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Abstract
The cysteine proteinases, μ- and m-calpain, along with their inhibitor, calpastatin, 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 was that the quantity and source of dietary protein could 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 in canine skeletal muscle. This study comprised eight diets with seven dogs per diet. A biopsy was taken from the biceps femoris of all 56 dogs before and after 10 wk on their respective diets. This experimental design allowed examination of change within individual dogs. Diets 1 to 4 contained 12% total protein derived from chicken and/or corn gluten meal in ratios of 100:0, 67:33, 33:67, and 0:100%, respectively. Diets 5 to 8 contained 28% total protein with protein sources and ratios identical to Diets 1 to 4. Differences in calpastatin were examined qualitatively using SDS-PAGE and immunoblotting, and semiquantitatively with densitometric analyses. The majority of the calpastatin blots showed three distinct calpastatin bands, the uppermost appearing at approximately 110 kDa. Diet 5 (28% CP, 100% chicken) resulted in an increase in the expression of the 110-kDa calpastatin band compared with the other two lower molecular weight bands in the same samples. Muscle from dogs fed Diet 5 showed greater increase in ($P < 0.05$) calpastatin intensity of the topmost band than those fed Diet 8 (0:100; chicken:corn gluten meal). Diet 5 (100:0; chicken:corn gluten meal) showed greater total calpastatin intensity than Diet 8 (0:100; chicken: corn gluten meal). These 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 than dogs fed diets containing corn gluten meal.

Keywords
Dogs, Dietary Protein, Proteinases, Skeletal Muscle

Disciplines
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Comments
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Effect of dietary protein on calpastatin in canine skeletal muscle[1]

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ABSTRACT: The cysteine proteinases, μ- and m-calpain, along with their inhibitor, calpastatin, 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 was that the quantity and source of dietary protein could 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 in canine skeletal muscle. This study comprised eight diets with seven dogs per diet. A biopsy was taken from the biceps femoris of all 56 dogs before and after 10 wk on their respective diets. This experimental design allowed examination of change within individual dogs. Diets 1 to 4 contained 12% total protein derived from chicken and/or corn gluten meal in ratios of 100:0, 67:33, 33:67, and 0:100%, respectively. Diets 5 to 8 contained 28% total protein with protein sources and ratios identical to Diets 1 to 4. Differences in calpastatin were examined qualitatively using SDS-PAGE and immunoblotting, and semiquantitatively with densitometric analyses. The majority of the calpastatin blots showed three distinct calpastatin bands, the uppermost appearing at approximately 110 kDa. Diet 5 (28% CP, 100% chicken) resulted in an increase in the expression of the 110-kDa calpastatin band compared with the other two lower molecular weight bands in the same samples. Muscle from dogs fed Diet 5 showed greater increase in ($P < 0.05$) calpastatin intensity of the topmost band than those fed Diet 8 (0:100; chicken:corn gluten meal). Diet 5 (100:0; chicken:corn gluten meal) showed greater total calpastatin intensity than Diet 8 (0:100; chicken: corn gluten meal). These 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 than dogs fed diets containing corn gluten meal.

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Introduction

Calpastatin is an inhibitor of the calpains, a family of calcium-dependent cysteine proteinases that are expressed ubiquitously (Carafoli and Molinari, 1998). Calpain enzymes are hypothesized to play a role in disassembly of sarcomeric proteins (Huang and Forsberg, 1998), which is proposed to be a necessary step for the breakdown of proteins through the ubiquitin-proteosome pathway (Wray et al., 2002). Through its inhibition of calpain, calpastatin could regulate this activity. Calpastatin expression in skeletal muscle is influenced by diet (van den Hemel-Grooten et al., 1997; Nikawa et al., 2002). Dietary protein content and quality are of critical importance in muscle growth and maintenance. A recent trial using canines (Wakshlag et al., 2003) reported that lean body mass is lost when protein quality is reduced. Wakshlag et al. (2003) reported that loss of lean body mass was correlated with increasing levels of vegetable-based protein in experimental diets. Because calpastatin, through inhibition of calpains, is hypothesized to play a role in muscle maintenance (Koohmaraie et al., 1995), we used tissue samples generated in the Wakshlag et al. (2003) study to determine the effects of dietary protein (amount and source) on the expression of calpastatin in canine skeletal muscle.

Materials and Methods

Animals

Fifty-six purpose-bred, reproductively intact, female mongrel dogs between the ages of 2 and 3 yr

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were used for this study. All dogs were housed and fed individually in 0.9- × 2.4-m indoor runs. The study was conducted under an IACUC-approved protocol and complied with all federal guidelines for conducting animal research. All dogs were fed Eukanuba Adult Maintenance Dog Formula during an initial 7-wk acclimation period to establish individual food intake and BW parameters. Diet amounts offered to each dog were adjusted on a weekly basis during this period to provide an adequate amount of food to satisfy their daily consumption needs and to maintain BW of the dogs. The final amount of food offered to each dog during this preliminary period was maintained during the subsequent experimental period. The dogs were fed once daily during both periods, with any feed refusals measured the following day.

For the experimental period, dogs were assigned to eight experimental diets containing either 12 or 28% protein comprised of varying ratios of animal- and vegetable-based protein sources (100:0; 67:33; 33:67; 0:100). Animal protein was provided as a 55:45 blend of chicken meal and fresh chicken, whereas corn gluten meal was used as the source of vegetable protein. Pregelatinized cornstarch was used to formulate the dry, extruded, isocaloric diets. Ingredient and nutrient composition of these diets is shown in Table 1. Throughout the study, food consumption was monitored on a daily basis and BW on a weekly basis. Fresh water was provided ad libitum throughout the study. Dogs were allocated to their respective dietary treatments based on BW and subjective BCS (Sunvold and Bouchard, 1998) obtained at the end of the acclimation period to ensure representative weight and condition across treatments.

A biopsy sample of the biceps femoris was obtained at wk 0 and 10 of the experimental period using standard surgical procedures. Each dog was preanesthetized using Atropine (0.022 mg/kg), induced with a combination of Xylazine/Telazol/Torbugesic (0.79/6.6/0.13 mg/kg) and maintained with 1% Isoflurane (Aerrane, Ohmeda Pharmaceutics; Liberty Corner, NJ) via mask delivery. The 1.0-g sample of muscle tissue was divided into three equal portions and snap frozen in liquid N. All biopsy samples were stored at –80°C until subsequent laboratory analysis. Penicillin G was administered subcutaneously to each dog following the biopsy procedure.

Sample Preparation

The procedure used for sarcoplasmic muscle protein extraction was modified from Doumit et al. (1996) to compensate for the size of the samples. A 0.1-g sample (weighed in the frozen state) was homogenized in five volumes of 4°C extraction buffer (10 m*M* EDTA, 0.1 mg/mL of ovomucoid, 2 μ*M* E-64, 2 m*M* PMSF, and 100 m*M* 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 Co., Barrington, IL) set on speed 5. Between intervals, the homogenizing tube was chilled on ice for 30 s. Homogenates were clarified by centrifugation at 22,000 × *g* for 20 min at 4°C. We previously determined that dialysis of low-molecular-weight protein allows for loading more protein, which is advantageous in detection on a Western blot (K. A. Cummins, personal communication). The supernatant was dialyzed overnight (10,000 MWCO; Pierce Chemical Co.; Rockford, IL) against a solution at 4°C containing 5 m*M* EDTA, 40 m*M* Tris, and 0.05% β-mercaptoethanol at pH 7.35. After dialysis, protein concentration was determined (Bradford, 1976) and samples were prepared for gel electrophoresis by diluting to a protein concentration of 3 mg/mL in gel sample buffer (3 m*M* EDTA, 3% SDS, 30% glycerol, 0.001% Pyronin Y, and 30 m*M* Tris-HCl, pH 8.0 [Wang, 1982]). Gel samples were then heated at 50°C for 20 min and subsequently stored at –80°C until analysis.

Immunoblotting

Ninety micrograms of protein from each sample was loaded into separate wells of a 10-cm × 12-cm × 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% SDS, 0.05% ammonium persulfate, and 0.05% *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, 0.125% TEMED, and 0.075% ammonium persulfate). The composition of the running buffer used was 25 m*M* Tris, 192 m*M* glycine, and 0.1% SDS, pH 8.3. Gels were run at 125 V for approximately 190 min at room temperature (until the dye front had run off the gel). At completion, the proteins on the gel were transferred immediately to Westran polyvinylidene fluoride membranes (Schleicher & Schuell, Inc.; Keene, NH) prewetted in 100% methanol and then in transfer buffer (25 m*M* Tris, 192 m*M* glycine, and 15% methanol). Gels were transferred for 90 min at 90 V at 4°C in a TE 22 Transphor electrophoresis unit (Hoefer Scientific Instruments; San Francisco, CA). Membranes were incubated in a blocking solution consisting of 5% nonfat dry milk dissolved in PBS-Tween (80 m*M* Na_2HPO_4, 20 m*M* NaH_2PO_4, 100 m*M* NaCl, and 0.1% polyoxyethylene 20-sorbitan monolaurate [Tween 20]) for 1 h at room temperature. After blocking, membranes were incubated in monoclonal primary antibody (mouse-anti-calpastatin, catalog No. MA3 945, Affinity Bioreagents, Inc., Golden, CO) diluted 1:10,000 in PBS-Tween with 1% nonfat dry milk for 1 h at room temperature. At the end of the primary incubation, the membranes were washed three times (10 min per wash) in PBS-Tween. Membranes were incubated for 1 h at room temperature in

Table 1. Ingredient composition, nutrient composition, and caloric distribution of experimental diets containing varying levels of animal and vegetable protein sources

Item	Experimental diet:	1	2	3	4	5	6	7	8
	Crude protein content:	12%				28%			
	Animal:vegetable:	100:0	67:33	33:67	0:100	100:0	67:33	33:67	0:100
Ingredient composition, %									
Chicken meal		12.8	8.5	4.2		29.0	19.6	9.6	
Chicken		10.5	7.0	3.5		24.2	16.2	8.0	
Corn gluten meal			6.3	12.7	18.7		15.3	31.2	46.5
Corn starch		49.0	48.0	48.0	47.0	29.0	26.0	23.0	21.0
Poultry fat		10.6	12.6	14.5	16.3	2.1	6.4	10.7	14.8
Minerals		7.1	7.4	6.4	7.9	5.8	6.2	7.2	7.2
Beet pulp		4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Poultry digest		3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Brewer's yeast		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Ground flax		0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Vitamins		0.8	0.9	0.8	0.8	0.7	0.8	0.8	0.7
Menhaden oil		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Methionine		0.2	0.2	0.2	0.3	0.1	0.3	0.5	0.8
Nutrient composition (laboratory analysis; DM basis)									
Proximate analysis									
Protein, %		13.2	12.8	12.1	14.4	29.4	30.8	31.3	31.5
Fat, %		19.2	19.7	19.2	20.3	19.8	19.9	19.3	19.0
Fiber, %		2.0	2.1	1.8	1.6	2.2	2.1	2.5	1.7
Moisture, %		7.8	7.4	8.9	10.1	9.8	6.8	6.5	7.9
Ash, %		7.8	7.7	7.4	6.7	8.5	11.5	7.7	7.6
Nitrogen-free extract, %[a]		61.2	60.9	63.2	61.3	46.0	40.0	43.1	45.0
Gross energy, kcal/kg		4,950	5,035	5,013	5,137	5,215	5,251	5,310	5,341
Caloric distribution, % of calories[b]									
Protein		12.3	12.3	12.2	12.4	28.2	28.3	28.3	28.4
Fat		39.8	40.3	40.1	40.6	40.2	40.6	40.9	40.6
Carbohydrate		47.9	47.4	47.7	47.3	31.6	31.1	30.9	31.0
Essential amino acids, % DM basis									
Arginine		0.85	0.53	0.46	0.42	1.85	1.75	1.14	0.98
Histidine		0.33	0.36	0.29	0.30	0.68	0.68	0.58	0.63
Isoleucine		0.52	0.44	0.34	0.42	1.23	1.31	1.00	1.10
Leucine		1.13	1.16	1.48	1.79	2.28	3.43	3.79	4.82
Lysine		0.87	0.64	0.45	0.26	2.01	1.66	0.87	0.53
Methionine		0.29	0.26	0.31	0.41	0.60	0.75	0.88	1.34
Cystine		0.25	0.31	0.33	0.40	0.67	0.82	0.94	0.96
Phenylalanine		0.54	0.51	0.57	0.68	1.15	1.51	1.50	1.82
Tyrosine		0.24	0.17	0.26	0.38	0.62	0.87	0.91	1.10
Threonine		0.66	0.52	0.47	0.48	1.34	1.37	1.13	1.13
Valine		0.57	0.52	0.49	0.51	1.31	1.43	1.20	1.25

[a]Nitrogen-free extract was calculated by difference.
[b]Calculation of the caloric distribution from protein, fat, and carbohydrate was based on modified Atwater factors of 3.8, 8.5, and 3.8 kcal/kg, respectively.

secondary antibody, goat-anti-mouse IgG conjugated with horseradish peroxidase, (catalog No. A2554, Sigma; St. Louis, MO) diluted 1:5,000 in PBS-Tween with 1% nonfat dry milk. After three 10-min washes in PBS-Tween, membranes were visualized using ECL Western blotting reagents (Amersham Life Science, Arlington Heights, IL) by exposure to film (Kodak Bio-Max Light-1 Film, 13 × 18 cm, Kodak No. 8689358, Rochester, NY) as directed by the manufacturer.

Densitometric analyses were performed on the calpastatin blots using the Kodak ID Image Analysis Software and a Kodak DC 120 camera. Western blots were examined with densitometry to provide a basis for statistical comparison. This comparison was made on samples from Diet 5 (28% protein, 100% chicken protein source) to Diet 8 (28% protein, 100% corn gluten meal). A reference from canine skeletal muscle sample that exhibited three high molecular weight bands (1, 2, and 3) was used to confirm migration of each band in each sample on gels used for densitometry analysis. Density of each band was measured. The post-trial biopsy value was divided by the pretrial biopsy value to provide a ratio indicating the change in calpastatin expression observed during the trial within an animal. For example, a value of 3 would indicate a threefold increase in the expression of calpastatin from the pretrial biopsy to the post-trial biopsy. This analysis allowed each animal to serve as its own control because the change over the trial was monitored. The two highest molecular weight bands (Bands 1 and 2) were examined separately, but the appearance of the lowest molecular weight band was

not found consistently in all samples examined. The intensity of all evident bands (1 to 3) in each sample was also added together for statistical comparison across the two diets examined (combined bands relative intensity ratio). The exact origin of the 70-kDa band is unknown. Although it is possible that this is a skeletal muscle calpastatin degradation product, it is more likely that it is erythrocyte calpastatin present in the biopsy sample (Takano et al., 1991). Because of the uncertainty of the origin of the 70 kDa band, only the "muscle type" calpastatin (bands 1 to 3) was used in the analysis.

Statistical Analysis

Body weight, food consumption, and subjective body condition score data were analyzed with ANOVA using protein source and level as independent factors. Densitometric data from calpastatin Western blots (Diets 5 and 8) were analyzed using an ANOVA to test the source of protein as the independent factor (SAS, 1998). Means separation was conducted using the Games-Howell procedure (SAS, 1998). The significance level was predetermined at $P < 0.05$.

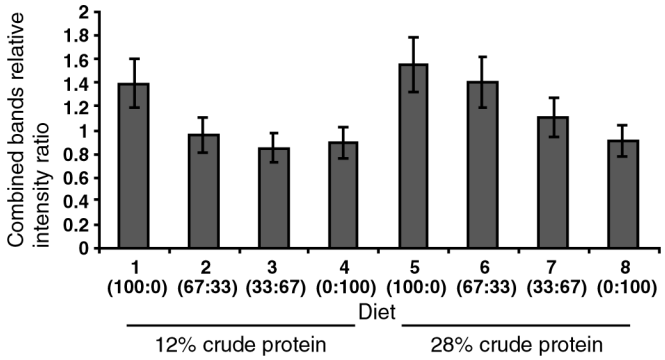

Figure 1. Treatment means (n = 7) for relative changes in high-molecular-weight calpastatin immunoreactive bands. The ratio was calculated as the density of the bands from the sample after 10 wk on trial divided by the density of the band at the end of the 7-wk acclimation period. The ratio indicates the change in that band within the biceps femoris of each dog. Treatments were 12% (Diets 1 to 4) or 28% CP diets (Diets 5 to 8) comprised of varying ratios of animal- and vegetable-based protein sources (100:0, Treatments 1 and 5; 67:33, Treatments 2 and 6; 33:67, Treatments 3 and 7; 0:100, Treatments 4 and 8). Bars indicate the standard error of the mean.

Results

Wakshlag et al. (2003) reported that loss of lean body mass was correlated with increasing levels of vegetable-based protein in experimental diets. Statistical analyses showed no significant diet-related differences in body weight, food consumption, or subjective BCS during the experimental period (data not shown). Average BW was 13.2 kg, and average food consumption (as-fed basis) was 312.5 g/d during the experimental period. Subjective BCS averaged 3.0 at the initiation of the project and 3.2 at wk 10. A score of 3.0 on the five-point rating scale represents an ideal body condition, in which ribs are palpable without excess fat covering, the waist can be observed behind the ribs when viewed from above, and the abdomen is tucked when viewed from the side (Sunvold and Bouchard, 1998).

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. Figure 1 summarizes the change in calpastatin expression over the trial period. The percentage of protein in the diet (Treatments 1 to 4 were 12% CP; Treatments 5 to 8 were 28% CP) did not have a significant effect on calpastatin ($P > 0.05$). Protein source as a main effect was not significant ($P = 0.13$); however, when individual diets were compared, changes in calpastatin were significantly greater in samples from dogs fed Diets 1 and 5 (100% animal protein source; combined bands ratio equals 1.53) compared with Diets 4 and 8 (100% corn gluten meal as the protein source; combined bands ratio equals 1.05) ($P < 0.05$). An interaction between protein source and amount was not detected. Regression analysis indicates a trend for a relationship between percentage of animal protein and calpastatin expression ($P = 0.086$). Samples from dogs fed in Diet 5 and 8 were chosen for more detailed analysis. These diets both contained 28% CP; Diet 5 had 100% chicken as the principal protein source and Diet 8 had 100% corn gluten meal as the protein source. Six of the seven dogs fed 28% CP, 100% chicken protein (Diet 5) responded with a greater intensity of the high-molecular-weight band of the calpastatin apparent in the biopsy sample taken 10 wk into the feeding period (Figure 2). Six of the seven samples from dogs fed Diet 8 (28% protein, 100% corn gluten meal) showed a decrease (Figure 2, lanes 4, 5, 9, and 10) or no change in calpastatin (Figure 2, lanes 13 and 14). Only one sample showed an increase in the expression of the high-molecular-weight calpastatin bands in the biopsy sample taken 10 wk into the feeding period. The highest molecular weight form of calpastatin was more abundant ($P = 0.0315$) in skeletal muscle of dogs fed Diet 5 compared with that of dogs fed Diet 8 (Table 2). An immunoreactive band migrating at approximately 70 kDa was observed in several samples. Because the secondary antibody did not bind to blots that had not been incubated with the anti-calpastatin antibody, we can conclude that the visible bands depict anti-calpastatin antibody binding. This band could be a degradation of the skeletal muscle calpastatin, but it is more likely erythrocyte calpas-

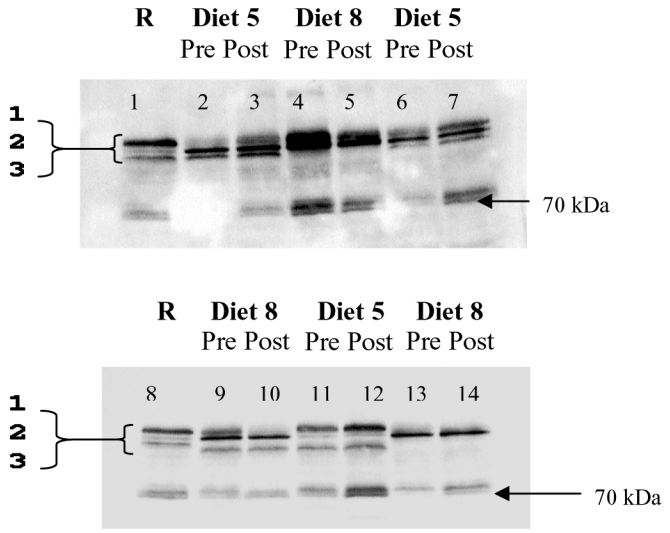

Figure 2. Western blot for calpastatin in canine biceps femoris from representative animals in Treatments 5 and 8. Diet 5 had 28% CP with protein sources of 100% chicken, 0% corn gluten meal. Diet 8 had 28% CP with protein sources of 0% chicken and 100% corn gluten meal. Lanes 1 and 8 are a reference sample (R) from canine biceps femoris to confirm separation of three high molecular weight calpastatin bands (labeled 1, 2, and 3; uppermost band is approximately 110 kDa). Each animal is represented by a pre- and post-trial sample. Samples preparation and immunoblotting procedure are detailed in the Material and Methods section.

tatin present in the biopsy sample (Takano et al., 1991).

Discussion

A large proportion of muscle protein degradation has been attributed to ubiquitin-proteosome dependent pathways (Taillandier et al., 1996). The direct contribution of the calpain enzyme system to myofibrillar protein degradation is not well defined. Calpains do not degrade actin or myosin (Goll et al., 1992). The contribution of calpains to overall protein degradation is minimal in hindlimb suspension (Taillandier et al., 1996) and denervation atrophy (Furuno et al., 1990) models. Calpain enzymes are hypothesized to play a role in disassembly of sarcomeric proteins (Huang and Forsberg, 1998). Calpastatin, the inhibitory component of the calpain proteolytic system, likely plays a role by inhibiting activation of calpain proteinases and thus decreasing calpain-induced proteolysis (Goll et al., 1992). This decrease in proteolysis causes decreased disassembly of myofibrils, which could affect muscle growth by allowing an increase in protein accretion (Goll et al., 1991). Williams et al. (1999) proposed that calpain-mediated release of myofilaments from Z-disks is the rate-limiting step in sepsis-induced protein degradation. Calpastatin expression has the potential to regulate this process. Nikawa et al. (2002) reported that, compared with a casein diet, a soy protein diet inhibited exercise-induced activation of calpains and the presence of myosin fragments in rat gastrocnemius. The soy protein diet also increased gastrocnemius calpastatin activity over the casein diet control group. Those authors attributed the soy protein effect to the higher cysteine:methionine ratios and higher content of arginine when compared to casein. In the current trial, the chicken protein source contained a higher cysteine:methionine ratio and greater amount of arginine than the corn gluten meal. It has been suggested that cysteine supplementation can increase muscle mass (Lands et al., 1999) or decrease protein catabolism (Droge and Holm, 1997).

In a companion study, Wakshlag et al. (2003) used dual X-ray absorptiometry to document that replacing the chicken protein source with corn gluten meal resulted in a loss in lean body mass over the feeding trial. These results showed that loss of lean body mass was correlated ($r = 0.56$) with increasing percentage of vegetable-based protein in the experimental diets. In this study, Diet 5 (100% chicken protein and 28% CP) contained the highest amount of animal protein, which contains a more optimal amino acid profile (Table 1). Wakshlag et al. (2003) reported that Diet 5 was the only diet that resulted in an increase in lean body mass. This treatment group also resulted in the most distinct increase in the high-molecular-weight band on calpastatin immunoblots. The physiological significance of the existence of different calpastatin immunoreactive bands in these samples is not clear. The observation that the high-molecular-weight band is more intense in samples from dogs in the 28% high-quality protein treatment group suggests that calpastatin is responsive to dietary treatments and may be associated with differences in lean body mass maintenance.

The appearance of more than one isoform of calpastatin could be a result of alternative splicing. Geesink et al. (1998) compared calpastatin from porcine heart and 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. This low-molecular-weight form was identical to that expressed in skeletal muscle. The low-molecular-weight isoform lacked exon 3, which encodes a portion of domain L, which is thought to aid in targeting calpastatin to membranes (Geesink et al., 1998). Recently, it has also been suggested that domain L plays a role in reactivating L-type calcium channels in guinea pig cardiac myocytes (Hao et al., 2000).

Parr et al. (1999; 2001) have demonstrated that beta agonists upregulate a 135-kDa form of calpastatin in

Table 2. Effect of source of protein on intensity of calpastatin immunoreactive bands[a]

	Diet 5 (100% chicken protein source; 28% protein) n = 7	Diet 8 (100% corn gluten meal protein source; 28% protein) n = 7	*P*-value
Band 1 relative intensity ratio	1.846 (0.180)[b]	0.708 (0.205)	0.0315
Band 2 relative intensity ratio	1.174 (0.185)	1.250 (0.125)	0.8359
Combined bands relative intensity ratio	1.551 (0.170)	0.914 (0.153)	0.0601

[a]The ratio was calculated as the density of the band from the sample after 10 wk on trial divided by the density of the band at the end of the 7-wk acclimation period. The ratio indicates the change in that band within the biceps femoris of each dog. Bands 1 and 2 are exhibited in Figure 2.
[b]Standard errors for each mean are noted in parentheses.

pig muscle. This response to beta agonists was accompanied by an increase in calpastatin activity (Parr et al., 1999). The increased appearance of the high-molecular-weight isoform of calpastatin in the current study was not as marked as those reported for pig muscle, but the change indicates that calpastatin expression or turnover were affected by diet. 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. However, a change in the bands detected on the immunoblots does suggest that protein source had an impact on calpastatin. Taken together with the result that the replacement of chicken protein with corn gluten meal reduced lean body mass, the change in calpastatin could offer insight into a possible mechanism by which muscle mass is maintained.

In summary, these data suggest that the dietary treatments elicited a change in calpastatin detected by immunoblotting. This was supported by 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 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 dogs that were fed corn gluten meal as the principal protein source may have been exhibiting a physiological response due to an increased need to mobilize amino acids from skeletal muscle to supplement an inadequate balance of amino acids. The observation that the high-molecular-weight band is more intense in the 28% high-quality protein diet and that that diet resulted in greater maintenance of lean body mass (Wakshlag et al., 2003) adds to evidence that the calpain system may have a role in muscle maintenance.

Implications

Dogs fed a higher percentage of total protein derived from corn gluten meal were less able to maintain muscle than those fed similar diets containing chicken protein. The loss in the ability to maintain muscle coincided with a decrease in a high-molecular-weight calpastatin band detected on western blots. The canines that were fed a 28% crude protein diet consisting of chicken as the sole protein source showed an increase in a high-molecular-weight form of skeletal muscle calpastatin in the biceps femoris, implying that these dogs may have had greater potential to regulate calpain-mediated degradation of muscle protein. Because calpastatin inhibits calpain, which partially degrades some skeletal muscle proteins, it is suggested that a change in calpastatin in muscle may function to maintain skeletal muscle mass and/or increase skeletal muscle protein accretion by minimizing proteolysis.

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