Effect of dietary protein on the calpain/calpastatin proteolytic system in canine skeletal muscle

by

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GENERAL INTRODUCTION

The calpains are a family of calcium-dependent cysteine proteinases that are expressed in skeletal muscle and all other tissues (Goll et al., 1992a). The calpain/calpastatin system has been implicated to play a role in muscle protein turnover (Goll et al., 1999). Calpain is suggested to initiate muscle protein turnover by degrading specific proteins for subsequent removal from the myofibril to allow replacement and repair of the proteins (Goll et al., 1999).

Calpastatin is a specific inhibitor of the calpains (Carafoli and Molinari, 1998). Calpastatin prevents the autoproteolysis of intact calpain, inhibits the expression of catalytic activity by autolyzed calpain, and competes with calpain for membrane binding (Melloni et al., 1992). Calpastatin is known to play a key role in the living animal in muscle protein turnover as a component of the calpain proteolytic system (Goll et al., 1992a). Calpastatin is hypothesized to play a role in muscle growth by inhibiting proteolysis by the calpains. This decrease in proteolysis causes decreased muscle protein degradation, which could affect muscle growth through an increase in protein accretion (Goll et al., 1991). Feeding ß-adrenergic agonists can increase calpastatin expression and activity in bovine skeletal muscle. In this particular case, observed calpastatin levels increased due to consumption of ß-agonist $L_{644,949}$ (Wheeler and Koohmarie, 1992). The

muscles in the hindquarters of callipyge sheep, which have an increase in muscle growth due to hypertrophy when compared to normal sheep, show higher calpastatin activity (Koohmarie et al., 1995). Levels of calpastatin in aging animals are also elevated when compared to young animals. In a comparative study using skeletal muscle in young and geriatric canines, Western blot analysis showed that the geriatric dogs had a significantly higher level of high molecular weight calpastatin than did the young dogs (Huff-Lonergan et al., 1999). The importance of calpastatin as a key element in the process of muscle protein turnover has consistently been observed, although it has proven to be one of the most variable components in the calpain system.

Recently the ubiquitously expressed calpain/calpastatin system has expanded to include tissue-specific calpains, adding a skeletal muscle specific form of calpain, p94 (Sorimachi et al., 1989). p94 is the first skeletal muscle specific calpain to be discovered. It was discovered during a cDNA screen for m- and μ-calpain (Sorimachi et al., 1989). The presence of p94 mRNA in skeletal muscle is tenfold higher than that of the ubiquitous calpains (Sorimachi et al., 1993). The exact function of p94 is unknown due to its apparent instability when it is separated from whole muscle. Since 1989, attempts at purifying p94 have been unsuccessful except by one research group (Kinbara et al., 1998). These results have not been confirmed by other laboratories. p94 is associated

with limb girdle muscular dystrophy type 2A (Spencer et al., 1997) in which the gene encoding p94 is mutated, producing absence or deficiency of the protein (Richard et al., 1995). p94 is proposed to have two binding sites on titin - one at the N_2 line and the other at the extreme C-terminus of titin (Sorimachi et al., 1995). Titin exhibits different isoforms which are present in different muscle types, including the two binding sites for p94 (Kinbara et al., 1997). These p94 specific binding sites may prove of great importance due to their association with known degradation points in titin, and therefore fragmentation in the myofibril. Because of these degradation sites in the myofibril, p94 could potentially play an important role in muscle protein turnover. Immunoblotting studies have shown that p94 has also been found in greater profusion within muscle fibers exhibiting fast, glycolytic metabolism, or type IIB fibers (Jones et al., 1999). This is also of interest due to the ability of muscle fibers to change metabolism based on exercise, innervation, or nutritional status.

Thesis organization

This thesis is organized according to an alternate style format. It is arranged beginning with a general introduction, followed by a general review of the literature, a publishable paper, and a concluding summary. References cited within each chapter are

listed at the end of that chapter. The paper will be submitted to Journal of Animal Science.

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GENERAL REVIEW OF LITERATURE

Calpain

Background

The calpain/calpastatin system is composed primarily of the ubiquitous cysteine proteinases m- and μ-calpain and their inhibitor, calpastatin (Goll et al., 1992a). These proteinases require calcium for activation (Goll et al., 1991). The calcium requirement for half-maximal activity for μ-calpain is 5-70 μM Ca^{2+}, whereas the calcium requirement for activation of m-calpain is 100-2000 μM Ca^{2+} (Goll et al., 1992a). The calpain/calpastatin system has been implicated to play a role in muscle protein turnover (Goll et al., 1999). The components of this system can be influenced by age (Huff-Lonergan et al., 1999), exercise (Arthur et al., 1999; Stauber and Smith, 1998; Spencer et al., 1997b), administration of epinephrine (Parr et al., 2000), injury (Belcastro et al., 1998), fiber type (Sultan et al., 2000), sepsis (Williams et al., 1999), cachexia (Busquets et al., 2000), genetic conditions (Koohmaraie et al., 1995; Duckett et al., 2000; Lorenzen et al., 2000; Delgado et al., 2001; Sensky et al., 1999), nutrition (Rosenvold et al., 2001; Wheeler and Koohmaraie, 1992; van den Hemel-Grooten et al., 1997) and combinations of these (Ertbjerg et al., 1999). All of these examples are interesting in regard to the calpain/calpastatin system, because they provide evidence that this system is responding

in a variety of different situations. Each instance of change within the components of this system add to the continued exploration of a more specific understanding of its physiological role. In order to define the mechanism and importance of this system on muscle protein accretion and degradation, it is vital to consider the composition of its elements including structure, expression and activation of the enzymes and calpastatin, and the interactions between system members.

Calpain domain structure

Calpain is composed of a large 80 kDa subunit and a small 30 kDa subunit (Ono et al., 1999) (Figure 1A). The large subunit is composed of four domains (I-IV) and the small subunit is made up of two domains (V and IV' or VI) (Carafoli and Molinari, 1998). Domain I contains the autolytic cleavage site and comprises the N-terminal part of the catalytic subunit. Domain II contains the active site residues Cys, His, and Asn. Domain III is suggested to play a role in amplifying the activation message originating from calcium binding to domain IV, but may possibly play a role in calcium-regulated phospholipid binding (Tompa et al., 2001). Domain IV is the calcium-binding domain and contains five EF-hand motifs (Carafoli and Molinari, 1998). A sixth EF-hand motif has also been predicted at the boundary between domains II and III. Domain V is the N-terminal region of the small subunit, which is glycine-rich and hydrophobic (Carafoli and

Molinari, 1998). Because of the hydrophobic nature of this domain, it has been suggested to be involved with membrane-binding. The last domain of the small subunit is termed domain IV' or VI. It is homologous to domain IV of the large subunit and contains five EF-hand motifs, although the fifth does not bind calcium and is thought to be involved in heterodimer formation with the catalytic subunit (Carafoli and Molinari, 1998).

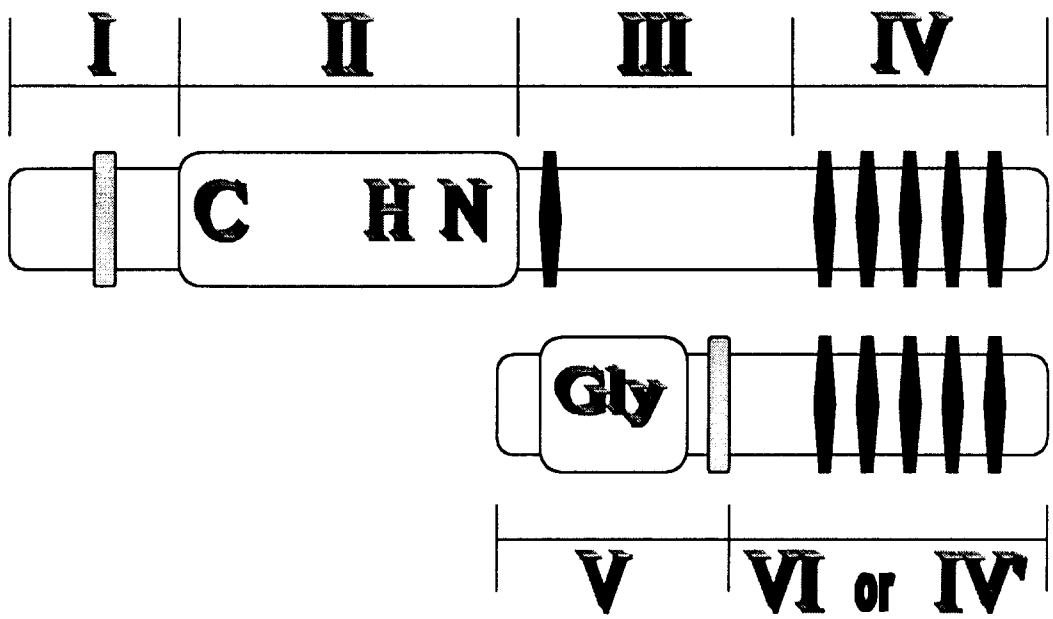

Figure 1A. Schematic representation of μ- and m-calpain adapted from Ono et al. (1999).

Autolysis of calpain

Autolysis is caused by limited proteolysis of m- or μ-calpain by itself. Autolysis lowers the calcium requirement for half-maximal activity from 3-50 to 0.5-2.0 μM Ca^{2+}

for μ-calpain, and from 400-800 to 50-150 μM Ca^{2+} for m-calpain (Goll et al., 1992b). Suzuki et al. (1981) examined autolysis in m-calpain. Upon addition of 6 mM Ca^{2+} m-calpain autolyzed from 82 kDa to 79 kDa in the first five minutes after calcium addition, but after 90 minutes the most evident band was at 60 kDa (Suzuki et al., 1981). At 100 μM Ca^{2+} both 79 kDa and 60 kDa fragments showed activity, whereas the 82 kDa form was only activated at 6 mM Ca^{2+}. Extended autolysis leads to a 30-33 kDa fragment that does not retain activity (Suzuki et al., 1981). Crawford et al. (1987) found that in the presence of 5 mM Ca^{2+} the small subunit of m-calpain autolyzed from 30 kDa to 28 kDa.

Because autolysis of calpain lowers the calcium concentrations required for activation, it has been speculated that intact calpain is actually a proenzyme. Calpastatin reduces the transition of calpain from the membrane-stable 78 kDa form to its cytosolic 75 kDa form, and also from the 80 kDa form to the 78 kDa form (Melloni et al., 1996). Crawford et al. (1998) demonstrated that intact calpain (80 kDa and 30 kDa subunits) with bound calcium is active. This was established by adding E-64, which is a known active-site inhibitor of cysteine proteinases, to intact m-calpain molecules. E-64 bound to the active site at pH 7.5 in a solution containing 5 mM Ca^{2+}. The results obtained from this experiment showed that E-64 can bind to the active site in m-calpain, even if it has not been autolyzed. The ability of E-64 to bind in the active site of calpain without

autolysis shows that the active site is available for proteolytic activity in the unautolyzed form of calpain. If intact, unautolyzed calpain were a proenzyme, then it would not be active unless it was autolyzed. Cong et al. (1993) obtained similar results when examining μ-calpain from bovine skeletal muscle. They came to the conclusion that calpain is not a proenzyme, because they observed proteolytic activity in unautolyzed μ-calpain.

It has been previously established that m- and μ-calpain participate in intermolecular autolysis (Goll et al., 1992a). Intermolecular autolysis implies that μ- and m-calpain are their own substrates – specifically cleaving in domains I and V. Thompson et al. (2000) examined the effect of μ-calpain on m-calpain. At concentrations of calcium that cause m-calpain to degrade other m-calpain molecules, μ-calpain did not degrade m-calpain. Activation of m-calpain requires a higher Ca^{2+} concentrations, so it was hypothesized that μ-calpain may be able to cleave m-calpain into a more calcium-sensitive species (Thompson et al., 2000). Although considered a possibility for activation of m-calpain, μ-calpain activating m-calpain by modification would not be an efficient mechanism in vivo. This mechanism would be more complex and less efficient if μ-calpain were required for activation of m-calpain because μ-calpain would have to be activated initially.

Calpain activation

Although there is a known calcium concentration required for activation of m- and μ-calpain, the process of calpain activation has not yet been fully elucidated. Two widely acknowledged models for calpain activation are the dissociation model and the autolysis model. A third factor that may play a role in calpain activation is the presence or absence of a calpain activator protein. The dissociation model of activation suggests that calpain becomes active after the large and small subunits have dissociated, whereas the autolysis model identifies unautolyzed calpain as a proenzyme, requiring autolysis for activation.

As demonstrated by Yoshizawa et al. (1995), calpain dissociates into subunits (80 and 30 kDa) in the presence of calcium concentrations required for activation of m- and μ-calpain. Specifically, m-calpain dissociated into subunits in the presence of 1 mM $CaCl_2$, and μ-calpain dissociated into subunits in the presence of 100 μM $CaCl_2$. Upon removal of Ca^{2+} the dissociation is reversed, reforming heterodimeric calpain. Interestingly, N-terminal autolysis of the 80 kDa and 30 kDa subunit occur in reverse order when comparing m- to μ-calpain (Crawford et al., 1987; Inomata et al., 1985). Autolysis of m-calpain occurs first in the 30 kDa subunit before autolysis of its 80 kDa subunit (Crawford et al., 1987) whereas μ-calpain autolyzes its N-terminal region first

(Inomata et al., 1985). The calpain large subunits have lower calcium requirements for activation when autolyzed, and also when separated from the small subunit (Yoshizawa et al., 1995). This is important in respect to both regulation and activation of calpain. Dissociation from the small subunit increases calcium sensitivity in both large subunits, which supports a regulatory role for the 30 kDa subunit in calpain activation. Calcium is required for autolysis and dissociation, although these two processes lower the calcium requirement. It almost seems paradoxical that calcium is necessary to initiate a process that results in a lower calcium requirement for activation.

The difference in order of modification between m- and μ-calpain suggests that the two large subunits differ in their mode of activation. This is interesting due to the structural similarity between m- and μ-calpain large subunits, as well as their shared identical small 30 kDa subunits. All could be indicative of the necessity for a separate activation mechanism due to distinct roles for m- and μ-calpain. Strobl et al. (2000) hypothesized that calpain undergoes a conformational change induced by calcium binding to the calmodulin-like domains, which arranges the catalytic sites into a position accessible to substrates. Furthermore, m-calpain has a larger number of acidic residues in this "switch loop" than μ-calpain, which could partially explain the different calcium requirements of these two molecules (Strobl et al., 2000). It should be noted however,

that some disagreement of the actual cause of subunit dissociation exists. Zhang and Mellgren (1996) argue that certain experimental procedures involving detergents in vitro could cause dissociation that is not calcium-mediated.

Although some research groups support the dissociation model for calpain activation, others support the autolysis model. Kitagaki et al. (2000) examined chicken μ/m-calpain to determine whether a combination of the two models could thoroughly explain calpain activation. They proposed the "autolysis-induced irreversible dissociation model." The hypothesis behind this model, as stated by Kitagaki et al. (2000), suggests that the autolyzed form of calpain (both 80 kDa and 30 kDa subunits) maintains activity for some time after it has autolyzed, along with dissociation of the large and small subunits for further regulation. This is an appealing model because it explains continued activity of calpain after autolysis has occurred, while leaving room for dissociation of subunits.

Calpain activator proteins have been characterized from human erythrocyte and bovine brain (Melloni et al., 1998). Melloni et al. (2000) have examined, specifically, two calpain activator proteins (CA) to determine their physiological function. The one specific to μ-calpain, which has a molecular mass of approximately 15 kDa, was described more thoroughly by this group (Melloni et al., 2000). Some evidence suggests

a possibility as a dimer in native conditions (Melloni et al., 1998). When associated with these proteins, the calcium concentration for activation decreases to lower than 1 μM for μ-calpain. This activator, which exists in erythrocytes, is localized on the inner surface of the plasma membrane (Melloni et al., 2000). A calpain activating protein found at the plasma membrane could be potentially important. Calpain associates at membranes in response to an increase in calcium concentration. This CA has been found at different levels in different cells depending on the level of proteinase activity required. Existence of calpain activating proteins could provide one more key to unlocking the mechanism of calpain activation.

Calpain expression

Calpain expression and activation can be influenced through several factors including nutrition, injury, exercise, and certain genetic conditions. For example, Busquets et al. (2000) demonstrated an increase in m-calpain expression in response to experimental cancer cachexia. This supports previous findings that cancer cachexia affects skeletal muscle protein through enhanced rates of protein turnover. It is of importance to examine the calpain system in such different situations to give us further insight into the mechanism of expression and activation. Through the knowledge gained

by such exploration, we will have a better ability to positively influence muscle growth and repair.

Epinephrine and exercise have an influence on the calpain system separately and in combination. Ertbjerg et al. (1999) produced conditions in pigs through exercise and administration of epinephrine that increased μ-calpain activity. Ertbjerg et al. (2000) examined the effect of epinephrine on calpastatin and calpain expression in C_2C_{12} cells with the result that μ- and m-calpain activity increased more than the increase in expression of calpastatin. Arthur et al. (1999) observed an increase in total (μ- and m-) calpain-like activity in rat plantaris after 60 minutes of exercise. Further observation suggested that calpain was predominantly present in a particulate form after exercise. This exercise consisted of running on a motor-driven treadmill for 60 minutes at a speed of 15 meters per minute. Calpain-like activity was determined via microplate assay with casein as a substrate.

In a study done to examine the effect of a protein-free diet on the calpain system in young pigs, van den Hemel-Grooten et al. (1997) measured mRNA levels of calpastatin, p94, and m- and μ-calpain. They found that mRNA levels of calpastatin and m-calpain did not change. Although μ-calpain and p94 mRNA levels decreased after 14 days on a protein-free diet, activity of μ-calpain did not change. The authors suggest that

their results imply an alternate mechanism for μ-calpain regulation under protein-free conditions. Rosenvold et al. (2001) demonstrated that feeding a low digestible carbohydrate diet to finishing pigs decreased the activity of μ-calpain and increased the activity of calpastatin. The combination of these two events favors a lower rate of protein turnover.

Changes in calpain activation have been observed in the situation of myocardial infarction. Kunimatsu et al. (1999) demonstrated, by using an antibody that recognized only m-calpain large subunits containing the active site His-containing sequence, that m-calpain is activated during myocardial infarction. This antibody would only be able to bind the active site if m-calpain were in its activated form. It is hypothesized that activated m-calpain, which has calcium bound to its calmodulin-like domains, has undergone a conformational change (induced by the bound calcium) that exposes its active site (Strobl et al., 2000). Without bound calcium, i.e. inactive m-calpain, this active site would not be available for binding with an antibody. During the experiment by Kunimatsu et al. (1999), m-calpain activity was observed predominantly in myocardial tissues that were damaged. Further, the observed tissue staining was stronger at the periphery of the cells. Their results support not only the hypothesis of calpain involvement in tissue damaging conditions, but also confirmation of calpain localization

at membranes during activation. Calpain could be playing a dual role in muscle damaging or healing conditions – in essence, calpain may be degrading proteins causing damage, or calpain could be removing damaged proteins to allow for repair and replacement of those polypeptides.

Decreased activity of m-calpain was observed in animals that exhibit the ryanodine receptor defect, although the activity of μ-calpain did not appear to be affected by this condition (Sensky et al., 1999). Alternatively, there was an observed increase in m-calpain and a decrease in μ-calpain activity in muscles affected by the callipyge phenotype (Delgado et al., 2001). These results obtained by Delgado et al. (2001) are in contrast to previous studies that did not find differences in μ-calpain activity associated with the callipyge phenotype (Koohmaraie et al., 1995; Lorenzen et al., 2000; Duckett et al., 2000).

Calpastatin

Calpastatin domain structure

The calpastatin domain structure comprises four homologous domains (1-4) preceded at the N-terminal by domain L (Figure 2.). Each domain is divided into three regions A, B, and C. Each domain has inhibitory activity, with region B exhibiting the inhibitory activity of the calpastatin molecule. The function of domain L is as yet

unknown, although recently Hao et al. (2000) has suggested a role for domain L in reactivating L-type calcium channels. For that particular study, calpastatin domain L was found to reactivate Ca^{2+} release channel activity from guinea pig cardiac muscle (Hao et al., 2000). Even when calpastatin has been fragmented by calpain it retains its calpain inhibitory activity because each domain contains an inhibitory sequence (Takano and Maki, 1999). This is interesting because proteolysis of calpastatin does not necessarily imply that it is no longer functional as an inhibitor.

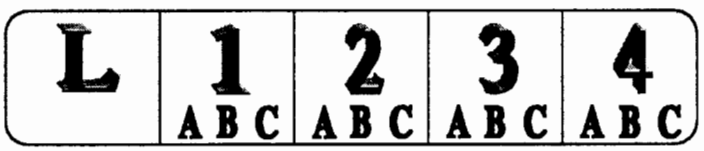

Figure 2. Schematic representation of calpastatin adapted from Takano and Maki (1999).

Calpastatin intracellular localization

Intracellular localization of calpastatin as described by De Tullio et al. (1999) in LAN-5 cells (a human neuroblastoma line) shows localization in two granular membrane-free structures near the nucleus. Alternatively, an increase in calcium concentration within the LAN-5 expression system causes calpastatin to relinquish aggregation and diffuse throughout the cytosol (De Tullio et al., 1999). Either PKA or PKC (Salamino et al., 1994) can accomplish post-translational modification of calpastatin

by phosphorylation. Phosphorylation by PKA drives aggregation of calpastatin molecules to localize into granules (Averna et al., 2001). According to Pontremoli et al. (1992) calpastatin phosphorylated by a homologous Ca^{2+}-independent protein kinase decreased its inhibitory efficiency when compared to a dephosphorylated form. Interestingly, phosphorylation of calpastatin by PKC in vitro does not result in aggregation (Averna et al., 2001).

The combination of phosphorylation and calcium sensitivity provide alternate routes for aggregation and diffusion of calpastatin within the cell. These mechanisms support evidence for a specifically regulated system to control proteolysis and inhibition within the cell. With an increase in calcium concentration sufficient enough to activate calpain, calpastatin abandons its aggregated state to diffuse through the cytosol where it has the ability to inhibit calpain activity. Phosphorylation by PKA but not PKC induces calpastatin aggregation, influencing localization. In contrast phosphorylation by PKC may affect the inhibitory efficiency of calpastatin (Pontremoli et al., 1992; Salamino et al., 1992).

Calpastatin interactions with calpain

Crawford et al. (1993) demonstrated that calpain can still bind calpastatin, even in the presence of E-64 or iodoacetic acid. However, binding efficiency is greatly decreased

by addition of these active-site inhibitors. This suggests that calpain binding to calpastatin requires access to the active sites in calpain, as well as lack of calpain inhibitors. Kawasaki et al. (1993) suggested, through experimental calpain binding to erythrocyte membranes, that calpain/calpastatin interactions occur through a site other than the calpain active site – possibly through a regulatory site. This experiment also demonstrated that the sequence of calpastatin responsible for calpain binding to membranes is different from the calpastatin inhibitory sequence.

Calpastatin expression

It has been shown that alternate forms of calpastatin are expressed in skeletal muscle and other tissues (Takano et al., 1986). More recently it has been suggested that these isoforms of calpastatin could be due to post-translational modification by phosphorylation or some other process not yet discovered. Pontremoli et al. (1991) found two different forms of calpastatin in rat muscle that seemed to correlate specifically with μ- or m-calpain. The two forms of calpastatin were designated calpastatin I and calpastatin II in reference to their ability to effectively inhibit μ- and m-calpain, respectively (Pontremoli et al., 1991). They demonstrated that μ-calpain was almost completely unable to degrade either of the two isoforms of calpastatin examined. (Although μ-calpain was fairly ineffective in degrading these two isoforms of calpastatin,

both isoforms were very sensitive to degradation by m-calpain.) Because calpastatin I is less effective at inhibiting m-calpain, Pontremoli et al. (1991) hypothesized that calpastatin II may be formed by post-translational modification (such as phosphorylation) of calpastatin I to make it more able to inhibit m-calpain.

Calpastatin expression in muscle can be affected by certain changes in nutrition, epinephrine administration, exercise, and certain genetic conditions. Feeding ß-adrenergic agonists to steers increased expression of calpastatin, including mRNA, protein levels, and calpain inhibitory activity (Wheeler and Koohmaraie, 1992). The $ß_2$-adrenergic receptor binds ß-agonists, which activates a signaling cascade involving cAMP-dependent protein kinase (Cong et al., 1998). Cong et al. (1998) identified a cAMP-responsive element in the gene promoter region of calpastatin, which could partially explain the change in calpastatin expression due to ß-adrenergic agonists.

Parr et al. (2000) examined the effect of epinephrine, which binds to $ß_2$-receptors, on porcine skeletal muscle. They hypothesized that because the bovine calpastatin gene promoter contains a cAMP-responsive component, which is activated by $ß_2$-receptors, that epinephrine may have a similar result (Cong et al., 1998). Parr et al. (2000) demonstrated that administration of epinephrine to pigs for 7 days increased calpastatin activity in skeletal muscle significantly (77% greater expression than placebo). Parr et al.

(2000) suggested that the increase in calpastatin activity may be due to post-translational modification (such as phosphorylation), which could be a mechanism for calpastatin mediation rather than a change in gene expression. Further conclusions from these studies suggest that epinephrine could be involved in the regulation of muscle protein turnover by influencing calpastatin expression. Because epinephrine can bind to $ß_2$-receptors, which activate a signaling cascade involving cAMP-dependent protein kinase, calpastatin expression can be influenced due to the cAMP-responsive element in its promoter region (Cong et al., 1998).

The calpain/calpastatin system is also altered in the callipyge phenotype in sheep. Muscles affected by the callipyge phenotype exhibit higher levels of calpastatin activity (Koohmaraie et al., 1995; Lorenzen et al., 2000; Duckett et al., 2000). The result of this condition is an increase in muscle weight due to hypertrophy in certain muscles, which are located mostly in the hindquarter (Koohmaraie et al., 1995). It has been suggested that the callipyge condition may be produced by a decrease in muscle protein degradation, causing increased protein accretion in those muscles (Koohmaraie et al., 1995; Lorenzen et al., 2000; Duckett et al., 2000).

In two muscles (*biceps femoris* and *longissimus dorsi*) that are affected by the callipyge phenotype calpastatin activity increased along with an increase in the activity of

m- and μ-calpain (Delgado et al., 2001). This experiment demonstrated that although m- and μ-calpain activity increase, calpastatin activity was overall greater than the activity of both calpains combined. Lorenzen et al. (2000) examined DNA, RNA, calpastatin expression, and fiber type in callipyge lambs, resulting in the suggestion that callipyge is most likely maintained through reduced protein degradation. Although all components of the calpain/calpastatin system increase in this condition, calpastatin activity is greater than that of m- and μ-calpain combined. The ratio of calpastatin activity to that of μ- and m-calpain in muscles affected by the callipyge condition has been observed to be greater than that of muscles unaffected by callipyge (Delgado et al., 2001). This suggests that muscle hypertrophy in the callipyge condition could be influenced by an imbalance of protein turnover with emphasis on the lack of protein degradation due to inhibition by calpastatin.

Protein metabolism

To demonstrate the role of m-calpain and calpastatin in skeletal muscle protein degradation, Huang and Forsberg (1998) overexpressed dominant-negative (DN) m-calpain and the calpastatin inhibitory domain (CID) in L8 cells. Overexpression of dominant-negative m-calpain was hypothesized to inhibit m-calpain activity by competing for substrate (Huang and Forsberg, 1998). They examined degradation of

fodrin and nebulin, known calpain substrates, as indicators of overall protein degradation. Although overexpression of both DN and CID reduced total protein degradation, CID caused about twice the reduction in degradation than overexpression of DN (Huang and Forsberg, 1998). These results support calpastatin in playing an important role as an inhibitor of protein degradation. This also supports that increasing calpastatin expression and activity has the potential to further decrease protein degradation, thereby increasing protein accretion.

Calpain/calpastatin system in myoblasts

As myoblasts fuse to form multinucleated myotubes, membranes and cytoskeletal framework are temporarily disrupted. Membrane protein degradation has been suggested to contribute to this reorganization of cell fusion. It has been suggested that the calpain system is involved with this fusion due to its known ability to degrade other skeletal muscle proteins. Barnoy et al. (1998) demonstrated that adding calpastatin, E-64d, calpeptin, or EGTA to myoblasts inhibited fusion into myotubes. Their results indicated that the proteins degraded in myoblast fusion are specific. For example, excess calpain added to cells did not degrade α-actinin, but did degrade talin. These findings support the calpain system as playing a key role in developing muscle in the fusion of myoblasts to myotubes.

p94

Background

The protein p94, also known as n-calpain, skm-calpain, nCL1, calpain 3, and CANP3, is an approximately 94 kDa muscle specific member of the cysteine proteinase family. p94 is the first tissue specific calpain to be discovered. It was characterized during a routine cDNA screen for m- and μ-calpain. Various rat organs were tested for the existence of p94 mRNA using a cDNA fragment of rat p94. p94 mRNA was detected only in skeletal muscle, including tongue. It was not found in other organs, including smooth muscle or heart muscle (Sorimachi et al., 1989). p94 is associated with limb-girdle muscular dystrophy type 2A (Richard et al., 1995; Spencer et al., 1997a) in which the gene encoding p94 is mutated, producing a complete absence or deficiency of the protein (Richard et al., 1995). p94 is proposed to have two binding sites on titin - one at the N_2 line and the other at the extreme C-terminus of titin (Sorimachi et al., 1995). These two proposed binding sites are also susceptible to degradation by the calpains (Suzuki et al., 1996). Because of these degradation sites in the myofibril, p94 could potentially play an important role in muscle protein turnover. p94 has also been found in greater abundance within muscle fibers exhibiting fast, glycolytic metabolism, or type

IIB fibers (Jones et al., 1999). This is of interest due to the ability of muscle fibers to change metabolism based on exercise, innervation, or nutritional status.

Sequence description of p94

Molecular features of p94, including cDNA sequence, were initially reported by Sorimachi et al. (1989). Initially a clone was isolated that hybridized to a cDNA fragment of the chicken large subunit but only under low stringent conditions, which involve high salt solutions. When cDNAs for both μ- and m-calpain were used as probes, the same results were attained. The library was rescreened, yielding a second clone. Both clones were subjected to restriction mapping and nucleotide sequence analyses. The two clones overlap by 1.4 kb, to cover a total length of 3.0 kb. The nucleotide sequence contains a single long open reading frame encoding 778 amino acid residues. Since the deduced amino acid sequence reveals significant homology to μ- and m-calpain of human and rabbit large subunits, the protein now designated p94 was considered to be a novel family member of the CANP large subunit. These two clones lacked the N-terminal part of the open reading frame including the initiation methionine codon. In order to determine the whole structure of the open reading frame and to examine the newly identified cDNA, human and rat muscle cDNA libraries were screened. Clones from the rat muscle cDNA library were obtained, some of which were long enough to

contain the total open reading frame. The restriction maps of the two long rat cDNA clones that had 3.2 kb inserts were identical and showed some similarity to the human clones, which they showed by restriction site mapping. The complete nucleotide sequence was determined and revealed that the cDNA contains a single open reading frame from 1 to 2466 bases. The deduced amino acid sequence of the rat cDNA (rat p94) consists of 821 amino acid residues with a molecular weight of 94,084. Comparison of the rat amino acid sequence to that of human p94 revealed that the two sequences are highly homologous (94%), although the human p94 lacks the N-terminal 43 residues found in the rat molecule. This is comparable to the similarity between human and rabbit m-types (93%) or μ-types (96%) of large subunits (Sorimachi et al., 1989).

p94 domain structure

p94 shows a high degree of sequence similarity to m- and μ-calpain large subunits (Figure 3.). It has three unique sequences, NS, IS1, and IS2, located within the domains comparable to those of the other calpain large subunits. These intervening sequences account for the extra molecular weight to total 94 kDa. The N-terminal of domain I contains the NS (novel sequence), IS1 (intervening sequence 1), is found in the middle of domain II, and IS2 (intervening sequence 2) is located at the C-terminal of domain III.

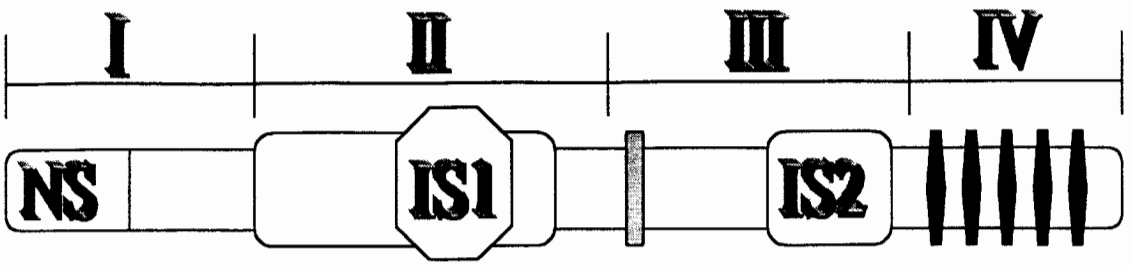

Figure 3. Schematic representation of p94 adapted from Sorimachi et al. (1996).

NS has a high quantity of proline residues and is about 60 residues long. IS1 is located just before the suggested active site histidine, suggesting that IS1 may be involved in p94 proteinase activity. IS2 has a lysine-rich sequence similar to the N-terminal nuclear localization signal (Sorimachi and Suzuki, 1992).

Comparison of full-length rat p94 (94 kDa) to various types of CANP large subunits (80 kDa) illustrates significant sequence similarity (Sorimachi et al., 1989). p94 is divided into four domains like m- and μ-calpain: domain I (residues 1-98), domain II (residues 99-400, 302 residues), domain III (residues 401-653, 253 residues), and domain IV (residues 654-821, 168 residues). Domain I of p94 includes 20-30 residues more than domain I of the large subunits of μ- and m-calpain, and the sequence is significantly different. The N-terminal 60 residues (NS) show no homology to other large subunits. Domain II is very homologous to other domains II (68, 69 and 66% to human m-type, μ-

type, and chicken, respectively) and contains Cys-129, His-332, and Asn-358 residues which possibly comprise the active site cysteine protease as found in other CANP large subunits. The regions around these residues are especially homologous to the corresponding regions in other large subunits. There are five tryptophan residues in the C-terminal region of domain II of all four large subunit sequences and are also a common feature in other cysteine proteases such as papain and cathepsins B, H, and L (containing 2-5 tryptophan residues in the corresponding regions). The structure of domain II suggests that p94 may possess proteolytic activity. However, p94 is distinct from other CANP large subunits in that it contains a unique 62-amino acid residue sequence (residues 268-329) in the C-terminal region of domain II (IS1). This region replaces 14 residues found in other CANP large subunits. Domain II also shows sequence similarity to other CANP large subunits, but the identity is low (41-46%), and lower than that for domains II and IV. p94 also contains a unique sequence of 77 residues (IS2) in the C terminus of domain III instead of the 30 residues found in the other molecules. Domain IV contains four E-F hand structures, which are comparable to those found in both human m- and μ-calpain. This suggests possible Ca^{2+}-binding activity in domain IV of p94. The sequence identity between domain IV of p94 and the calcium-binding domain of the CANP small subunit is 58%, which is higher than that between p94 and human μ- or m-

type or chicken (52, 45, or 56%). Eight continuous C-terminal residues (813-820) of p94 and the small subunit are identical, while these residues are variable among other large subunits. The mRNA for the CANP small subunit is expressed ubiquitously. Because p94 contains a short repetitive sequence in domain III, which is similar to a sequence found in the CANP small subunit in front of the calcium-binding domain, a close evolutionary relationship is suggested for p94 and the CANP small subunit (Sorimachi et al., 1989).

p94 autolysis

It was originally thought that p94 autolyzed quickly after translation due to its absence in rat skeletal muscle extracts even though the presence of p94 mRNA in skeletal muscle is tenfold higher than that of the ubiquitous calpains (Sorimachi et al., 1993). To investigate the expression and localization of p94 in vitro, Sorimachi et al. (1993) expressed wild-type p94 in COS cells. The authors constructed a mutated form of p94, lacking the IS2 region. When inserted into COS cells, the mutated form was expressed at a level of at least 100 times that of wild-type p94. They also produced similar results to the deletion of the IS2 region by replacing the active site cysteine with serine or alanine. This "active site" is hypothesized to be active at this point because the function of p94 is

not yet known. From these experiments it was hypothesized that p94 autolyzed very rapidly after its translation, and that the IS2 region played a role in this degradation.

Because p94 autolysis has been tied to the IS2 region, and the N_2 line (within the I band) portion of titin binds to the IS2 region, research was conducted to examine whether the presence of this N_2 portion of titin would affect p94 autolysis (Kinbara et al., 1998). During this study wild-type p94 was expressed with the titin N_2 fragment in COS cells. The results showed that p94 autolyzed even in the presence of this titin fragment. Further, they found that autolysis of p94 was not affected by either EDTA or calcium, but was accelerated by NaCl. The other main point of significance was the isolation of the p94 degradation sites to the IS1 region, supporting the role of IS1 as essential along with IS2 for autolysis. In addition, this group was able to purify p94 at significant quantities from rabbit skeletal muscle, which indicates that p94 does not autolyze immediately after translation.

Federici et al. (1999) identified two autolytic cleavage sites in p94. This was accomplished by expressing p94 in E. coli, and Sf9 cells, as well as examining p94 in homogenized muscle. The first site found was near the N-terminal region of p94, similar to the autolytic cleavage sites in m- and μ-calpain. The second site was located near the

N-terminal region of domain III. The authors suggested that these autolytic cleavage sites could be part of a process leading to regulation of p94 protease function.

The ubiquitous calpains, m- and μ-calpain, both require calcium for activation. Autolysis of μ-calpain occurs at 50-150 μM calcium (Cong et al., 1993ss), whereas autolysis of m-calpain occurs at 90-400 μM calcium (Thompson et al., 2000). This autolysis decreases the Ca^{2+} requirement for activation. Although p94 has a domain structure suggesting calcium-binding ability, it is not currently agreed upon if p94 has a calcium requirement for activation and/or autolysis. Previously described studies by Kinbara's group have indicated that p94 is not affected by calcium concentration (1998). Although, other research has shown that expression of His-tagged p94 in Sf9 cells, which are an insect line, has suggested calcium-dependent autolysis (Branca et al., 1999). These authors reported that p94 expressed in Sf9 cells did not autolyze in the absence of calcium. The presence of 500 nM calcium induced a detectable appearance (by western blotting) of the previously recognized 55 kDa degradation product of p94 autolysis. This seems to suggest that p94 activity is stimulated by calcium under certain conditions. Although it is interesting to observe a calcium-related effect on p94, it is still necessary to consider the constraints of the cell line used to examine such conditions, as well as inconsistent data from other research involving calcium (Kinbara et al., 1998). The other

important point to keep in mind is that these experiments are not examining effects in native p94. Establishing a calcium requirement, or lack thereof, for p94 is of importance because it will give us insight toward possible functions of the molecule. It could also show us a more complete view of the conditions in which p94 may become active, and whether it requires interaction with other myofibrillar proteins such as titin to stabilize or inactivate p94 in vivo.

p94 purification

Although m-calpain, μ-calpain, and calpastatin were originally characterized in polypeptide form, p94 was found at the cDNA level initially. Purification of this protein has proven to be somewhat of a challenge (Sorimachi et al., 1993; Shevchenko et al., 1998; Branca et al., 1999). Attempts at purifying full-length active p94 have been unsuccessful because of its reported rapid autolysis except by one research group (Kinbara et al., 1998). During this study Kinbara's group established a partial purification procedure for recombinant p94, which had its active site cysteine replaced with serine, to enable its application to native p94. While their attempts at purifying recombinant p94 were indeed successful, their procedure was unsuccessful in purifying active full-length p94. p94 was purified with NaCl, but in an autolyzed form consisting of 58 and 55 kDa fragments.

Associations with other proteins

Poussard et al. (1996) demonstrated that p94 may play an important role in myoblast differentiation. During this experiment p94 mRNA expression was shown to be present in cultured muscle cells, especially at later stages of differentiation. The consequences resulting from the lack of p94 expression were examined due to the results of this initial study. To remove the ability of differentiated cells to express p94, an antisense oligonucleotide treatment was applied to myotubes (Poussard et al., 1996). There were two main types of disorganization that resulted from this treatment. The first type showed that some of the myofibrils remained in register in the center of the myotubes, but this alignment was lost toward the periphery of the cell. The A and M bands were clearly visible, suggesting that the thick filaments were in register. The I and H bands were well defined, proving that the sarcomeres were not in a contracted state. The H bands also had distinct edges, which further supports the evidence for fixed length of the sarcomere thin filaments. The most obvious structural disruptions were seen in the Z lines, which were not continuous, but were an electron-dense and discontinuous material in the center of the I band. The second case of myofibrillar disruption entailed a complete disorganization within the myofibril. A and I bands could not be identified along contractile bundles.

p94 is has two binding sites on titin - one at the N_2 line and the other at the extreme C-terminus of titin (M-is7) as stated previously by Sorimachi et al. (1995). Titin exhibits different isoforms that are present in different muscle types (Kinbara et al., 1997). Titin has two alternative exons encoding IgC2 repeats, N_2A and N_2B, but only N_2A is found in skeletal muscle titin (Kinbara et al., 1997). The N_2A region is found in the portion of titin located in the I band (Sorimachi et al., 1995). The C-terminal region of titin has an exon (Mex5) that encodes M-is7 (located in the M line [Sorimachi et al., 1995]), which is also alternatively spliced. The form of titin found predominantly in fast-twitch muscles does not contain the M-is7 sequence (Kinbara et al., 1997). These p94 specific binding sites may prove of great importance due to their association with known degradation points in titin (Suzuki et al., 1996), and therefore fragmentation in the myofibril. During examination of p94 autolysis, p94 was coexpressed in COS cells with the N_2 region of titin, which resulted in no proteolysis of titin by p94 (Kinbara et al., 1998). This suggests that titin, or more specifically this portion of titin, is not a substrate for p94 under these conditions. These results suggest that p94 may be associated with titin to protect it from degradation by the ubiquitous calpains or some other proteinase.

Fiber type expression of p94 was examined because of its known binding with titin, which exhibits different isoforms in different muscles (Jones et al., 1999). This

study included examination of p94 in three main fiber types (I, IIA, and IIB), both within muscles of those predominant types and individual fibers. The results showed that p94 expression increased with an increase in fast glycolytic fibers. Fast glycolytic fibers, or type IIB fibers, are associated with muscle hypertrophy (Mozdziak et al., 1998). During pharmacologically and surgically induced hypertrophy in rat soleus, evidence of hypertrophy in type IIB fibers was observed (Mozdziak et al., 1998). Association of p94 with type IIB fibers is intriguing considering the ability of these fibers to undergo growth in a hypertrophic manner, which involves protein accretion. Type IIB fibers also show more hypertrophic growth than other fibers. Because possible functions for titin include a role in the assembly of myofibrils, it is an interesting observation to find p94 associated with fibers that are able to undergo hypertrophic growth.

p94 expression

Although it has not yet been determined what role p94 plays in muscle protein turnover, its classification as a muscle specific form of calpain implies a possible role for p94 in muscle protein turnover. In a study done to examine the effect of a protein-free diet on the calpain system in young pigs, van den Hemel-Grooten et al. (1997) measured a decrease in p94 mRNA when expressed per units of total RNA or per gram of muscle tissue in the pigs that were fed the protein-free diet. Busquets et al. (2000) demonstrated

a decrease in p94 expression due to experimental cancer cachexia. This is interesting due to the fact that cachexia is associated with a higher rate of protein turnover, and increased m-calpain expression. Such a finding, as suggested by the authors, could imply that down-regulation of p94 occurs due to a possible regulatory role in muscle proteolysis.

Functions

The exact function of p94 is unknown in part due to rapid autolysis, which is encountered when attempting to separate it from whole muscle. One particular research group suggested possible functions that include signal transduction and a regulatory, rather than a degradative role in muscle protein maintenance (Shevchenko et al., 1998). During that particular study, a 94 kDa thiol protease, thought to be p94, was found to have the ability to cleave the ryanodine receptor/Ca^{2+} release channel. Modification of the Ca^{2+} release channel would increase intracellular calcium levels, which would activate m- and μ-calpain. Assuming this protein was indeed p94, it could play a regulatory role in conjunction with m- and μ-calpain in regulating muscle protein degradation just by influencing the state of the Ca^{2+} release channel within a muscle cell. As stated previously, Kinbara et al. (1998) suggested that p94 may play a protective role in association with titin from other proteases.

p94 and LGMD2A

Richard et al. (1995) examined the state of p94 in 38 families expressing some form of limb girdle muscular dystrophy (LGMD) because the position of the p94 gene fit the criteria as a possible candidate as a causative agent of this disease. During the course of this study, Richard et al. (1995) found 15 different mutations at DNA level that were associated with LGMD2A. Eight of these mutations caused the production of a truncated and assumed to be inactive form of p94. Various point mutations encompassed the remaining individuals. The conclusions from this study suggested a disruption in the higher order of the protein structure when the protein was expressed in an active form. They hypothesized that p94 has an active role in signal transduction, which supports the suggestion that inactivation of p94 causes the pathological conditions of LGMD2A.

Sorimachi et al. (2000) did a study with LGMD2A mutants which focused on point mutations of the p94 gene. Ten LGMD2A point mutants were constructed to examine how p94 was affected by each. The results from these mutations gave a range of results from losing binding ability to the N_2 region of titin to no apparent defects in the autolytic activity of p94. Their conclusions supported the hypothesis that all regions of p94 have specific and necessary functions for p94 to work in its expected form.

Summary

The calpain/calpastatin system in skeletal muscle is composed primarily of μ- and m-calpain (Goll et al., 1992a). Recently skeletal muscle-specific calpain, p94, has been added to this family of cysteine proteinases (Sorimachi et al., 1989). Numerous studies have been conducted on the components of this system. Goll et al. (1999) hypothesized that this system plays a role in skeletal muscle protein turnover. It has been observed that the major components are affected by certain changes including diet, genetics, and age (Wheeler and Koohmaraie, 1992; Koohmaraie et al., 1995; Huff-Lonergan et al., 1999). An increase in the expression and activity of calpastatin has been implicated in a decrease in the amount of protein degradation in muscle, thereby maintaining the existing proteins and allowing for the possibility of more protein accretion within the myofibril (Lorenzen et al., 2000).

Although little is known about p94, it is suggested to be associated with titin (Sorimachi et al., 1995). Titin is thought to play a role in myofibrillar assembly (Poussard et al., 1996), and if p94 is associated with titin this could lend insight into a function for p94 in relation to the myofibril. Were p94 to be involved in an assembly-type relationship with titin, it could also be important in restructuring the myofibril during protein turnover. Because of the connection with skeletal muscle protein turnover

and the observed physiological responses in these elements due to various dietary change, we have hypothesized that the expression of two components, calpastatin and p94, may be affected in canine skeletal muscle due to percentage and source of dietary protein.

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EFFECT OF DIETARY PROTEIN ON THE CALPAIN/CALPASTATIN SYSTEM IN CANINE SKELETAL MUSCLE

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ABSTRACT

The cysteine proteinases μ- and m-calpain along with their inhibitor, calpastatin, and possibly skeletal muscle specific p94, have been hypothesized to play a role in skeletal muscle protein degradation. Because nutrition has previously been shown to influence the expression of calpastatin, the working hypothesis of this study is that the quantity and source of dietary protein can influence regulation of the calpain system in muscle. The objectives to support this hypothesis were to determine the effects of dietary protein (amount and source) on the expression of calpastatin and p94 in canine skeletal muscle. This study consisted of 8 diets with 7 dogs per diet. A biopsy was taken from the semitendinosis of all 56 dogs prior to and after 12 weeks on their respective diets. This experimental design allowed examination of change within individual dogs. Diets 1-4 were 12% total protein and contained ratios of chicken to corn gluten protein of 100:0, 67:33, 33:67, and 0:100%, respectively. Diets 5-8 were 28% total protein with identical protein ratios to diets 1-4. The differences in calpastatin and p94 were examined

qualitatively using SDS-PAGE and immunoblotting, and quantitatively with densitometric analyses and DEXA. Western blots to examine calpastatin were probed with an anti-calpastatin antibody (MA3945, Affinity Bioreagents). p94 blots were examined with a monoclonal anti-p94 antibody (NCL-CALP-12A2, Novocastra Labs). The majority of the calpastatin blots showed an expression of three distinct calpastatin bands, the uppermost appearing at approximately 110 kDa. Diet 5 (28% crude protein, 100% chicken) resulted in an increase in the expression of the110 kDa calpastatin band compared to the other two lower molecular weight bands in the same samples. A significant difference ($P < 0.05$) was obtained from comparison of the ratio of relative intensity in the topmost band when comparing two of the 28% crude protein diets. Diet 5 (100:0) showed greater calpastatin intensity than diet 8 (0:100). No treatment differences in detection of p94 were observed. DEXA data showed that all animals in this trial lost lean body mass. However, the dogs that were fed diet 4 (100% corn gluten meal,12% total protein) lost significantly more lean body mass than those fed all other diets except diet 3 (33% chicken, 67% corn gluten meal,12% total protein). The calpastatin data suggest that dogs fed a diet containing a higher total percentage of chicken protein may have a greater potential to regulate calpain-mediated degradation of muscle protein. The data collected from DEXA measurements suggest that dogs fed a

higher percentage of protein from chicken are better able to maintain lean body mass than dogs fed a lower percentage of protein entirely from corn gluten meal.

Key words: Calpastatin, p94, Canine, Skeletal muscle, DEXA

Introduction

Calpastatin is an inhibitor of the calpains, which are a family of calcium-dependent cysteine proteinases that are expressed ubiquitously (including skeletal muscle) (Carafoli and Molinari, 1998). Calpastatin prevents the activation of the catalytic activity of calpain (Melloni et al., 1992). Calpastatin is known to play a key role in the living animal in muscle protein turnover as a regulatory component of the calpain proteolytic system (Goll et al., 1992). Calpastatin also retains the ability to be post-translationally modified by phosphorylation by PKA and PKC (Salamino et al., 1994). Although phosphorylation by PKA causes calpastatin to aggregate, phosphorylation by PKC does not cause aggregation (Averna et al., 2001). Aggregated calpastatin may have decreased inhibitory activity because it may be less available for localization with calpain. According to Pontremoli et al. (1992) calpastatin phosphorylated by a homologous Ca^{2+}-independent protein kinase decreased its inhibitory efficiency when compared to a dephosphorylated form.

Calpastatin expression or activity can be affected by certain nutritional changes, by some genetic conditions, and age of the animal. Calpastatin activity in bovine skeletal muscle can increase in response to feeding some ß-adrenergic agonists (Wheeler and Koohmarie 1992). In this particular case, observed calpastatin activity increased due to consumption of the ß-agonist $L_{644,949}$. The muscles in the hindquarters of callipyge sheep, which have a large increase in muscle growth due to hypertrophy, show higher calpastatin activity and reduced protein degradation (Koohmarie et al., 1995). Levels of calpastatin in geriatric animals are also elevated when compared to young animals. A comparative study using skeletal muscle from young and geriatric canines was conducted to examine the effect of age on calpastatin expression. Western blot analysis showed that the geriatric dogs had a significantly higher level of high molecular weight calpastatin in muscle than did the young dogs (Huff-Lonergan et al., 1999). These data suggest that calpastatin in muscle exhibits a physiological response to certain changes in the animal, whether they are diet, genetics, or the aging process. This physiological response may enable an animal to increase protein degradation to draw needed energy from its muscles due to malnutrition, or slow down protein degradation to enable muscle growth.

p94 is the first skeletal muscle specific calpain to be discovered. It was discovered during a cDNA screen for m- and μ-calpain (Sorimachi et al., 1989). The

presence of p94 mRNA in skeletal muscle is tenfold higher than that of the ubiquitous calpains (Sorimachi et al., 1993). The exact function of p94 is unknown due to its apparent instability when it is isolated from whole muscle. Since 1989, attempts at purifying p94 have been unsuccessful except by one research group, which was only able to partially purify p94 (Kinbara et al., 1998). A mutation in p94 is associated with limb girdle muscular dystrophy type 2A (Spencer et al., 1997a) in which p94 is mutated, producing absence or deficiency of the protein (Richard et al., 1995). Evidence suggests that p94 has two binding sites on titin - one at the N_2 line and the other at the extreme C-terminus of titin (Sorimachi et al., 1995). Titin exhibits different isoforms which are present in different muscle types, including the two binding sites for p94 (Kinbara et al., 1997). These p94 specific binding sites may prove of great importance due to their association with known degradation points in titin, and therefore fragmentation of the myofibril. Because of its association with these degradation sites in the myofibril, p94 could potentially play an important role in muscle protein turnover. Muscle fibers exhibiting fast, glycolytic metabolism, or type IIB fibers show higher expression of p94 than do type I or IIA (Jones et al., 1999). Type IIB fibers show more hypertrophic growth than other fiber types, which is a process requiring increased protein accretion

(Mozdziak et al., 1998). This is also of interest due to the ability of muscle fibers to change metabolism based on exercise, innervation, or nutritional status.

The working hypothesis of this study is that the source and level of protein in the diet can influence the state of the components of the calpain/calpastatin system in canine muscle. To investigate this hypothesis, calpastatin and p94 were examined in biopsies taken from the semitendinosis of fifty-six canines fed 8 diets (7 animals per diet). The objectives of this study were to determine 1) the state of the inhibitor of the calpains, calpastatin, in the semitendinosis from dogs fed diets containing protein from different sources fed at different levels in the diet; 2) the effect of the administration of high or low protein diets on the predominant posttranslational state of calpastatin in the semitendinosis of dogs; 3) the effect of dietary treatments on p94 in canine semitendinosis biopsy samples.

Materials and Methods

Materials

Samples for this project were obtained from an ongoing research project at The Iams Co., Lewisburg, OH. Fifty-six dogs of similar age were fed a 12% (n=28) or 28% (n=28) protein diet consisting of different levels (n=4) and different sources (n=2) of dietary protein for twelve weeks. The ratios of the two protein sources (chicken: corn

gluten) within each percentage group were 100:0, 67:33, 33:67, and 0:100. (Table 1) Biopsies were taken from the semitendinosis of each dog before the feeding trial began and again after twelve weeks on the experimental diets. Samples were stored at -80° C until they were subjected to analysis.

Methods

Sarcoplasmic muscle protein extraction. To determine the state of calpastatin, the inhibitor of the calpains, the sarcoplasmic protein fraction was isolated from the biopsy samples. This fraction was used for chemiluminescent immunoblotting analyses. The procedure used for biopsy muscle protein extraction was modified from Doumit et al. (1996) to compensate for the size of the samples. A 0.1 gram sample was homogenized in 5 volumes of 4° C extraction buffer (10 mM EDTA, 0.1 mg/mL ovomucoid, 2 μM E-64, 2 mM PMSF, and 100 mM Tris, pH 8.3) in a 1 mL Kontes Duall tissue grinding tube with a PTFE pestle. Samples were homogenized for three intervals of 10 strokes each with the pestle attached to a Barnant Series 10 Mixer (model # 700-5400, Barnant Company; Barrington, IL) set on speed 5. Between intervals, the homogenizing tube was chilled on ice for 30 seconds. Once the samples were homogenized, they were removed from the homogenizing tube with a plastic transfer pipette and placed in a 1.5 mL

microfuge tube. Homogenizing tubes were rinsed with 100 µL of extraction buffer, which was then added to the sample.

Samples were clarified by centrifugation at 22,000 x g in the ST-MICRO rotor of the Sorvall Super T21 centrifuge (Sorvall Products, L.P.; Newtown, CT) for 20 minutes at 4° C. The supernatant was removed from the pellet, which continued to chill on ice until extraction, with a 5 cc plastic syringe and a 20 gauge needle. The supernatant was then dialyzed overnight (Pierce Slide-a-lyzer Dialysis Cassettes, 10,000 MWCO, 0.5-3.0 mL; cat. # 66425ZZ, Pierce Chemical Company; Rockford, IL) against a solution at 4° C containing 5 mM EDTA, 40 mM Tris, and 0.05% ß-mercaptoethanol at pH 7.35. After dialysis, the protein concentration of the supernatant was determined using the method described by Bradford (1976) using premixed reagents (Bio-Rad Laboratories; Hercules, CA). Samples were prepared for gel electrophoresis by diluting to a protein concentration of 3 mg/mL in gel sample buffer (3 mM EDTA, 3% SDS, 30% glycerol, 0.001% Pyronin Y, and 30 mM Tris-HCl, pH 8.0 [Wang et al., 1982]). Gel samples were then heated at 50° C for 20 minutes. Heated samples were aliquotted into four separate microcentrifuge tubes and stored at -80° C until loaded onto gels.

Myofibrillar muscle protein extraction. To determine how p94, a skeletal muscle specific calpain, was affected by dietary treatments in canine biopsy samples the

myofibrillar fraction of each sample was solubolized for chemiluminescent immunoblotting analyses. The pellet from the initial extraction in low ionic strength buffer was homogenized in a 1 mL Kontes Duall tissue grinding tube with a PTFE pestle. Extraction was completed in ten volumes of room temperature whole muscle extraction buffer (2% SDS, 10 mM phosphate, pH 7.0) for 20 strokes, or until most of the visible tissue was in solution. Samples were homogenized with a Barnant Series 10 Mixer (model # 700-5400, Barnant Company; Barrington, IL) set on speed 2. After homogenizing, samples were transferred to microcentrifuge tubes with a plastic transfer pipette. Samples were clarified at 10,000 x g in the ST-MICRO centrifuge rotor of the Sorvall Super T21 centrifuge (Sorvall Products, L.P.; Newtown, CT) for 20 minutes at 20° C. The protein concentration of the supernatant obtained by this second extraction was determined using the method described by Lowry et al. (1951) with the Bio-Rad DC assay kit (Bio-Rad Laboratories; Hercules, CA). Samples for gel electrophoresis were adjusted to 4 mg/mL in gel sample buffer (3 mM EDTA, 3% SDS, 30% glycerol, 0.001% Pyronin Y, and 30 mM Tris-HCl, pH 8.0). Samples were then heated at 50° C for 20 minutes prior to being aliquotted into 4 separate microcentrifuge tubes for storage at -80° C until analysis.

SDS PAGE for calpastatin. Ninety μg of protein from each sample were loaded into separate wells of a 10 cm x 12 cm x 1.5 mm Hoefer SE 260B Mighty Small II (Pharmacia Biotech; San Francisco, CA) 15% acrylamide separating gel (15% acrylamide/bis [100:1 acrylamide: bisacrylamide], 0.375 M Tris-HCl, pH 8.8, 0.1% sodium dodecyl sulfate (SDS), 0.005% ammonium persulfate, and 0.0005% N,N,N',N'-Tetramethylenediamine (TEMED). The 15% separating gel had a 5% acrylamide stacking gel (5% acrylamide/bis [100:1 acrylamide: bisacrylamide], 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.001% TEMED, and 0.007% ammonium persulfate). The composition of the running buffer used was 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3. Gels were run at 125 volts for approximately 190 minutes at room temperature (until the dye front had run off the gel). At completion, the proteins on the gel were transferred immediately to Westran PVDF (polyvinylidene fluoride) Protein Transfer and Sequencing membranes (Schleicher & Schuell, Inc.; Keene, NH), prewet in 100% methanol and then in transfer buffer (25 mM Tris, 192 mM glycine, and 15% methanol). Gels were transferred for 90 minutes at 90 volts at 0.4° C in a TE 22 Transphor Electrophoresis Unit (Hoefer Scientific Instruments; San Francisco, CA) cooled with a Lauda ECO-Line RE-106 Refrigerating Circulator (Brinkmann Instruments, Inc.; Westbury, NY) set at 0.4° C. After transfer, the PVDF membranes

were dried for approximately 90 minutes. Membranes were then wrapped in cellophane and stored in the refrigerator until immunoblotting.

Western blotting for calpastatin. Dried membranes were rewet in 100% methanol for approximately 3 seconds and placed into a blocking solution consisting of 5% non-fat dry milk dissolved in PBS-Tween (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Polyoxyethylene 20-sorbitan monolaurate [Tween 20]) for one hour at room temperature. After blocking, the membranes were incubated in monoclonal primary antibody (mouse-anti-calpastatin cat. # MA3 945, Affinity Bioreagents, Inc.; Golden, CO) at a dilution of 1:10,000 (antibody: PBS-Tween) for one hour at room temperature. At the end of the primary incubation, the membranes were washed three times (ten minutes per wash) in PBS-Tween. Upon completion of the third wash, the membranes were incubated for one hour at room temperature in secondary antibody, goat-anti-mouse IgG conjugated with horseradish peroxidase, (cat. # A2554, Sigma; St. Louis, MO) at a dilution of 1:5,000 (antibody: PBS-Tween with 1% non-fat dry milk). After secondary incubation, the membranes were washed three times (ten minutes per wash) in PBS-Tween. A fourth wash of PBS-Tween was applied before detection. The presence of calpastatin was detected using ECL Western Blotting reagents (Amersham Life Science,

Arlington Heights, IL) as directed by the manufacturer by exposure to film (Kodak BioMax Light-1 Film, 13x18 cm, Kodak No. 8689358).

Densitometry. Densitometric analyses were performed on the calpastatin blots using the Kodak ID Image Analysis Software (Rochester, NY) and a Kodak DC 120 camera. This procedure was done to give a numerical value to the apparent differences seen by visual evaluation. Each animal was used as its own standard to create the ratios used for statistical analysis. These ratios were developed by dividing the post-diet biopsy calpastatin intensity values by the pre-diet biopsy values. For example if the ratio of animal XYZ was two, then the band intensity from the post-diet biopsy was twice as intense as the pre-diet biopsy.

SDS PAGE for p94. One hundred twenty µg of protein from each sample were loaded into separate wells of a 15% acrylamide separating gel (15% acrylamide/bis [100:1 acrylamide: bisacrylamide], 0.375 M Tris-HCl, pH 8.8, 0.1% sodium dodecyl sulfate (SDS). The 15% separating gel had a 5% acrylamide stacking gel (5% acrylamide/bis [100:1 acrylamide: bisacrylamide], 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.001% TEMED, and 0.007% ammonium persulfate). The gel apparatus used was the Hoefer SE 260B Mighty Small II (Pharmacia Biotech; San Francisco, CA). The composition of the running buffer used was 25 mM Tris, 192 mM glycine, and 0.1%

SDS, pH 8.3. Gels were run at 125 volts for approximately 190 minutes at room temperature (until the dye front had run off the gel). At completion, the proteins on the gel were transferred immediately to Westran PVDF (polyvinylidene fluoride) Protein Transfer and Sequencing membranes (Schleicher & Schuell, Inc.; Keene, NH) prewet in 100% methanol and then in transfer buffer (25 mM Tris, 192 mM glycine, and 15% methanol). Gels were transferred for 90 minutes at 90 volts in a TE 22 Transphor Electrophoresis Unit (Hoefer Scientific Instruments; San Francisco, CA) with an Ecoline RE106 circulating water bath (Lauda Brinkman) set at 0.4° C. After transfer, the PVDF membranes were dried for approximately 90 minutes. Then they were wrapped in cellophane and stored in the refrigerator until immunoblotting.

Western blotting for p94. Dried blots were rewet in 100% methanol for 3 seconds before being placed into a blocking solution consisting of 5% non-fat dry milk dissolved in PBS-Tween (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Polyoxyethylene 20-sorbitan monolaurate [Tween 20]) for one hour at room temperature. After blocking, membranes were incubated in monoclonal primary antibody (mouse anti-p94 cat. #NCL-CALP-12A2, Novocastra Labs, UK) at a dilution of 1:1,000 (antibody: PBS-Tween with 1% BSA) for one hour at room temperature. Three ten minute washes in PBS-Tween were performed before secondary incubation. Membranes were incubated

in secondary antibody, sheep-anti-mouse IgG conjugated to horseradish peroxidase, (cat. # NA931, Amersham Pharmacia Biotech; Piscataway, NJ) at a dilution of 1:5,000 (antibody: PBS-Tween) for one hour. Three washes (ten minutes each) were completed with addition of a fourth wash before detection. The presence of p94 was detected using ECL Western Blotting reagents (Amersham Life Science, Arlington Heights, IL) as directed by the manufacturer by exposure to film (Kodak BioMax Light-1 Film, 13x18 cm, Kodak No. 8689358, Fisher cat. # 05-728-47, Fisher Scientific; Itasca, IL).

DEXA. Dual Electron X-ray Absorptiometry (DEXA) data was collected at the Iams Co. (Lewisburg, OH). DEXA provides measurements for lean body mass (LBM), fat content (FC), and bone mineral content (BMC) (Sunvold and Bouchard, 1998). The concept of DEXA includes two energy beams (6.4 and 11.2 fJ), which pass through the body to give different values based on mass and type of tissue (Svendsen et al., 1993). DEXA is appealing because it is relatively quick, determines LBM, FC, and BMC in a noninvasive manner, and it is highly repeatable (Sunvold and Bouchard, 1998). DEXA measurements were taken before animals began dietary treatments and at the completion of the feeding trial.

Statistical analysis

Densitometric data from calpastatin Western blots were analyzed using an ANOVA to test treatment effects, with source and percentage of protein as the independent factors. DEXA data were analyzed with an ANOVA to test treatment, percent crude protein, and source of protein effects. The significance level was predetermined at $P < 0.05$.

Results

Calpastatin

Upon examination of the immunoblots, three isoforms of calpastatin were visible in most of the animals with the uppermost isoform appearing at approximately 110 kDa. The diets which most clearly produced differences in the presence of calpastatin in the muscles of the dogs fed in this trial were diets 5 and 8. These were both 28% crude protein - diet 5 was composed of 100% chicken protein, and diet 8 was 100% corn gluten protein. In six out of the seven animals fed 28% crude protein, 100% chicken protein (diet 5), there was a greater expression of the high molecular weight isoform of the calpastatin apparent in the second biopsy sample (Figures 1A and 1B), with one showing a decline in the second sample. The animals fed the 28% protein diet, 100% corn gluten meal showed a decrease in apparent calpastatin three out of seven times (Figure 1A,

Figure 1B, lanes 2 and 3), with three animals showing no change (Figure 1B, lanes 6 and 7) and one showing an increase in the second biopsy sample. Comparisons to examine the protein source effect at the 12% crude protein level (Figure 2), and the percent crude protein effect within the same protein source (Figures 3 and 4) were also completed with no consistent results.

Densitometry

Western blots depicting calpastatin were examined with densitometry to provide a basis for statistical comparison. Samples were standardized against a sample from an animal fed diet 6. This particular animal was chosen for a standard because it showed a clear separation of all three bands evident in most of the samples, and the dogs fed diet 6 did not show any observable differences due to the diet. Then the post-diet biopsy value was divided by the pre-diet biopsy to provide a ratio indicating the change in calpastatin expression within an animal. For example, a number of 3 would indicate a three-fold increase in the expression of calpastatin from the pre-diet biopsy to the post-diet biopsy. The two highest molecular weight isoforms were examined separately, but the appearance of the lowest molecular weight isoform was not found consistently in all samples examined. The intensity of all evident isoforms in each sample were also added together for statistical comparison across the two diets examined. Upon examination of

the blots depicting calpastatin in the dogs fed the 28% crude protein 100% chicken diet and the 28% crude protein 100% corn gluten meal diet, a significant difference (P = 0.0315) was obtained from comparing the highest molecular weight isoform of calpastatin from the dogs that were fed diet 5 to the dogs that were fed diet 8 (Table 2). There was a significant increase in the intensity of the high molecular weight isoform in the dogs that were fed diet 5. The samples taken from dogs fed diet 5 showed a greater intensity of calpastatin expression than those fed diet 8.

p94

Gel samples made from the myofibrillar protein fraction were examined through immunoblotting to determine the effects of dietary protein on p94 in canine skeletal muscle. All samples produced clear, consistent evidence of the presence of 94 kDa protein detected with an anti-p94 antibody. Although all animals showed the apparent expression of this 94 kDa protein, there were no differences seen between diets, within animals due to diet, or between animals (Figure 5).

DEXA

According to the DEXA data collected, all animals in this feeding trial lost lean body mass (on average, 5.27% per animal). There were significant differences observed due to percent protein fed, source of protein, and treatment effect. When the percent

protein effect was examined, it was apparent that the animals fed the 28% protein diets (diets 5-8) lost significantly less lean body mass than those fed the 12% protein diets (diets 1-4), (Table 3). The effect of corn gluten meal also produced a significant difference. Animals fed 0% corn gluten meal, or 100% chicken meal (diets 1 and 5), protein lost less lean body mass than those fed 100% corn gluten meal (diets 4 and 8), (Table 4). A comparison of diet 4 to diets 1, 2, 5, 6, 7, and 8 showed a significant difference in that animals fed diet 4, which was 100% corn gluten meal and 12% crude protein, lost more lean body mass than those fed the other 6 diets (Table 5). Upon comparison of diet 3 (12% crude protein 33% chicken protein and 67% corn gluten meal) to diet 5, diet 3 showed a greater loss in lean body mass than did diet 5 (Table 5).

Discussion

Calpastatin is known to play a key role in the living animal in muscle protein turnover as a component of the calpain proteolytic system (Goll et al., 1992). Calpastatin is thought to play a role in muscle growth by inhibiting proteolysis by the calpains. This decrease in proteolysis causes decreased muscle protein degradation, which could affect muscle growth by allowing an increase in protein accretion (Goll et al., 1991). Feeding ß-adrenergic agonists can increase calpastatin levels in bovine skeletal muscle. In this particular case, observed calpastatin activity increased due to consumption of ß-agonists

(Wheeler and Koohmarie, 1992). This increase in calpastatin is interesting because it is also associated with muscle growth. Rosenvold et al. (2001) demonstrated that feeding a low digestible carbohydrate diet to finishing pigs increased calpastatin activity. This could be a situation where calpastatin is responding in a preservative manner to try to protect the amino acids within the existing muscle in these animals. It could also be a response to an initial increase in calpain activity, if the animal was trying to retrieve more amino acids to supplement its lack of nutrition.

In this study diet 5 (100% chicken protein and 28% crude protein) had the highest amount of animal protein, which is most easily digested, and resulted in the most distinct increase in the high molecular weight isoform of calpastatin. The appearance of a significant dietary effect on the expression of calpastatin could have a number of explanations. These isoforms could be the result of post-translational modification such as phosphorylation, or possibly a change in mRNA expression of the protein itself. Calpastatin can be phosphorylated post-translationally by PKA or PKC (Salamino et al., 1994). The possibility of phosphorylation causing the change in isoform of calpastatin is interesting due to calpastatin aggregation, which requires phosphorylation by PKA in neuroblastoma LAN-5 cells (Averna et al., 2001). Phosphorylated, aggregated calpastatin is thought to be inactive, as it is not associated with the membrane where

activated calpain localizes. A study done by Pontremoli et al. (1992) found that a phosphorylated form of calpastatin showed less inhibitory efficiency than a dephosphorylated form. This further suggests a change in calpastatin activity due to phosphorylation, or lack thereof.

Pontremoli et al. (1991) found two different forms of calpastatin in rat muscle (calpastatin I and calpastatin II) that seemed to correlate specifically with μ- or m-calpain. They demonstrated that μ-calpain was almost completely unable to degrade either of the two isoforms of calpastatin examined. Although μ-calpain was fairly ineffective in degrading these two isoforms of calpastatin, both isoforms of calpastatin were very sensitive to degradation by m-calpain. Pontremoli et al. (1991) suggested that the formation of calpastatin II by post-translational modification could be important to allow calpastatin to be more able to inhibit m-calpain.

Another possibility of the appearance of more than one isoform of calpastatin could be a result of alternative splicing. Geesink et al. (1998) examined calpastatin in porcine heart as compared to porcine skeletal muscle. When calpastatin was purified by Affigel blue chromatography, they observed two separate peaks of calpastatin, with a low molecular weight form eluting earlier than a high molecular weight form. Upon further examination of porcine cardiac and skeletal muscle, the two isoforms found in cardiac

were determined to be the result of alternative splicing. It appears that the low molecular weight isoform lacked exon 3, which encodes a portion of domain L. This low molecular weight form was identical to that expressed in skeletal muscle (Geesink et al., 1998). Recently it has been suggested that domain L plays a role in reactivating L-type calcium channels in guinea pig cardiac myocytes (Hao et al., 2000).

The increase in the appearance of the high molecular weight isoform of calpastatin could be interpreted to suggest that a chicken based diet was more efficiently utilized by skeletal muscle for maintaining those proteins than the corn gluten based diet. Conversely, the corn gluten based diet may have been less easily utilized as an energy source to maintain the existing skeletal muscle proteins. This may have been observed as a change in calpastatin isoform. In this study we cannot definitively state that the increase in a high molecular weight isoform of calpastatin coincides with an increase in calpastatin activity. An increase in calpastatin activity has the potential to decrease muscle protein degradation by slowing down protein degradation. This can result in increased growth through accretion or at the least, maintenance of the existing muscle proteins by halting degradation of those proteins.

Although the results of the Western blots to examine p94 did not show any observable differences in the 94 kDa protein we detected, the appearance of this 94 kDa

protein in dog skeletal muscle biopsies is of interest in itself. Because we used an anti-p94 antibody to examine these blots, and the protein detected was at 94 kDa, it is likely that the protein we observed is p94. The presence of p94 mRNA in skeletal muscle is tenfold higher than that of the ubiquitous calpains (Sorimachi et al., 1993). It is suggested that p94 autolyzes quickly after translation (Sorimachi et al., 1993), causing difficulty in characterizing its properties. The exact function of p94 is unknown due to its apparent instability when it is separated from whole muscle. Attempts at purifying p94 have been unsuccessful except by one research group, which was only able to partially purify p94 (Kinbara et al., 1998). These results have not been reproduced by anyone else. The samples used in our trial were biopsies taken from 56 animals at two different time points. This provides 112 different scenarios in which this 94 kDa protein was detected by Western blotting. It seems that if p94 autolyzed quickly after translation it would not be easily detected in biopsies. This study also shows that this 94 kDa protein can be detected in a crude pellet extraction from muscle biopsies.

According to the DEXA data, the animals that were fed diet 4, which was a low percentage of protein from strictly vegetable sources, lost significantly more lean body mass than the animals fed all of the other diets except diet 3 where there was no significant difference. Diet 3 was very similar to diet 4 in that it was a low percentage of

crude protein, but it had 33% chicken protein added to the corn gluten meal. There were also significant effects from percentage of protein and source of protein. When compared to the lower percent crude protein strictly from corn gluten meal, the dogs fed a higher percent of total protein from chicken sources lost less lean body mass. This implies that the animals that were fed the higher crude protein chicken-based diets were better able to maintain lean body mass than those fed a lower percent of crude protein from strictly from corn gluten meal. Calpastatin data from the 28% crude protein that was entirely chicken protein (diet 5) supports a decrease in protein degradation, i.e. muscle protein accretion or at the least muscle protein maintenance.

Along with the calpastatin data, there could be a connection with the increase in calpastatin expression in the animals fed diet 5. The animals fed diet 5 conserved a significant amount of lean body mass when compared to diet 4, as well as showing the greatest increase in the highest molecular weight isoform of calpastatin. It cannot be said that calpastatin caused an increase in lean body mass in this trial. There is an interesting implication for calpastatin to have a role in maintaining lean body mass for this particular dietary trial. These data suggest that skeletal muscle calpastatin has elicited a physiological response in this trial to dietary protein. This is exemplified by the evidence of an increase in a higher molecular weight isoform of calpastatin associated with

biopsies from the skeletal muscle of dogs fed a higher percentage of chicken protein (more highly digestible) as well as a higher percentage of total protein. The observation of an increase in calpastatin expression leads to the question of whether it is increasing protein accretion in the muscles examined. Although we did not examine protein degradation and accretion, the DEXA data show that animals fed the diet which elicited such a response in calpastatin lost less lean body mass when compared to the dogs fed a lower percentage of total protein strictly from corn gluten (less digestible). The dogs that were fed strictly corn gluten may be exhibiting a physiological response due to an increasing need to mobilize amino acids from skeletal muscle to supplement the lack of nutrition.

Implications

The DEXA analyses showed that the dogs fed 12% crude protein 100% of which was corn gluten meal (diet 4), lost the most lean body mass when compared to all the other diets except the diet composed of 12% crude protein with 33% chicken and 67% corn gluten meal (diet 3). The implications from the DEXA data suggest that the animals that were fed a lower percentage of total protein strictly from corn gluten meal were less able to maintain muscle than those that were fed diets of higher total protein from entirely chicken sources. The canines that were fed a diet consisting entirely of chicken protein at

a level of 28% crude protein showed an increase in a high molecular weight form of skeletal muscle calpastatin in the semitendinosis. Because calpastatin inhibits calpain, which partially degrades some skeletal muscle proteins, it is suggested that an increase in calpastatin in the live animal may increase skeletal muscle protein accretion by minimizing proteolysis. This muscle protein accretion could, in turn, produce an increase in overall skeletal muscle growth.

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Table 1. Composition of dietary treatments

Diet	% Crude protein	% Chicken protein	% Corn gluten meal
1	12	100	0
2	12	67	33
3	12	33	67
4	12	0	100
5	28	100	0
6	28	67	33
7	28	33	67
8	28	0	100

Table 2. Densitometric data from diets 5 and 8

	5 - (100 % animal proteins source)	8- (100% vegetable protein source)	P-Value
Band 1 Relative Intensity Ratio	1.846 *(.280)*	.708 *(.3054)*	.0315
Band 2 Relative Intensity Ratio	1.174*(.285)*	1.250 *(.225)*	.8359
Combined Bands Relative Intensity Ratio	1.551 *(.270)*	.914 *(.153)*	.0601

Table 3. Percent crude protein effect for percent lean body mass gained

Percent crude protein	Diets	Mean	Standard error
12	1-4	-7.087[a]	0.905
28	5-8	-3.461[b]	0.864

Means with different superscripts indicate significant differences at $P < 0.05$.

Table 4. Corn gluten meal effect for percent lean body mass gained

Percent corn gluten meal	Diets	Mean	Standard error
0	1, 5	-3.467[a]	0.927
33	2, 6	-5.092[ab]	1.365
67	3, 7	-5.547[ab]	1.398
100	4, 8	-7.262[b]	1.549

Means with different superscripts indicate significant differences at $P < 0.05$.

Table 5. Dietary treatment effect for percent lean body mass gained

Diet	% Crude protein	% Corn gluten meal	Mean	Standard error
1	12	0	-5.490^{bc}	0.878
2	12	33	-5.546^{bc}	1.986
3	12	67	-6.457^{ab}	1.693
4	12	100	-10.856^{a}	1.975
5	28	0	-1.444^{c}	1.259
6	28	33	-4.639^{bc}	2.015
7	28	67	-4.637^{bc}	2.308
8	28	100	-3.068^{bc}	0.695

Means with different superscripts indicate significant differences at $P < 0.05$.

Figure 1A. Western blot depicting dogs from diets 5 and 8

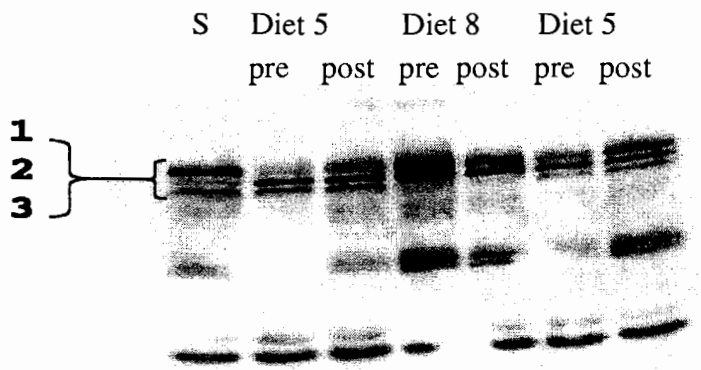

Figure 1B. Western blot depicting dogs from diets 5 and 8

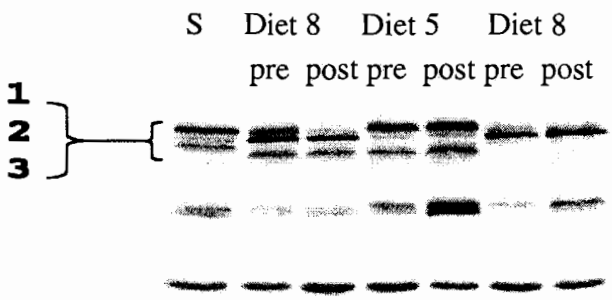

Figure 2. Western blot depicting calpastatin in dogs fed diets 1 and 4

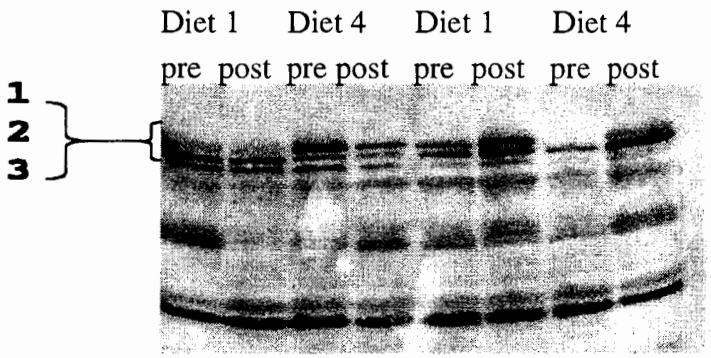

Figure 3. Western blot depicting calpastatin in dogs fed diets 1 and 5

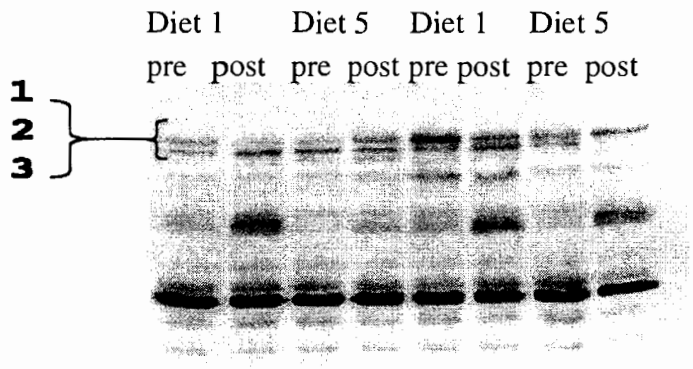

Figure 4. Western blot depicting calpastatin in dogs fed diets 4 and 8

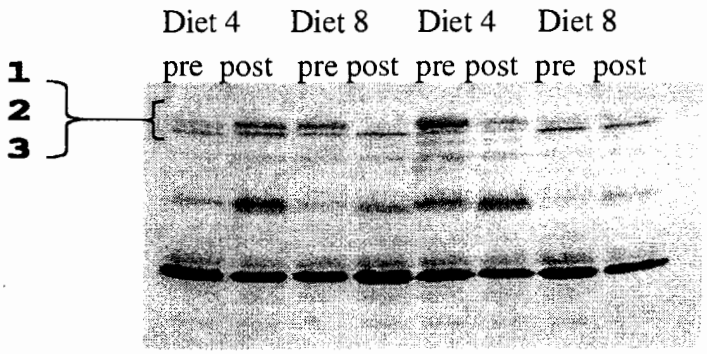

Figure 5. Western blot depicting p94 in dogs fed diets 5 and 8

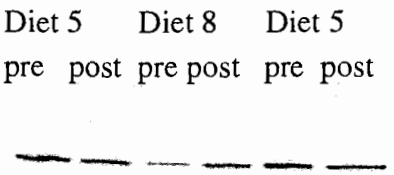

Figure 1A. Figure 1A depicts calpastatin in animals from diets 5 and 8. Diet 5 contained 28% crude protein with protein sources of 100% chicken, 0% corn gluten meal. Diet 8 contained 28% crude protein with protein sources of 0% chicken and 100% corn gluten meal. This represents a dietary protein source comparison due to both diets containing identical quantities of crude protein. The extract of the sarcoplasmic fraction was used to examine calpastatin. This blot was incubated in an anti-calpastatin primary antibody (MA3 945) at a concentration of 1:10,000 (antibody: PBS Tween). The blot was incubated in secondary antibody (Goat ant-mouse HRP) at a concentration of 1:5,000 (antibody: PBS Tween with 1% non-fat dry milk). Lane 1 is a sample from animal HJAMGT (which was fed diet 6) to give a standard for densitometric analyses. Lanes 2 and 3 correspond to animal HILMEH (which was fed diet 5), pre- and post-diet biopsy samples, respectively. Lanes 4 and 5 are samples taken from animal HIJTAB (which was fed diet 8), pre- and post-diet biopsy samples, respectively. Lanes 6 and 7 are samples taken from animal HILMIM (which was fed diet 5), pre- and post-diet biopsy samples, respectively. The uppermost three bands seen in the standard sample represent calpastatin, with the top band appearing at approximately 110 kDa.

Figure 1B. Figure 1B also represents calpastatin in animals from diets 5 and 8. The samples from the sarcoplasmic fraction were used to examine calpastatin. This blot was treated with the same procedure as that in Figure 1A. Lane 1 is the same animal, HJAMGT, depicted as the standard in Figure 1A. Lanes 2 and 3 represent pre- and post-diet biopsy samples from animal HILMIS, which was fed diet 8. Animal HJBMHI, fed diet 5, is represented in lanes 4 and 5. Lanes 6 and 7 were loaded with samples from animal HJDTAR, which was fed diet 8. The uppermost three bands correspond to calpastatin.

Figure 2. Figure 2 is an example of the comparison of calpastatin from animals fed diets 1 and 4. The same procedure for Figure 1A was used for this figure, including use of the samples made from the sarcoplasmic extraction. Diet 1 was 12% crude protein – 100% of which was from chicken sources. Diet 4 was also 12% crude protein, but the protein source was 100% corn gluten meal. The comparison of diet 1 to diet 4 is another source comparison, as both diets are 12% crude protein. Lanes 1 and 2 correspond to animal HJGMBB, which was fed diet 1. Lanes 3 and 4 represent animal HJDMFS, fed diet 4. Lanes 5 and 6 are samples from animal HJFMAF, fed diet 1, and lanes 7 and 8 are samples from animal HJAMFL, fed diet 4. The three top bands show calpastatin.

Figure 3. Figure 3 shows a comparison of calpastatin from animals fed diet 1 and diet 5. The samples from the low ionic strength extraction were used to examine calpastatin. This comparison was done to examine the differences seen from the percentage of crude protein within the same protein source. Diet 1 was 12% crude protein from 100% chicken sources and diet 5 was 28% crude protein, also from 100% chicken sources. Lanes 1 and 2 are samples from animal HJBMEX (fed diet 1), lanes 3 and 4 are samples from animal HILMEH (fed diet 5) lanes 5 and 6 are samples from animal HJBMHX (fed diet 1), and lanes 7 and 8 are samples from animal HILMIM (fed diet 5). The three top bands represent calpastatin.

Figure 4. Figure 4 is a comparison of calpastatin from the dogs fed the diets composed of 100% corn gluten protein. The samples made from the sarcoplasmic extraction were used to examine calpastatin. Diet 4 was 12% crude protein and diet 8 was 28% crude protein. Lanes 1 and 2 are from animal HJBMLN (fed diet 4), lanes 3 and 4 are from animal HILMIS (fed diet 8), lanes 5 and 6 are from animal HJDMFS (fed diet 4), and lanes 7 and 8 are from animal HJDTAR (fed diet 8). The uppermost three bands correspond to calpastatin.

Figure 5. Figure 5 depicts p94 in samples from animals fed diets 5 and 8 that were examined using an anti-p94 antibody. The myofibrillar samples made from the second extraction were used to examine the state of p94 in the dogs. Lanes 1 and 2 were loaded with samples from animal HJHMCE (fed diet 5), lanes 3 and 4 were loaded with samples from animal HJHMAK (fed diet 8), and lanes 5 and 6 were loaded with samples from animal HJHMDX (fed diet 5). This blot was incubated in an anti-p94 primary antibody (NCL-CALP-12A2) at a concentration of 1:1,000 (antibody: PBS Tween with 1% BSA). After primary incubation, the blot was incubated in secondary antibody (Sheep anti-mouse HRP) at a concentration of 1:5,000 (antibody: PBS Tween). The only evident bands seen on this blot correspond to a 94 kDa protein to which the anti-p94 primary antibody bound.

GENERAL CONCLUSION

Calpastatin has been hypothesized to play a key role in the living animal in muscle protein turnover as a component of the calpain proteolytic system (Goll et al., 1992). Calpastatin is thought to be involved in muscle growth by inhibiting proteolysis by the calpains. This decrease in proteolysis causes decreased muscle protein degradation, which could affect muscle growth through an increase in protein accretion (Goll et al., 1991). Calpastatin activity can be increased through certain changes in nutrition such as feeding ß-adrenergic agonists to bovines (Wheeler and Koohmarie 1992), or feeding a low digestible carbohydrate diet to finishing pigs (Rosenvold et al., 2001). This change in calpastatin activity is significant because of its associations with differences in skeletal muscle growth.

Calpastatin can be post-translationally modified by phosphorylation with either PKA or PKC (Salamino et al., 1994), although only PKA affects aggregation (Averna et al., 2001). Phosphorylated, aggregated calpastatin is thought to be inactive because it is not associated with the membrane where activated calpain localizes. A study done by Pontremoli et al. (1992) found that a phosphorylated form of rat skeletal muscle calpastatin showed less inhibitory efficiency than a dephosphorylated form.

Calpastatin has also been shown to exhibit different isoforms in porcine cardiac muscle due to alternative splicing (Geesink et al., 1998). Porcine cardiac and skeletal muscle were compared, with expression of a high molecular weight form found only in cardiac muscle and a low molecular weight isoform that was found in both cardiac and skeletal muscle. The low molecular weight form did not contain exon 3, which encodes a portion of domain L (Geesink et al., 1998). Until recently domain L did not have a known function, but Hao et al. (2000) suggest that domain L is involved in the reactivation of Ca^{2+} L-type channels in guinea pig cardiac myocytes.

Calpastatin can be influenced through nutrition, it can be modified post-translationally by phosphorylation, and it can be expressed in different isoforms through alternative splicing. Given these three points, the results from the Western blots done for this project have some interesting implications for calpastatin as an influential role in muscle growth. There was a significant difference in the expression of the high molecular weight isoform observed in our Western blots when comparing diet 5, which was 28% crude protein entirely from chicken, to diet 8, which was 28% crude protein composed completely of corn gluten meal. This suggests that calpastatin expression was affected by the different dietary sources of protein.

The DEXA data collected showed that the dogs fed the lowest percentage total protein taken completely from corn gluten meal (diet 4) lost significantly more lean body mass than those fed diet 5 (28% total protein, 100% chicken protein). This is interesting in regard to the higher digestibility of the chicken based diet, as well as the increase in total protein. Although it can not be said that the greater intensity of the high molecular weight isoform of calpastatin is associated with increased lean body mass, there appears to be an association with greater total protein (from chicken) and maintenance of lean body mass. It is possible that the observed physiological response (exemplified as an increase in a high molecular weight isoform of calpastatin) in this study may partly explain the dogs' ability to maintain lean body mass in the form of decreased skeletal muscle protein degradation.

There is no way to determine whether the differences that we observed were due to post-translational modification of the protein, or at the translational level in this trial. It would be an interesting addition to this study to examine if the differences observed are due to alternative splicing in skeletal muscle in these dogs with RT-PCR (Geesink et al., 1998). Another way to examine the post-translational state of calpastatin would be to examine calpastatin in Western blots using antibodies against phosphorylated residues

(Kaufmann et al., 2001). This would give us a measure if the three separate bands that were observed were affected by phosphorylation or not.

The Western blots used to examine p94 in this study did not detect an observable difference in the expression of the 94 kDa protein detected by the ant-p94 antibody we used, although Van den Hemel-Grooten et al. (1997) observed that p94 mRNA in young pigs decreased after feeding them a protein-free diet. There have been even fewer trials to examine p94 because of its recent discovery, and the difficulty of examining wild type p94 due to its apparent autolytic features. It is an interesting subject to explore because of its possible role in muscle protein turnover, which is suggested due to its close association with titin degradation regions.

The appearance of a 94 kDa protein with the anti-p94 antibody in the canine samples we examined is interesting in itself, merely due to the possibility that it could be p94. Because p94 exhibits extensive autolytic characteristics, it could be an interesting indication of the state of p94 in biopsy samples from canine skeletal muscle. The samples taken from the myofibrillar fraction for this study all showed a clear expression of this 94 kDa band. If this 94 kDa protein was p94, and p94 does autolyze quickly after translation as suggested previously, it would be seemingly difficult to find it in an intact state in a muscle at any given point in time.

In addition to further examination of calpastatin and p94, investigating μ- and m-calpain would give a more complete picture of the functions of the calpain/calpastatin system in skeletal muscle. If m- and/or μ-calpain were also affected by this feeding trial, the system of protein turnover could be influenced toward more degradation, or more accretion. By limiting the number of diets to two – either high and low protein or only different sources, it might have given a clearer idea of the greater influence to this protein system. Examination of more than one muscle would also have given us a broader view of whether the changes we observed in the semitendinosis of the dogs in this trial are duplicated in other skeletal muscle. Another interesting addendum to this trial could have been examination of fiber type in these dogs by examining myosin heavy chain isoforms (Talmadge and Roy, 1993). Fiber type can be a useful indication of metabolic changes which are occurring in a muscle. It would also be useful to identify whether or not the proteins from the diet were utilized by the animal. This could be examined through labeling the diet and examining for amino acids that were not utilized by the animal (Hess et al., 2000).

The results of our trial support a role for calpastatin in skeletal muscle maintenance, in respect to the observed physiological response to dietary influence. The DEXA data provided a basis for evaluation of lean body mass, and although it did not

reflect any increase in lean body mass, the dogs that were fed a higher protein (from chicken) diet appeared to maintain lean body mass better than those fed a lower protein corn gluten meal diet. This seems to imply that the higher protein chicken-based diet, which is more highly digestible than corn gluten meal, was utilized more effectively to maintain canine lean body mass.

The next step in the quest for the physiological functions of this system is to determine why the components of the calpain/calpastatin system respond to factors such as dietary change. We also need to determine if calpain and calpastatin are responding to changes in physiological conditions, or if they are initiating those changes. For example, does increased calpastatin activity cause the callipyge condition, or is it a response to an initial change in another physiological component within those muscles affected by the condition? Our trial gives insight into calpastatin responses to dietary influence in canine skeletal muscle. Much remains to be learned regarding the relationships that have been documented here, but each new study continues to build our understanding of this system.

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