellet compared to control. Calpain activity in quadriceps in both control and mdx pellet fractions decreased with age, while supernatant activities increased, possibly related to muscle fiber development. The same increase in diaphragm supernatant activity across age was also present. In the diaphragm, calpain activity in the pellet was greatest in the absence of calcium, suggesting either p94 activity, or that another protease (or inhibitor) was present. There was no difference in calpain or calpastatin content between mdx and control quadriceps or diaphragm at any age. These results suggest that calpain may not play a major role in the mechanism of onset in DMD, but that it could be a secondary effect of the disease process.

In the present study, the calpain activity assay assessed global calpain activity, but if calpain was locally increased at membrane “hot spots” as Alderton et al. (2000b) suggests, our method would not detect these subtle changes. Western analysis of supernatant and pellet fractions could detect larger, more general changes in total calpain

and calpastatin distribution, but this method is not precise enough for localization of calpains. However, potential localization of calpains at the membrane may be a key determinant of the dystrophic phenotype.

Apoptosis and Calpain

The DGC is hypothesized to participate in signaling activities (Blake et al., 2002a) and the absence of DGC may initiate a signal cascade for apoptosis. For example, as early as age 14 days, dystrophic muscle demonstrated a significant increase in the number of apoptotic nuclei in mdx muscle compared to control. These nuclei were apparent in muscle that demonstrated no other signs of evident pathology (Tidball et al. (1995).

In addition to its presence in the cytosol and being structure-bound, calpain is found in the nucleus (Kumamoto et al., 1992, Nori et al., 1993) as is p53, a transcription factor that activates expression of genes that lead to apoptosis (Lodish et al, 2003). p53 has been shown *in vitro* to be a substrate of calpain (Kubbutat et al, 1997). Because calpain does not completely degrade, but rather modifies its substrates, it may act as a modulator of p53 function (Sorimachi et al., 1997). This modulation of p53 function may suggest a role for calpain in the regulation of apoptosis.

Calpains have been shown to cleave several caspases, major proteases involved in apoptosis (Chua et al., 2000, Nakagawa et al., 2000). By cleaving several of the caspases, calpain could be a positive and negative regulator of apoptosis (Goll et al., 2003). The increased levels of apoptosis noted by Tidball et al. (1995) could in part be attributed to calpain activity.

The significant increase in calcium-dependent activity at age 7 days in the mdx quadriceps pellet may result in modulation of either p53 or caspases resulting in increased apoptosis in the dystrophic muscle that may initiate muscle death, therefore, contributing to the onset of DMD. By age 14 days in the dystrophic muscle, calpain activity may return to control levels, but the possible activation of apoptosis at age 7 days may be enough to initiate the dystrophic process. Although nuclei are most likely not present in our pellet fraction, caspases have been located in the endoplasmic recticulum (Nakagawa et al., 2000) that would be found in the pellet fraction.

Summary

In the present study, the role of calpain in the onset of DMD appears to be minor if global cellular calcium-dependent activity is evaluated. There were similar patterns of calpain activity and total calpain and calpastatin content in both control and mdx samples, suggesting a similar pattern of development in control and mdx muscle from ages 7-21 days. There was a significant difference between control and mdx calpain activity only in mdx quadriceps pellet at age 7 days that may be due to differences in regulation and/or distribution of calpains early in mdx maturation compared to control. This difference in calpain activity may also suggest a possible role for calpain in the onset of DMD in the regulation of p53 and caspases in apoptosis. Finally, the methods employed in this study did not allow determination of localized calpain activity, and it is the determination of the specific targets that may reveal the role of calpain in the onset of DMD.

Research Hypotheses Conclusions

The null hypothesis H_{01} was rejected at age 21 days because quadriceps calpain activity in the control supernatant was significantly greater compared to mdx. The null hypothesis H_{02} was rejected at age 7 days because quadriceps activity in the mdx pellet was significantly greater than control. The null hypotheses H_{03} and H_{04} were not rejected, as there was no difference between control and mdx activity in the diaphragm supernatant or pellet at any age. The null hypothesis H_{05} was rejected because of significant increases in the pellet compared to the supernatant at age 7 and 14 days in both quadriceps and diaphragm. The null hypotheses H_{06}, H_{07}, H_{08}, and H_{09} were not rejected because there were no significant differences between control and mdx total calpain and calpastatin content at any age in either the quadriceps or diaphragm. The null hypothesis H_{10} was rejected because the supernatant in age 21 day control quadriceps had significantly more total calpain and calpastatin than the pellet and the supernatant in age 7 and 21 days mdx diaphragm had more total calpain than the pellet.

Future Directions

This investigation suggested that calpain activity may not play a major role in the onset of DMD, but this conclusion may be due to methodological limitations. For example, it was not possible to determine if calpain activity was specific to m- and ì-calpain and/or p94. However, if both an isoform-specific calpain antibody and a positive

control for each of the proteins were used, it may then be possible to determine if the calpain:calpastatin ratio could indicate calpain activity.

Muscle homogenates in this study were separated into supernatant and pellet fractions. The pellet fraction contained several membranes, including those of the sarcolemma, the sarcoplasmic recticulum, and the mitochondria. A purified sarcolemmal preparation could be used to determine calpain activity specifically at the membrane.

Calpain activity could be observed *in situ* in whole muscle fibers in the presence of the fluorogenic substrate. Reversible permeabilization of the sarcolemma would allow the substrate to enter the cell and a change in fluorescence could be monitored in intact muscle fibers. Calpain content could also be observed *in situ* by immunohistochemistry. These two techniques would allow more specific locations of calpains and calpain activity to be determined.

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Appendix A
Methods

Appendix A

SDS-PAGE

Sodium dodecyl sulfate (SDS) is an anionic detergent that disrupts noncovalent interactions within proteins and binds to proteins at a ratio of 1.4 grams SDS per gram of protein which gives the denatured proteins a negative net charge proportional to their size. Separation is accomplished by using a polyacrylamide gel, which has pores that permit migration of proteins according to their size. During separation on the gel, the negatively charged proteins migrate based on their size from the cathode (negative electrode) to the anode (positive electrode). Smaller proteins move through the pores more quickly and therefore migrate further compared to the larger ones.

A polyacrylamide gel forms from the co-polymerization of acrylamide and bis-acrylamide initiated by formation of free radicals in the acrylamide solution. Pore size is inversely proportional to the percentage of acrylamide and bis-acrylamide in the gel. For example, more acrylamide results in a smaller pore size, which gives better resolution for smaller proteins. Polymerization is initiated by formation of free radicals. Ammonium persulfate is used to start the free radical formation and TEMED (N-, N'-tetramethylenediamine) is used as a catalyst for the polymerization. It is important that oxygen is excluded from the solution because it is a free radical scavenger and will hinder the polymerization process.

The Laemmli (1970) system consists of a stacking gel and separating gel. The purpose of the stacking gel is to concentrate or stack the proteins to get thin bands and better resolution in the separating gel. Stacking occurs because the low percentage of acrylamide (4%) in the stacking gel yields a large pore size to permit proteins of all sizes mobility in the gel. Once the proteins move into the separating gel they begin to "unstack". The amount of acrylamide in the separating gel is selected according to the size of the protein of interest.

The separating gel is poured first into a glass sandwich made of two glass plates that have a spacer between them that determines the thickness of the gel, and that stands on a rubber gasket to prevent the liquid acrylamide from leaking from the plates before it polymerizes. After the separating gel has polymerized, the stacking gel is poured. A comb is inserted into the stacking gel before it polymerizes to form wells. When the

stacking gel is polymerized, the comb is removed and the protein samples are loaded into the wells.

Solutions were allowed to come to room temperature and then were degassed for twenty minutes to remove air and facilitate polymerization. The initiators, ammonium persulfate and TEMED were added and the solution swirled. The7.5% separating gel, which optimally separates proteins between 30-120kD (Bio-Rad), was poured using a transfer pipette filling the cassette up to one centimeter below the well-forming comb. The gel was overlaid with 20% EtOH to prevent exposure to oxygen. Polymerization was complete after 30 min. The EtOH was decanted and the gel washed with 0.1%SDS and then excess SDS was absorbed with filter paper. The degassed 4% stacking gel solution was poured over the separating gel to the top of the cassette and the well-forming comb was inserted. Polymerization was complete in 90 min.

Enhanced Chemiluminescence

Chemiluminescence is a process by which a significant fraction of intermediates or products of a chemical reaction are produced in an excited state yielding measurable emission of photons (e.g. light) from the excited molecules. Various manufacturers have developed substrates to enhance light emission and duration. In this case, the substrates were luminol and hydrogen peroxide from Super Signal West Luminol/Enhancer Solution and Super Signal West Pico Stable Peroxide Solution from Pierce.

Appendix B
Raw Data

Appendix B

Quadriceps			Mass (g)		Activity		Content	
Sample	Age	Genotype	Body	Muscle	Ca^{2+}	$-Ca^{2+}$	Total Calpain	Calpastatin
1485Ap10-12	7	mdx	4.6	0.0338	9.7	6.48	1.542199	0.57151
1485Ap1-3	7	mdx	4.6	0.0313	10.07	6.1	6.502509	4.680593
1485Ap4-6	7	mdx	3.9	0.03	10.88	4.8	1.783855	1.036572
1485Ap7-9	7	mdx	5	0.023	11.1	4.51	1.405022	1.861472
1497Ap1-3	7	mdx	5.9	0.0399	6.632	4.504	2.928295	7.264845
1497Ap4-6	7	mdx	6.1	0.0861	7.575	5.738	2.196507	18.33465
Mean			5.0	*0.0407	9.3262	5.3553	2.7264	5.6249
SEM			0.3	*0.0094	0.7434	0.3518	0.7878	2.7439
1485Ap10-12,p100	7	mdx	4.6	0.0338	58.81	21.42	1.749516	12.73528
1485Ap1-3,p100	7	mdx	4.6	0.0313	60.79	21.63	9.624125	5.078632
1485Ap4-6,p100	7	mdx	3.9	0.03	65.49	13.85	0.87257	0.921289
1485Ap7-9,p100	7	mdx	5	0.023	46.31	16.4	0.995742	1.055022
1497Ap1-3,p100	7	mdx	5.9	0.0399	18.42	36.16	1.779078	0
1497Ap4-6,p100	7	mdx	6.1	0.0861	19.8	31.497	2.270485	4.119433
Mean			5.0	*0.0407	44.9367	23.4928	2.8819	3.9849
SEM			0.3	*0.0094	8.5695	3.5383	1.3653	1.929
1447Cp1-3	7	control	4.5	0.0324	9.45	5.73	5.33183	2.821454
1447Cp4-6	7	control	4.5	0.0335	7.96	4.96	0.635872	4.054697
1448Cp1-3	7	control	5.8	0.0398	8.335	4.65	1.410596	0.989949
1448Cp4-6	7	control	5.5	0.0392	10.431	7.06	0.506498	3.074252
1561Ap1-3	7	control	5.2	0.0728	12.688	8.016	3.013891	11.60394
1561Cp1-3	7	control	5.9	0.0697	8.653	8.072	2.263564	10.70369
Mean			5.3	*0.0479	9.5862	6.4147	2.1937	5.5413
SEM			0.2	*0.0075	0.7173	0.6172	0.7396	1.824
1447Cp1-3,p100	7	control	4.5	0.0324	27.821	38.99	5.991308	4.509753
1447Cp4-6,p100	7	control	4.5	0.0335	23.46	21.53	0.216505	0.930011
1448Cp1-3,p100	7	control	5.8	0.0398	22.62	23.31	1.380965	2.478164
1448Cp4-6,p100	7	control	5.5	0.0392	19.531	24.81	1.585341	4.905191
1561Ap1-3,p100	7	control	5.2	0.0728	19.931	32.68	2.468752	1.58243
1561Cp1-3,p100	7	control	5.9	0.0697	27.719	37.761	1.643344	2.598879
Mean			5.3	*0.0479	23.5137	29.6802	2.2144	2.8341
SEM			0.2	*0.0075	1.4803	3.0361	0.8111	0.6448

1) In the 'Sample' column, the supernatant data is presented first for each genotype, followed by the pellet data designated by p100. This is consistent for all ages and muscles.

2) Each sample at age 7 days is represented by tissue pooled from 3 pups (e.g., 1485Ap10-12 is pooled tissue from pups 10, 11, and 12). The body mass for each sample is the mean body mass of the 3 pups.

3) ***** Mass and SEM of pooled muscle tissue from 3 pups.

4) Total calpain and calpastatin content was considered to be 0 when content was less than 0.1.

Quadriceps			Mass (g)		Activity		Content	
Sample	Age	Genotype	Body	Muscle	Ca^{2+}	$-Ca^{2+}$	Total Calpain	Calpastatin
1452Ap1	14	mdx	8	0.0361	5.643	3.784	2.658352	0.540828032
1452Ap2	14	mdx	6.8	0.0334	4.031	3.889	18.77979	1.37382672
1467Ap1	14	mdx	10	0.0624	7.631	3.848	1.686169	2.074973162
1467Ap2	14	mdx	7.5	0.0496	13.318	12.136	0.1984734	0.544551594
1468Ap1	14	mdx	8	0.051	12.282	12.268	3.763278	0.605478328
1468Ap2	14	mdx	7.3	0.0498	21.297	31.099	2.309177	0.776784802
Mean			7.9	0.0471	10.6703	11.1707	4.8992	0.9861
SEM			0.5	0.0044	2.6011	4.3224	2.8173	0.2528
1452Ap1,p100	14	mdx	8	0.0361	15.887	28.264	1.442196	0.376870736
1452Ap2,p100	14	mdx	6.8	0.0334	13.131	14.613	14.3823	0.321284037
1467Ap1,p100	14	mdx	10	0.0624	11.582	13.87	0.625831	0.816316292
1467Ap2,p100	14	mdx	7.5	0.0496	20.388	34.398	0.087522	0.213352637
1468Ap1,p100	14	mdx	8	0.051	29.603	50.567	0	0.572753682
1468Ap2,p100	14	mdx	7.3	0.0498	13.851	26.1	0	0
Mean			7.9	0.0471	17.407	27.9687	2.7563	0.3856
SEM			0.5	0.0044	2.7374	5.576	2.3363	0.1159
1447Dp1	14	control	4.8	0.0238	14.659	11.225	1.249392	0.780486629
1447Dp2	14	control	5	0.0358	15.641	9.56	14.74143	0.376199431
1561Bp5	14	control	9.4	0.0864	22.112	13.023	3.506562	1.138270531
1561Bp4	14	control	9.2	0.0663	15.636	16.811	2.677577	1.050411255
1562Bp1	14	control	8.8	0.0657	17.774	14.467	0	0
1562Bp2	14	control	8	0.0567	13.745	10.635	0.45345	0.83018388
Mean			7.5	0.0558	16.5945	12.6202	3.7714	0.6959
SEM			0.9	0.0093	1.2319	1.1013	2.2598	0.1764
1447Dp1,p100	14	control	4.8	0.0238	20.602	19.218	0.698363	0.198015994
1447Dp2,p100	14	control	5	0.0358	22.2	21.175	0.609907	0.205306884
1561Bp5,p100	14	control	9.4	0.0864	20.186	19.501	0.77183	0.645707059
1561Bp4,p100	14	control	9.2	0.0663	16.266	15.341	0.519067	0.194471051
1562Bp1,p100	14	control	8.8	0.0657	19.843	14.8	3.403497	0.598058645
1562Bp2,p100	14	control	8	0.0567	16.116	18.322	0	0.032817632
Mean			7.5	0.0558	19.2022	18.0595	1.0004	0.3124
SEM			0.9	0.0093	1.0078	1.02	0.4934	0.1015

Quadriceps			Mass (g)		Activity		Content	
Sample	Age	Genotype	Body	Muscle	Ca^{2+}	$-Ca^{2+}$	Total Calpain	Calpastatin
1467Ap3	21	mdx	13.5	0.071	16.025	12.475	1.411438	0.22466
1467Ap4	21	mdx	12.9	0.0671	15.23	12.246	2.646875	1.587749
1467Ap5	21	mdx	13.2	0.1211	17.716	11.585	2.010227	0.75835798
1468Ap5	21	mdx	10.7	0.1056	21.207	16.759	0.418694	X
1472Ap4	21	mdx	12.1	0.0636	15.12	14.99	1.111919366	0.48492
1472Ap5	21	mdx	12.8	0.0704	18.51	16.97	7.398079	4.582604
Mean			12.5	0.0831	17.3013	14.1708	2.4995	1.5277
SEM			0.4	0.0098	0.9586	0.9742	1.0279	0.7973
1467Ap3,p100	21	mdx	13.5	0.071	13.251	14.745	0	0.088115
1467Ap4,p100	21	mdx	12.9	0.0671	14.3	20.692	2.302104	0.231946
1467Ap5,p100	21	mdx	13.2	0.1211	12.953	9.017	0	0.321952418
1468Ap5,p100	21	mdx	10.7	0.1056	14.842	23.296	0.008901	X
1472Ap4,p100	21	mdx	12.1	0.0636	36.28	10.51	0.354595221	0.385474
1472Ap5,p100	21	mdx	12.8	0.0704	36.11	8.73	2.560315	0.700308
Mean			12.5	0.0831	21.2893	14.4983	0.871	0.3456
SEM			0.4	0.0098	4.7219	2.5498	0.4977	0.1018
1448Dp1	21	control	7.8	0.0772	21.453	11.146	1.941346	2.362894
1448Dp2	21	control	7	0.0646	19.514	90.77	3.19157	5.039848
1561Ap4	21	control	11.7	0.1196	20.092	10.836	1.281347664	9.371292
1561Ap5	21	control	13.1	0.136	17.426	10.776	5.987614	2.071733
1561Bp6	21	control	12	0.1221	24.287	18.387	2.741153	0.690635064
1561Bp7	21	control	10.8	0.1153	20.299	13.463	0.879386	X
Mean			10.4	0.1058	20.5118	12.2808	2.6704	3.9073
SEM			1.0	0.0115	0.92875	1.3489	0.7516	1.5369
1448Dp1,p100	21	control	7.8	0.0772	15.722	18.506	1.077303	0.067138
1448Dp2,p100	21	control	7	0.0646	14.173	15.415	0.723328	0
1561Ap4,p100	21	control	11.7	0.1196	15.898	11.758	0.226776441	0.354802
1561Ap5,p100	21	control	13.1	0.136	16.056	12.196	0.112837	0
1561Bp6,p100	21	control	12	0.1221	10.21	9.287	0.025313	0
1561Bp7,p100	21	control	10.8	0.1153	9.636	7.062	0.073926	X
Mean			10.4	0.1058	13.6158	12.3707	0.3732	0.0844
SEM			1.0	0.0115	1.2021	1.6833	0.1749	0.0688

X- No sample for Western Analysis

Diaphragm			Mass (g)		Activity		Content	
Sample	Age	Genotype	Body	Muscle	Ca^{2+}	$-Ca^{2+}$	Total Calpain	Calpastatin
1485Ap10-12D	7	mdx	4.6	0.054	6.328	21.037	2.20373	0.250003
1485Ap1-3	7	mdx	4.6	0.0536	9.958	4.029	2.317819	1.329693
1485Ap4-6	7	mdx	3.9	0.0567	10.128	3.897	1.663901	0.018496
1485Ap7-9D	7	mdx	5	0.0549	5.969	6.356	3.15139	3.15139
1497Ap1-3D	7	mdx	5.9	0.0655	8.856	13.216	3.129069	1.847371
1497Ap4-6D	7	mdx	6.1	0.0641	9.547	5.37	3.139292	0.336951
Mean			5.0	*0.0581	8.4643	8.9842	2.6009	1.557
SEM			0.3	*0.0022	0.75532	1.9794	0.2574	0.4925
1485Ap10-12,p100D	7	mdx	4.6	0.054	18.802	44.291	0.528084	0
1485Ap1-3,p100	7	mdx	4.6	0.0536	23.784	31.95	2.698242	0.440469
1485Ap4-6,p100	7	mdx	3.9	0.0567	26.924	34.345	0.810334	0
1485Ap7-9,p100D	7	mdx	5	0.0549	16.069	16.447	0.521493	0
1497Ap1-3,p100D	7	mdx	5.9	0.0655	14.067	43.08	1.393613	0.468727
1497Ap4-6,p100D	7	mdx	6.1	0.0641	18.079	45.576	1.810673	0
Mean			5.0	*0.0581	19.6208	35.9482	1.2937	0.1515
SEM			0.3	*0.0022	1.9763	4.5167	0.3496	0.0959
1447Cp1-3	7	control	4.5	0.0564	7.513	3.317	2.636314	0.536242
1447Cp4-6	7	control	4.7	0.0525	7.457	3.828	0.939979	0
1448Bp1-6D	7	control	5.1	X	12.781	16.511	2.278711	0.675925
1448Cp1-3D	7	control	5.8	0.0541	9.562	5.857	2.741212	0.700142
1448Cp4-6D	7	control	5.5	0.0642	12.895	8.397	6.030101	5.017983
1561Ap1-3D	7	control	5.2	0.0519	11.832	6.077	2.314915	5.924954
Mean			5.1	*0.0558	10.34	7.3312	2.8235	2.1425
SEM			0.2	*0.0022	1.0267	1.9794	0.6935	1.0642
1447Cp1-3,p100	7	control	4.5	0.0564	17.714	23.699	0.792497	0.072714
1447Cp4-6,p100	7	control	4.7	0.0525	24.796	34.084	0.094164	0.016968
1448Bp1-6,p100D	7	control	5.1	X	16.985	55.757	0.440566	0.03829
1448Cp1-3,p100D	7	control	5.8	0.0541	19.611	37.848	0.862497	0
1448Cp4-6,p100D	7	control	5.5	0.0642	22.083	44.225	3.831302	1.519462
1561Ap1-3,p100D	7	control	5.2	0.0519	21.765	33.847	1.671779	0.409565
Mean			5.1	*0.0558	20.4923	38.2433	1.2821	0.3428
SEM			0.2	*0.0022	1.2037	4.4378	0.5533	0.2435

***** Mass and SEM of pooled samples from 3 pups.
X- No sample for Western Analysis

Diaphragm			Mass (g)		Activity		Content	
Sample	Age	Genotype	Body	Muscle	Ca^{2+}	$-Ca^{2+}$	Total Calpain	Calpastatin
1452Ap1D	14	mdx	8	0.0311	10.741	9.253	4.544123	4.537635
1452Ap2D	14	mdx	6.8	0.0296	10.95	9.969	2.484734	4.418411
1467Ap1D	14	mdx	10	0.0321	11.422	10.149	4.715974	10.11219
1467Ap2D	14	mdx	7.5	0.0426	14.726	12.095	5.654851	35.61062
1468Ap1D	14	mdx	8	0.0355	19.168	18.812	1.032178	1.721002
1468Ap2D	14	mdx	7.3	0.0301	24.809	28.784	2.060509	0.36772
Mean			7.9	0.0335	15.3027	14.8437	3.4154	9.4613
SEM			0.5	0.002	2.3127	3.1342	0.7385	5.4052
1452Ap1,p100D	14	mdx	8	0.0311	20.486	44.529	2.614615	1.123408
1452Ap2,p100D	14	mdx	6.8	0.0296	23.198	48.74	2.006418	2.783586
1467Ap1,p100D	14	mdx	10	0.0321	20.843	36.168	2.800186	6.745921
1467Ap2,p100D	14	mdx	7.5	0.0426	22.287	39.262	1.84907	3.667211
1468Ap1,p100D	14	mdx	8	0.0355	20.453	26.791	0.157207	1.188323
1468Ap2,p100D	14	mdx	7.3	0.0301	14.12	19.707	1.514631	0.286125
Mean			7.9	0.0335	20.2312	35.8662	1.8237	2.6324
SEM			0.5	0.002	1.3021	4.4568	0.3867	0.9641
1447Dp1D	14	control	4.8	0.015	14.398	10.14	4.94011	2.732475
1447Dp2D	14	control	5	0.018	14.47	15.168	4.739627	39.76351
1561Bp4D	14	control	9.4	0.0306	11.035	24.284	1.878818	1.471059
1561Bp5D	14	control	9.2	0.0255	17.29	10.157	X	X
1562Bp1D	14	control	8.8	0.0315	23.82	17.992	3.925987	1.874586
1562Bp2D	14	control	8	0.0271	31.142	22.854	1.580991	5.378669
Mean			7.5	0.0246	18.6925	16.7658	3.4131	10.2441
SEM			0.9	0.0027	2.045	2.4848	0.7094	7.4112
1447Dp1,p100D	14	control	4.8	0.015	22.757	47.609	4.285665	11.9701
1447Dp2,p100D	14	control	5	0.018	12.452	28.529	7.345393	23.84334
1561Bp4,p100D	14	control	9.4	0.0306	21.833	40.019	1.556581	0.525846
1561Bp5,p100D	14	control	9.2	0.0255	16.48	22.076	1.822401	0.499566
1562Bp1,p100D	14	control	8.8	0.0315	12.122	19.746	0.764071	0.52302
1562Bp2,p100D	14	control	8	0.0271	22.53	29.842	0.487297	1.574089
Mean			7.5	0.0246	18.029	31.3035	2.7102	6.4893
SEM			0.9	0.0027	2.0451	4.3604	1.0773	3.926

X- No sample for Western Analysis

Diaphragm			Mass (g)		Activity		Content	
Sample	Age	Genotype	Body	Muscle	Ca^{2+}	$-Ca^{2+}$	Total Calpain	Calpastatin
1467Ap3D	21	mdx	13.5	0.0417	32.654	28.794	1.565624	0.776173
1467Ap4D	21	mdx	12.9	0.0462	25.218	22.07	1.903655	2.69503
1467Ap5D	21	mdx	13.2	0.0485	28.216	15.316	4.066518	2.287855
1468Ap3D	21	mdx	11.1	0.05	27.027	19.959	1.828806	1.06238
1472Ap4D	21	mdx	12.1	0.0309	30.46	34.397	1.280076	0.128897
1472Ap5D	21	mdx	12.8	0.0489	38.357	32.988	3.92864	6.72144
Mean			12.6	0.0444	30.322	25.5873	2.4289	2.2786
SEM			0.4	0.003	1.9267	3.121	0.5048	0.9707
1467Ap3,p100D	21	mdx	13.5	0.0417	14.597	20.964	0.084278	0
1467Ap4,p100D	21	mdx	12.9	0.0462	23.037	38.248	0.761779	1.011874
1467Ap5,p100D	21	mdx	13.2	0.0485	19.33	33.996	0.059106	0.050285
1468Ap3,p100D	21	mdx	11.1	0.05	26.393	41.564	0.142201	0.341465
1472Ap4,p100D	21	mdx	12.1	0.0309	31.855	34.588	0.263154	0
1472Ap5,p100D	21	mdx	12.8	0.0489	16.605	28.498	0.410732	0
Mean			12.6	0.0444	21.9695	32.9763	0.2869	0.2339
SEM			0.4	0.003	2.6365	2.9974	0.1088	0.1648
1448Dp1D	21	control	7.8	0.0364	19.27	10.06	2.221074	0.768168
1448Dp2D	21	control	7	0.0432	22.964	15.143	2.563227	0.828275
1561Ap4D	21	control	11.7	0.0479	29.333	20.419	0.946879	0.157529
1561Ap5D	21	control	13.1	0.0381	43.463	29.8	4.335162	50.51078
1561Bp6D	21	control	12	0.0455	29.505	22.504	3.25248	0.544477
1561Bp7D	21	control	10.8	0.0491	41.956	23.715	1.813395	0.952061
Mean			10.4	0.0434	31.0818	20.2735	2.522	8.9602
SEM			1.0	0.0021	4.0104	2.818	0.4795	8.3109
1448Dp1,p100D	21	control	7.8	0.0364	28.532	22.187	0.671726	0
1448Dp2,p100D	21	control	7	0.0432	29.837	23.303	0.601736	0
1561Ap4,p100D	21	control	11.7	0.0479	12.723	21.379	0.061144	0.015568
1561Ap5,p100D	21	control	13.1	0.0381	14.695	26.556	3.959789	8.134841
1561Bp6,p100D	21	control	12	0.0455	37.185	30.492	0.302491	0.254697
1561Bp7,p100D	21	control	10.8	0.0491	39.904	28.305	0.632387	0.92963
Mean			10.4	0.0434	27.3127	25.3703	1.0382	1.5558
SEM			1.0	0.0021	4.6786	1.4898	0.5922	1.3239

Appendix C
Statistical Analysis

Appendix C

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0883	.0083	7.93	.0085
Age (days)	2	.5383	.2691	24.18	<.0001
Genotype x Age	2	.04815	.0241	2.16	.1326
Residual	30	.3339	.0111		
Total	35	1.0086			

Two-way Analysis of Variance for Quadriceps Supernatant Activity+Ca^{2+}

Two-way Analysis of Variance for Quadriceps Supernatant Activity - Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0003	.0003	.02	.8986
Age (days)	2	.3671	.1836	7.92	.0017
Genotype x Age	2	.0200	.0100	.43	.6534
Residual	30	.6951	.0232		
Total	35	.0011			

Two-way Analysis of Variance for Quadriceps Pellet Activity + Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	.7454	.7454	6.91	.0134
Age (days)	2	2.1420	1.0710	9.93	.0005
Genotype x Age	2	.8178	.4089	3.79	.0341
Residual	30	3.2364	.1079		
Total	35	6.9416			

Two-way Analysis of Variance for Quadriceps Pellet Activity - Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0342	.0342	.54	.4676
Age (days)	2	1.1100	.5550	8.78	.0010
Genotype x Age	2	.3888	.1944	3.08	.0609
Residual	30	1.8961	.0632		
Total	35	3.4291			

Two-way Analysis of Variance for Quadriceps Supernatant Total Calpain Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	2.2189	2.2189	.14	.7108
Age (days)	2	26.3834	13.1917	.83	.4445
Genotype x Age	2	2.5357	1.2678	.08	.9232
Residual	30	474.9870	15.8329		
Total	35	506.1250			

Two-way Analysis of Variance for Quadriceps Pellet Total Calpain Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	8.5331	8.5331	1.00	.3244
Age (days)	2	22.9454	11.4727	1.35	.2747
Genotype x Age	2	2.7961	1.3981	.16	.8491
Residual	30	255.0596	8.5019		
Total	35	289.3343			

Two-way Analysis of Variance for Quadriceps Supernatant Calpastatin Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	3.7720	3.7720	.27	.6062
Age (days)	2	136.6539	68.3269	4.92	.0147
Genotype x Age	2	11.6879	5.8439	.42	.6603
Residual	28	388.4898	13.8746		
Total	33	539.5737			

Two-way Analysis of Variance for Quadriceps Pellet Calpastatin Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	2.0620	2.0620	.46	.5025
Age (days)	2	75.7791	37.8896	8.48	.0013
Genotype x Age	1	1.9649	.9825	.22	.8040
Residual	28	125.1187	4.4685		
Total	33	205.0569			

Two-way Analysis of Variance for Diaphragm Supernatant Activity +Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	2.7967	1.3984	38.80	<.0001
Age (days)	2	.0363	.0363	1.01	.3236
Genotype x Age	2	.0105	.0052	.15	.8656
Residual	30	1.0812	.0360		
Total	35	3.9247			

Two-way Analysis of Variance for Diaphragm Supernatant Activity -Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0255	.0255	.56	.4598
Age (days)	2	1.3099	.6549	14.43	<.0001
Genotype x Age	2	.0785	.0393	.87	.4311
Residual	30	1.3615	.0454		
Total	35	2.7754			

Two-way Analysis of Variance for Diaphragm Pellet Activity +Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0161	.0161	.4	.5310
Age (days)	2	.2089	.1045	2.61	.0903
Genotype x Age	2	.0864	.0432	1.08	.3532
Residual	30	1.2022	.0401		
Total	35	1.5137			

Two-way Analysis of Variance for Diaphragm Pellet Activity -Ca^{2+}

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0975	.0975	1.08	.1405
Age (days)	2	.3782	.1891	2.10	.3068
Genotype x Age	2	.1543	.0772	.86	.4351
Residual	30	2.7052	.0902		
Total	35	3.3352			

Two-way Analysis of Variance for Diaphragm Supernatant Total Calpain Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	.0951	.0951	.05	.8280
Age (days)	2	5.3844	2.6922	1.36	.2723
Genotype x Age	2	.0733	.0366	.02	.9817
Residual	29	57.3709	1.9873		
Total	34	62.9603			

Two-way Analysis of Variance for Diaphragm Pellet Total Calpain Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	2.6448	2.6448	1.26	.2708
Age (days)	2	15.6950	7.8476	3.740	.0356
Genotype x Age	2	1.4070	.7035	.33	.7181
Residual	30	63.0286	2.1009		
Total	35	82.7754			

Two-way Analysis of Variance for Diaphragm Supernatant Calpastatin Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	69.1200	69.1200	.49	.4909
Age (days)	2	384.5949	192.2975	1.35	.2739
Genotype x Age	2	66.1030	33.0515	.23	.7938
Residual	29	4116.6490	141.9534		
Total	34	4638.0967			

Two-way Analysis of Variance for Diaphragm Pellet Calpastatin Content

Source of Variation	DF	SS	MS	F	P
Genotype	1	28.8374	28.8374	1.59	.2177
Age (days)	2	129.8685	64.9342	3.57	.0407
Genotype x Age	2	21.1412	10.5906	.58	.5655
Residual	30	545.7306	18.1910		
Total	35	725.5777			

Appendix D
Calpain Activity Assay + Inhibitors

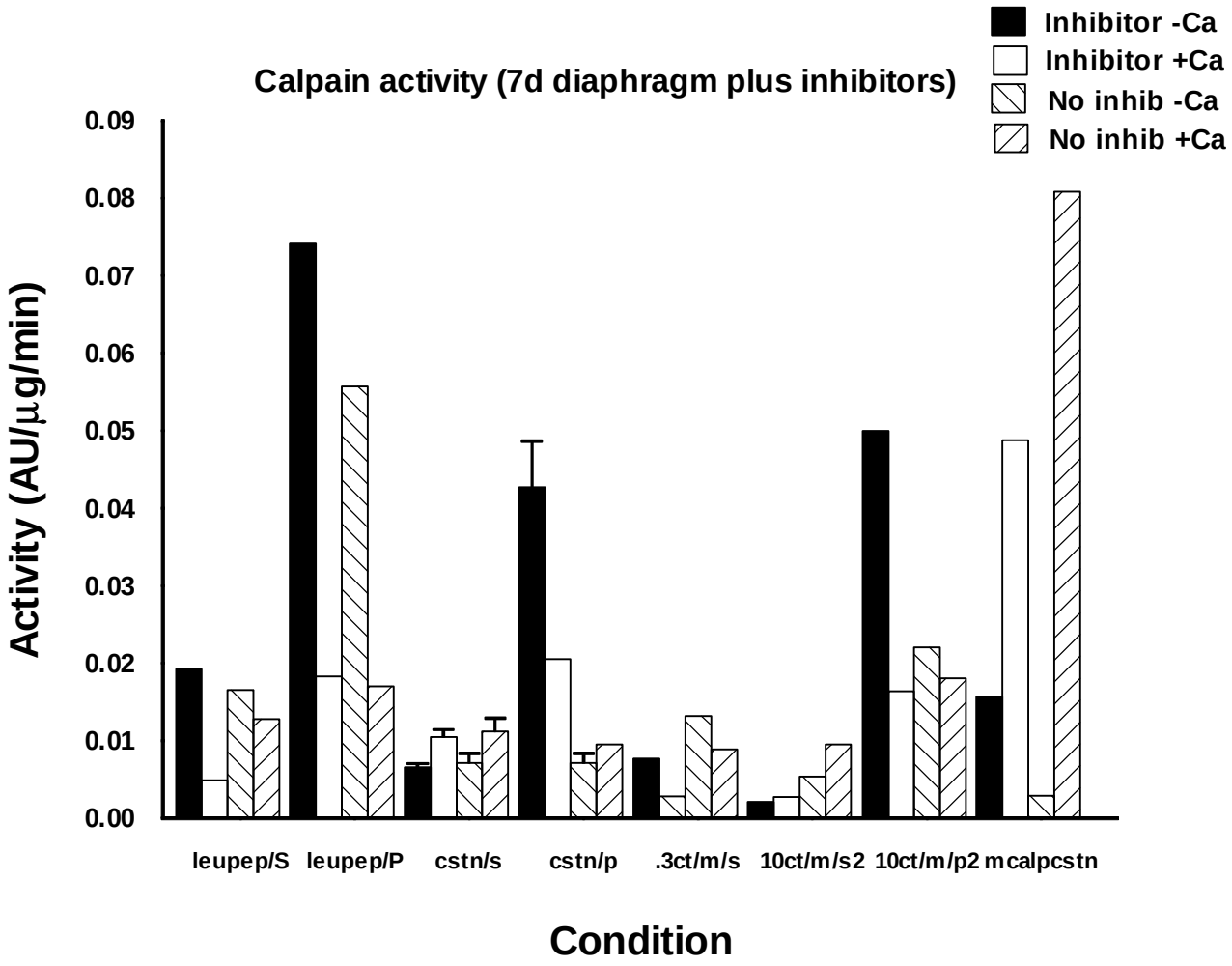

Figure 18. Seven day diaphragm activity with inhibitors. Leupep=leupeptin, s=supernatant, p=pellet, cstn=calpastatin, .3ct/m/s=3ìl of inhibitor cocktail with mdx supernatant, 10ct/m/s2=10ìl of inhibitor cocktail with mdx supernatant, 10ct/m/p=10ìl of inhibitor cocktail with mdx pellet, m calp cstn= m-calpain standard +calpastatin. The inhibitor cocktail contained in mM: 104 AEBSF, 0.08 aprotinin, 2.1 leupeptin, 3.6 bestatin, 1.5 pepstatin A, 1.4 E-64. This inhibitor cocktail had a wide specificity for the inhibition of serine, cysteine, aspartic, and amino-peptidases.

Vita

Kati E. Draper, the daughter of Tom and Judy Draper, was born on November 16, 1979 in Youngstown, Ohio. She graduated from Boardman High School in June 1998. She attended Westminster College in New Wilmington, Pennsylvania where she received her Bachelor of Science in Biology in May 2002. Kati will graduate with a Master of Science in Human Nutrition, Foods and Exercise from Virginia Polytechnic Institute and State University in May 2004.