The activity and content of calpains in maturing dystrophic muscle membranes

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ABSTRACT

Increased calcium-activated calpain proteolysis in the sarcolemma membrane is thought to be a primary mechanism in the pathophysiology of Duchenne Muscular Dystrophy (DMD). However, few studies have tested this possibility prior to the overt signs of the dystrophy. The purpose of this study was to test the hypothesis that there is greater calpain content and total relative calpain activity in membranes obtained from dystrophic (mdx; mdx:utrophin-deficient (mdx:utrn$^{-/-}$)) compared to wildtype (wt) mouse skeletal muscles during maturation at ages 7- and 21-d,and at a post-maturation age of 35-d. Calpain activity was determined as the calcium-dependent cleavage of the flurogenic substrate SLY-AMC, and content was determined by Western analysis with an anti-calpain antibody. There were several intriguing findings:

1. There was an inverse relationship between calpain content and relative activity in the whole muscle in both wt and mdx mice from age 7- to 35-d: calpain content decreased, and relative calpain activity increased as the mice aged. This suggests a similar role for calpain in both genotypes, which might relate to specific maturation processes, possibly up to age 21-d. Although the inverse relation was evident at 35-d, the targets for calpain in mdx compared to wt likely differed.

2. The increased relative calpain activity in the membrane fraction of mdx mice at age 35-d (26.73 Arbitrary Units, (AU)) compared to that of age 7-(4.9AU; $p<0.05$) and 21-d (8.74AU; $p<0.05$) is temporally related to degeneration and regeneration processes, and may also indicate activation of apoptosis, in mdx muscles at this age.
3. At age 7-d, there were no significant differences in either calpain content or relative calpain activity in all subcellular fractions for wt and mdx mice. This result might suggest similar calpain distribution and activities that are related to the regulation of muscle maturation and differentiation in both genotypes. (Note:data were not obtained for the mdx:utrn$^{-/-}$ mice at age 7-d because of insufficient animals).
4. At age 21-d, there was greater relative calpain activity in the myofibrillar supernatant fraction in mdx (15.13AU) than wt mice (1.18AU; $p<0.05$). This could indicate calpain's role in the initiation of myofibrillar protein turnover and the proteolysis of submembranous networks in the mdx muscles.
5. At age 21-d, greater calpain content in the mdx (1.40µg) compared to wt (0.23 µg; $p<0.05$) membrane fraction might suggest a broader distribution of calpain along membranes that contributes to the onset of dystrophy in the mdx muscles.
6. At age 35-d, there was greater calpain content in the mdx:utrn$^{-/-}$ compared to the wt membrane (0.48µg vs 0.13 µg), cytosolic (0.88µg vs 0.30µg), and myofibrillar supernatant (0.49µg vs 0.17µg; $p<0.05$) fractions This

increased content and broad distribution across several subcellular fractions may reflect degeneration and regeneration processes, and potentially activation of apoptosis, in the mdx:utrn$^{-/-}$ muscles.

These data suggest that calpain activity contributes to dystrophic pathophysiology mainly in the membrane fraction of mdx skeletal muscles at age ~21-d, but appears to contribute later at 35-d and in more subcellular fractions in mdx:utrn$^{-/-}$ skeletal muscles.

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List of Important Definitions

DMD: Duchenne Muscular Dystrophy

DGC: Dystrophin Glycoprotein Complex

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

ECM: Extracellular Matrix

SR: Sarcoplasmic reticulum

SLY-AMC: Suc-Leu-Tyr-7-Amido-4-methylcoumarin, a specific fluorogenic calpain substrate

EDTA: ethylene diamine tetraacetic acid, a calcium chelator

EGTA: ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N' -tetraacetic acid, a calcium chelator

DTT: Dithiothreitol, reduces disulfide bonds and maintain monothiols.

PMSF: Phenyl methyl sulfonyl fluoride, a serine protease inhibitor

Chapter 1: Introduction

Introduction

Duchenne Muscular Dystrophy (DMD) is a recessive X-linked, progressive muscle-wasting disease affecting ~1 in 3,500 boys (Emery, 1989). It is characterized by broad protein proteolysis and subsequent cell necrosis, which results in the progressive wasting of skeletal muscle (Blake et al., 2002).

Generally, DMD patients are clinically normal at birth. The first symptoms are observed between ages 2~5 years, with the children first exhibiting with a waddling gait, followed by progressive impaired mobility, and the eventual need for a wheelchair before the age of 12 (Blake et al., 2002). In addition to impaired mobility, there is also intellectual impairment and a steady deterioration of pulmonary function. Eventually, patients die of cardiac failure and/or respiratory insufficiency in their late teens.

DMD is caused by mutations in the *dystrophin* gene that encodes the cytoskeletal protein, dystrophin. Dystrophin is a membrane-associated protein, present in vertebrate skeletal, cardiac and smooth muscle. Multiple independent nonsense point mutations in the dystrophin gene result in either the complete absence or greatly reduced levels of the protein product in human DMD and the dystrophic mdx mouse model (Blake et al., 2002).

The dystrophin glycoprotein complex (DGC) is a main component of the sarcolemma membrane. It associates with dystrophin to form a physical connection between the intracellular skeleton (via actin filaments) and the extracellular basal lamina (via laminins). A submembrane network is established through dystrophin by

the interactions of the N-terminal sites with F-actin (Levine et al., 1992) and the C-terminal with the DGC (Ervasti and Campbell, 1991). Therefore, dystrophin serves as a structural element in muscle fibers. The membrane hypothesis of DMD suggests that loss of dystrophin leads to defects in the cytoskeleton, which could render the muscle membrane more vulnerable to mechanical damage (Hutter, 1992). This view is supported by the demonstration of discontinuities in muscle plasma membrane from electron-microscopic studies (Rowland, 1976). However, it is still unclear if loss of dystrophin leads to fragility of the sarcolemma membrane and loss of the actin-filament association with the plasma membrane during early maturation or following maturation.

The precise mechanisms leading to onset of DMD have not yet been defined. A number of studies support the hypothesis that calcium-activated proteolysis as a result of elevated intracellular calcium levels (Turner et al., 1988), leads to an increased myofibrillar protein degradation rate in dystrophic muscle (Blake et al., 2002; Spencer et al., 1995; Zeman et al., 1985). Membrane lesions have been suggested as a mechanism for disrupting intracellular calcium homeostasis (Alderton and Steinhardt, 2000b; McCarter and Steinhardt, 2000), which could result from an increased frequency of transient sarcolemma wounds that occur during contraction in the dystrophic muscle fibers (Alderton and Steinhardt, 2000b), and thereby activate broad calcium-dependent proteolysis (Alderton and Steinhardt, 2000b; Spencer et al., 1995). Thus, calcium-dependent proteolysis is considered largely responsible for the elevated calcium-sensitive proteolysis in mdx myotubes (Alderton and Steinhardt, 2000a). An abnormal calcium level may also act as a

primary trigger in the progressive downstream events that lead to muscle weakness, by mediating an elevated rate of protein degradation (Spencer et al., 1995). In an environment of increased intracellular calcium, it is likely the calpains are responsible for the increased calcium-dependent proteolysis.

Calpains, or calcium-activated neutral proteases, are a large family of calcium dependent cysteine/thiol proteolytic enzymes expressed ubiquitously (Carafoli and Molinari, 1998). The two major isoforms, μ-calpain (calpain I) and m-calpain (calpain II) are both calcium-dependent, but differ in their calcium requirement for half-maximal activation when assayed in vitro, with ranges for μ-calpain of 1-100μM and m-calpain of 0.1-1 mM (Berchtold et al., 2000). Both isoforms consist of a large 80 kD catalytic subunit and a small 30 kD regulatory subunit. A third isoform, p94, is calcium-independent (Carafoli and Molinari, 1998).

Calpains are not solely cytosolic proteases, as they can translocate to cytoskeletal structures for activation. These structures include plasma membrane, Endoplasmic Reticulum (ER) and Golgi apparatus (Hood et al., 2003). The C2-like lipid-binding domain in calpain is considered responsible for calpain binding to phosphatidylinositol bisphosphate (PIP2), which is enriched in the plasma membrane (Hood et al., 2003). When localized in the close vicinity of their potential substrates (i.e., cytoskeletal–associated molecules and cell signaling molecules), calpains likely undergo a conformational change that increases their sensitivity to activation by intracellular calcium (Hood et al., 2003). Once activated, the calpains are capable of cleaving substrates near these subcellular structures. Calpains have a relatively strict specificity for their substrates so that they usually cleave at only a

limited number of sites, which is different from digestive proteases with broad target specificities (Berchtold et al., 2000). An important aspect of calpains is their ability to modify rather than completely degrade substrates by cleaving between functional domains rather than within each domain, providing fragments of substrate proteins that may alter cell structural integrity and/or signaling pathways (Carafoli and Molinari, 1998; Perrin and Huttenlocher, 2002). Many calcium channels are calpain substrates, including Ca^{2+}-ATPase, L-type calcium channels, and the ryanodine receptor (RyR) (Berchtold et al., 2000).

Recent studies have reported that calpain concentration and/or levels of auto-proteolytic modification vary over the course of the disease in *mdx* mice, which coincides with the particular stage of the disease process (Spencer et al., 1995): total calpain is increased in peak necrotic *mdx* mice (4 weeks of age) and in completely regenerated *mdx* mice (14 weeks of age), but not in prenecrotic mice (2 weeks of age); the auto-proteolytic activation of μ-calpain was highest during peak necrosis, and returned to levels near controls in regenerated muscle. Further studies showed that calpains translocation also corresponded to the state of pathophysiology of each dystrophic fiber (Spencer and Tidball, 1996).

Collectively, these studies suggest a potentially important role for calpains in the pathogenesis of DMD. However, calpain activities and content in maturing dystrophic muscle fibers (e.g., up to and including a weaning age of 21-d) have not been well studied. The purpose of this study, therefore, was to evaluate the calcium-dependent calpain activity and content in different subcellular fractions of wild type (wt) and dystrophic mouse genotypes during early maturation at ages 7- and 21-d,

and for comparison during a more advanced stage of disease, at a post weaning age of 35-d, to determine if calpain proteolysis contributes to DMD onset. The two dystrophic mouse models used in this study were mdx which lacks dystrophin, and mdx:utrn$^{-/-}$, which lacks both dystrophin and utrophin (a protein homolog of dystrophin). The overall aim was to determine if total calpain activity and content were greater in the dystrophic compared to the wild type quadriceps muscles during early maturation to determine if calpain activity was an early contributor to DMD pathogenesis.

Statement of Problem

The relation between the absence of dystrophin and the onset of DMD has not yet been clearly defined. Absence of dystrophin and the DGC from skeletal muscle sarcolemma membranes could potentially lead to muscle membrane fragility, and/or increased leakage of calcium from outside to inside the fiber. The membrane hypothesis suggests that calcium influx through membrane defects and/or leaky channels could activate calcium-dependent calpain proteolysis. If such defects were evident during maturation, activation of calcium-dependent calpains could represent an onset mechanism for DMD.

To test this possibility, calpain activity should be assessed during maturation (e.g., age 7-d to 21-d). Previously in our lab, we used an ultra-centrifugation method to separate homogenized dystrophic quadriceps muscles into supernatant and pellet fractions following a 100,000 g spin at ages 7-, 14- and 21-d. A primary result was increased calcium-dependent calpain activity in the dystrophic (*mdx*) pellet fraction

at age 7-d (Draper, unpublished). The pellet fraction from this method should contain myofibrillar proteins, sarcolemmal and SR plasma membranes as well as microsomes (Sultan et al., 2000). Thus, the proteolytic activity in the sarcolemmal membrane fraction alone could not be discriminated. The determination of increased calpain activity in the membrane fraction of maturing dystrophic muscle could help determine calpain's role in the onset of DMD. Calpain content should also be determined to better understand if calpain activity and content are closely related.

Therefore, the purpose of this study was to determine if greater calcium-dependent calpain activity and content were present in the membrane fraction of dystrophic compared to wild type quadriceps muscles at ages 7-, 21-, and 35-d using a calpain activity assay and Western analysis, respectively. The primary aim of the study was to determine if calpain activity was predominant in the membrane compared to the myofibrillar fraction of dystrophic skeletal muscle, and to determine if this occurred before (7-d) or at weaning age (21-d), or following weaning (35-d).

Significance of study

Duchenne Muscular Dystrophy is a severe and fatal muscle-wasting disease affecting 1 in 3,500 boys worldwide (Emery, 1989). Patients usually present with a waddling gait or difficulties in climbing stairs at ages 2~5 years, are confined to a wheelchair before the age of 12, and die in their late teens or early twenties of cardiac and/or respiratory failure.

The identification of the DMD gene, *dystrophin*, on the X-chromosome was a major accomplishment in human disease research. However, currently there is no

treatment available to significantly increase the lifespan of DMD patients, although the genetic basis of the disease has been known for over 18 years (Hoffman et al., 1987).

The onset mechanisms of the disease are presently not known. However, the membrane hypothesis suggests that the loss of dystrophin will lead to lesions in the sarcolemma membrane, which renders the muscle membrane more vulnerable to mechanical damage (Hutter, 1992). The lesions may also cause disrupted intracellular calcium homeostasis (Alderton and Steinhardt, 2000b; McCarter and Steinhardt, 2000). Unregulated intracellular calcium could activate calcium-dependent proteolysis in muscle fibers (Alderton and Steinhardt, 2000b; Spencer et al., 1995). Calpains are a large family of calcium-dependent cysteine/thiol proteolytic enzymes, that are expressed ubiquitously (Carafoli and Molinari, 1998). Increased calpain activity has been demonstrated in *mdx* mice and *mdx* myotubes (Alderton and Steinhardt, 2000a; Spencer et al., 1995), suggesting a role for calpain in the pathophysiology of DMD, but the time course of the increased calpain activity, particularly during early maturation (e.g., age 7-21 d) is not well defined. If calpain is found to be an important determinant in the onset of DMD, therapeutic treatments could be developed to delay or prevent the onset of DMD, and thereby ameliorate the severity of DMD and elongate the lifespan of patients.

Specific Aims

1. To test the hypothesis that calpain activity in maturing skeletal muscle membranes is temporally related to the onset of DMD.
2. To test the hypothesis that greater calcium-dependent calpain content and activity are evident in the membrane fraction of skeletal muscles obtained from maturing *mdx* and *mdx:utrn*$^{-/-}$ dystrophic mice.

Research Hypotheses

Main Hypotheses:

1. Increased calcium-dependent calpain proteolytic activity in maturing dystrophic skeletal muscle membranes contributes to the onset of DMD.
2. The increased calcium-dependent calpain proteolytic activity is predominantly in the dystrophic skeletal muscle membrane fraction.

Specific Hypotheses:

H_0: Calpain activity will not be different between the mouse genotypes or the quadriceps muscles at any of the ages.

H_1: Calpain content will be greater in the membrane compared to the myofibrillar or cytosolic fractions.

H_2: Calpain activities will be greater in the presence compared to absence of calcium in all subcellular fractions, and this will be true for both dystrophic and control muscles obtained from maturing mice.

H_3: Calcium-dependent calpain activities will be greater in the dystrophic (*mdx* and *mdx:utrn* $^{-/-}$) compared to the control membrane fractions at each age.

H_4: Calcium-dependent calpain activity will be increased in the dystrophic membrane fraction as mice mature.

H_5: Calcium-dependent calpain activities in the mdx:utrn$^{-/-}$ membrane fraction will be greater than those of the mdx membrane fraction at each 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 and mdx:utrn$^{-/-}$ mice are suitable models for the study of DMD.
4. The muscle homogenates and resultant fractions retained calpain activity.
5. The fluorogenic substrate SLY-AMC was specific only to calpain proteolysis.
6. The total calpain antibody used in this study was specific to detect the μ- and m- isoforms of calpain, and the band intensities on Western blots determined by densitometry reflected the content of calpain in each sample, and thus could be normalized to the intensity of the band representing a known amount of purified μ-calpain on the same blot.
7. The affinity of μ- and m-calpain for the antibody was the same; therefore, the densities of both bands could be summed and normalized to the purified μ-calpain.
8. Calpain activities measured in this study in vitro reflect calpain activity in vivo.
9. The contribution of the calpain isoform, p94, to calpain proteolysis was negligible.

Limitations

The following were limitations of the current study:

1. The current study was limited to the quadriceps of mice due to the sample mass requirement for the calpain assay.
2. The membrane fraction following the ultra-spin speed (135,000 g) was likely composed of sarcolemma and other cellular membranes, therefore it was not a purified sarcolemmal membrane preparation.
3. P94, a third isoform of calpain, is calcium–independent, therefore not considered in total calpain activity or content.
4. Calpain activity measured at a specific calcium concentration in this study does not truly reflect its real activity in vivo.

Chapter 2: Literature review

Duchenne Muscular Dystrophy (DMD) is a severe recessive X-linked, progressive muscle-wasting disease affecting ~1 in 3,500 boys. It is characterized by the absence of dystrophin and the dystrophin-glycoprotein complex (DGC) that yields muscle membrane fragility and/or loss of signaling (Blake et al., 2002). The onset mechanisms of DMD and their time course remain undefined.

Clinical characteristics of DMD

The onset of DMD starts at age 2~5 years in 90% of the cases. These children are characterized by physical and mental development retardation. A delay in speech and motor development are most noticeable; walking is delayed for at least 5 months compared to a normal child. Muscles undergoing in progressive wasting are bilateral and symmetrical, starting from the lower limbs. Weakened hip abductor muscles leads to a waddling gait, and the weakened knee and hip extensors cause difficulties in climbing stairs. Contractures arise at the joints, such as elbows, knees, and hips. By the age of 12, 95% of patients are confined to a wheelchair (Emery, 1989).

Necrotic or degenerating muscle fibers are characteristically seen in all postnatal DMD muscle biopsies even before muscle weakness is clinically observed (Blake et al., 2002). Serum levels of the muscle isoform of creatine kinase are elevated after birth (Blake et al., 2002). Another sign in early dystrophic pathophysiology is the active regeneration of muscle to replace or repair lost or damaged fibers. Regenerating fibers are characterized by small fiber diameter, and large, centrally placed myonuclei (Bell and Conen, 1968). Eventually, regeneration

ceases, and muscle fibers are gradually replaced by adipose and fibrous connective tissue, giving rise to the clinical appearance of pseudohypertrophy, which is followed by atrophy (Emery, 1977). The combination of progressive fibrosis and muscle fiber loss results in muscle wasting and ultimately muscle weakness.

Dystrophin gene and its mutations in DMD

The dystrophin gene was completely cloned in 1987 (Koenig et al., 1987), and the protein dystrophin was identified in 1987 (Hoffman et al., 1987). The dystrophin gene is one of the largest genes described to date, spanning ~2.5Mb of genomic sequence. The full-length mRNA transcript is 14kb, and is predominantly expressed in skeletal and cardiac muscle with smaller amounts in brain.

Besides a full-length gene transcription product, the *dystrophin* gene also has at least four internal promoters, which give rise to shorter dystrophin transcripts that encode truncated C-terminal isoforms. These isoforms are primarily expressed in nonmuscle tissues including the brain, central and peripheral nervous system, and lung, and are thought to provide the necessary binding sites for a number of dystrophin-associated proteins (Schofield et al., 1994). However, the molecular and cellular function of these isoforms has not yet been elucidated.

Dystrophin proteins (Figure 1) can be categorized into four separate domains: (1) the NH_2 terminus, (2) the central rod domain, (3) the cysteine-rich domain, and (4) the COOH-terminal domain. The high mutation frequency in *dystrophin* ($1X10^{-4}$ genes/generation) gives rise to hundreds of independent mutations. Most mutations result in the complete absence, or much reduced levels of the protein product, while

some generate partially functional protein products as in Becker muscular dystrophy (BMD), a milder case of muscular dystrophy that is genetically allelic with DMD (Blake et al., 2002).

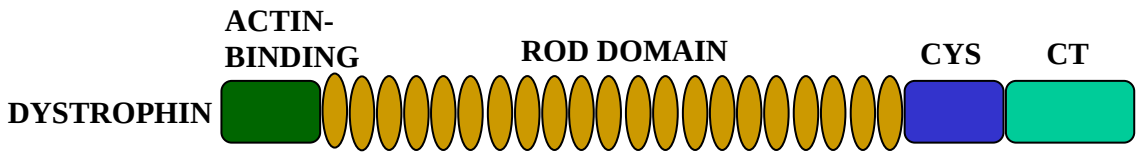

Figure 1. Domain structure of the dystrophin protein. (Modified from Blake et al., 2001)

The mutations in the *dystrophin gene* can be classified into two main categories according to the size of the deletion: large and small. The vast majority of large deletions are clustered around two mutation "hot spots", which result in the loss of part of the rod domain and/or the actin-binding domain (Kunkel et al., 1989; Liechti-Gallati et al., 1989). The rod domain can have large in-frame deletions without serious clinical consequences, which has facilitated the design of dystrophin "mini-genes" for gene therapy approaches. One third of DMD cases are caused by very small deletions and point mutations, most of which generate premature stop codons (Lenk et al., 1993; Roberts et al., 1994). These small mutations are distributed evenly throughout the gene (Gardner et al., 1995), but because only a few protein products have been detected so far, it is likely that the corresponding transcripts or the truncated proteins are unstable (Hoffman et al., 1987).

Sarcolemma membrane and its associated structures

Muscle fibers are coated by a layer of extracellular matrix material called the basement membrane (Figure 2). The lipid bilayer of the plasma membrane of muscle fibers is called the sarcolemma. There are several large protein complexes

(i.e., the dystrophin-glycoprotein complex, the focal adhesion complex), and specialized regions/structures (i.e., T-tubule, costamere) localized at or associated with the sarcolemma that are important for its integrity and provide for the cell communication between the muscle fiber and extracellular matrix.

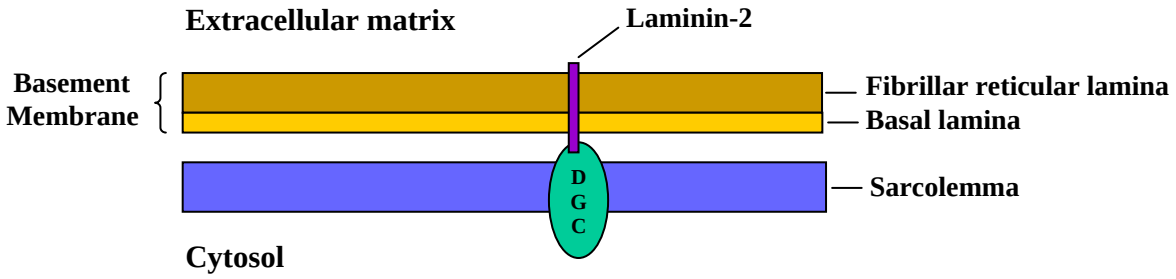

Figure 2. Schematic structure of the skeletal muscle plasma membrane. (DGC: dystroglycan complex)

• Basement membrane

The function and maintenance of tissue integrity are dependent on specific interactions of cells with the surrounding extracellular matrix (Mayer, 2003). Skeletal muscle fibers are coated by a layer of extracellular matrix material called the basement membrane (BM). The BM is composed of two layers: an internal basal lamina (BL), which lies approximately 25 nm from the plasma membrane, and an external fibrillar reticular lamina (Bloch et al., 2002; Sanes, 2003). BMs contain protein and carbohydrate, but no lipid or nucleic acid. The fibrils of the reticular lamina are collagenous, and are embedded in an amorphous proteoglycan-rich ground substance (Sanes, 2003).

Trans-membrane receptors are involved in polymerization and assembly of the extracellular matrix and in addition provide both a mechanical link to the cytoskeleton and a means of transducting signals from the extracellular matrix to the nucleus (Mayer, 2003). Such transmembrane receptors include the DGC, integrin receptors, ion receptors, such as the Na^+/K^+-ATPase, and many others (Sanes, 2003).

- Laminin

The lamina is a group of glycoproteins present in the basal lamina (Figure 3). Each laminin is a heterotrimer assembled from α, β, and γ chain subunits. There are currently 10 known laminin chains: α1-5, β1-3, γ1 and 2 (Colognato and Yurchenco, 2000). The lamina can self-assemble, and constitutes major ligands for cell surface receptors (i.e., α-dystroglycan) involved in the transmission of force from the cell interior (Gullberg et al., 1999). Laminin binds to matrix macromolecules, and interacts with integrins, dystroglycan, and other receptors. In this way, laminins contribute to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival (Colognato and Yurchenco, 2000). The importance of the lamina for muscle integrity is specifically demonstrated in merosin-deficient congenital muscular dystrophy, which is caused by defects in the laminin α2 chain (merosin) (Patton et al., 1999).

Recent advances in understanding the function and distribution of various laminin subunits have revealed a difference between normal and dystrophic membranes. In control muscles, α2, β1 and gamma 1 were abundant in the basal lamina surrounding the fibers; α1, α3-5, β3 and γ2 were undetectable; and, β2 was

present at a low level. Laminin α2, α4 and α5 are the major laminin chains in the basement membrane during muscle formation, but laminin α4 and α5 chains are absent in adult muscle (Gullberg et al., 1999). However, in dystrophic patients, levels of α5 were increased, which might reflect the regenerative process (Patton et al., 1999).

- Lipid composition of sarcolemma

Mammalian cell membranes consist of a lipid bilayer composed primarily of phospholipids and cholesterol (Spector and Yorek, 1985). There are three major kinds of membrane lipids: phospholipids, glycolipids and cholesterol, with their percentage distribution variable in animal membranes. The lipids in skeletal muscle membranes are made up of one-third neutral lipid and two-thirds phospholipids, and are predominantly located in membrane structures such as, the sarcolemma. Neutral lipids (i.e., cholesterol esters and triglycerides) are not charged, and serve not only as a source of energy but also as a depot of components required for the formation of biological membranes and thus contribute to maintenance and balanced formation of subcellular structures (Buhman et al., 2001). The main fatty acids in muscle phospholipids are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and arachidonic acids (20:4) (the numbers in parantheses indicate the chain length and the degree of saturation, e.g., palmitic (16:0) denotes a sixteen carbon (C_{16}) fatty acid with no double bonds (0)) (Fiehn et al., 1971; Spector et al., 1979).

Owens and Hughes (1970) characterized the lipid composition in several muscle subcellular fractions (myofibrillar, mitochondrial, and microsomal) of normal and dystrophic mice aged 2-4 months. They found no differences in the fatty acid

composition of neutral lipid extracts from normal and dystrophic preparations, but there was a significant decrease in the percentage of 22:6 phospholipids in the dystrophic microsomal fraction, which could reflect an altered phospholipid pattern (Owens and Hughes, 1970).

- DGC

The relation between dystrophin, the DGC, and the onset of DMD has not been defined. However, the membrane hypothesis suggests changes in the sarcolemma membrane are central to the development of muscular dystrophy. The membrane hypothesis states that the loss of dystrophin and the disruption of the DGC leads to defects in the cytoskeleton, which renders the muscle membrane more vulnerable to mechanical damage (Hutter, 1992). The outcomes are sarcolemmal instability, membrane tears, and eventual muscle fiber necrosis (Campbell, 1995; Mendell et al., 1995). Therefore, a better understanding of the structure and function of the membrane protein complex and its associated proteins is fundamental to the understanding of the pathophysiology of DMD.

One of the primary functional components of the sarcolemma is the dystrophin-glycoprotein complex (DGC, Figure 3), also known as the dystrophin-associated protein complex (DAPC). The DGC along with associated dystrophin, forms a critical membrane cytoskeletal structure, spanning the sarcolemma membrane in a mosaic pattern (Watkins et al., 2000). The complex functions as a scaffold to connect the cytoplasmic myofibrillar contractile elements to the extracellular basal lamina and to maintain muscle integrity for force generation (Blake et al., 2002).

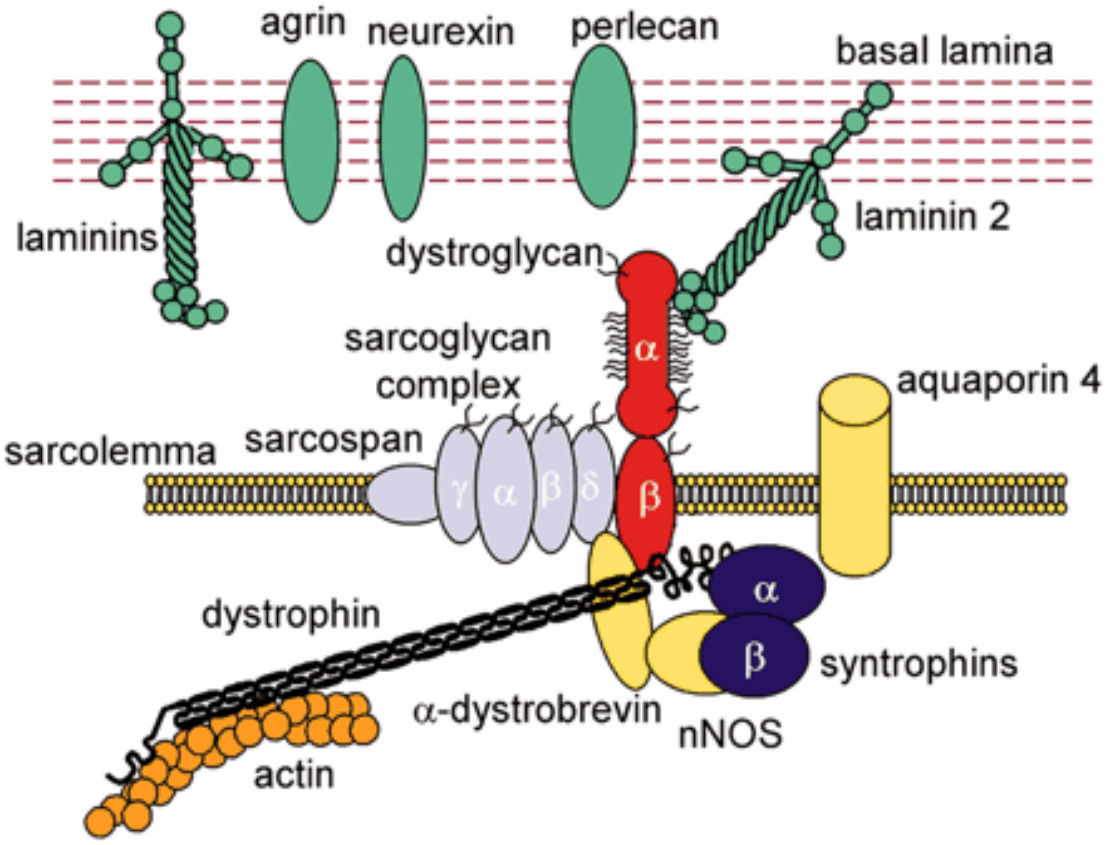

Figure 3. A general view of the skeletal muscle membrane complex (Modified from Michele DE and Kampbell KP, 2003)

The DGC is a multi-molecular, transmembrane link between the intracellular cytoskeleton and the extracellular basal lamina (Ervasti and Campbell, 1993). It is comprised of a heterodimer protein, α- and β-dystroglycan (DG), the sarcoglycan-sarcospan complex, and the syntrophin-dystrobrevin complex (Towler et al., 2004). DG are encoded by a single gene and cleaved into two subunits (α,β) by posttranslational processing (Ibraghimov-Beskrovnaya et al., 1992). α-DG is a highly glycosylated extracellular peripheral membrane protein that binds laminin-2 in the surrounding basal lamina, and it is anchored to the sarcolemma membrane by the

cytoplasmic domain of β-DG. β-DG, in turn, is connected to intracellular cytoskeletal F-actin and the intermediate filament component desmin, through dystrophin and the syntrophin-dystrobrevin complex, respectively (Carlsson and Thornell, 2001) (Figure 3).

Dystroglycan (Figure 3) is a ubiquitously expressed laminin receptor, serving to initially bind to laminin on the cell surface followed by binding of laminin with β1 integrins (Spence et al., 2002). A network of laminin, dystroglycan and integrins then undergoes rearrangements with associated reorganization of the actin cytoskeleton (Spence et al., 2002). It has been hypothesized that the DG are functionally responsible for the organization of the basement membrane, and the β-subunit plays a role in the assembly of other membrane protein complexes, such as the sarcoglycan-sarcospan complex (Michele and Campbell, 2003). However, in the myodystrophy (*myd*) mouse, an autosomal recessive model of muscular dystrophy, despite large disruptions of basement membrane in the brain, the skeletal muscle basement membrane remains morphologically intact. This suggests that integrins containing β1 isoforms are required in concert with dystroglycan as a basement membrane assembly and may in part compensate for the loss of dystroglycan (Michele and Campbell, 2003).

- Integrins

Integrins are transmembrane receptors composed of α and β subunits that play critical roles in both cell-matrix and cell-cell adhesion. They are heterodimeric, transmembrane glycoproteins consisting of an alpha and a beta chain that are non-covalently associated. The increase in integrin members is due to the expression of

splice variants (Mayer, 2003). The localization of integrin subunits to regions of cell adhesion in muscle, i.e., myotendinous junction (MTJ), neuromuscular junction (NMJ) and costameres, strongly support a role for integrins in maintaining adhesion links in muscle (Spence et al., 2002). Integrins are also concentrated in focal adhesions. For example, β1 integrin interacts with two cytoskeletal elements: α-actinin and talin (Otey et al., 1990). These proteins interact with several other focal adhesion-associated proteins including actin and vinculin, thereby establishing a direct, physical link between the ECM and the cytoskeleton (Jones et al., 1989). Studies have implicated α7β1 integrin in the organization of the muscle basement membrane (Colognato et al., 1999). In addition, enhanced expression of the α7β1 integrin alleviates the development of Duchenne-like muscular dystrophy in mdx/utrn$^{-/-}$ mice and significantly extends longevity (Burkin et al., 2001). Integrins have also been implicated in the signaling control of intracellular pH and intracellular calcium concentration (Schwartz et al., 1991), and in tyrosine phosphorylation of numerous cellular proteins (Kornberg et al., 1991). This evidence may suggest an influence of integrin on intracellular calcium levels in dystrophic muscle fibers.

- T-tubules

The transverse tubules (t-tubules) are specialized invaginations of the sarcolemma membranes that surround myofibrils and consist of a complex system of branching tubules in both transverse and longitudinal orientation to the myofibrils (Towler et al., 2004). T-tubules have a unique protein/lipid make-up and are enriched in cholesterol (Rosemblatt et al., 1981).

T-tubules are essential for the coordinated contraction of muscle fibers (Towler et al., 2004). Some loss of contractile strength could conceivably arise from shear damage to T-tubules, where dystrophin is not distributed (Byers et al., 1991). Recent studies have suggested that the structure and function of the t-tubules are more complex than previously believed. For example, the t-tubules are an important determinant of cardiac cell function, and may have a similar role in skeletal muscle fibers. Many proteins involved in cellular calcium cycling concentrate at the t-tubule, especially at the main site of excitation-contraction coupling, which ensures a harmonized calcium release throughout the cell, both spatially and temporally (Brette and Orchard, 2003).

Glucose is a major energy source for muscle. It can be used directly as a fuel in glycolysis or be stored as glycogen. The observation that transverse tubule membranes contain approximately five times more glucose transporter than sarcolemma raised the question about where glucose transport occurs in muscle (Towler et al., 2004). The t-tubule membrane system is continuous with the surface sarcolemma and is a tubule system in which extracellular fluid is in close contact with the interior of the muscle fiber (Dohm et al., 1993). Thus, the t-tubule membrane may represent a major site of glucose transport in skeletal muscle. The GLUT family consists of tissue-specific isoforms that facilitate the transportation of glucose across cell membranes. GLUT4 is the major isoform that responds to insulin and exercise in mammalian skeletal muscle. GLUT4 is recruited from distinct intracellular storage compartments to the plasma membrane and to the t-tubules in response to either

insulin or exercise and the effects of these two stimuli are additive (Towler et al., 2004).

- Costameres

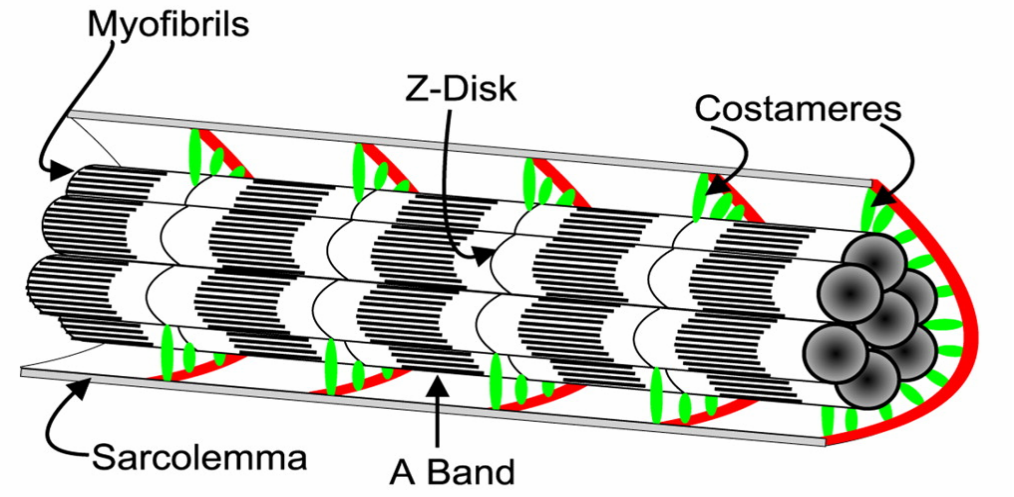

Figure 4. Costameric organization in skeletal muscle. (Modified from Ervasti, 2003)

Costameres (Figure 4) are subsarcolemmal protein assemblies that circumferentially align in register with the Z lines of peripheral myofibrils, and physically couple force-generating sarcomeres with the sarcolemma in striated muscle cells (Ervasti, 2003). Costameres contain three main components: the focal adhesion-type complex, the spectrin-based complex, and the dystrophin/DGC. The focal adhesion-type complex is composed of cytoplasmic proteins, including vinculin, talin, α-actinin, tensin, paxillin, and zyxin. It connects with both cytoskeletal actin filaments and the transmembrane proteins α-and β-integrin. The spectrin-based complex is a group of anchor proteins that is essential for the electrical activity of the

muscle fiber, including Na^+/K^+-ATPase, and most probably the voltage gated Na^+ channel (Bloch et al., 2002).

Costameres have two major functions. First, costameres are sites of interconnection between the sarcomere and the sarcolemma and therefore are thought to function as anchor sites for stabilization of the sarcolemma and for the integration of pathways involved in mechanical force transduction (Fatkin and Graham, 2002). In this way, costameres keep the sarcolemma aligned against contraction-induced damage and in-register with nearby contractile structures (Bloch et al., 2002). Second, costameres physically couple force-generating sarcomeres with the sarcolemma in striated muscle cells, and thus might be responsible for lateral contractile force transmission from sarcomeres to the extracellular matrix (Ervasti, 2003). These functions require that costameres link the contractile apparatus through the membrane to the extracellular matrix.

Costameres are connected with the contractile apparatus by intermediate filaments, which are formed by desmin and its associated proteins, synemin and paramemin at Z lines and M lines. Milner et al. (1996) demonstrated that desmin could influence costameric organization. Costameres link to the extracellular matrix by laminin, which is a major component of the basal lamina of all cells.

Defects in costameres would likely compromise muscle strength either directly, by reducing the efficiency of lateral force transmission, or indirectly, by increasing the potential for sarcolemma damage (Bloch et al., 2002). Studies of mdx mouse muscles, which lack dystrophin, support this idea. For example, muscle fibers in the mdx mouse aged 90-110 days are more susceptible to contraction-induced

sarcolemmal rupture (Petrof et al., 1993). However, this membrane weakness might be age-dependent. Grange et al. (2002) found that there was no additional damage to sarcolemma membranes of EDL muscles from mdx and mdx:utrn$^{-/-}$ mice aged 9-12-d after, as compared with before, a stretch injury protocol based on uptake of the fluorescent dye, procion orange (Grange et al., 2002). This finding suggests that fast-twitch skeletal muscles of dystrophic mouse pups were resistant to injury from acute mechanical stress during early maturation. Therefore, during early maturation, other mechanisms may be responsible for the onset of muscular dystrophy (e.g., calpain proteolysis).

Dystrophin was the first disease-relevant protein shown to be enriched at the costamere (Porter et al., 1992). Dystrophin connects to the DGC through the C-terminal. The DGC complex functions to anchor the sarcolemma to costameres and stabilize the sarcolemma against physical forces transduced through costameres during muscle contraction or stretch. The absence of dystrophin in humans and mice leads to a disorganized costameric lattice and disruption of sarcolemmal integrity (Ervasti, 2003).

In summary, the DGC and its associated protein complexes are important for the maintenance of sarcolemma integrity. A defect in one or more of these components could either lead to or contribute to muscular dystrophy. Therefore, understanding sarcolemma properties and its associated structures is critical to understand how breakdown of sarcolemma membranes might contribute to the onset of pathophysiology in DMD.

Mouse models of DMD

Two mouse models for DMD include dystrophin-deficient (mdx) and dystrophin- and utrophin- deficient (mdx:utrn$^{-/-}$) mice. The mdx mouse has an X-linked point mutation in the dystrophin gene that results in a truncated protein (Bulfield et al., 1984; Sicinski et al., 1989), which is 27% of the normal length. DMD was initially identified from its abnormally elevated levels of pyruvate kinase and creatine kinase in plasma (Bulfield et al., 1984). Mdx mice have a near normal lifespan, which differs from the shortened lifespan of human patients. These mice show histological signs of muscular dystrophy between 2~8 weeks, but show little weakness after age 6 weeks (Blake et al., 2002; Durbeej and Campbell, 2002), and do not develop the severe myofibrosis that is characteristic of end-stage DMD (Grady et al., 1997). Rounds of muscle fiber degeneration and regeneration in mdx muscles, which characterize the dystrophic process, peak between weeks 3~4 (Bulfield et al., 1984; Collins and Morgan, 2003) and decline to very low levels thereafter (Grady et al., 1997). Regenerating fibers are characterized by the presence of centralized nuclei. Reduced force production and power output of mdx skeletal muscles are evident from age 9-12-d (Grange et al., 2002), but overt signs of muscular dystrophy were not evident. This seemed to suggest that membrane weakness was not directly related to the onset of muscular dystrophy, at least not at this early maturing age. The most affected muscle in the mdx mouse is the diaphragm (Blake et al., 2002; Durbeej and Campbell, 2002), likely because of its constant use for breathing.

Compensation by utrophin may explain the difference in lifespan and dystrophic severity between the mdx mouse and human patients (Grady et al., 1997). Utrophin, the autosomal homolog of dystrophin, is confined to the subsynaptic membrane at the NMJ in normal skeletal muscle. It is up-regulated in mdx mice and DMD patients in regenerating muscle fibers to compensate partially for the functional loss of dystrophin, but compensation for dystrophin is more effective in mice (Grady et al., 1997; Helliwell et al., 1992). Therefore, the mdx mouse is not an ideal model for DMD.

The mdx:utrn$^{-/-}$ mouse, is derived from crossing mdx (dystrophin$^{-/-}$) with utrophin$^{-/-}$ mice (Grady et al., 1997). This model is characterized by a reduced lifespan (<20 weeks), weight loss, severe muscle weakness with joint contractures, obvious growth retardation, diminished mobility, and premature death (Durbeej and Campbell, 2002; Grady et al., 1997). The onset of the degeneration and regeneration cycles is evident at age 2-weeks, a week earlier than those in the mdx mouse, and continues to about age 10-weeks. Eventually, failure of regeneration results in replacement of muscle fiber with adipose cell and connective tissue (Grady et al., 1997). The severity of dystrophy in this model resembles DMD more closely than in the mdx mouse.

Although the precise mechanism by which utrophin attenuates the effects of dystrophin deficiency in mdx mice is not clear, the differences in the time course of the dystrophic process between the mdx and the mdx:utrn$^{-/-}$ mice could suggest the stabilization of the membrane by utrophin may confine degeneration and regeneration cycles to a limited period in mdx mice, and may limit the extent of

fibrosis. Grady et al. (1997) also reported that mdx:utrn$^{-/-}$ muscles have a more than 2-fold prolonged relaxation time after contraction compared to mdx mice. This may reflect a more severe impairment of calcium homeostasis in mdx:utrn$^{-/-}$ than in mdx mice (Grady et al., 1997). However, it is not presently known if utrophin modulates calcium homeostasis.

Intracellular calcium homeostasis

Calcium is a primary regulatory ion for skeletal muscle contraction, and also represents an important signal for many cellular processes. For example, calcium homeostasis is critical to muscle contraction as well as calcium-dependent proteolysis in muscle fibers (Blake et al., 2002). At rest, cytosolic free-calcium concentration in the normal fiber (hereafter, calcium concentrations imply the free concentration, and cytosolic and intracellular are interchangeable) is maintained at ~50nM (Berchtold et al., 2000). After membrane depolarization, the cytosolic calcium concentration is transiently increased ~100 fold to initiate contraction. If membrane depolarization ceases, calcium is quickly resequestered by the sarcoplasmic reticulum to restore a low intracellular calcium concentration. The membrane hypothesis states that dystrophic muscle membranes are more vulnerable to mechanical damage (Hutter, 1992). This damage could result in calcium influx into dystrophic muscle fibers, which may also have a reduced ability to regulate intracellular calcium levels (Turner et al., 1988).

• Increased intracellular calcium

Several observations suggest calcium homeostasis is perturbed in dystrophin-deficient muscles. By using fluorescently labeled calcium chelators, spectroscopic studies have demonstrated that the total calcium content in both DMD patients and fetuses was elevated (Bertorini et al., 1982; Bertorini et al., 1984). A marked increase in the intracellular calcium concentration was also found in cultured human dystrophic muscle fibers and mdx muscle fibers (Turner et al., 1988), and mdx myotubes later (Bakker et al., 1993).

Additional studies revealed that increased intracellular calcium is likely to occur in specific regions of muscle fibers. In *mdx* myofibers, local calcium increases occurred more often at the sarcolemma peripherally than deep inside fibers when challenged by increased external calcium (Turner et al., 1991). Using the patch-clamp technique, Allard et al. (2000) found a 3-fold greater calcium level in the subsarcolemma region of *mdx* compared to wild-type fibers at ages 3-5 weeks (Mallouk et al., 2000). Calcium level at the sarcoplasmic reticulum (SR) is almost 50% greater in *mdx* than in control myotubes (Robert et al., 2001). Taken together, these data indicate increased intracellular calcium in dystrophic muscle fibers and myotubes, potentially localized near the sarcolemma and SR.

• Calcium pools

There are two calcium pools for muscle fibers, an extracellular calcium pool and an intracellular calcium reservoir, the SR. Increases in intracellular calcium could arise from either or both pools.

1) Extracellular calcium pool

Increased intracellular calcium could come from the increased calcium channel activity and/or transient ruptures in dystrophic membranes. The higher occurrence of membrane ruptures in dystrophic muscle may allow localized transient calcium influxes that lead to increased calcium-dependent proteolysis. Activation of calcium-dependent proteases might in turn activate calcium leak channels near the wounds (McCarter and Steinhardt, 2000). Given intracellular calcium homeostasis was not restored to normal after acute treatment with tetrodotoxin, which should prevent any calcium rise due to transient tears but would not affect a long-term defect in calcium homeostasis (Hopf et al., 1996), the chronically raised intracellular calcium seems mainly to result from increased calcium channel activity rather than the membrane ruptures (McCarter and Steinhardt, 2000). Abnormal calcium influx in adult mdx muscle fibers is thought due to storage-dependent channels that are voltage-independent (Vandebrouck et al., 2002). These channels belong to the transient receptor potential channel (TRPC) family (Vandebrouck et al., 2002). Excess calcium influx can also occur in mdx muscle fibers through abnormal L-type calcium channels (Yoshida et al., 1997) and mechanosensitive leak channels (Franco-Obregon and Lansman, 2002). If these calcium channels were proteolytically activated near ruptures in a calcium-dependent manner in the dystrophic membrane, this might lead to a chronically raised intracellular calcium level, increased calcium-dependent proteolysis and eventually, muscle fiber necrosis.

2) Intracellular calcium pool, the Sarcoplasmic Reticulum (SR)

The SR (Figure 5) is a large source of intracellular calcium. The Ca^{2+}-ATPase pump in the SR membrane is responsible for restoring intracellular calcium levels after muscle contraction. The ryanodine receptor (Ryr) and SR Ca^{2+}-ATPase are the primary means of release and uptake of calcium by the SR, respectively. Upon depolarization, the Ryr through interactions with the dihydropyridine receptor (DHPR) on the t-tubule membrane releases calcium from the SR transiently. Although the Ca^{2+}-ATPase continues to pump calcium into the SR even during depolarization, the release of calcium results in increased intracellular calcium. When depolarization ceases, the calcium is then pumped back into SR by the Ca^{2+}-ATPase. Studies with mdx mice aged 3 months showed that the calcium uptake by the sarcoplasmic reticulum was decreased because of the down-regulation of ryanodine receptor type 1 (RyR1) and the presence of a negative regulator of Ca^{2+}-ATPase (Tkatchenko et al., 2000). This suggests that the function of both the Ryr receptor and the SR calcium pump may be impaired in mdx muscle fibers at this age.

- Intracellular calcium binding proteins

There are many calcium-binding proteins both inside (i.e., calsequestrin and calsequestrin-like proteins) and outside the SR (i.e., parvalbumin), which help to restore a low intracellular calcium level in addition to the Ca^{2+}-ATPase. Culligan et al. (2002) showed a drastic decrease of calsequestrin-like protein concentration and impaired calcium binding in 8-week old dystrophic *mdx* muscle. Calcium binding assays of SR vesicles showed that calcium binding was significantly reduced in *mdx* preparations compared with normal control. These changes are accompanied by a distinct reduction in calcium binding proteins. Using calcium-activated K^+ channels

in the sarcolemma as a calcium sensor, there was a significant increase in the free calcium level to 115nM in the subsarcolemmal area (the cytosolic area that is in close vicinity to the sarcolemma) of mdx fibers compared to 40nM in controls (Mallouk et al., 2000). Using the inside-out patch-clamp technique, the same group confirmed that the 3-fold overload of calcium present in mdx muscle fibers was restricted to the subsarcolemmal compartment (Mallouk et al., 2000). These data suggest that the sequestering mechanism in mdx muscle was unable to handle the abnormally increased calcium concentration. This might result from the lower level of calcium-binding proteins in mdx fibers, which might represent an additional factor that contributes to the progressive functional decline of dystrophic muscle fibers.

The extracellular compared to the intracellular calcium pool is likely to play a more important role in dystrophic muscle fibers, by affecting the calcium leaky channel in the muscle membrane itself. Calcium influx could arise from transient membrane lesions under mechanical stress or through calcium leak channels. Calcium channels that are responsible for the influx of calcium can be categorized as storage-dependent (e.g., the TRPC family), voltage-gated (storage-independent) channel (e.g., the L-type calcium channels) and mechanosensitive calcium channels. It is likely that calcium influx through more activated calcium channels is mainly responsible for the calcium-dependent proteolysis in dystrophic muscle fibers. Increased calcium influx into muscle fibers and the reduced ability of calcium handling in dystrophic muscle fiber could lead to the broad calcium-dependent proteolysis and therefore facilitate the dystrophic muscle fiber necrosis.

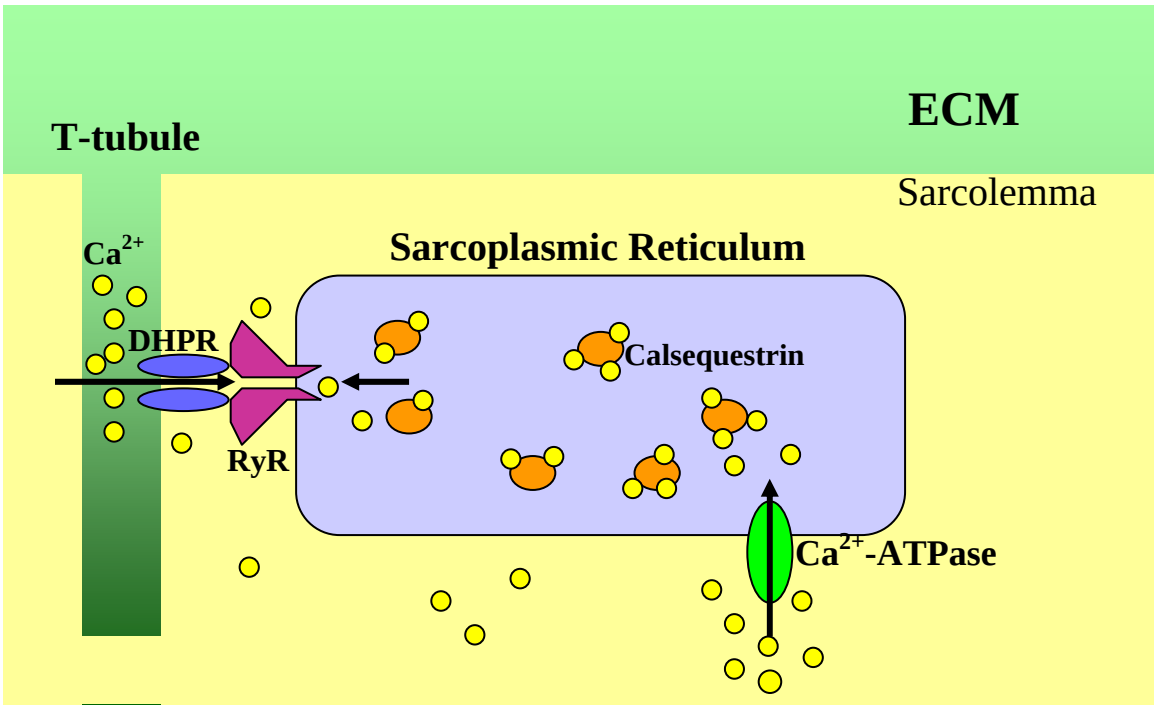

Figure 5. Calcium channels and calcium-binding proteins related to the sarcoplasmic reticulum.

Proteolysis

The three major proteolytic pathways in muscle are: the lysosomal cathepsin, the calcium-dependent calpain, and the ATP-dependent ubiquitin proteasome pathways (Cooney et al., 1997). Calcium-dependent proteolysis appears largely responsible for the elevated calcium-sensitive proteolysis in mdx myotubes because pretreatment of myotubes with ammonia, an inhibitor of lysosomal proteolysis, still yielded protein degradation (Alderton and Steinhardt, 2000a). ATP-dependent ubiquitin proteolysis pathway was only suggested to be involved in p94 (a third calpain isoform) deficient muscle (Combaret et al., 2003). However, p94 is calcium-independent and, therefore, was excluded from calcium-dependent proteolysis.

Increased intracellular calcium levels are likely to initiate many of the progressive downstream events that lead to muscle weakness by mediating

elevated rates of protein degradation (Spencer and Tidball, 1992). In non-contracting mdx myotubes, increased calcium influx through abnormally activated calcium leak channels was considered primarily responsible for most of the elevated proteolysis (Alderton and Steinhardt, 2000b).

In summary, an increased resting intracellular calcium level is evident in dystrophic compared to control myotubes and fibers, and it appears to arise mainly from the extracellular pool via either transient membrane lesions or abnormally opened calcium channels. The increased calcium could lead to the local activation of proteases, which may modify calcium leak channels to cause further calcium ingress (Blake et al., 2002). A deteriorating cycle of increased proteolysis is formed and this eventually leads to muscle fiber necrosis. Calpains are calcium-activated proteases and these are thought to play an important role in the pathophysiology of DMD.

Calpains

- General characteristics of calpain

Calpains, or calcium-activated neutral proteases, are a large family of calcium dependent cysteine/thiol proteolytic enzymes, that are expressed ubiquitously (Carafoli and Molinari, 1998). The two major isoforms, μ-calpain (calpain I) and m-calpain (calpain II) are both calcium-dependent for activity, but differ in their calcium requirement for half-maximal activation when assayed *in vitro*, which is 1-100μM and 0.1-1 mM, respectively (Berchtold et al., 2000). Both isoforms consist of a large 80 kD catalytic subunit and a small 30 kD regulatory subunit. Another muscle-specific isoform, p94 (or calpain 3, 94kD) is calcium-independent, and lacks the small

regulatory subunit (Carafoli and Molinari, 1998). μ- and m-calpain are mainly present in the cytosol before activation, whereas p94 is concentrated in the nucleus to regulate muscle cell growth and differentiation through the modulation of muscle determination factors (MDF) (e.g., MyoD) (Berchtold et al., 2000).

- Mechanisms for calpain activation

Calpains exist in an "inactive" proenzyme form before activation. The first step in calpain activation is a calcium-dependent translocation to the muscle membrane in the heterodimeric form. The hydrophobic NH_2 terminus of the small subunit is responsible for membrane interaction (Suzuki et al., 1995). μ- and m-calpains are regulated both by binding calcium and through interaction with inhibitors (e.g., calpastatin) or activators (e.g., membrane phospholipids).

Based on crystal structure analysis, the positions of the catalytic triad residues (Cys105, His 262 and Asn286) at the interface between Domain I and II are similar in calpains and other cysteine proteases (Groves et al., 1998). In cysteine proteases, the cysteine residue is usually activated by the histidine residue first, and then makes a nucleophilic attack on the carbonyl carbon atom of the substrate. The peptide bond is then hydrolyzed. However, there is a specific calcium requirement for activation of the calpains because the atomic distance between Cys105 and His262 (~10.5 A) is much greater than the typical distance found in other cysteine proteases (~3.7A) (Hosfield et al., 1999), and this hinders the formation of a competent catalytic triad, Therefore, calpains require a certain concentration of intracellular calcium for activation.

However, although there is a distinct calcium requirement for calpain activation, the calcium concentration in a resting myofiber is maintained at ~50nM, which increases ~100 fold when the membrane is depolarized (Berchtold et al., 2000). The levels of calcium required for calpain activation are difficult to achieve under *in vivo* physiological conditions (Carafoli and Molinari, 1998). One of the mechanisms that could possibly decrease the calcium requirement for calpain activation is interaction with phospholipids (Berchtold et al., 2000; Imajoh et al., 1986; Pontremoli et al., 1985). It has been suggested that phospholipids might be an activator of calpains because phospholipids lower the calcium requirement for calpain activation *in vitro* (Coolican and Hathaway, 1984). Phospholipids may be necessary for calpain activation to overcome the lower physiological intracellular calcium levels (generally <1μM) even during fiber activation (Berchtold et al., 2000).

The activities of μ- and m-calpain are also thought to be regulated by the specific inhibitor calpastatin, a protein of 110kD, whose inhibition of calpain is calcium-dependent (Carafoli and Molinari, 1998). Calpastatin regulates calpain conversion from an inactive to active form at the membrane, and the translocation of calpain (Berchtold et al., 2000). Less is known about the activation and inhibition of p94 because its half-life is extremely short (25min) and is not inhibited by calpastatin (Carafoli and Molinari, 1998).

- Calpain substrates

Calpains cleave at the carboxyl side of a tyrosine (Tyr), methionine (Met), or arginine (Arg) residue in the P1 position if the P2 position is occupied by a

hydrophobic amino acid (Sasaki et al., 1984). Calpains have a relatively strict specificity for their substrates so that they usually cleave at only a limited number of sites, which is different from digestive proteases with broad target specificities (Berchtold et al., 2000). Many calcium channels are calpain substrates, including the Ca^{2+}-ATPase, L-type calcium channels (including DHPR), and the ryanodine receptor (RyR) (Berchtold et al., 2000). Another well-known group of substrates are enzymes, such as protein kinase C and phospholipase C. In addition, the levels of a number of transcription factors are regulated by calpains (e.g., c-Jun, MyoD) (Wang and Yuen, 1999). An important aspect of calpain action is its ability to modify rather than completely degrade its substrates. This results in fragments of substrate proteins that may alter cell structural integrity and/or signaling pathways (Carafoli and Molinari, 1998; Perrin and Huttenlocher, 2002). The function of calpains is determined by the distribution of their substrates in different compartments of the cell and the cell types. In red blood cells, calpain activation is associated with platelet activation (Spencer and Tidball, 1996). In muscle cells, calpains play a role in the initiation of myofibrillar protein turnover, myoblast fusion and cell proliferation (Wang and Yuen, 1999). The down-regulation of protein kinase C in a variety of cell types has been shown to be blocked by calpastatin (Hong et al., 1995; Kishimoto et al., 1983). Hence, it is likely calpains are involved in both modifying cell structural integrity as well as signaling pathways.

- Cellular functions

    Breakdown of cytoskeletal structures

The μ- and m-calpain isoforms were initially thought to be localized within the Z disks of skeletal muscle cells (Dayton and Schollmeyer, 1981), which suggested a role in breaking submembranous networks of actin by cleaving cytoskeletal proteins. Calpain mediates increased plasma membrane permeability and hydrolysis of cytoskeleton-associated paxillin, vinculin, and talin during renal cell death (Liu and Schnellmann, 2003). These cytoplasmic proteins are also the components of the focal adhesion complex (FAC), and the association of focal adhesion kinase (FAK) with integrin-based focal adhesions of cultured cells is through paxillin-mediated interaction between the C-terminal domain of FAK, an important protein tyrosine kinase (Fluck et al., 2002). The major biological function of FAK is the control of FAC turnover, and localization of FAK is in many cell types restricted to FACs (Cary and Guan, 1999). FAK also mediates several integrin signaling pathways, including the promotion of cell migration, proliferation and spreading, and the prevention of cell apoptosis (Cary and Guan, 1999). If the components of FAC, such as paxillin, vinculin and talin, are hydrolyzed because of the mediation of calpain proteolytic activity, the structural interaction between the cytoskeleton network and sarcolemma will be compromised, lateral force transduction will likely be reduced, and the integrin-mediated signal cascades and associated signal molecules may also be affected.

Calpains and glucose transport

Calpain-mediated pathways have also been found to regulate muscle mass and glucose transporter GLUT4 turnover (Otani et al., 2004). The results of

additional experiments that demonstrated GLUT4 is a substrate for m-calpain *in vitro* are consistent with this idea (Otani et al., 2004). Based on these results, it can be inferred that the activation of calpains will facilitate the breakdown of their substrates, including the glucose transporter, GLUT4, which could affect the normal metabolism and performance of skeletal muscle because glucose is a main energy source of muscle movement.

- The localization of calpains

Where calpains localize in a muscle cell, and whether there is cellular translocation, have not yet been clarified. For example, μ- and m-calpain were thought to be localized within the Z disks of skeletal muscle fibers (Dayton and Schollmeyer, 1981), however, Hood et al. (2003) suggested μ-calpain and m-calpain were cytosolic proteins that translocated to both focal complexes/adhesions and the plasma membrane (Hood et al., 2003). Using confocal microscopy and isopycnic density centrifugation, μ- and m-calpain and the 30kDa regulatory subunit were found associated with the endoplasmic reticulum and Golgi apparatus in cultured fibroblasts (Hood et al., 2003). This evidence suggested that calpains were associated with the cytoskeletal matrix, which could be a possible mechanism to avoid interaction with their primary inhibitor, calpastatin. At present, the specific localization sites for, and the requirement for translocation of the calpains remain unclear.

Calpains and DMD

Calpains as calcium-dependent proteases may represent an active component in the pathophysiology of dystrophic muscle. Membrane disruptions in mdx fibers caused by long-term contractions resulted in influx of intracellular free

calcium into the sarcoplasm (Alderton and Steinhardt, 2000a), which may lead to the abnormal activation of calcium-specific transient receptor potential channels (TRPC) (Vandebrouck et al., 2002) by calcium-dependent proteases, such as calpains. Modification of the channels near the wound sites could then cause further calcium ingress into the dystrophic cell (Blake, et al., 2002), setting up a debilitating degenerative cycle. Although it is not clear where calpains are localized, if μ- and m-calpain are localized within the Z disks of skeletal muscle fibers (Dayton and Schollmeyer, 1981), they could degrade submembranous networks of actin by cleaving cytoskeletal proteins. Recent studies have reported that calpain concentration and/or levels of auto-proteolytic modification vary over the course of the disease in *mdx* mice, which coincides with the particular stage of the disease process (Spencer et al., 1995). For example, total calpain content is increased in peak necrotic mdx mice at age 4 and 14 weeks, but not in prenecrotic mice at age 2 weeks. In addition, the auto-proteolytic activation of μ-calpain was highest during peak necrosis and returned to levels near controls in regenerated muscle.

Furthermore, studies by Spencer and Tidball showed evidence of abnormal calpain translocation, which also corresponded to the state of pathophysiology of each dystrophic fiber (Spencer and Tidball, 1996). They found calpain present throughout the cytosol, but more concentrated near the plasma membrane in healthy, fully differentiated muscle fibers, whereas calpain showed only a homogeneous cytosolic distribution in degenerating mdx fibers. Therefore, calcium-dependent calpain proteolysis could yield at least two detrimental outcomes: (1) modification of TRPC channels, which leads to further Ca^{2+} entry; and (2) degradation of the

cytoskeleton. These outcomes could therefore lead to ultimate degradation and necrosis of the muscle fibers.

Calpains could also play a regulatory role in cellular functions because calpains cleave between rather than within functional domains, and therefore do not affect the functional domain of their substrates (Berchtold et al., 2000; Carafoli and Molinari, 1998). Calpains do not just respond to signaling cues but may, once activated, help regulate signaling. Potential regulation could be achieved by the cleavage of specific transcription factors, cytosolic enzymes and/or signaling molecules. These effects could influence gene expression, cell function and signaling pathways. C-Fos and c-Jun transcription factors are highly sensitive to calpains in vitro (Pariat et al., 2000). Calpains are known to down-regulate protein kinase C (PKC) in a variety of cell types (Wang and Yuen, 1999). Many of the PKC target substrates are components of signal transduction pathways and include proteins that regulate ion channels, growth factor receptors, structural and regulatory proteins of the cytoskeleton (Wang and Yuen, 1999).

Calpain may also be involved in the signaling pathway of apoptosis, because cleavage of p53 (a tumor-suppressor protein) leads to a small number of defined fragments, a step considered necessary to trigger apoptosis (Carafoli and Molinari, 1998). In addition to the potential role of calpain in DMD, Limb Girdle Muscular Dystrophy type 2A (LMGD2A) provides a direct example of calpain involvement in another muscular dystrophy. LMGD2A is a dystrophic disease in which the structural sarcolemma proteins are normally expressed, but there is a deficiency in the muscle specific calpain p94 (Richard et al., 1995).

Based on the above findings, it is reasonable to suggest that calpains could also be involved in the pathogenesis of DMD through disruption of signaling pathways, which eventually lead to the degradation and necrosis of the muscle fiber. However, the mechanisms and time course of these pathways are not presently known.

Summary

The relation between dystrophin deficiency and the onset of DMD has not yet been clarified. It is suggested that increased calcium-dependent proteolysis caused by increased intracellular calcium is a contributor to the pathophysiological processes of DMD, though it is not clear whether the proteolysis is a primary or secondary effect. A limiting factor to understanding the role of calcium-dependent proteolysis in DMD onset is that many studies were conducted either with dystrophic mice at post-weaning ages (beyond the age of onset) or in cultured myotubes (which may not reflect the in vivo state). Therefore, it is necessary to study the role of calcium-dependent proteolysis as a potential onset mechanism in dystrophic mouse models during maturation.

Chapter 3: Materials and Methods

Rationale for experimental approach

Previously in our lab, calpain activities were assessed in the structure-bound and supernatant fractions of mdx dystrophic quadriceps muscles at ages 7, 14 and 21 d (Draper, unpublished). The structure-bound fraction contained membrane and myofibrillar fractions, whereas the supernatant contained the cytosolic fraction. Results revealed that the structure-bound activity was greater than that of the supernatant at these ages, but the overall calpain activity in the presence or absence of calcium did not vary much across age or genotype, except for a higher calpain activity in the structure-bound pellet fraction at age 7-d. The methods employed in the Draper study did not discriminate calpain activity between the myofibrillar and membrane components of the structure bound fraction. This discrimination is necessary to determine if calpain activity is localized to the membrane during early maturation and therefore represents an early onset mechanism of DMD.

Mice

All methods were approved by the Animal Care Committee of Virginia Tech. Dystrophic models used in this study were mdx mice, which are dystrophin-deficient, and mdx:utrophin-deficient (*mdx: utrn*$^{-/-}$) mice, which lack both dystrophin and its protein homolog, utrophin. C57BL/6 mice were used as controls.

Mice were housed in the Virginia Tech Laboratory Animal Resource facility with a 12 hour light/dark cycle and were provided food and water *ad libitum*.

Quadriceps muscles were obtained from mice of each genotype at ages 7-, 21- and 35-d. At age 21- and 35-d, 6 mice were collected as individual samples. At 7 days, 4 mice were pooled for each sample because of the small size of the quadriceps muscles at this age. Note: muscles from mdx:utrn$^{-/-}$ mice at age 7-d were not obtained because there were insufficient animals to pool.

Muscle sample preparation

Control C57BL/6, mdx, and mdx/utrn$^{-/-}$ mice were anesthetized with 2mg xylazine and 20mg ketamine per 100g body mass. Quadriceps muscles from control and dystrophic mice at ages 7, 21 and 35 days were harvested and cut into thin strips along the length of the muscle (~1-2 mm). Muscle strips were tied to glass capillary tubes and soaked in cold fiber glycerination buffer [FGB, (60mM KPr), 0.5% Triton, 0.005M EGTA, 24% glycerol] at 4 ºC for 2 days with a buffer change every 24 hours. This is a common method to “skin” skeletal muscles to isolate the contractile apparatus, thus the membranes should dissociate from the contractile proteins and become soluble in the glycerination buffer together with cytosolic proteins. The membrane fraction was concentrated as a pellet after centrifuging the glycerination buffer at 315,000 g for 60 min at 2˚C (TL-100 Beckman ultracentrifuge).

The myofibrillar protein fraction was homogenized in 1 mL homogenization buffer [20mM Tris (pH7.4), 5mM EDTA, 5mM EGTA, 1mM DTT, 10µg/ml PMSF, and 10µg/mL pepstatin A] using a ground glass homogenizing tube and pestle, and a 3 x 1 min cycle with a 1 min interval between each pulse. The pestle was clamped in the chuck of a variable speed drill fixed to a stand. The speed of the motor was set

on a rheostat at 40V on a 150V scale. Homogenates were centrifuged at 1,000 g for 10min at 4ºC. The myofibrillar protein pellet fraction was resuspended in 1mL homogenizing buffer. All fractions were kept on ice and assayed for calpain activity the same day.

This sample preparation was different from that used previously (Draper, unpublished) to separate the membrane from the myofibrillar subcellular fraction. The specific differences in the protocols are compared in Figure 5.

Calpain activity assay

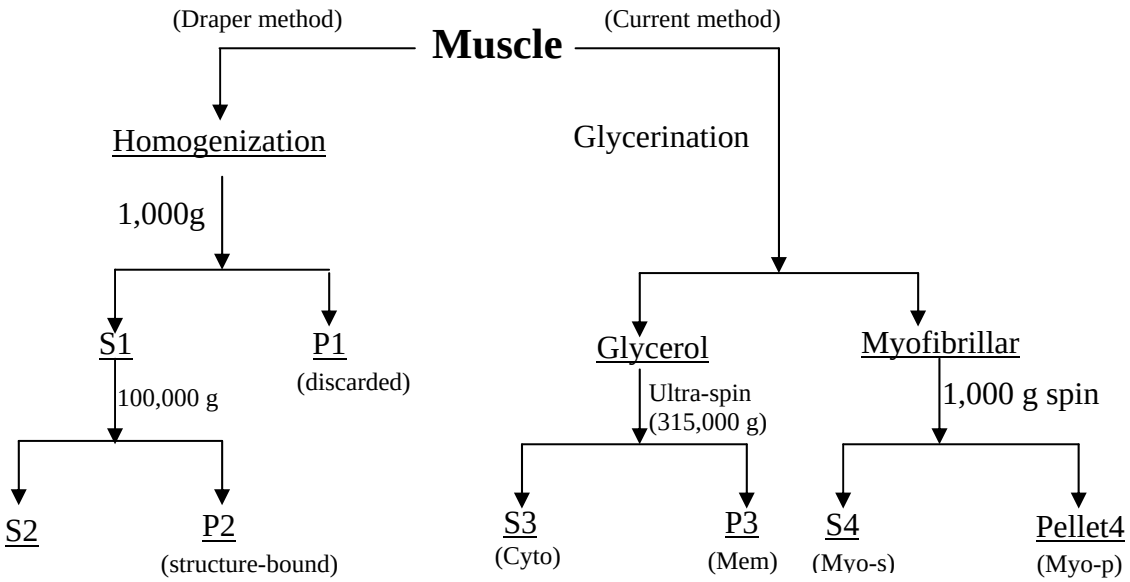

Figure 6. Flowchart of sample preparation. Comparison of the previous (Draper, unpublished) and current methods. (S: supernatant; P:pellet; Cyto: cytosolic fraction; Mem: membranes; Myo-s: myofibrillar supernatant; Myo-p: Myofibrillar pellet)

Ten µL of each sample fraction 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 was similar to the control buffer with the exception that 5mM $CaCl_2$ was added, and EDTA and EGTA were excluded. 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. When SLY-AMC is cleaved by calpain, electrons in the conjugated diene of SLY-AMC fluoresce at an emission wavelength of 440-460nm when excited at a wavelength of 360-380nm. Fluorescence was monitored for 15 min at 37ºC at an excitation wavelength of 380nm and an emission wavelength of 460nm (Perkin Elmer LS50B fluorometer). The calpain activity was determined as the calcium-dependent cleavage of SLY-AMC over a minimum period of 50 sec and expressed as arbitrary units normalized to both the total protein content (AU/min/mg) in each sample and total calpain content (AU/min/µg) for each sample.

In a pilot study, the activity assay for each sample fraction was run in duplicate. The results from the duplicates for any of the fractions showed little variation. Based on this limited variation and because the sample volume for each fraction was limited, the activity data for this study were obtained from a single activity assay for each sample fraction. This ensured enough of each sample fraction was available to determine protein concentration and calpain content.

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 standard or sample was added

to each cuvette. The cuvette was vortexed to mix the protein sample and Bradford reagent well, and then incubated for ~10 min at room temperature. Absorbance at 595nm was determined on a Hewlett-Packard 8453 UV-vis spectrophotometer. A standard curve was constructed using bovine serum albumin as the standard (0.1mg/mL~5.0mg/mL).

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

SDS-PAGE analysis is typically used to separate proteins in a sample according to their relative molecular weight. Proteins can be visualized in the gel using Coomassie blue stain (refer to Appendix A) or following Western transfer by use of specific primary and secondary antibodies.

Samples were mixed with 2X sample buffer (240mM β-mercaptoethanol, 1M Tris (pH 6.8), 20% glycerol, 0.1% bromophenol blue, and 10% SDS). Samples were boiled for three minutes to denature proteins. If the protein concentrations of samples, especially the membrane preparations, were lower than 1.0mg/mL, samples were evaporated in a Labconco Centrivap for approximately 30 min until the total volume was decreased to less than 40µL before being boiled. This typically yielded a protein concentration greater than 1.0mg/mL.

A mini-PROTEIN® 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 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 (7-206kDa, Bio-Rad) were loaded in the first well. Thirty µg of each sample protein were loaded in the remaining wells, one sample per well. Samples were run on a 4% stacking and 12% separating gel, each 1.5mm thick. The voltage was initially set at a constant 150V while the samples were in the stacking gel, and then decreased to 120V when samples were in the 12% separating gel. Voltage was applied for about 2 hours or until the bromophenol blue tracking dye ran off the bottom of the gel. To minimize the heat developed during the gel run, the gel apparatus was placed in an ice bath.

Western analysis

Western analysis was used to detect the presence of a specific protein in a sample by use of specific antibodies. In our study, Western analysis was used for two purposes: 1) to assess the purity of each prepared cellular fraction using antibodies against specific cellular fraction markers (results reported in Appendix B), and 2) to measure the total calpain content in each prepared muscle sample by probing with a total calpain antibody (Table 1). Once the sample proteins were separated on an SDS-PAGE gel, they were transferred to a nitrocellulose membrane, and detected by their specific antibodies.

Before transfer in a Bio-Rad Trans-blot® cell, a piece of nitrocellulose membrane (6x8 cm), two sheets of trans-blot paper, and the gel were soaked in transfer/blotting buffer (3g/L Tris, 14g/L glycine, and 300mL/L methanol) for 15 min. The transfer sandwich was assembled as follows. From anode to cathode: sponge, 1 sheet of filter paper, nitrocellulose membrane, gel, 1 sheet of filter paper, sponge. 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 inserted into the transfer cell chamber reduced heat accumulation in the transfer buffer. The transfer was run at a constant voltage of 100V for 100 min.

Table 1. Primary antibodies

Protein	1º Antibody	Notation	Dilution	Host	Manufacturer
Calpain	NCL-CALP-11B3	Target protein	1:50	Mouse	Novo Castra
Actin	Anti-α1actin	Myofibrillar protein marker	1:1000	Mouse	Affinity Bioreagents
Calpastatin	Calpastatin antibody	Calpain inhibitor	1:1000	Rabbit	Sigma
Na^+/K^+-ATPase	Na^+/K^+-ATPase antibody	Sarcolemma marker	1:1000	Mouse	Abcam

Table 2. Secondary antibodies

2º Antibody	Dilution	Host	Manufacturer
HRP-conjugated Affinity-pure Anti-mouse IgG	1:10,000 - 1:200,000	Goat	Jackson Immuno Research laboratories
HRP-conjugated Affinity-pure Anti-rabbit IgG	1:10,000 - 1:200,000	Goat	Jackson Immuno Research laboratories
HRP-conjugated Affinity-pure Anti-goat IgG	1:10,000 - 1:200,000	Donkey	Santa Cruz

Following transfer, the nitrocellulose was incubated in blocking buffer (3-5% non-fat dry milk in TBST (TBS is 100 mM Tris-base, 1500 mM NaCl, pH7.6 + 0.05% Tween20) for an hour with shaking. The nitrocellulose membrane was washed 3 times, each for 10 min, in TBST at room temperature. The membrane was then incubated with primary antibody (Table 1) in blocking buffer with shaking overnight at 4°C. The low temperature overnight always provided a clear background because lower temperatures minimize the non-specific binding of primary antibody to proteins. After this treatment, the membrane was incubated with a secondary antibody

conjugated with horseradish peroxidase (HRP) (Table 2) in blocking buffer for 2 hours, followed by 3 washes of TBST, each for 15 min.

The presence or absence of the protein of interest was visualized using enhanced chemiluminescence (ECL) according to the manufacture's instructions (Pierce). HRP conjugated to the secondary antibody catalyzed the oxidation of luminal by hydrogen peroxide. The membrane was incubated in 3 mL of peroxide solution and 3 mL of the luminol solution for 5 min, and then taken out of the solution mixture. The membrane was briefly dried, wrapped in plastic wrap (Saran wrap), and placed in the film developer cassette for development. In the dark room, the membrane was exposed to Kodak X-Omat film for varying durations of time from 5 sec to 5 min, and then processed in a Konica SRX-101A Medical Film Processor.

Densitometry

Following Western transfer, relative protein content was determined by densitometry using 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. The band density of the control protein (μ-calpain) was used to normalize the calpain activity in each sample band. Because autolyzed small fragments of calpains were also revealed on the film, total calpain content was determined from the sum of individual band densities.

Relative calpain activity was determined to resolve whether increased calpain activity was due to 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.

Statistics

Descriptive statistics, e.g., mean ± standard error of the mean (SEM), were determined for body mass, total protein concentration, calpain activity and total calpain content.

Data were analyzed by Statistical Analysis System (SAS Version9.1). A two-way ANOVA was used to determine the main effect of two 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 ($p<0.05$).

Chapter 4: Results

In this study, we used an enzyme activity assay to measure calpain proteolytic activity in different subcellular fractions (i.e., membrane, myofibrillar and cytosol fractions) of wild type (wt) and dystrophic (mdx, mdx:utrn$^{-/-}$) quadriceps muscles at ages 7-, 21- and 35-d. Calpain content in these fractions at each age and for each genotype were determined by Western analysis.

Mice

At each age, the numbers of each mouse genotype and their average body mass are shown in Table 3. Note, at age 7-d, the quadriceps mass was so small that tissue from 4 pups were pooled for one sample for the wt and mdx mice. Because of insufficient mdx:utrn$^{-/-}$ animals to pool at age 7-d, these data were not obtained.

Table 3. The number and genotype of mice and their average body mass (g). *No data at age 7-d were obtained. [a]The body mass of wt mice is significantly less than that of dystrophic mice at age 21-d, $p<0.05$. [b]The body mass of mdx:utrn$^{-/-}$ was less than that of wt and mdx, $p<0.05$.

	wt	**mdx**	**mdx:utrn$^{-/-}$**
7d	4.07±0.14g; n=6 (24)	5.33±0.29g; n=6 (24)	*
21d	[a]7.33±0.38g; n=6	10.7±0.57g; n=6	10.40±0.43g; n=4
35d	19.37±1.18g; n=6	19.90±1.10g; n=6	[b]14.58±1.25g; n=4

Real-Time Calpain Activity Assay

Calpain activity was determined as the calcium-dependent cleavage of the flurogenic substrate SLY-AMC over a minimum period of 50 sec, and expressed as arbitrary units per minute per milligram of total protein (AU/mg/min).

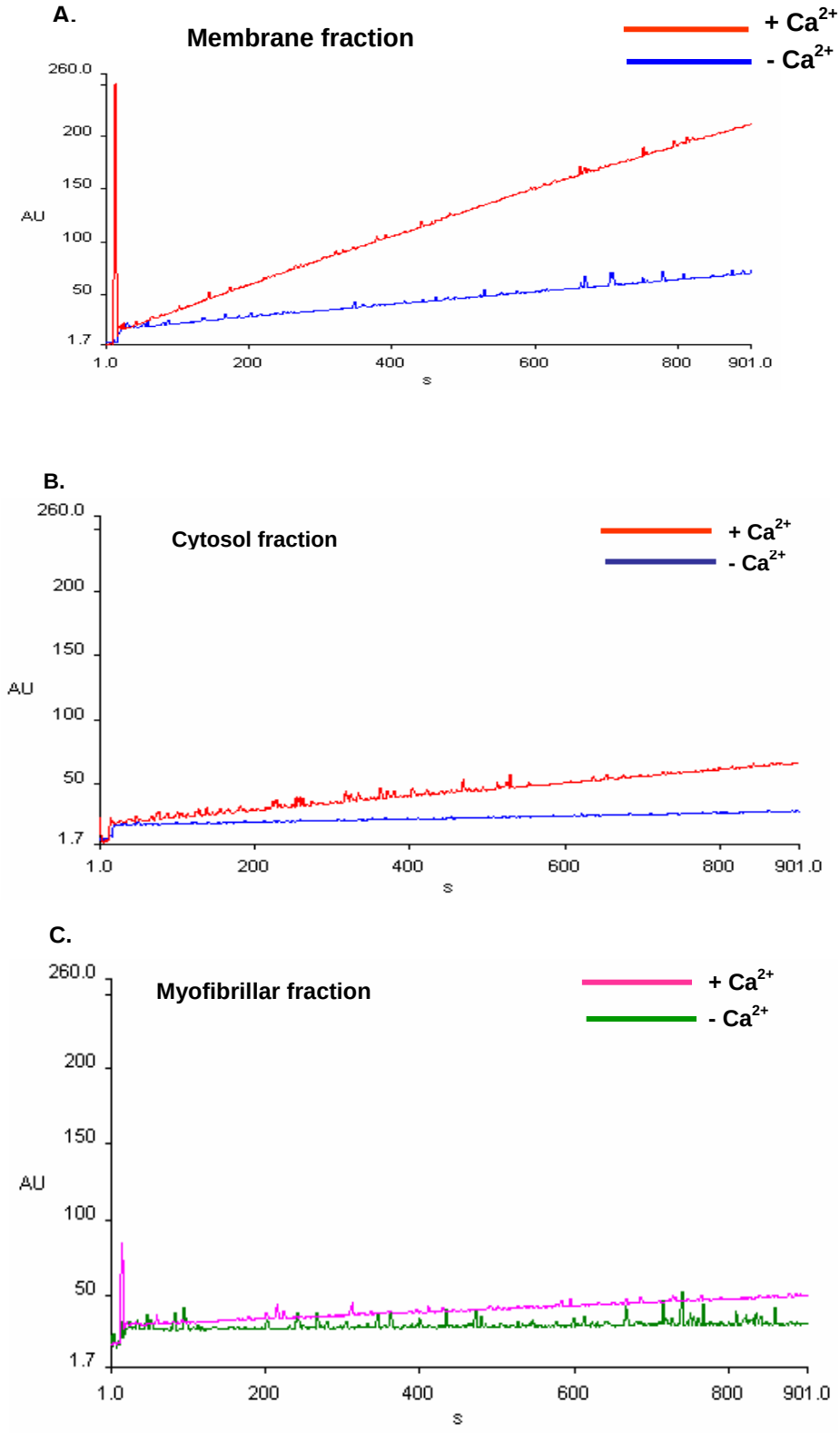

Figure 7. Examples of calpain activity (in arbitrary units, AU) in each subcellular fraction of 21-d mdx mice in the presence and absence of calcium.

As shown in Figure 7, calpain activity was increased in the presence compared to the absence of calcium. This difference was most apparent in the membrane (A) compared to either the cytosol (B) or myofibrillar (C) fractions. Calpain activity in the presence of calcium was always greater than in the absence of calcium across both age and genotype.

Absolute calpain activity

Across age within genotype, there were no significant differences in calpain activity in the membrane fraction of mdx and mdx:utrn$^{-/-}$ mice (Figure 8), however, calpain activity in the membrane fraction of wt mice at age 21-d was less than that at age 7- and 35-d ($p<0.05$).

Across genotype within age, calpain activity in the membrane fraction of mdx mice was greater than that of wt mice ($p<0.05$), and calpain activity in the myofibrillar supernatant and cytosolic fractions of mdx mice were greater than that of wt and mdx:utrn$^{-/-}$ mice ($p<0.05$) (Figure 8).

Calpain activity normalized to total protein content

- Total protein content determined by Bradford assay (Table 4)

Total protein content in the cytosol and myofibrillar pellet fractions of all genotypes increased significantly from age 21- to 35-d. However, total protein content in the membrane fraction did not change across age for any of the genotypes, and was significantly less than all the other subcellular fractions across age within genotype.

Table 4. Total protein content *No data at age 7-d were obtained. [a]Total protein content at age 35-d were greater than that of age 21-d within each subcellular fraction, $p<0.05$. [b]Total protein content were greater than that of age 21-d within genotype, $p<0.05$. [c]Total calpain content in the cytosolic fraction of mdx mice were greater than that of age 7- and 21-d, $p<0.05$. [d]Total calpain content in the myofibrillar supernatant fraction were greater than that of age 7-d, $p<0.05$.

		Total protein concentration (mg/ml)		
		Age		
Genotype	**Fraction**	**7d**	**21d**	**35d**
wt	myo-s	1.19 ± 0.22	1.21 ± 0.08	[c]2.56 ± 0.16
	myo-p	2.41 ± 0.24	2.28 ± 0.21	[ac]5.27 ± 0.18
	membrane	0.70 ± 0.08	0.74 ± 0.05	1.10 ± 0.18
	cytosol	3.11 ± 0.39	3.50 ± 0.18	[ac]4.92 ± 0.37
mdx	myo-s	2.02 ± 0.37	2.56 ± 0.19	[d]3.25 ± 0.35
	myo-p	3.24 ± 0.36	3.58 ± 0.19	[ac]5.12 ± 0.47
	membrane	0.97 ± 0.27	0.30 ± 0.08	1.12 ± 0.36
	cytosol	2.88 ± 0.19	1.80 ± 0.17	[ac]4.78 ± 0.84
mdx:utrn$^{-/-}$	myo-s	*	1.25 ± 0.22	1.85 ± 0.38
	myo-p	*	2.83 ± 0.63	[a]3.65 ± 0.77
	membrane	*	1.07 ± 0.01	1.00 ± 0.24
	cytosol	*	2.90 ± 0.23	[ab]4.31 ± 0.29

• Calpain activity normalized to total protein content.

Calpain activity in the presence and absence of calcium was measured and normalized to the total protein content of each sample and expressed as an arbitrary unit per min per mg (AU/min/mg) (Figure 9).

Calpain activity was calcium-dependent. Calpain activity in the presence of calcium was significantly greater than that in the absence of calcium in all subcellular fractions across age and genotype.

Calpain activity was predominant in the membrane fraction both in the presence and absence of calcium, with very little activity evident in the other three subcellular fractions.

Within genotype across age, calpain activity in the membrane fraction of wt mice at age 7-d was significantly greater than that of age 21- and 35-d. However, there was no significant difference between calpain activities in the membrane fraction of mdx mice across age.

Within age across genotype, calpain activity in the membrane fraction of wt mice at age 7-d was significantly greater than that of age-matched mdx mice. At age 21-d, calpain activity in the membrane fraction of mdx was significantly greater than that of wt and mdx:utrn$^{-/-}$. At age 35-d, there is no significant difference in calpain activity in each subcellular fraction.

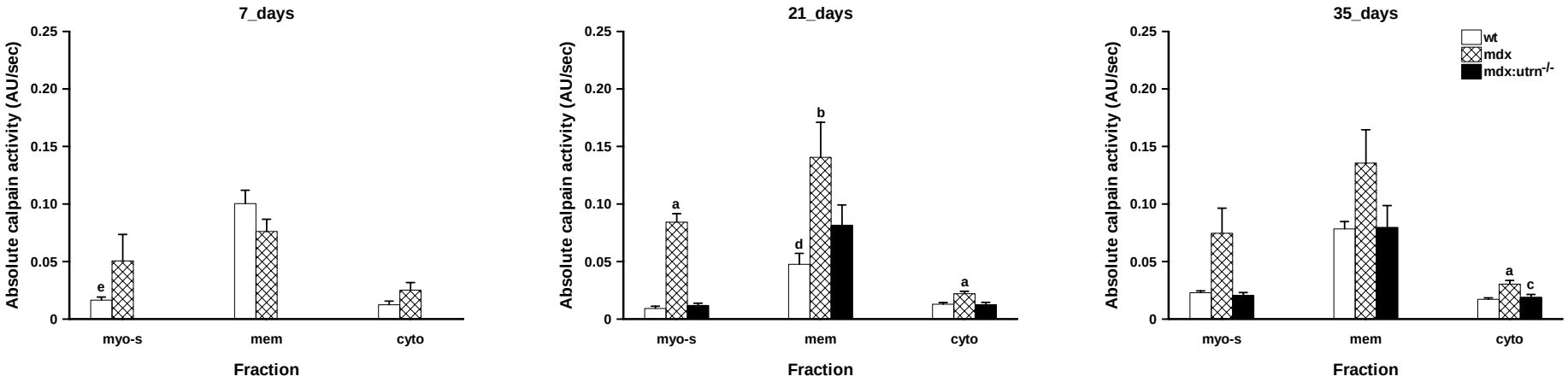

Figure 8. Absolute calpain activity of quadriceps subcellular fractions obtained from each mouse genotype at each age in the presence of Ca^{2+}. Calpain activity of mdx:utrn$^{-/-}$ mice at age 7-d was not available as there were insufficient numbers of this genotype. [a] Calpain activity is significantly greater than that of wt and mdx:utrn$^{-/-}$ mice within age ($p<0.05$); [b] Calpain activity is significantly greater than that of wt mice within age ($p<0.05$); [c] Calpain activity is significantly greater than that of age 21-d within genotype ($p<0.05$); [d] Calpain activity is significantly less than that of mice at age 7- and 35-d within genotype; [e] Calpain activity is significantly less than that of 35-d and greater than that of 21-d within genotype ($p<0.05$).

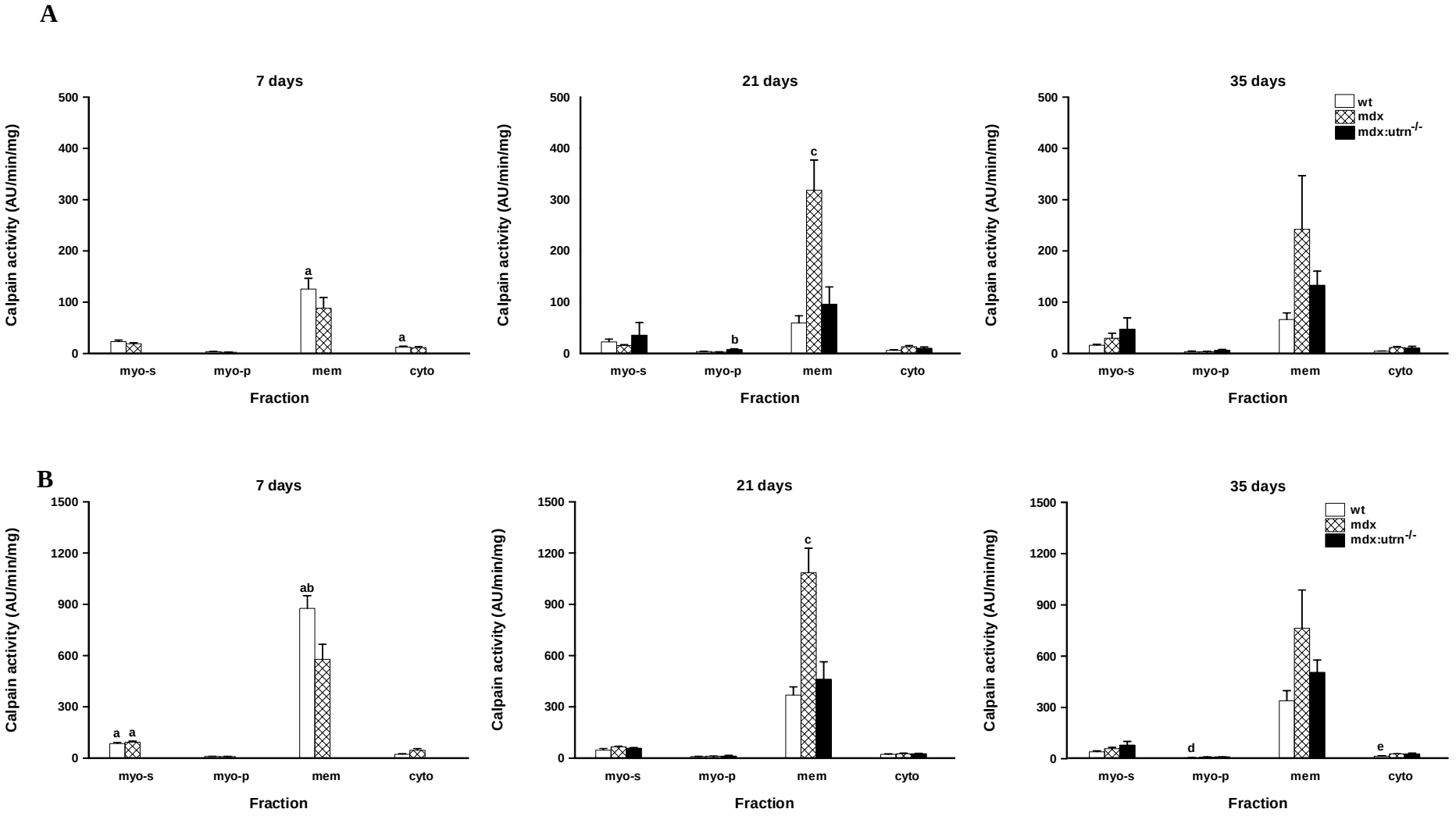

Figure 9. (A) Calpain activity (normalized to total protein) of quadriceps subcellular fractions obtained from each mouse genotype at each age in the absence of Ca^{2+}. Calpain activity of mdx:utrn$^{-/-}$ mice at age 7-d was not available as there were insufficient numbers of this genotype. The range of calpain activity in the absence of Ca^{2+} is one-third of that in the presence of Ca^{2+} (panel B). [a] Calpain activity is significantly greater than that of 21- and 35-d within genotype ($p<0.05$); [b] Calpain activity is significantly greater than that of wt and mdx mice within age ($p<0.05$); [c] Calpain activity is significantly greater than that of wt and mdx:utrn$^{-/-}$ mice within age ($p<0.05$);
(B) (normalized to total protein) of quadriceps subcellular fractions obtained from each mouse genotype at each age in the presence of Ca^{2+}. Calpain activity of mdx:utrn$^{-/-}$ mice at age 7-days was not available as there were insufficient numbers of this genotype. [a] Calpain activity is significantly greater than that of 21- and 35-d within genotype ($p<0.05$); [b] Calpain activity is significantly greater than that of mdx mice within age ($p<0.05$); [c] Calpain activity is significantly greater than that of wt and mdx:utrn$^{-/-}$ mice within age ($p<0.05$); [d] Calpain activity is significantly greater less than that of mice at age 7- and 21-d within genotype ($p<0.05$). Note: the range of relative calpain activity in the presence of Ca^{2+} was 3-fold of that in the absence of calcium in panel A.

Calpain activity normalized to total calpain content

Calpain activity was normalized to total calpain content to determine if increased calpain activity was due to either increased total calpain content or more activity per unit of calpain.

Total calpain content in each sample was determined by Western analysis for mice at each age (7-, 21-, and 35-d) and for each genotype (wt, mdx, mdx:utrn$^{-/-}$). Densitometry was used to assess sample calpain content. The density of each protein band was measured and normalized to the density of a control μ-calpain (Sigma) band on the same blot. To determine the optimal amount of control protein to load, a loading curve for the control protein was performed (Figure 10).

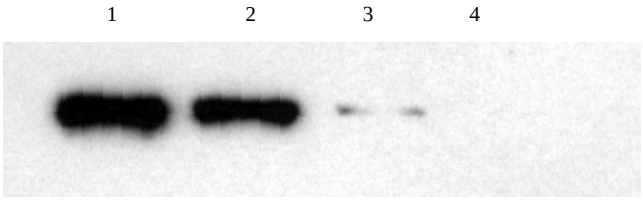

Figure 10. Western analysis of a loading curve of purified μ-calpain. (Lane: 1, 2μg; 2, 1μg; 3, 0.5μg; 4, 0.2μg).

In many of our western blots (12 of 16), our initial aliquot of purified μ-calpain was used successfully at 0.2 -0.5 μg (note: no loading curve was run). However, when we purchased additional purified protein to complete our blots (4 of 16), the sensitivity to the antibody was reduced, and therefore we ran a loading curve. Based on the loading curve (Figure 10), a range of 1~2μg μ-

calpain was used thereafter. The reason for the difference between the two aliquots of purified μ--calpain is not clear.

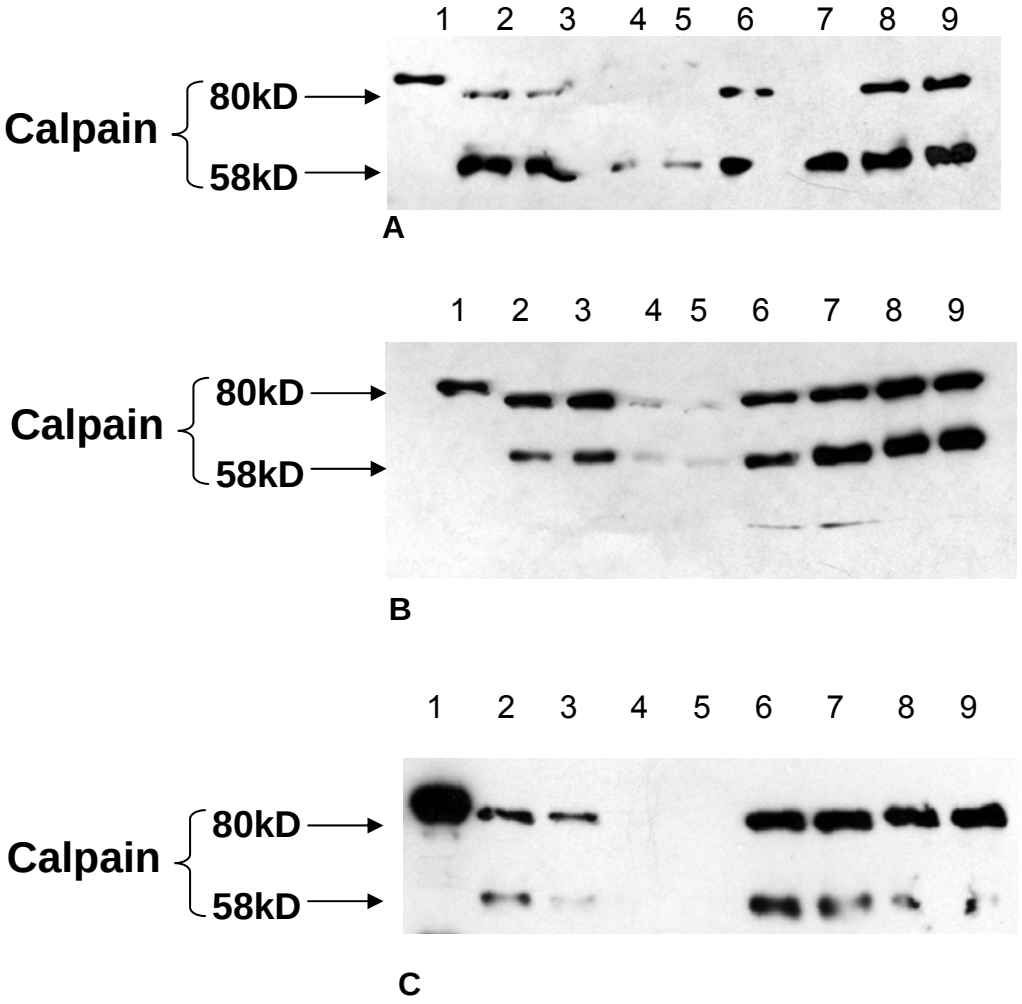

Figure. 11 Western analysis for total calpain in quadriceps. Lane:1 Control μ-calpain (0.2μg for blot A &B; 1.5μg for blot C), 2&3 Myofibrillar supernatant fractions; 4&5, Myofibrillar pellet fractions; 6&7, Membrane fractions; 8&9, Cytosol fractions. (A). Total calpain in wild type mice at age 21-d; (B) Total calpain in mdx mice at age 21-d; (C) Total calpain in mdx:utrn$^{-/-}$ mice at age 21-d.

Examples of immunoblots of total calpain in each subcellular fraction obtained from wild type, mdx and mdx:utrn$^{-/-}$ mice of age 21-d are shown in Figure 11. Calpain was detected in each subcellular fraction. However, there was

a relatively small amount of calpain evident in the myofibrillar pellet fraction of the two dystrophic genotypes. Both dystrophic genotypes displayed autolyzed calpain fragments, 80kD and 58kD.

Total calpain content

- General trends across age and genotype

In general, total calpain content in the whole quadriceps muscle decreased between age 7- and 35-d in both the control and mdx mice (Figure 12). Calpain content was significantly greater at age 7-d compared to age 35-d for both control and mdx mice, respectively ($p<0.05$). Calpain content was predominant in the membrane and cytosol fractions, with little in the myofibrillar pellet fraction. There was greater calpain content in the membrane fractions across age (7-35d) and/or across genotype ($p<0.05$), but no significant difference in calpain content of the pellet fraction was evident across age and/or genotype.

- Within genotype and across age

In wild type mice, mean total calpain content in the membrane fraction (1.28μg) at age 7-d was significantly greater than that at age 21- (0.23μg) and 35-d (0.13μg; $p<0.05$). Calpain content in the other subcellular fractions (myofibrillar and cytosol) were not different across age. In mdx mice, total calpain content in the membrane and cytosol fractions were significantly different across age. Mean total calpain content in the cytosol fraction at age 7-d (1.86μg) was about 4-fold that at age 21- (0.58μg) and 35-d (0.44μg; $p<0.05$). Total calpain content in the membrane fraction at age 35- d (0.34μg) was significantly less

than that of age 7- (1.03μg) and 21-d (1.40μg; $p<0.05$). However, calpain content in the myofibrillar fractions were not different across age.

In mdx:utrn$^{-/-}$ mice, calpain content decreased in the membrane fraction from age 21- (1.02μg) to 35-d (0.48μg; $p<0.05$). There were no differences in the other subcellular fractions across age.

- Within age and across genotype

Calpain content was not different within each age group across genotype for the myofibrillar pellet fraction. There was no difference in the calpain content of the membrane fraction at age 7-d between wt and mdx mice.

At age 21-d, calpain content in the membrane fraction of wt mice (0.23μg) was less than that of mdx (1.40μg; $p<0.05$) and mdx:utrn$^{-/-}$ mice (1.02μg; $p<0.05$), but there was no difference in calpain content between mdx and mdx:utrn$^{-/-}$ mice.

At age 35-d, calpain content in the membrane fraction of wt mice (0.13μg) was less than that of mdx:utrn$^{-/-}$ mice (0.48μg; $p<0.05$). Calpain content in the cytosol fraction of mdx:utrn$^{-/-}$ mice (0.88μg) was greater than that of wt (0.30μg; $p<0.05$) at age 35-d. Calpain content in the myofibrillar supernatant fraction of mdx:utrn$^{-/-}$ mice (0.49μg) was greater than that of wt mice (0.17μg; $p<0.05$).

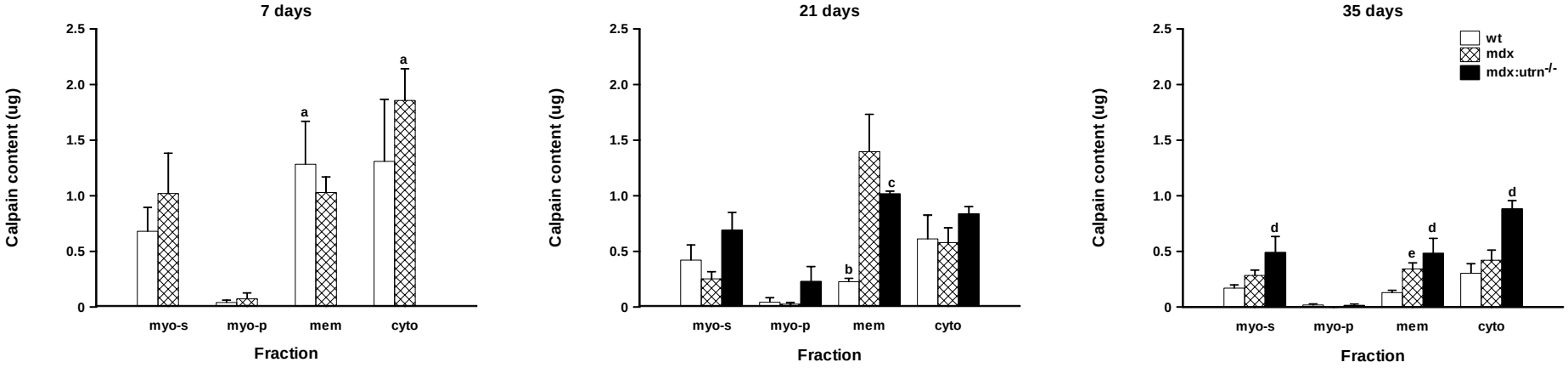

Figure 12 Total calpain content of quadriceps subcellular fractions obtained from each mouse genotype at each age. Calpain content was determined from 30µg of total protein loaded on an SDS gel, and then quantified by densitometry following Western anaylsis. Calpain content of mdx:utrn$^{-/-}$ mice at age 7-d was not available as there were insufficient numbers of this genotype. [a] Calpain content significantly greater than that at age 21- and 35-d within genotype ($p<0.05$); [b] Calpain content significantly less than that of mdx and mdx:utrn$^{-/-}$ mice within age group($p<0.05$); [c] Calpain content significantly greater than that at age 35 days within genotype ($p<0.05$); [d] Calpain content significantly greater than that of wt mice within age ($p<0.05$); [e] Calpain content significantly less than that of mice at age 7- and 21-days within genotype ($p<0.05$).

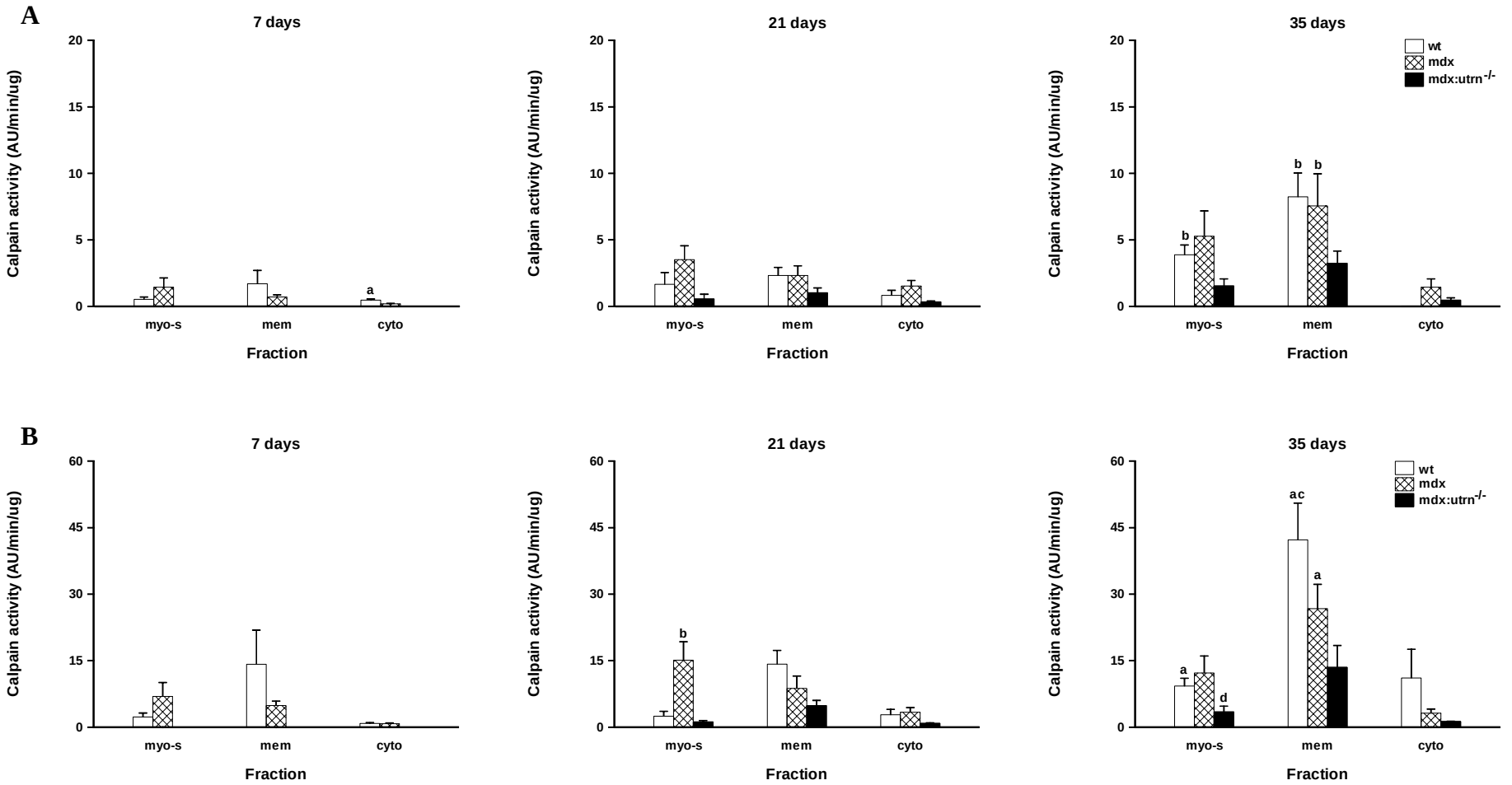

Figure 13 (A) Relative calpain activity of quadriceps subcellular fractions obtained from each mouse genotype at each age in the absence of Ca^{2+}. Relative calpain activity of mdx:utrn$^{-/-}$ mice at age 7-d was not available as there were insufficient numbers of this genotype. [a] Calpain activity was significantly greater than that of mdx within age ($p<0.05$); [b] Calpain activity was significantly greater than that of mice at age 7- and 21-d within age ($p<0.05$).

(B) Relative calpain activity of quadriceps subcellular fractions obtained from each mouse genotype at each age in the presence of Ca^{2+}. Relative calpain activity of mdx:utrn$^{-/-}$ mice at age 7-d was not available as there were insufficient numbers of this genotype. [a] Calpain activity was significantly greater than that of 7- and 21-d within genotype ($p<0.05$); [b] Calpain activity was significantly greater than that of wt and mdx:utrn$^{-/-}$ mice within age ($p<0.05$); [c] Calpain activity was significantly greater than that of mdx:utrn$^{-/-}$ mice within age ($p<0.05$); [d] Calpain activity was significantly greater than that of mice at age 21-d within genotype ($p<0.05$). Note: the range of relative calpain activity in the presence of Ca^{2+} was 3-fold of that in the absence of Ca^{2+} in panel A.

Relative calpain activity

Relative calpain activity was determined by expressing each sample's total calpain activity relative to its total calpain content as estimated by Western analysis (Figure 13). Because calpain content was not detected in the myofibrillar pellet fractions in many samples independent of age and genotype, we did not calculate calpain activity for this fraction.

- General trends across age and genotype

In general, relative calpain activity was predominant in the membrane fraction, and was greater in the presence compared to the absence of calcium. Age and genotype both had significant effects on calpain activity in the membrane fraction in the presence of calcium.

- Within genotype and across age

In wt mice, relative calpain activity in the membrane fraction in the presence of calcium was greater at age 35-d (42.25AU) than that at age 7-(14.17AU; $p<0.05$) and 21-d (14.21AU; $p<0.05$), and this held true with relative calpain activity in the myofibrillar supernatant fraction 9.30AU (35-d) vs 1.21 (7-d) and 2.48AU (21-d; $p<0.05$).

In mdx mice, age only affected relative calpain activity in the membrane fraction. Calpain activity in the membrane fraction of mice aged 35-d (26.73AU) was greater than that of 21d (8.74AU; $p<0.05$) and 7d (4.9AU; $p<0.05$).

In mdx:utrn$^{-/-}$ mice, calpain activity in the cytosol fraction was less at age 21-d (0.88AU) than at age 35-d (1.27AU; $p<0.05$). There were no differences in calpain activity across age in any of the other subcellular fractions.

• Within age and across genotype

At age 7-d, there were no differences in calpain activity in any of the subcellular fractions across genotype.

At age 21-d, relative calpain activity of the myofibrillar supernatant fraction of mdx mice (15.13AU) was greater than that of wt (2.49AU; $p<0.05$) and mdx:utrn$^{-/-}$ mice (1.18AU; $p<0.05$). There were no differences in calpain activity across genotype in any of the other subcellular fractions.

At age 35-d, relative calpain activity in the membrane fraction of wt (42.15AU) was significantly greater than that of mdx:utrn$^{-/-}$mice (13.49AU; $p<0.05$). There were no differences in calpain activity across genotype in any of the other subcellular fractions.

Chapter 5: Discussion

Calcium influx through skeletal muscle membrane defects and/or calcium channels could activate calcium-dependent calpain proteolysis (Hutter, 1992; Vandebrouck et al., 2002). If such defects were evident during maturation, activation of calcium-dependent calpains could represent an onset mechanism for DMD. Previously in our lab, Draper evaluated calpain activity in the structure-bound protein fraction of quadriceps obtained from maturing mdx mice to determine if increased calpain activity played a role in the onset of DMD (Draper, unpublished), and found a greater calpain activity in the structure-bound pellet fraction of mdx mice at age 7-d. However, in this study, calpain activity in the membrane compared to the myofibrillar fractions could not be discriminated because both were considered present in the structure-bound fraction (refer to Figure 6). Therefore, the purpose of this study was to determine if greater calcium-dependent calpain content and activity were present in the membrane fraction of quadriceps muscles of dystrophic *mdx* and *mdx:utrn*$^{-/-}$ mice obtained at maturing ages of 7- and 21-d prior to or in the initial stages of dystrophic process, respectively, and at a post-weaning age of 35-d, at a time following the onset of disease. Calpain activity was determined by an enzyme activity assay and calpain content by Western analysis. Calpain activity was normalized to total protein and total calpain content (relative calpain activity).

Major findings

Studies conducted in our lab are among the first to study the distribution of calpain content and activity in subcellular fractions of dystrophic skeletal muscle

during early maturation. Our results indicate that: (1) relative calpain activity was predominant in the membrane and myofibrillar supernatant fractions for all genotypes at each age; (2) total calpain content in each subcellular fraction decreased as the mice aged from 7- to 35-d, but relative calpain activity in the membrane fraction increased; (3) across age within genotype, there was an inverse relation between relative calpain activity and content in the membrane fraction of wt and mdx mice from age 7- to 35-d; (4) within age across genotype, total calpain content in each subcellular fraction of mdx:utrn$^{-/-}$ mice at age 35-d was significantly greater than wt, whereas relative calpain activity in the wt compared to mdx:utrn$^{-/-}$ membrane fraction at age 35-d was significantly greater; (5) at age 21-d, there was greater calpain content in the mdx compared to the wt in the membrane fraction and greater relative calpain activity in the mdx compared to the wt myofibrillar supernatant fraction.

Within genotype across age

Within genotype, calpain content of both membrane and cytosolic fractions of wt mice decreased significantly from age 7- to 35-d, and calpain content of the membrane fractions of mdx mice at age 7- and 21-d were significantly greater than that of 35-d. In contrast, relative calpain activity in the membrane fraction of wt and mdx mice at age 35-d was significantly greater than those at age 7- and 21-d within genotype. This suggests that, as mice matured from age 7- to 35-d, overall calpain content in the muscle decreased, but there was greater proteolytic activity per unit calpain. This greater activity might reflect regulation associated with specific maturation processes, such as myoblast fusion and muscle

differentiation (Moyen et al., 2004). Because this distinct inverse relation was true for both the wt and mdx genotypes, it suggests the role of calpain was similar in both, possibly up to age 21-d, as there is little overt dystrophy present in the mdx mice at this age. However, between ages 21- and 35-d, although the inverse relation is maintained, the roles for increased calpain activity in mdx compared to wt muscle likely differed.

The increased relative calpain activity in the membrane fraction of mdx mice at the post-dystrophic onset age of 35-d is temporally related to the rounds of degeneration and regeneration cycles in the mdx muscle (Grady et al., 1997). During muscle regeneration, there is formation of new myotubes (Anderson and Vargas, 2003), and calpain is involved in myoblast fusion (Schollmeyer, 1986). Inhibition of calpain activity in L6 and C2C12 myoblasts prevents myotube formation (Colby-Germinario et al., 2004). Alderton and Steinhardt (2000) suggest that calpain proteolysis is high in both normal and mdx myotubes during myotube alignment and fusion. At age 35-d in wt, it would be reasonable to assume that the increased calpain activity is not likely involved in the process of degeneration and regeneration. However, if the continuous alignment and fusion of satellite cells were evident in regenerating dystrophic muscle fibers at age 35-d, the greater relative calpain activity in the membrane fraction of mdx mice could reflect this function.

Relative calpain activity in the absence of calcium was significantly greater in the membrane fraction of wt and mdx mice at age 35-d compared to activities at age 7- and 21-d. This increased calcium-independent protease activity at age

35-d might be due to another protease that could cleave the calpain substrate used in this study. Based on our data, neither the identity of this protease nor the significance of this activity can be determined.

Within age across genotype

At age 7-d, there were no significant differences in either calpain content or relative calpain activity in all subcellular fractions for the wt and mdx mice. The high level of calpain content but low level of relative activity in all mdx subcellular fractions, except the myofibrillar pellet fraction, suggests that calpain was broadly distributed throughout the maturing muscle fibers playing an active role in the regulation of muscle maturation and differentiation. For example, the increased calpain content in the myofibrillar supernatant fraction might suggest a role of calpain in myofibrillar protein turnover (Wang and Yuen, 1999), whereas the high calpain content in the cytosolic fraction may reflect the need for remodeling of cytosolic proteins during maturation, because calpain can cleave a number of soluble substrates, e.g., protein kinase A, protein kinase C (Hong et al., 1995; Reyland et al., 2000), and transcription factors, e.g., c-Jun, MyoD (Wang and Yuen, 1999). Although we did not identify the specific substrate targets for calpain in the two genotypes, on the basis of similar calpain content and activity, we interpret these results to indicate the role of calpain for both was related to maturation and, therefore, the calpain activity in the mdx muscles was unlikely to be involved in the pathogenesis of muscular dystrophy at this age. Mdx:utrn$^{-/-}$ mice exhibit an earlier onset of dystrophic pathophysiology (age ~7-10-d; Grady et al, 1997). Unfortunately, we were unable to obtain data for the mdx:utrn$^{-/-}$ mice

at 7-d, so it is not known if calpain content and activity were different from wt and, therefore, if calpain was potentially involved in dystrophic onset.

At age 21-d, relative calpain activity in the myofibrillar supernatant fraction in mdx mice was significantly greater than that of wt mice. This could indicate initiation of proteolysis of submembranous networks of actin by cleavage of cytoskeletal proteins in submembranous networks (Dayton and Schollmeyer, 1981). Hydrolysis of cytoskeletal proteins by calpain could compromise plasma membrane permeability (Liu and Schnellmann, 2003) and affect the signaling pathway(s) from membrane receptors to the cytoskeletal network (Fluck et al., 2002). This could reflect an early event in dystrophic onset.

At age 21-d, relative calpain activity in the membrane fraction was not significantly different across genotype. However, calpain content in the membrane fraction of dystrophic mice was significantly greater than that of wt mice at this age; therefore, the absolute calpain activity before normalization to total calpain content was significantly greater than that of the age-matched control. This is consistent with evidence that calcium-dependent proteolysis in mdx muscle fibers was greater than controls at ages 3-6 weeks (Turner et al., 1993). Assuming the method used to isolate the membrane fraction reflected the true calpain content, the greater content in the mdx membrane fraction suggests a broader distribution of calpain among the muscle membranes at age 21-d. Although the membrane fraction did not represent sarcolemmal membranes only, it would be reasonable to assume that sarcolemmal membranes represented the greatest proportion of the membrane fraction. Calpains can bind phospholipids

(Berchtold et al., 2000). Thus, it is possible the increased calpain content could indicate a greater distribution of calpain along and/or represent concentrated foci of calpain content at the sarcolemmal membrane. Increased calpain content and activity in the membranes could lead to detrimental modifications of transient receptor channels (TRPC) in the plasma membrane (Vandebrouck et al., 2002), or the Ryr receptor and/or the Ca^{2+}-ATPase pump in the SR membrane (Rardon et al., 1990). One or all of these disruptions could cause unwanted calcium influx into the cytosol. In addition to the reduced proteolysis, the greater calcium influx through damaged dystrophic membranes might also serve as a signaling event to turn on additional expression of calpain (Deshpande et al., 1995), leading to further membrane damage.

At age 35-d, calpain content in mdx:utrn$^{-/-}$ mice were significantly greater than wt mice in the myofibrillar supernatant and both the membrane and cytosolic fractions. This increase did not appear to be a function of increased total protein because there were no significant differences in the total protein concentration of these fractions between the mdx:utrn$^{-/-}$ and wt mice. Therefore, it implies a broader distribution and/or more focused localization of calpain in the mdx:utrn$^{-/-}$ muscles.

The increases in calpain content and relative activity in the quadriceps muscles from mdx mice at 21-d and mdx:utrn$^{-/-}$ mice at 35-d supports the idea that calpains do play a role in dystrophic pathophysiology (Spencer et al., 1995).

Calpains as active contributors to the pathophysiology of DMD

To the author's knowledge, calpain content and activity have not been carefully studied at ages close to the onset of the dystrophic process. Calpain content and relative activity in all subcellular fractions were not different between wt and mdx mice at age 7-d, which suggested that calpains were unlikely to be involved in the pathophysiology of dystrophy in the mdx mouse at this age.

Increased expression of calpain has been found associated with apoptosis in rats (Ray et al., 2000). Many calpain substrates (e.g., fodrin, p53) are elements in the apoptotic pathway (Carafoli and Molinari, 1998), and the use of calpain inhibitors prevents apoptosis (Squier et al., 1994). Elevated apoptosis was noted in mdx muscle fibers at age 4-weeks (Tidball et al., 1995). Therefore, one possibility for the increased calpain expression at age 21-d in mdx and 35-d in mdx:utrn$^{-/-}$ could be related to apoptosis.

The onset of dystrophic process is believed to begin at age ~21-d in mdx mice (Shavlakadze et al., 2004). Thus, the greater calpain activity could represent broad proteolysis in the mdx compared to wt muscle fibers, coincident with or just following the onset of the dystrophic process (Mokhtarian et al., 1999). At age 21-d, there was a significantly greater absolute calpain activity in the membrane and myofibrillar supernatant fractions in mdx compared to wt mice. The increased mdx absolute calpain activity in these two subcellular fractions might reflect increased intracellular calcium in specific regions of the muscle fibers, which has been suggested by others (Robert et al., 2001; Turner et al., 1991). Calcium influx through transient membrane lesions or calcium leak

channels (Hutter, 1992; Vandebrouck et al., 2002) are thought responsible for the reduced ability of calcium handling in dystrophic muscle fibers, and thereby can lead to calcium-dependent proteolysis and ultimate fiber necrosis. However, it is not clear if this increased absolute calpain activity in the membrane and myofibrillar supernatant fractions of mdx mice started at age 21-d or earlier. Our data at age 7-d suggested calpain proteolysis activity in mdx mice compared to wt mice were similar and related to maturation. Draper's study (unpublished) indicated that there was no significant difference in calpain content in the structure-bound fraction between mdx and wt mice at age 14-d. Based on the present data, we can't determine if calpain proteolytic activity is a primary or a secondary event in the pathogenesis of the disease, but our data together with the Draper data suggest that increases in calpain proteolytic activity likely occurred between 14 and 21-d in the mdx muscles. However, because the content and activity in the mdx:utrn$^{-/-}$ mice of age 7-d were not obtained, we can't predict the potential age of onset in mdx:utrn$^{-/-}$ mice.

Regulation of calpain activity

The activation of calpains is regulated by binding of calcium and interactions with protein inhibitors, i.e., calpastatin (Carafoli and Molinari, 1998) or activators, i.e., membrane phospholipids (Coolican and Hathaway, 1984).

Calpains are calcium-dependent cysteine proteases, and the required concentrations of calcium for activation in vitro of ~1-100um for u-calpain and 0.1-1mM for m-calpain (Berchtold et al., 2000) are considerably higher than physiological calcium levels (<1uM) (Goll et al., 1992).

In addition to a required calcium level, calpain activation in vivo depends partially on calcium-dependent interactions with cytoskeletal structures (Mellgren, 1987), where calpains would have access to relevant substrates associated with the cytoskeletal matrix (Hosfield et al., 1999). These structures include the plasma membrane, ER and Golgi apparatus (Hood et al., 2003). In our study, we found significantly more calpain present in the myofibrillar supernatant fraction of mdx mice compared to wt mice at age 21-d. This might indicate the association of calpains with the cytoskeletal matrix for activation. This association may represent a potential mechanism for calpain to avoid its cytosolic inhibitor, calpastatin, and thereby facilitate activation (Hosfield et al., 1999).

Phospholipids are major components of membranes, including the sarcolemma, and activate calpain by reducing the amount of calcium required for activation (Saido et al., 1992). The interaction between calpain and phospholipids is thought to be mediated by the D-III domain, which is considered the calcium-dependent lipid-binding domain (Sutton et al., 1995). The interaction between calpain and phospholipids would position calpain in the close vicinity of potential substrates in the membranes and could induce a conformational change that would result in an increased sensitivity to activation by intracellular calcium (Hood et al., 2003). In dystrophic mice, damaged membranes likely expose phospholipids binding sites for the calpain lipid-binding domain (Hood et al., 2003), which would therefore influence calpain activation and proteolytic activity near membranes. Calcium entry through these local membrane wounds (hot spots) may also promote increased influx of calcium through calcium leak

channels, which in turn could increase calcium-activated proteolysis (Alderton and Steinhardt, 2000b).

Calpastatin is an endogenous calpain inhibitor (Carafoli and Molinari, 1998). The presence or absence of calpastatin could be another factor that accounts for the discrepancy between a similar level of calpain content and significantly less calpain activity in the cytosolic fraction of all ages and genotypes in this study. For example, at age 35-d, there was about the same amount of calpain in the membrane (0.34μg) and cytosolic fractions (0.44μg); however, the corresponding relative calpain activity in the membrane fraction (26.73±5.48AU) was much greater than that in the cytosolic fraction (3.16±0.92AU). To assess the potential inhibitory potential of calpastatin, a single experiment was conducted to determine calpastatin distribution in each subcellular fraction (refer to Appendix C). The result revealed approximately equal amounts of calpastatin present in both membrane and cytosolic fractions, but no detectable content in either of the myofibrillar fractions. Given the dominant calpain activity in the membrane compared to the cytosolic fraction, this result suggests that calpain activity in the membrane fraction was not fully inhibited by endogenous calpastatin. Therefore, the increased activity in the membrane fraction was likely due to activation by phospholipids.

Summary

In the present study, the data support the idea that calpains are active contributors to the pathophysiology of muscular dystrophy. We found significantly

greater absolute calpain activity in the membrane fraction of mdx compared to wt mice at age 21-d. Although this age is typically cited as the age of dystrophic onset in mdx mice, the specific age at which calpain content and activity are increased can't be delineated based on the present data. However, the results of the Draper and current studies suggest the onset of increased calpain activity likely lies between ages 14- and 21-d. Thus, it is reasonable to conclude that increased calpain activity in maturing mdx skeletal muscle membranes is closely related to the onset of DMD. Unfortunately, we were unable to obtain data for the mdx:utrn$^{-/-}$ mice at 7-d, therefore, it is not known if calpain was potentially involved in dystrophic onset in mdx:utrn$^{-/-}$ mice.

The similar pattern for relative calpain content and activity in wt and mdx mice from age 7- to 35-d suggested at least some of the mdx calpain activity was associated with the regulation of muscle maturation and differentiation, likely between 7- and 21-d. What is not known is if calpain activity in the mdx muscles was regulating maturation processes in a similar way to wt during this period or if these processes were disrupted and thereby contributed to the dystrophic pathophysiology. However, at 35-d in mdx:utrn$^{-/-}$ muscles, it is likely the increased calpain content and modest relative activity may have been in part due to regeneration of muscle fibers.

Calpains are calcium-activated. However, our results show that calpains were present in other subcellular fractions, such as the myofibrillar supernatant and cytosolic fractions, but showed little activity compared to the membrane fraction. These results might suggest that calpain is activated by interactions with

phospholipids in addition to calcium. This interaction could explain the increased activity in the membrane fractions.

Research hypotheses conclusions

The null hypothesis H_0 was rejected because there was significantly greater activity in the membrane fraction of mdx mice at age 21-d. The main hypothesis 1 was partially rejected, because we could only conclude that calpain proteolysis was closely related to the onset of DMD. The main hypothesis 2 was partially rejected because increased calpain activity was not always predominant in the membrane fraction. H_1 was partially rejected, because calpain content was not always greater in the membrane than myofibrillar and cytosolic fractions at all ages. H_2 was accepted, because calpain activity was calcium-activated in all fractions for all genotypes. H_3 was rejected because the membrane fraction did not always contain the majority of calpain content at all ages. H_4 was accepted because one of our major findings was that relative calpain activity increased because mice matured. H_5 was rejected because calpain activity in the membrane fraction of mdx:utrn$^{-/-}$ mice was not always greater than that of mdx mice.

Future research directions

This investigation found that relative calpain activity in the membrane fraction increased significantly at age 35-d in both wt and mdx mice compared to that of 7- and 21-d. We think this increased activity in mdx mice was associated with the temporally related degeneration and regeneration cycles, and this role of

calpain is likely different from that in wt mice. To determine if calpain is involved in the degeneration and regeneration cycles in mdx mice, a future study could subject wt mice muscles to injury to trigger regenerative processes for comparison to mdx muscles at the same age. For, example, if this was done at age 14-d, then the wt injured muscles and the mdx muscles could be examined to determine if calpain content and activity change in a similar way between age 14- and 21-d. A similar study could be conducted at an age corresponding to the cycles of degeneration and regeneration occurring in mdx mice (e.g., 21-42-d). If there was no significant difference between calpain activity in the injured wt and mdx mice at the same age, it could suggest that calpain is indeed temporally related to the regenerative process in mdx mice, and this role of calpain is not evident in the age matched wt mice.

Phospholipids have been suggested to activate calpains near membranes. However, the activation mechanism is still unclear. Determination of potential differences in phospholipid composition between dystrophic and normal sarcolemmal membranes might provide some insights into the underlying mechanism.

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Appendix A:

Sample protein profiles by SDS-PAGE analysis

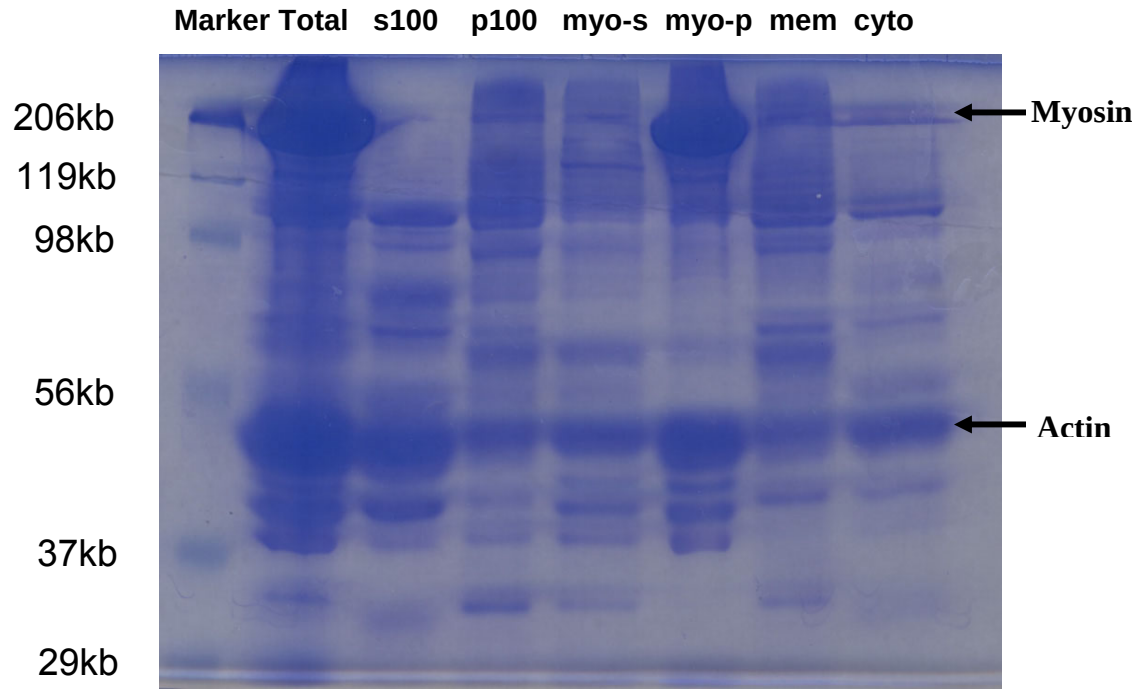

Figure 14 Protein profiles of quadriceps muscle subcellular fractions Thirty µg of protein (190-d mdx mice) from each fraction were loaded onto a 12% gel to assess the protein profiles of each sample fraction. (Total: total muscle homogenate; s100/p100: the supernatant/pellet fraction of the structure-bound fraction as described in Draper's study; Myo-s: myofibrillar protein supernatant; myo-p: myofibrillar protein pellet; Mem: membrane proteins; Cyto: cytosol fraction).

According to the SDS protein profile, one of the major myofibrillar proteins, myosin (~200 kD), was found predominantly in the myo-p fraction following the 1,000 g spin, whereas another major protein, actin, was present in all fractions, but had the least content in the membrane fraction. Content of both these myofibrillar proteins are greatly reduced in the membrane fraction as expected. It suggests that our sample preparation method primarily separated myofibrillar protein from other cellular fractions.

Appendix B:

Subcellular fraction identification by marker proteins

1. Myofibrillar fraction identification

α1- actin was selected as a marker of the myofibrillar fraction. Thirty µl of muscle sample was loaded in each well, and bovine vascular aortic endothelial cell lysate was used as a reference. The primary anti-α1-actin was used at a 1:1000 dilution (Affinity Bioreagents), and a secondary anti-mouse antibody was used at a 1:2500 dilution (Jackson Immunoresearch).

As shown in Figure 15, actin was predominant in the myofibrillar supernatant and pellet fractions. There was also some actin present in the membrane fraction, but no detectable actin band in the cytosol fraction. Given actin is a component in membrane cytoskeletal structures, its presence in the membrane fractions seems reasonable. Therefore, this immunoblot indicated that our preparation separated the myofibrillar fraction from other cellular fractions successfully.

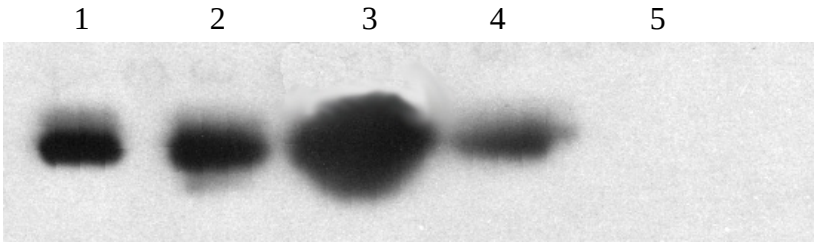

Figure 15. Western analysis of α1-actin in each subcellular fraction. Lane1. Bovine vascular aortic endothelial cell lysate as a reference; 2. Myofibrillar supernatant; 3 Myofibrillar pellet; 4. Membrane fraction; 5. Cytosol fraction.

2. Membrane fraction identification

Na^+/K^+-ATPase was selected as a membrane marker. Thirty µl of muscle sample was loaded in each well, and 1µg of rat brain microsomal preparation (Upstate) was used as a control on the same blot. The primary anti-Na^+/K^+-ATPase was applied at a 1:1000 dilution (Abcam, ab7671), and secondary anti-mouse antibody at a 1:2500 dilution (Jackson Immunoresearch).

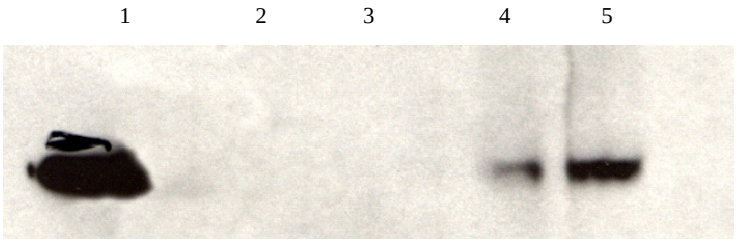

Figure 16. Western analysis of Na^+/K^+-ATPase in each subcellular fraction Lane1. Rat brain microsomal preparation as a control; 2. Myofibrillar supernatant; 3 Myofibrillar pellet; 4. Membrane fraction; 5. Cytosol fraction.

As shown in Figure 16, Na^+/K^+-ATPase was almost equally present in both membrane and cytosol fractions, but was not present in either the myofibrillar supernatant or pellet fractions. This confirmed our membrane fraction was totally separated from the myofibrillar proteins. However, the presence of Na^+/K^+-ATPase in the membrane and cytosol fractions could have been caused by: 1) the dissociation of Na^+/K^+-ATPase from the sarcolemmal membranes into the cytosol fraction; or 2) residual sarcolemmal membranes in the cytosol fraction (i.e., incomplete separation of the membrane from the cytosol fraction after ultra-centrifugation). Based on relative calpain activity data, it was more likely that the presence of Na^+/K^+-ATPase in the cytosol fraction was caused by dissociation rather than incomplete separation, because there was very low relative calpain

activity in the cytosol fraction (i.e., the presence of phospholipids from membranes would have likely activated calpain in the cytosol fraction, but activity in this fraction was low).

Appendix C:

Relative calpastatin and calpain content in different subcellular fractions

To determine if calpain activity in each subcellualr fraction was inhibited by calpastatin, after probing with the total calpain antibody, the same blot was "stripped" (i.e., original primary and secondary antibodies removed chemically), and then the blot was probed for the presence of calpastatin.

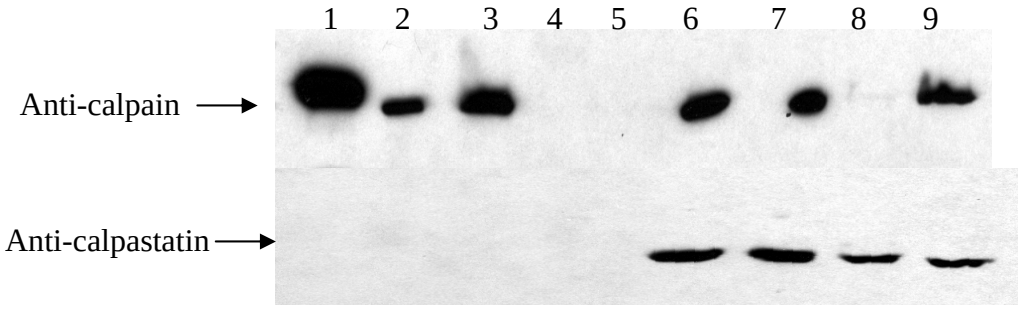

Figure 17. Western analysis of calpain and calpastatin in each subcellular fraction. Samples were obtained from mdx mice of age 21-d. Primary anti-calpastatin (Sigma) was applied at a dilution of 1:5000 over night at 4°C, and the secondary anti-rabbit was applied at a dilution of 1:25,000 for 2 hours. Lane1.μ-calpain; 2&3: myofibrillar supernatant; 4&5: myofibrillar pellet; 6&7: membrane fraction; 8&9: cytosol fraction.

Calpain is present in myofibrillar supernatant, membrane and cytosol fractions, but the calpain inhibitor, calpastatin, is not present in either myofibrillar supernatant or pellet fractions. This suggested that calpastatin was not associated with myofibrillar structure, but more likely present as a cytosolic inhibitor.

Appendix D Raw data

Mouse age and body mass

Mouse ID	Age (day)	Genotype	Body mass (g)
326p1-4	7	wt	4.05
1226a-p1-4	7	wt	4.53
1226b-p1-4	7	wt	4.33
1229a-p1-4	7	wt	4.13
1229b-p1-4	7	wt	3.88
1221p1-4	7	wt	3.58
Mean			4.07
SEM			0.14
326a	21	wt	7.00
2028p1	21	wt	7.00
2028p2	21	wt	6.50
1214p1	21	wt	8.00
1214p2	21	wt	8.90
1220p1	21	wt	6.60
Mean			7.33
SEM			0.38
347p1	35	wt	15.90
347p2	35	wt	15.80
347p3	35	wt	19.30
128p1	35	wt	21.80
128p2	35	wt	21.50
1978	35	wt	21.90
Mean			19.37
SEM			1.18
2017ap1-4	7	mdx	4.38
2017bp1-4	7	mdx	4.63
216p1	7	mdx	5.08
216p2	7	mdx	5.13
6057p1-4	7	mdx	8.20
828p1-4	7	mdx	4.60
Mean			5.33
SEM			0.29
2034p1	21	mdx	9.60
6507	21	mdx	12.50
2034p3	21	mdx	10.30
6831p1	21	mdx	10.30
6831p2	21	mdx	12.30
6830p2	21	mdx	9.20
Mean			10.70
SEM			0.57

Mouse ID	Age (day)	Genotype	Body mass (g)
2031p1	35	mdx	20.30
2031p2	35	mdx	20.00
1971p1	35	mdx	21.40
1971p2	35	mdx	21.20
1976	35	mdx	21.90
1984	35	mdx	14.60
Mean			19.90
SEM			1.10
114a	21	mdx:utrn$^{-/-}$	9.60
114b	21	mdx:utrn$^{-/-}$	10.10
216p1	21	mdx:utrn$^{-/-}$	10.30
216p2	21	mdx:utrn$^{-/-}$	11.60
Mean			10.40
SEM			0.43
1997a-p1	35	mdx:utrn$^{-/-}$	14.70
1997a-p2	35	mdx:utrn$^{-/-}$	17.00
111	35	mdx:utrn$^{-/-}$	11.10
302	35	mdx:utrn$^{-/-}$	15.50
Mean			14.58
SEM			1.25

Relative calpain activity and content

∗Calpain activity in the myofibrillar pellet fraction was not determined due to the absence of calpain content in some of the myofibrillar pellet fractions. S1000: myofibrillar pellet fraction; p1000: myofibrillar pellet fraction; p85K (the pellet from the 315,000g (i.e., 85Krpm) centrifugation step): membrane fraction; gly: cytosol fraction. Sample calculation for 1226a-s1000: 0.025AU/sec* 60sec/min / (0.01ml*1.59mg/ml) ≅ 94AU/min/mg Notes: (1) 0.01 ml was the sample volume used in the assay; (2) only Absolute calpain activity in the presence of Ca^{2+} is presented in the tables below.

wt mice at age 7-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain content (ug)
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$		
1226a-s1000	0.025	94.92	24.42	0.99	0.26	1.59	1.52
1226b-s1000	0.021	92.34	29.21	1.15	0.36	1.36	1.09
1229a-s1000	0.015	86.57	14.38	2.81	0.47	1.04	0.32
1229b-s1000	0.013	105.78	27.91	1.25	0.33	0.73	0.62
1221-s1000	0.006	74.78	29.72	1.01	0.40	0.51	0.38
326-s1000	0.018	57.05	11.96	0.02	1.38	1.91	0.17
Mean	0.016	85.24	22.93	1.2	0.5	1.19	0.7
SEM	0.003	7.00	3.19	0.4	0.2	0.22	0.2
1226a-p1000	0.005	10.39	4.55	4.69	2.05	2.80	0.06
1226b-p1000	0.003	8.43	5.19	1.47	0.90	2.38	0.14
1229a-p1000	0.004	9.36	3.19	∗	∗	2.47	0.00
1229b-p1000	0.005	14.03	2.40	∗	∗	1.93	0.00
1221-p1000	0.003	11.42	0.44	25.14	0.97	1.60	0.01
326-p1000	0.003	5.03	3.39	4.65	3.13	3.26	0.04
Mean	0.004	9.78	3.19	∗	∗	2.41	0.04
SEM	0.000	1.23	0.68	∗	∗	0.24	0.02
1226a-p85K	0.100	941.85	118.33	2.69	0.34	0.64	2.24
1226b-p85K	0.098	967.20	170.02	2.50	0.44	0.61	2.36
1229a-p85K	0.087	702.74	113.46	3.81	0.61	0.74	1.37
1229b-p85K	0.087	1131.70	190.45	3.85	0.65	0.46	1.35
1221-p85K	0.155	880.09	119.08	49.36	6.68	1.06	0.19
326-p85K	0.074	633.86	41.31	22.81	1.49	0.70	0.19
Mean	0.100	876.24	125.44	14.2	1.7	0.70	1.3
SEM	0.012	74.54	21.23	7.7	1.0	0.08	0.4
1226a-gly	0.023	33.79	21.01	0.40	0.25	4.08	3.43
1226b-gly	0.021	28.56	13.90	0.48	0.23	4.40	2.62
1229a-gly	0.010	22.42	10.72	0.84	0.40	2.72	0.73
1229b-gly	0.005	9.90	8.30	0.68	0.57	2.90	0.42
1221-gly	0.005	15.26	8.25	1.26	0.68	1.90	0.23
326-gly	0.011	24.45	10.83	1.50	0.66	2.67	0.44
Mean	0.012	22.40	12.17	0.9	0.5	3.11	1.3
SEM	0.003	3.56	1.96	0.2	0.1	0.39	0.6

wt mice at age 21-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$		
1214p1-s1000	0.010	39.28	44.13	4.80	5.39	1.59	0.13
1214p2-s1000	0.014	66.66	31.58	6.70	3.18	1.25	0.12
1220p1-s1000	0.012	74.21	21.31	0.74	0.21	1.01	1.02
326a-s1000	0.011	39.44	16.70	1.40	0.59	1.20	0.32
2028p1-s1000	0.001	55.04	7.56	1.22	0.17	1.07	0.54
2028p1-s1000	0.007	3.30	14.44	0.09	0.39	1.13	0.40
Mean	0.009	46.32	22.62	2.5	1.7	1.21	0.4
SEM	0.002	10.35	5.40	1.1	0.9	0.08	0.1
1214p1-p1000	0.004	10.39	4.55	*	*	3.24	0.00
1214p2-p1000	0.004	8.43	5.19	*	*	2.13	0.00
1220p1-p1000	0.003	9.36	3.19	0.64	0.39	2.06	0.25
326a-p1000	0.004	14.03	2.40	*	*	2.42	0.00
2028p1-p1000	0.001	11.42	0.44	3.41	0.94	1.72	0.08
2028p2-p1000	0.005	5.03	3.39	1.22	0.70	2.12	0.07
Mean	0.004	9.39	3.70	*	*	2.28	0.07
SEM	0.001	1.36	0.65	*	*	0.21	0.04
1214p1-p85K	0.047	377.69	58.35	18.62	2.88	0.74	0.15
1214p2-p85K	0.047	378.09	56.32	20.56	3.06	0.75	0.14
1220p1-p85K	0.090	558.39	100.18	22.47	4.03	0.97	0.24
326a-p85K	0.030	416.93	92.92	13.24	2.95	0.72	0.22
2028p1-p85K	0.023	251.21	44.94	5.39	0.96	0.60	0.34
2028p2-p85K	0.049	229.07	1.71	4.97	0.04	0.70	0.28
Mean	0.048	368.56	59.07	14.21	2.32	0.75	0.23
SEM	0.010	48.91	14.53	3.12	0.61	0.05	0.03
1214p1-gly	0.009	33.79	21.01	3.53	2.36	3.79	0.16
1214-2-gly	0.012	28.56	13.90	8.53	1.60	3.83	0.09
1220p1-gly	0.010	22.42	10.72	0.40	0.14	3.27	1.55
326a-gly	0.019	9.90	8.30	1.09	0.19	4.05	0.65
2028p1-gly	0.015	15.26	8.25	1.89	0.20	3.09	0.61
2028p2-gly	0.012	24.45	10.83	1.41	0.46	2.97	0.62
Mean	0.013	22.23	6.04	2.81	0.82	3.50	0.61
SEM	0.001	2.28	1.18	1.22	0.38	0.18	0.21

wt mice at age 35-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$		
347p1-s1000	0.022	45.63	19.42	6.13	2.61	2.02	0.18
347p2-s1000	0.029	52.16	14.51	4.95	1.38	2.40	0.29
347p3-s1000	0.025	37.69	13.51	10.61	3.80	2.62	0.11
1978-s1000	0.018	56.39	18.64	16.29	5.39	2.38	0.09
128p1-s1000	0.024	21.95	20.90	6.65	6.34	2.72	0.20
128p2-s1000	0.020	24.46	8.22	11.15	3.75	3.19	0.16
Mean	0.023	39.71	15.87	2.49	1.65	2.56	0.42
SEM	0.002	5.83	1.93	1.08	0.88	0.16	0.14
347p1-p1000	0.007	3.75	0.11	4.99	0.14	4.88	0.04
347p2-p1000	0.008	6.27	1.73	0.00	0.00	4.82	0.00
347p3-p1000	0.009	6.85	1.53	5.75	1.28	5.92	0.07
1978-p1000	0.003	8.71	10.75	*	*	5.06	0.00
128p1-p1000	0.006	2.68	1.31	*	*	5.31	0.00
128p2-p1000	0.006	3.36	1.52	*	*	5.63	0.00
Mean	0.006	5.26	2.82	*	*	5.27	0.02
SEM	0.001	0.97	1.6	*	*	0.18	0.01
347p1-p85K	0.056	299.28	51.81	36.22	6.27	0.65	0.13
347p2-p85K	0.081	350.95	64.93	49.31	9.12	0.74	0.11
347p3-p85K	0.066	414.98	81.44	32.57	6.39	0.69	0.18
1978-p85K	0.076	575.33	120.86	19.44	4.08	1.53	0.20
128p1-p85K	0.094	172.59	32.19	37.31	6.96	1.60	0.09
128p2-p85K	0.097	220.07	46.37	78.62	16.57	1.40	0.06
Mean	0.078	338.87	66.27	42.25	8.23	1.10	0.13
SEM	0.006	59.21	12.88	8.26	1.79	0.18	0.02
347p1-gly	0.021	14.85	3.31	2.14	0.48	3.95	0.37
347p2-gly	0.021	14.39	5.00	2.00	0.70	3.83	0.41
347p3-gly	0.017	16.83	3.57	1.73	0.37	4.63	0.58
1978-gly	0.013	22.61	5.60	2.99	0.74	5.38	0.35
128p1-gly	0.014	10.70	4.02	41.16	15.48	5.77	0.03
128p2-gly	0.017	10.80	5.05	16.71	7.81	5.93	0.07
Mean	0.017	15.03	4.42	11.12	4.26	4.92	0.30
SEM	0.001	1.81	0.38	6.46	2.54	0.37	0.09

mdx mice at age 7-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$		
828p1-s1000	0.162	97.57	21.94	17.19	3.87	3.32	0.57
6057p1-s1000	0.054	108.47	25.87	1.31	0.31	3.01	2.49
2017a-s1000	0.017	82.43	17.62	0.78	0.17	1.21	1.28
2017b-s1000	0.016	74.91	15.29	0.68	0.14	1.27	1.40
216p1-s1000	0.026	94.46	20.39	15.71	3.39	1.67	0.10
216p2-s1000	0.028	104.62	12.61	5.96	0.72	1.63	0.29
Mean	0.051	93.74	18.95	6.94	1.43	2.02	1.02
SEM	0.023	5.27	1.95	3.12	0.70	0.37	0.36
828p1-p1000	0.024	11.94	2.49	11.00	2.29	3.96	2.29
6057p1-p1000	0.008	10.29	1.40	1.47	0.20	4.49	0.20
2017a-p1000	0.003	8.54	2.25	*	*	2.44	0.00
2017b-p1000	0.004	9.94	2.47	*	*	2.16	0.00
216p1-p1000	0.003	5.88	1.56	*	*	3.33	0.00
216p2-p1000	0.005	9.03	3.86	*	*	3.08	0.00
Mean	0.008	9.27	2.34	*	*	3.24	0.42
SEM	0.003	0.83	0.36	*	*	0.36	0.38
828p1-p85K	0.107	372.98	57.24	7.75	1.19	1.72	0.83
6057p1-p85K	0.100	321.34	46.60	6.87	1.00	1.86	0.87
2017a-p85K	0.054	499.63	85.07	1.88	0.32	0.65	1.72
2017b-p85K	0.038	715.38	126.65	2.38	0.42	0.32	0.96
216p1-p85K	0.076	882.54	171.53	5.00	0.97	0.52	0.92
216p2-p85K	0.081	674.58	41.10	5.56	0.34	0.72	0.87
Mean	0.076	577.74	88.03	1.03	0.71	0.97	1.03
SEM	0.011	88.51	21.09	0.14	0.16	0.27	0.14
828p1-gly	0.015	12.31	3.65	0.40	0.12	2.47	2.26
6057p1-gly	0.058	93.15	20.69	1.14	0.25	3.72	3.05
2017a-gly	0.015	31.98	9.35	0.54	0.16	2.84	1.69
2017b-gly	0.017	39.99	12.06	0.62	0.19	2.52	1.62
216p1-gly	0.022	49.00	12.36	1.16	0.29	2.71	1.14
216p2-gly	0.024	46.79	8.07	1.04	0.18	3.02	1.36
Mean	0.025	45.54	11.03	0.82	0.20	2.88	1.86
SEM	0.007	10.95	2.32	0.14	0.03	0.19	0.28

mdx mice at age 21-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$		
2034p1-s1000	0.066	67.45	14.99	13.49	3.00	1.95	0.29
2034p3-s1000	0.074	60.68	11.64	13.24	2.54	2.45	0.34
6831p1-s1000	0.086	58.88	16.95	16.11	4.64	2.93	0.32
6831p2-s1000	0.117	72.66	14.01	16.28	3.14	3.22	0.43
6507p1-s1000	0.074	76.53	22.19	0.00	0.00	2.52	0.00
6830p2-s1000	0.088	59.01	14.43	31.65	7.74	2.29	0.14
Mean	0.084	65.87	15.70	15.13	3.51	2.56	0.25
SEM	0.007	3.08	1.47	4.12	1.05	0.19	0.06
2034p1-p1000	0.013	9.27	2.28	42.76	10.54	2.89	0.02
2034p3-p1000	0.017	10.86	2.70	84.14	20.90	3.18	0.01
6831p1-p1000	0.021	11.72	2.89	14.08	3.47	3.65	0.09
6831p2-p1000	0.025	12.84	4.29	39.16	13.10	3.87	0.04
6507p1-p1000	0.013	12.22	2.16	*	*	4.17	0.00
6830p2-p1000	0.023	6.17	0.41	*	*	3.73	0.00
Mean	0.019	10.51	2.46	*	*	3.58	0.03
SEM	0.002	1.01	0.51	*	*	0.19	0.01
2034p1-p85K	0.120	802.37	192.56	7.34	1.76	0.30	0.98
2034p3-p85K	0.199	632.42	162.07	20.09	5.15	0.63	0.60
6831p1-p85K	0.041	1651.93	565.87	0.86	0.29	0.05	2.89
6831p2-p85K	0.080	1136.71	371.20	3.09	1.01	0.14	1.55
6507p1-p85K	0.160	1181.29	295.34	9.48	2.37	0.29	1.53
6830p2-p85K	0.242	1105.41	321.76	11.59	3.37	0.41	0.83
Mean	0.140	1085.02	318.14	8.74	2.33	0.30	1.40
SEM	0.031	143.58	59.12	2.79	0.71	0.08	0.34
2034p1-gly	0.018	28.23	12.85	1.49	0.68	1.29	0.73
2034p3-gly	0.021	24.06	15.84	2.18	1.43	1.77	0.59
6831p1-gly	0.026	38.17	20.36	2.34	1.25	1.37	0.67
6831p2-gly	0.030	29.51	15.65	6.58	3.49	2.04	0.27
6507p2-gly	0.018	14.96	9.13	1.03	0.63	1.91	1.05
6830p2-gly	0.018	19.21	4.76	6.67	1.65	2.42	0.17
Mean	0.022	25.69	13.10	3.38	1.52	1.80	0.58
SEM	0.002	3.35	2.25	1.04	0.43	0.17	0.13

mdx mice at age 35-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$		
1971p1-s1000	0.123	51.50	14.33	7.80	2.17	4.05	0.22
1971p2-s1000	0.152	58.30	11.29	9.61	1.86	3.53	0.25
2031p1-s1000	0.023	60.79	29.85	28.79	14.14	2.23	0.26
2031p2-s1000	0.080	86.05	22.76	17.71	4.68	2.21	0.51
1976-s1000	0.029	35.83	20.49	4.44	2.54	3.33	0.18
1984-s1000	0.041	62.90	77.91	5.06	6.27	4.20	0.28
Mean	0.075	59.23	29.44	12.23	5.28	3.26	0.28
SEM	0.022	6.69	10.05	3.84	1.90	0.35	0.05
1971p2-p1000	0.022	6.16	1.57	*	*	3.72	0
1971p2-p1000	0.032	10.08	3.64	*	*	4.46	0
2031p1-p1000	0.006	11.90	5.31	*	*	4.69	0
2031p2-p1000	0.004	14.50	4.48	*	*	4.90	0
1976-p1000	0.006	5.38	0.81	*	*	6.17	0
1984-p1000	0.011	7.18	4.48	*	*	6.81	0
Mean	0.014	9.20	3.38	*	*	5.12	0
SEM	0.005	1.46	0.73	*	*	0.47	0
1971p1-p85K	0.176	316.41	63.08	33.55	6.69	0.23	0.17
1971p2-p85K	0.249	384.70	80.67	18.02	3.78	0.36	0.51
2031p1-p85K	0.060	1532.03	623.99	25.80	10.51	0.59	0.41
2031p2-p85K	0.080	1380.79	517.39	47.47	17.79	1.32	0.31
1976-p85K	0.097	361.93	70.16	27.42	5.31	1.84	0.17
1984-p85K	0.153	606.26	97.09	8.08	1.29	2.38	0.44
Mean	0.136	763.69	242.06	26.73	7.56	1.12	0.34
SEM	0.029	223.68	104.93	5.48	2.40	0.36	0.06
1971p1-gly	0.033	20.86	6.78	4.38	1.42	2.35	0.33
1971p2-gly	0.042	30.37	9.04	2.60	0.77	2.86	0.84
2031p1-gly	0.021	27.70	14.71	*	*	3.96	0
2031p2-gly	0.027	29.14	19.21	6.72	4.43	5.38	0.37
1976-gly	0.024	29.56	9.55	3.20	1.03	6.95	0.50
1984-gly	0.036	31.41	10.13	2.08	0.67	7.19	0.60
Mean	0.030	28.17	11.57	3.16	1.39	4.78	0.44
SEM	0.003	1.55	1.86	0.92	0.64	0.84	0.12

mdx:utrn$^{-/-}$ mice at age 21-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		+Ca^{2+}	-Ca^{2+}	+Ca^{2+}	-Ca^{2+}		
114a-s1000	0.008	54.91	19.84	0.75	0.27	0.88	0.65
114b-s1000	0.009	56.34	1.76	2.02	0.06	0.93	0.26
216p1-s1000	0.016	69.54	108.11	1.05	1.64	1.37	0.90
216p2-s1000	0.014	48.23	13.22	0.91	0.25	1.80	0.96
Mean	0.012	57.26	35.73	1.18	0.56	1.25	0.69
SEM	0.002	4.46	24.41	0.29	0.36	0.22	0.16
114a-p1000	0.003	9.33	4.28	*	*	1.78	0.00
114b-p1000	0.008	25.28	5.21	*	*	1.78	0.00
216p1--p1000	0.001	1.56	11.32	0.11	0.79	3.52	0.50
216p2-p1000	0.005	7.61	9.11	0.77	0.93	4.26	0.42
Mean	0.004	10.95	5.06	*	*	2.83	0.23
SEM	0.001	5.06	1.65	*	*	0.63	0.13
114a-p85K	0.072	404.74	1.35	4.05	0.01	1.07	1.07
114b-p85K	0.133	759.95	160.78	8.30	1.76	1.05	0.96
216p1-p85K	0.067	384.07	89.19	4.09	0.95	1.05	0.99
216p2-p85k	0.054	296.87	130.12	3.08	1.35	1.09	1.05
Mean	0.082	461.41	95.36	4.88	1.02	1.07	1.02
SEM	0.018	102.22	34.60	1.16	0.37	0.01	0.03
114a-gly	0.009	23.74	15.16	0.74	0.47	2.34	0.75
114b-gly	0.008	17.33	9.40	0.63	0.34	2.78	0.77
216p1-gly	0.016	31.90	3.05	1.22	0.12	3.09	0.81
216p2-gly	0.016	28.05	12.46	0.93	0.41	3.40	1.03
Mean	0.012	25.25	10.02	0.88	0.34	2.90	0.84
SEM	0.002	3.12	2.60	0.13	0.08	0.23	0.06

mdx:utrn$^{-/-}$ mice at age 35-d

Sample	Absolute calpain activity (AU/sec)	Calpain activity normalized to total protein (AU/min/mg)		Calpain activity normalized to total calpain (AU/min/ug)		Protein concentration (mg/ml)	Calpain Content (ug)
		+Ca^{2+}	-Ca^{2+}	+Ca^{2+}	-Ca^{2+}		
111-s1000	0.020	145.47	106.08	1.55	1.13	0.84	0.79
302-s1000	0.021	62.85	11.19	2.54	3.08	1.97	0.35
1997a-s1000	0.027	59.73	16.33	7.31	1.30	2.67	0.17
1997b-s1000	0.015	46.19	56.05	2.40	0.66	1.90	0.66
Mean	0.021	78.56	47.41	3.45	1.54	1.85	0.49
SEM	0.002	22.59	21.97	1.31	0.53	0.38	0.14
111-p1000	0.005	10.68	8.51	5.42	4.32	2.82	0.06
302-p1000	0.008	9.58	5.06	*	*	1.89	0.00
1997a-p1000	0.003	8.04	1.89	*	*	4.82	0.00
1997b-p1000	0.006	9.67	9.06	*	*	5.08	0.00
Mean	0.006	9.49	6.13	*	*	3.65	0.01
SEM	0.001	0.54	1.67	*	*	0.77	0.01
111-p85K	0.040	650.04	147.83	2.76	0.63	0.37	0.87
302-p85K	0.101	581.38	96.13	7.55	4.88	1.04	0.44
1997a-p85K	0.122	470.68	83.59	22.93	3.79	1.55	0.26
1997b-p85K	0.056	316.51	204.45	20.74	3.68	1.06	0.35
Mean	0.080	504.65	133.00	13.49	3.24	1.01	0.48
SEM	0.019	72.79	27.58	4.93	0.91	0.24	0.14
111-gly	0.026	41.59	11.27	1.44	0.39	3.73	1.07
302-gly	0.015	21.13	1.30	1.33	0.87	4.27	0.88
1997a-gly	0.015	17.58	9.55	1.26	0.08	5.12	0.72
1997b-gly	0.020	28.61	18.71	1.05	0.57	4.12	0.86
Mean	0.019	27.22	10.21	1.27	0.48	4.31	0.88
SEM	0.003	5.31	3.57	0.08	0.17	0.29	0.07

Densitometry data

Sample calculation for 1226a-s1000: 0.2µg * (1035544.75AU / 136391.50AU) ≅ 1.52µg

wt 7-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	136391.50	1.00	0.20
Track 2	1226a-s1000		
Number	Raw vol.		
1	327352.00	1035544.75	1.52
2	708192.75		
Track 3	1226b-s1000		
Number	Raw vol.		
1	125703.20	745812.80	1.09
2	599979.81		
3	20129.78		
Track 4	1226a-p1000		
Number	Raw vol.		
1	5232.60	42293.31	0.06
2	32000.46		
3	5060.25		
Track 5	1226b-p1000		
Number	Raw vol.		
1	16352.91	93404.11	0.14
2	77051.20		
Track 6	1226a-p85K		
Number	Raw vol.		
1	548212.75	1526926.78	2.24
2	942074.69		
3	18892.48		
4	17746.86		
Track 7	1226bp85K		
Number	Raw vol.		
1	618039.56	1607544.46	2.36
2	938939.25		
3	31956.48		
4	18609.17		
Track 8	1226a-gly		
Number	Raw vol.		
1	801691.69	2337676.29	3.43
2	232887.41		
3	1182995.25		
4	62351.30		
5	57750.65		
Track 9	1226b-gly		
Number	Raw vol.		
1	637175.19	1784391.81	2.62
2	1147216.63		

wt 7-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	118383.98	1.00	0.20
Track 2	1229a-s1000		
Number	Raw vol.		
1	168685.91	189525.87	0.32
2	20839.96		
Track 3	1229b-s1000		
Number	Raw vol.		
1	309426.81	366239.84	0.62
2	56813.03		
Track 4	1229b-p1000		
Number	Raw vol.	0.00	0.00
Track 5			
Number	Raw vol.	0.00	0.00
Track 6	1229a-p85K		
Number	Raw vol.		
1	485616.94	808625.91	1.37
2	323008.97		
Track 7	1229b-p85K		
Number	Raw vol.		
1	479950.53	800822.56	1.35
2	320872.03		
Track 8	1229a-gly		
Number	Raw vol.		
1	334685.34	431919.55	0.73
2	97234.20		
Track 9	1229b-gly		
Number	Raw vol.		
1	237620.23	248656.17	0.42
2	11035.94		

wt 7-d

	Control	Sample/ref density ratio	calpain (ug)
Number	**Raw vol.**		
1	274924.56	1.00	0.20
Track 2	**1221-s1000**		
Number	**Raw vol.**		
1	14305.39	517314.88	0.38
2	379617.81		
3	123391.67		
Track 3	**1221-p1000**		
Number	**Raw vol.**		
1	9992.01	9992.01	0.01
Track 4	**1221-p85K**		
Number	**Raw vol.**		
1	225383.94	266715.36	0.19
2	37830.21		
3	3501.21		
Track 5	**1221-gly**		
Number	**Raw vol.**		
1	310966.44	316011.67	0.23
2	5045.24		
Track 6	**326-s1000**		
Number	**Raw vol.**		
1	220178.17	227836.80	0.17
2	7658.63		
Track 7	**326p1000**		
Number	**Raw vol.**		
1	2020.14	48453.01	0.04
2	9972.14		
3	15274.79		
4	12379.08		
5	2503.72		
6	6303.13		
Track 8	**326-p85K**		
Number	**Raw vol.**		
1	5613.21	413458.37	0.19
2	391697.59		
3	16147.57		
Track 9	**326-gly**		
Number	**Raw vol.**		
1	589751.44	598011.18	0.44
2	8259.75		

wt 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1.00	914889.94	1.00	0.30
Track 2	1214p1-s1000		
Number	Raw vol.		
1.00	396686.75	0.13	0.13
Track 3	1214p2-s1000		
Number	Raw vol.		
1.00	379112.91	0.12	0.12
Track 4	1214p1-p1000		
Number	Raw vol.	0.00	0.00
Track 5	1214p2-p1000		
Number	Raw vol.		
1.00	0.00	0.00	0.00
Track 6	1214p1-p85K		
Number	Raw vol.		
1.00	457778.91	0.15	0.15
Track 7	1214p2-p85K		
Number	Raw vol.		
1.00	105710.20	0.03	0.14
Track 8	1214p1-gly		
Number	Raw vol.		
1.00	473451.56	0.16	0.16
Track 9	1214-2-gly		
Number	Raw vol.		
1.00	263402.38	0.09	0.09

wt 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1.00	77568.07	1.00	0.20
Track 2	1220p1-s1000		
Number	Raw vol.		
1.00	79256.41	0.20	1.02
2.00	57624.13	0.15	
3.00	258370.19	0.67	
Track 3	1220p1-p1000		
Number	Raw vol.		
1.00	4968.67	0.01	0.25
2.00	91606.05	0.24	
Track 4	1220p1-p85K		
Number	Raw vol.		
1.00	49087.96	0.13	0.24
2.00	54117.55	0.14	
3.00	345247.31	0.89	
Track 5	1220p1-gly		
Number	Raw vol.		
1.00	157082.41	0.41	1.55
2.00	445632.94	1.15	
Track 6	326a-s1000		
Number	Raw vol.		
1.00	91649.63	0.24	0.32
2.00	11285.60	0.03	
3.00	20560.15	0.05	
Track 7	326a-p1000		
Number	Raw vol.		
1.00	0.00	0.00	0.00
Track 8	326a-p85K		
Number	Raw vol.		
1.00	85503.51	0.22	0.22
Track 9	326a-gly		
Number	Raw vol.		
1.00	207320.73	0.53	0.65
2.00	43645.47	0.11	

wt 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1.00	276537.47	1.00	0.20
Track 2	**2028p1-s1000**		
Number	**Raw vol.**		
1.00	145956.06	0.11	0.54
2.00	602832.81	0.44	
Track 3	**2028p1-s1000**		
Number	**Raw vol.**		
1.00	90913.48	0.07	0.40
2.00	11156.74	0.01	
3.00	449041.97	0.32	
Track 4	**2028p1-p1000**		
Number	**Raw vol.**		
1.00	20285.46	0.01	0.08
2.00	87047.39	0.06	
Track 5	**2028p2-p1000**		
Number	**Raw vol.**		
1.00	15499.33	0.01	0.07
2.00	84520.33	0.06	
Track 6	**2028p1-p85K**		
Number	**Raw vol.**		
1.00	6783.03	0.00	0.34
2.00	184315.31	0.13	
3.00	273019.72	0.20	
Track 7	**2028p2-p85K**		
Number	**Raw vol.**		
1.00	382026.66	0.28	0.28
Track 8	**2028p1-gly**		
Number	**Raw vol.**		
1.00	278496.94	0.20	0.61
2.00	558978.38	0.40	
Track 9	**2028p2-gly**		
Number	**Raw vol.**		
1.00	333919.53	0.24	0.62
2.00	528125.50	0.38	

wt 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	214207.02	1.00	0.50
Track 2	347p1-s1000		
Number	Raw vol.		
1	75904.49	0.18	0.18
Track 3	347p2-s1000		
Number	Raw vol.		
1	122750.90	0.29	0.29
Track 4	347p1-p1000		
Number	Raw vol.		
1	16270.23	0.04	0.04
Track 5	347p2-p1000		
Number	Raw vol.	0.00	0.00
Track 6	347p1-p85K		
Number	Raw vol.		
1	54162.30	0.13	0.13
Track 7	347p2-p85K		
Number	Raw vol.		
1	48785.53	0.11	0.11
Track 8	347p1-gly		
Number	Raw vol.		
1	159616.73	0.37	0.37
Track 9	347p2-gly		
Number	Raw vol.		
1	177615.69	0.41	0.41

wt 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	219525.11	1.00	0.50
Track 2	347p3-s1000		
Number	Raw vol.		
1	49761.78	0.11	0.11
Track 3	347p3-p1000		
Number	Raw vol.		
1	29466.71	0.07	0.07
Track 4	347p3-p85K		
Number	Raw vol.		
1	147173.02	0.34	0.18
Track 5	347p3-gly		
Number	Raw vol.		
1	253091.69	0.58	0.58
Track 6	1978-s1000		
Number	Raw vol.		
1	39819.75	0.09	0.09
Track 7	1978-p1000		
Number	Raw vol.	0.00	0.00
Track 8	1978-p85K		
Number	Raw vol.		
1	204972.05	0.47	0.20
2	8846.34	0.02	
Track 9	1978-gly		
Number	Raw vol.		
1	153911.58	0.35	0.35

wt 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	696342.69	1.00	0.50
Track 2	128p1-s1000		
Number	Raw vol.		
1	278367.56	0.20	0.20
Track 3	128p2-s1000		
Number	Raw vol.		
1	219865.34	0.16	0.16
Track 4	128p1-p1000		
Number	Raw vol.	0.00	0.00
Track 5	128p2-p1000		
Number	Raw vol.	0.00	0.00
Track 6	128p1-p85K		
Number	Raw vol.		
1	125613.53	0.09	0.09
Track 7	128p2-p85K		
Number	Raw vol.		
1	86545.90	0.06	0.06
Track 8	128p1-gly		
Number	Raw vol.		
1	42894.99	0.03	0.03
Track 9	128p2-gly		
Number	Raw vol.		
1	103390.95	0.07	0.07

Mdx 7-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	21723.57	1	0.20
Track 2	828p1-s1000		
Number	Raw vol.		
1	61399.82	61399.82	0.57
Track 3	828p1-p1000		
Number	Raw vol.		
1	7308.53	14009.30	0.13
2	6700.77		
Track 4	828p1-p85K		
Number	Raw vol.		
1	40462.88	89939.34	0.83
2	49476.45		
Track 5	828p1-gly		
Number	Raw vol.		
1	87282.14	245959.72	2.26
2	158677.58		
Track 6	6057p1-s1000		
Number	Raw vol.		
1	93509.28	270717.89	2.49
2	177208.61		
Track 7	6057p1-p1000		
Number	Raw vol.		
1	34116.30	34116.30	0.31
Track 8	6057p1-p85K		
Number	Raw vol.		
1	34365.41	94503.52	0.87
2	60138.10		
Track 9	6057p1-gly		
Number	Raw vol.		
1	123635.07	331507.79	3.05
2	207872.72		

Mdx 7-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	173315.84	1.00	0.20
Track 2	2017a-s1000		
Number	Raw vol.		
1	459757.75	1106163.63	1.28
2	646405.88		
Track 3	2017b-s1000		
Number	Raw vol.		
1	442681.00	1210162.00	1.40
2	767481.00		
Track 4	2017a-p1000		
Number	Raw vol.		
1	0.00	0.00	0.00
Track 5	2017b-p1000		
Number	Raw vol.		
1	0.00	0.00	0.00
Track 6	2017a-p85K		
Number	Raw vol.		
1	627031.94	1493725.13	1.72
2	866693.19		
Track 7	2017b-p85K		
Number	Raw vol.		
1	347459.44	835202.44	0.96
2	487743.00		
Track 8	2017a-gly		
Number	Raw vol.		
1	657926.69	1467154.22	1.69
2	149764.59		
3	659462.94		
Track 9	2017b-gly		
Number	Raw vol.		
1	674227.25	1406548.25	1.62
2	732321.00		

mdx 7-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	119132.27	1.00	1.00
Track 2	216p1-s1000		
Number	Raw vol.		
1	11961.94	11961.94	0.10
Track 3	216p2-s1000		
Number	Raw vol.		
1	34065.86	34065.86	0.29
Track 4	216p1-p1000		
Number	Raw vol.	0.00	0.00
Track 5	216p2-p1000		
Number	Raw vol.	0.00	0.00
Track 6	216p1-p85K		
Number	Raw vol.		
1	7012.67	109425.15	0.92
2	102412.48		
Track 7	216p2-p85K		
Number	Raw vol.		
1	104035.39	104035.39	0.87
Track 8	216p1-gly		
Number	Raw vol.		
1	21483.71	136263.54	1.14
2	114779.83		
Track 9	216p2-gly		
Number	Raw vol.		
1	10381.05	162520.91	1.36
2	152139.86		

Mdx 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	231598.80	1.00	0.20
Track 2	2034p1-s1000		
Number	Raw vol.		
1	241512.22	0.21	0.29
2	97231.72	0.08	
Track 3	2034p3-s1000		
Number	Raw vol.		
1	231238.80	0.20	0.34
2	158796.16	0.14	
Track 4	2034p1-p1000		
Number	Raw vol.		
1	21760.97	0.02	0.02
Track 5	2034p3-p1000		
Number	Raw vol.		
1	14260.40	0.01	0.01
Track 6	2034p1-p85K		
Number	Raw vol.		
1	307555.00	0.27	0.98
2	349460.19	0.30	
3	26796.65	0.02	
Track 7	2034p3-p85K		
Number	Raw vol.		
1	390861.81	0.34	0.59
2	263850.41	0.23	
3	34275.27	0.03	
Track 8	2034p1-gly		
Number	Raw vol.		
1	468769.31	0.40	0.73
2	378473.41	0.33	
Track 9	2034p3-gly		
Number	Raw vol.		
1	372875.81	0.32	0.59
2	307303.56	0.27	

Mdx 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	396853.00	1.00	0.20
Track 2	**6831p1-s1000**		
Number	**Raw vol.**		
1	396745.59	0.20	0.32
2	240658.73	0.12	
Track 3	**6831p2-s1000**		
Number	**Raw vol.**		
1	506032.03	0.26	0.43
2	349232.38	0.18	
Track 4	**6831p1-p1000**		
Number	**Raw vol.**		
1	75760.40	0.04	0.09
2	59410.42	0.03	
3	45657.88	0.02	
Track 5	**6831p2-p1000**		
Number	**Raw vol.**		
1	36516.11	0.02	0.04
2	39012.41	0.02	
Track 6	**6831p1-p85K**		
Number	**Raw vol.**		
1	419996.56	0.21	2.89
2	404205.44	0.20	
3	35088.11	0.02	
Track 7	**6831p2-p85K**		
Number	**Raw vol.**		
1	551117.63	0.28	1.55
2	673401.81	0.34	
3	66063.65	0.03	
Track 8	**6831p1-gly**		
Number	**Raw vol.**		
1	671902.19	0.34	0.67
2	659802.19	0.33	
Track 9	**6831p2-gly**		
Number	**Raw vol.**		
1	544934.63	0.27	0.27
2	663989.81		

Mdx 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	81090.34	1.00	0.20
Track 2	6507p1-s1000		
Number	Raw vol.		
1	0.00	0.00	0.00
Track 3	6507p1-p1000		
Number	Raw vol.	0.00	0.00
Track 4	6507p1-p85K		
Number	Raw vol.		
1	229831.03	480343.00	1.53
2	250511.97		
Track 5	6507p2-gly		
Number	Raw vol.		
1	211120.16	425907.44	1.05
2	214787.28		
Track 6	6830p2-s1000		
Number	Raw vol.		
1	57107.99	57107.99	0.14
Track 7	6830p2-p1000		
Number	Raw vol.	0.00	0.00
Track 8	6830p2-p85K		
Number	Raw vol.		
1	175087.55	321747.88	0.83
2	146660.33		
Track 9	6830p2-gly		
Number	Raw vol.		
1	66901.49	66901.49	0.17

Mdx 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	112026.16	1.00	0.50
Track 2	1971p1-s1000		
Number	Raw vol.		
1	39000.25	0.17	0.22
2	10285.54	0.05	
Track 3	1971p2-s1000		
Number	Raw vol.		
1	38470.68	0.17	0.25
2	18647.33	0.08	
Track 4	1971p1-p1000		
Number	Raw vol.		
Track 5	1971p2-p1000	0.00	0.00
Number	Raw vol.		
Track 6	1971p1-p85K	0.00	0.00
Number	Raw vol.		
1	32563.14	0.15	0.17
Track 7	1971p1-p85K		
Number	Raw vol.		
1	59023.77	0.26	0.51
2	54816.61	0.24	
Track 8	1971p1-gly		
Number	Raw vol.		
1	61563.89	0.27	0.33
2	12672.53	0.06	
Track 9	1971p2-gly		
Number	Raw vol.		
1	118279.94	0.53	0.84
2	70027.40	0.31	

Mdx 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	470300.06	1.00	1.00
Track 2	2031p1-s1000		
Number	Raw vol.		
1	120645.06	0.26	0.26
Track 3	2031p2-s1000		
Number	Raw vol.		
1	241957.47	0.51	0.51
Track 4	2031p1-p1000		
Number	Raw vol.		
Track 5	2031p2-p1000	0.00	0.00
Number	Raw vol.		
Track 6	2031p1-p85K	0.00	0.00
Number	Raw vol.		
1	192683.52	0.41	0.41
Track 7	2031p2-p85K		
Number	Raw vol.		
1	147728.13	0.31	0.31
Track 8	2031p1-gly		
Number	Raw vol.	0.00	0.00
Track 9	2031p2-gly		
Number	Raw vol.		
1	174876.27	0.37	0.37

Mdx 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	477579.47	1.00	0.50
Track 2	1976-s1000		
Number	Raw vol.		
1	170284.30	0.18	0.18
Track 3	1976-p1000		
Number	Raw vol.		0.00
Track 4	1976-p85K		
Number	Raw vol.		
1	173025.69	0.18	0.17
Track 5	1976-gly		
Number	Raw vol.		
1	474387.56	0.50	0.50
Track 6	1984-s1000		
Number	Raw vol.		
1	258272.17	0.27	0.28
2	6580.97	0.01	
Track 7	1984-p1000		
Number	Raw vol.		0.00
Track 8	1984-p85K		
Number	Raw vol.		
1	411767.63	0.43	0.44
2	10855.50	0.01	
Track 9	1984-gly		
Number	Raw vol.		
1	572477.56	0.60	0.60

Mdx:utrn$^{-/-}$ 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	448480.47	1.00	1.50
2	86627.66	535108.13	
Track 2	**114a-s1000**		
Number	**Raw vol.**		
1	155606.30	230283.45	0.65
2	74677.15		
Track 3	**114b-s1000**		
Number	**Raw vol.**		
1	80530.02	92359.66	0.26
2	11829.64		
Track 4	**114a-p1000**		
Number	**Raw vol.**	0.00	0.00
Track 5	**114b-p1000**		
Number	**Raw vol.**	0.00	0.00
Track 6	**114a-p85K**		
Number	**Raw vol.**		
1	246593.84	381401.08	1.07
2	134807.23		
Track 7	**114b-p85K**		
Number	**Raw vol.**		
1	264476.97	343147.14	0.96
2	78670.17		
Track 8	**114a-gly**		
Number	**Raw vol.**		
1	230359.25	268508.65	0.75
2	38149.40		
Track 9	**114b-gly**		
Number	**Raw vol.**		
1	250710.17	274939.30	0.77
2	24229.13		

Mdx:utrn$^{-/-}$ 21-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	968547.06	1.00	1.50
2	89557.02		
3	234336.00	1292440.08	
Track 2	**216p1-s1000**		
Number	**Raw vol.**		
1	335031.25	779053.34	0.90
2	444022.09		
Track 3	**216p2-s1000**		
Number	**Raw vol.**		
1	10313.79	823289.17	0.96
2	295196.41		
3	517778.97		
Track 4	**216p1--p1000**		
Number	**Raw vol.**		
1	124913.07	432033.07	0.50
2	307120.00		
Track 5	**216p2-p1000**		
Number	**Raw vol.**		
1	83276.88	361477.32	0.42
2	278200.44		
Track 6	**216p1-p85K**		
Number	**Raw vol.**		
1	429067.56	849296.84	0.99
2	420229.28		
Track 7	**216p2-p85k**		
Number	**Raw vol.**		
1	381792.63	904165.16	1.05
2	522372.53		
Track 8	**216p1-gly**		
Number	**Raw vol.**		
1	445155.19	697825.48	0.81
2	252670.30		
Track 9	**216p2-gly**		
Number	**Raw vol.**		
1	442548.91	883444.09	1.03
2	440895.19		

Mdx:utrn$^{-/-}$ 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	385656.28	1.00	1.50
2	118002.09	503658.37	
Track 2	111-s1000		
Number	Raw vol.		
1	234069.55	263966.17	0.79
2	29896.63		
Track 3	111-p1000		
Number	Raw vol.		
1	18651.18	18651.18	0.06
Track 4	111-p85K		
Number	Raw vol.		
1	293095.00	293095.00	0.87
Track 5	111-gly		
Number	Raw vol.		
1	360536.31	360536.31	1.07
Track 6	302-s1000		
Number	Raw vol.		
1	116142.55	116142.55	0.35
Track 7	302-p1000		
Number	Raw vol.	0.00	0.00
Track 8	302-p85K		
Number	Raw vol.		
1	149195.17	149195.17	0.44
Track 9	302-gly		
Number	Raw vol.		
1	296714.16	296714.16	0.88

Mdx:utrn$^{-/-}$ 35-d

Track 1	Control	Sample/ref density ratio	calpain (ug)
Number	Raw vol.		
1	393727.69	1.00	1.50
Track 2	**1997a-s1000**		
Number	**Raw vol.**		
1	44443.88	44443.88	0.17
Track 3	**1997b-s1000**		
Number	**Raw vol.**		
1	156626.48	174521.18	0.66
2	17894.70		
Track 4	**1997a-p1000**		
Number	**Raw vol.**	0.00	0.00
Track 5	**1997b-p1000**		
Number	**Raw vol.**	0.00	0.00
Track 6	**1997a-p85K**		
Number	**Raw vol.**		
1	69214.35	69214.35	0.26
Track 7	**1997b-p85K**		
Number	**Raw vol.**		
1	92347.79	92347.79	0.35
Track 8	**1997a-gly**		
Number	**Raw vol.**		
1	188490.00	188490.00	0.72
Track 9	**1997b-gly**		
Number	**Raw vol.**		
1	225795.75	225795.75	0.86

Appendix E: Statistical analysis

Two-way ANOVA of calpain content for myofibrillar supernatant fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	0.6513	0.3256	1.76	0.1860
Age (days)	2	2.7353	1.3676	7.41	0.0020
Genotype x Age	3	0.3968	0.1323	0.72	0.5488
Residual	36	6.6484	0.1847		
Corrected Total	43	9.9425			

Two-way ANOVA of calpain content for myofibrillar pellet fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	0.1546	0.0773	0.61	0.5481
Age (days)	2	0.3940	0.1970	1.56	0.2245
Genotype x Age	3	0.3750	0.1250	0.99	0.4091
Residual	36	4.5529	0.1265		
Corrected Total	43	5.4094			

Two-way ANOVA of calpain content for membrane fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	1.5724	0.7862	3.21	0.0521
Age (days)	2	5.6644	2.8322	11.57	0.0001
Genotype x Age	3	3.1633	1.0544	4.31	0.0108
Residual	36	8.8154	0.2449		
Corrected Total	43	19.0310			

Two-way ANOVA of calpain content for cytosol fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	1.2820	0.6410	1.61	0.2133
Age (days)	2	9.8120	4.9060	12.35	<.0001
Genotype x Age	3	0.6401	0.2134	0.54	0.6600
Residual	36	14.3042	0.3973		
Corrected Total	43	25.2394			

Two-way ANOVA of calpain activity +Ca^{2+} for myofibrillar supernatant fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	788.9025	394.4513	10.18	0.0003
Age (days)	2	294.0488	147.0244	3.79	0.0320
Genotype x Age	3	149.7309	49.9103	1.29	0.2934
Residual	36	1395.2393	38.7566		
Corrected Total	43	2496.1665			

Two-way ANOVA of calpain activity -Ca^{2+} for myofibrillar supernatant fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	55.6722	27.8361	4.97	0.0124
Age (days)	2	75.1588	37.5794	6.71	0.0033
Genotype x Age	3	2.8886	0.9629	0.17	0.9147
Residual	36	201.6615	5.6017		
Corrected Total	43	318.1771			

Two-way ANOVA of calpain activity +Ca^{2+} for membrane fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	2056.9154	1028.4577	6.60	0.0036
Age (days)	2	4004.0119	2002.0060	12.86	<.0001
Genotype x Age	3	465.5987	155.1996	1.00	0.4055
Residual	36	5605.6861	155.7135		
Corrected Total	43	12014.2685			

Two-way ANOVA of calpain activity -Ca^{2+} for membrane fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	56.0074	28.0037	2.98	0.0635
Age (days)	2	275.4515	137.7257	14.65	<.0001
Genotype x Age	3	18.3704	6.1235	0.65	0.5873
Residual	36	338.4695	9.4019		
Corrected Total	43	679.6540			

Two-way ANOVA of calpain activity +Ca^{2+} for cytosol fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	153.4251	76.7125	2.04	0.1453
Age (days)	2	208.3701	104.1851	2.76	0.0764
Genotype x Age	3	155.8731	51.9577	1.38	0.2648
Residual	36	1356.4796	37.6800		
Corrected Total	43	1837.6602			

Two-way ANOVA of calpain activity -Ca^{2+} for cytosol fraction

Source of Variation	DF	SS	MS	F	P
Genotype	2	21.2098	10.6049	1.77	0.1849
Age (days)	2	32.4122	16.2061	2.70	0.0805
Genotype x Age	3	23.8808	7.9603	1.33	0.2803
Residual	36	215.7458	5.9929		
Corrected Total	43	287.8856			

Vita

Ms Qiong Wang was born in Shenyang, China in 1980. She earned her B.S. in Biotechnology at Wuhan University, China in 2003, and was recognized with the “Excellent Graduate” award. Qiong came to the United States to pursue her Masters degree in muscle physiology at Virginia Tech. During her Masters training, she maintained a GPA of 4.0/4.0, and was awarded both the “Hepler Summer Research Fellowship” in 2004, and the “Raville Outstanding Graduate Student” in April, 2005 by the Department of Human Nutrition, Foods, and Exercise. After earning her Masters degree, she will pursue her PhD in the Program of Biomedical Science at the University of Michigan in the Fall semester of 2005.