

Comparison Between 3-Methylhistidine Production and Proteinase Activity as Measures of Skeletal Muscle Breakdown in Protein-Deficient Growing Barrows¹

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ABSTRACT: This experiment was conducted to determine the relationship between 3-methylhistidine (3MH) production and proteinase activity in skeletal muscles of growing barrows. Barrows at 13 wk of age were randomly assigned to either control diet available on an ad libitum basis (21% of ME consisted of protein; control group), control diet fed restricted (pair-fed with barrows in protein-free group; intake-restricted group), or protein-free diet available on an ad libitum basis (protein-free group) for 14 d. During the last 3 d, blood samples were collected for determination of 3MH production rate, which is a measure of myofibrillar protein breakdown. At slaughter, two muscles were taken: masseter (M) and longissimus (L) muscles. The muscle samples were analyzed for calpastatin, μ -calpain, m-calpain, mul-

ticatalytic proteinase (MCP), cathepsin B, cathepsins B+L, and cystatins activities. Both muscles were also analyzed for amounts of DNA, RNA, total protein, and myofibrillar and sarcoplasmic proteins. Growth rate (kilograms/day) was influenced by dietary treatments ($P < .05$). Fractional breakdown rate (FBR, percentage/day) of skeletal muscle, as calculated from 3MH production rate (micromoles-kilogram⁻¹·day⁻¹), was 27% higher for the protein-free group than for the control group. However, no differences in proteinase activities were observed, except for lower MCP activity in the M muscle of the protein-free group than in that of the other groups ($P < .05$). In the present study, no direct relation was observed between myofibrillar protein degradation rate and proteinase activities in skeletal muscle during a protein-free feeding strategy.

Key Words: Pigs, Dietary Protein, Methylhistidine, Proteinases, Growth, Skeletal Muscle

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Introduction

Insight into the mechanisms and regulation of protein metabolism in skeletal muscle is essential for interventions to increase efficiency of protein deposition in meat animal production. Protein deposition is

dependent on two opposite processes: synthesis and breakdown of protein. However, little is known about mechanisms and regulation of protein breakdown of skeletal muscle. In pigs, determining urinary excretion of 3-methylhistidine (3MH) is not a valid method for estimating skeletal muscle protein breakdown, because 3MH is mainly retained in the body as the dipeptide balenine (Harris and Milne, 1981). This problem can be circumvented with the recently developed procedure employing a bolus injection of labeled 3MH, methyl-²H₃-N⁷-methylhistidine (**d₃-3MH**), to measure de novo production rather than excretion of 3MH (Rathmacher et al., unpublished data).

Several proteinase systems are thought to be involved in skeletal muscle protein breakdown. These

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proteinases can be divided into non-lysosomal proteinases, such as the calpain system (μ - and m-calpain and their natural inhibitor calpastatin) and multicatalytic proteinase (MCP), and the lysosomal proteinases, such as cathepsins, which are inhibited by cystatins (Beynon and Bond, 1986). Calpains are probably involved in the initial step of myofibrillar breakdown by weakening of the Z-disc, but not in breakdown of myosin and actin (Goll et al., 1992; Koohmaraie, 1992). The MCP is probably part of a larger complex, which is capable of degrading proteins into oligopeptides (Rivett, 1989; Orłowski, 1990). However, MCP does not degrade myofibrillar proteins (Koohmaraie, 1992). Cathepsins seem to play a role in proteolysis under poor nutritional conditions (Kettelhut et al., 1988). Ballard et al. (1988) found no relationship between the calpain system and growth rates in chickens. Morgan et al. (1993) found a negative relationship between calpastatin activity and protein breakdown in steers.

The purpose of this study was to investigate the relationship between 3MH production and proteinase activity in growing pigs under a protein-free feeding strategy.

Experimental Procedures

Experimental Design

The study was carried out at the Roman L. Hruska U.S. MARC, Clay Center, NE. The Animal Care and Use Committee of this institute approved the use and treatment of animals in this study according to guidelines established by the USDA. Barrows from a crossbred foundation with equal genetic contributions from Chester White, Landrace, Large White, and Yorkshire were used. On d -16, at an age of approximately 11 wk, barrows were moved to individual pens (1.22 m \times 1.22 m) equipped with feeders and automatic nipple waterers in a slotted-floor building that was enclosed and temperature-controlled. The experiment involved three different treatments with 10 barrows each: the control group had free access to the control diet (21% of metabolizable energy consisted of protein); the intake-restricted group also received the control diet, but was pair-fed with those in the protein-free group; the protein-free group had free access to the protein-free diet. The composition of the experimental diets is presented in Table 1. On d -10, barrows were randomly assigned to one of the three treatments, so that their mean body weights were similar between treatments. On d -3, barrows received the experimental diets gradually (50% nursery diet mixed with 50% experimental diet). From d 0, complete experimental diets were offered. Feed intake was recorded daily and body weight was measured on d 0, 5, 10, and 14.

Table 1. Composition of the experimental diets

	Control	Protein-free
Ingredient, g/100 g		
Corn	69.0	—
Soybean meal	25.4	—
Cornstarch	—	81.2
Soybean oil	2.0	4.7
Corn cobs	—	10.5
Constant components ^a	3.6	3.6
Calculated ^b		
Protein, g/100 g	17.0	3
Fat, g/100 g	4.8	4.8
Carbohydrates, g/100 g	56.3	85.1
Crude fiber, g/100 g	3.4	3.4
Analyzed		
Gross energy, MJ/kg	16.4	15.4
Protein, g/100 g	15.7	—
Dry matter, g/100 g	88.1	87.2

^aConstant components are as follows: dicalcium phosphate, 2.0 g; limestone, .6 g; iodized salt, .4 g (supplied .28 mg of iodine per kilogram of diet); vitamin premix, .2 g; trace minerals, .2 g; and choline chloride .2 g (supplied 868 mg of choline per kilogram of diet). The composition of the vitamin premix was as follows: vitamin A, 529.1 IU; vitamin D, 70.55 IU; vitamin E, 3.533 IU; vitamin K, .35 mg; riboflavin, .53 mg; d-pantothenic acid, 2.12 mg; niacin, 2.82 mg; vitamin B₁₂, 2.64 μ g; thiamine, .22 μ g; biotin, 22.0 μ g; and folic acid 88.2 μ g. Composition of trace mineral premix was as follows: ferrous sulphate heptahydrate, 16.0 mg; cupric oxide, 1.0 mg; manganese oxide, 2.0 mg; zinc oxide, 10.0 mg; and calcium carbonate was used as a carrier.

^bData are calculated according to Nutritional Data for United States and Canadian Feeds (3rd Ed.). National Academy Press, Washington, DC, 1982.

Cannulation of the Jugular Vein

On d 7, a catheter was surgically inserted into the internal jugular vein similar to the method for cannulation of the carotid artery (Yen and Killefer, 1987). Micro-Renathane tubing (Braintree Scientific, Braintree, MA) of .66 mm i.d. and .95 mm o.d. was inserted into the internal jugular vein. After surgery, Biozide gel (polyvinylpyrrolidone-iodine complex, Performance Products, St. Louis, MO) was applied to the outside of the wounds to prevent infection.

3-Methylhistidine Production

During the final 3 d of this experiment (d 11 to d 14) 3MH production was measured according to the method of Rathmacher et al. (unpublished data). Briefly, a standard amount of d₃-3MH (.1371 mg/kg BW) was dissolved in 25 mL of sterile saline, injected into the jugular vein, and flushed with 10 mL of saline. This was followed by serial blood sampling (10 mL) at 1, 2, 5, 15, 30, 60, 90, 180, 360, 540, 720, 1,440, 2,160, 2,880, 3,600, and 4,320 min. Each syringe contained 15 mg of EDTA as the anticoagulant. Additional blood samples were collected before the injection of deuterated 3MH to correct for background enrichment of the experimental samples. Blood samples were centrifuged for 20 min at 500 \times g

and plasma was stored at -70°C for later analysis of $\text{d}_3\text{-3MH}$ and 3MH by gas chromatography/mass spectrometry (**GC/MS**) using the method of Rathmacher et al. (1992).

Calculation of Fractional Breakdown Rate

The de novo production rate of 3MH (micromoles per kilogram per day) was calculated as described by Rathmacher et al. (1992). The fractional breakdown rate (**FBR**) can be calculated if the total amount of 3MH in the body is known. Therefore, assumptions were made that 8% of the total body consists of muscle protein (Mulvaney et al., 1985) and 3MH content in porcine skeletal muscle is $3.8742 \mu\text{mol/g}$ of muscle protein (Rathmacher et al., unpublished data).

Collection of Muscle and Liver Samples

After taking the last blood sample on d 14, barrows were killed by electric stunning and exsanguination. The left longissimus (**L**) muscle, both masseter (**M**) muscles, and the liver were dissected, trimmed of visible fat, and weighed. Both muscles were cut into pieces and visible fat and connective tissue were removed. Within 30 min after slaughter, 10-g samples of either muscle were prepared for assays of both of the calpains, calpastatin, and MCP as described below. In addition, an aliquot of both muscles was frozen in liquid nitrogen and stored at -70°C for later analysis of other components as described below.

Calpains and Calpastatin Assay

Proteinase activities in **M** and **L** muscles were determined according to the method described by Koohmaraie (1990) with some minor modifications. Briefly, 10 g of muscle was homogenized in 6 volumes of extraction buffer that consisted of 50 mM Tris, 10 mM EDTA, 10 mM β -mercaptoethanol (**MCE**), and 10 mM phenylmethanesulfonyl fluoride (**PMSF**); pH was adjusted to 8.3 with 6 N HCl at 4°C . After centrifugation for 2 h at $36,000 \times g$, the supernatant was filtered through four layers of cheesecloth and then glass wool and loaded onto a 1.5-cm \times 20-cm column of DEAE-Sephacel, which had been equilibrated with elution buffer (40 mM Tris, 5 mM EDTA, and 10 mM MCE, pH 7.35). After removing the unbound proteins with elution buffer, the bound proteins were eluted with a linear salt gradient from 25 to 350 mM NaCl in elution buffer (230 mL of each). One unit of calpain activity was defined as the amount of proteinase that catalyses an increase of 1.0 absorbance unit at $A_{278} \text{ nm}$ in 60 min at 25°C using casein as a substrate. Calculation for total inhibitor activity of calpastatin is described by Koohmaraie (1990). One unit of calpastatin activity was defined

as the amount of calpastatin that inhibits one unit of m-calpain activity.

Multicatalytic Proteinase Assay

Activity of MCP in either muscle was measured according to the method described by Arbona and Koohmaraie (1993). Briefly, chymotrypsin-like activity of the proteinase was determined by the amount of p-nitroaniline (**pNA**) released from the synthetic substrate, N-CBZ-Gly-Gly-Leu-pNA. Activity of MCP was assayed in the same fractions after DEAE-Sephacel chromatography as that used for calpains and calpastatin activity. The reaction mixture consisted of 90 μL of assay buffer (50 mM Tris, .5 mM EDTA, 1 mM NaN_3 , pH 8.0), 100 μL of fraction, and 10 μL of 10 mM substrate. After incubation at 55°C for 30 min, the reaction was stopped with .3 mL of 1% SDS and 1 mL of .1 M sodium borate, pH 9.1. The amount of pNA released was measured at 410 nm. One unit of peptidase activity was defined as the amount of proteinase required to release 1 μmol of pNA from the substrate in 60 min at 55°C .

Cystatins and Cathepsins B and B+L Assay

The activity of cystatins and cathepsins B and B+L were determined in either muscle according to the method described by Koohmaraie and Kretchmar (1990).

DNA, RNA, and Protein Assay

Both **M** and **L** muscles were assayed for RNA, DNA, and protein content. The procedure of Labarca and Paigen (1980) using Hoechst 33258 reagent (bisbenzimidazole; Sigma Chemical, St. Louis, MO) was used for determining muscle DNA concentrations. Content of RNA in muscle was determined according to Munro and Fleck (1969). Protein content was determined using the biuret procedure (Gornall et al., 1949).

Myofibrillar, Sarcoplasmic, and Total Protein Content Assay

Myofibrillar proteins were isolated as described by Solaro et al. (1971). Briefly, approximately 200 mg of muscle were homogenized using a Duall grinder in a buffer containing 60 mM KCl, 30 mM imidazole, 3 mM MgCl_2 , .5 mM EGTA, 1 mM dithiothreitol (**DTT**), 2 mM PMSF, pH 7.0. An aliquot of the homogenate was analyzed for total protein. The remainder of the homogenate was centrifuged at $3,000 \times g$, and the precipitate containing myofibrillar and stromal proteins was washed twice in the homogenization buffer. Supernatants containing the sarcoplasmic proteins were pooled and their protein content measured. The precipitate was then rehomogenized (with the Duall

grinder) in the homogenization buffer containing 1% Triton X-100 and then allowed to incubate for 60 min. The sample was centrifuged, the pellet was washed to remove Triton X-100, and the protein content of the pellet containing the myofibrils was determined. All samples for protein measurements were solubilized in .1 M NaOH, and protein was determined by the method of Lowry et al. (1951). The total protein recovery ([soluble + myofibrillar protein]/total protein concentrations) was the same between the three treatment groups ($84 \pm 2\%$, $P > .1$ for the M muscle; $84 \pm 2\%$, $P > .5$ for the L muscle).

Statistical Analyses

Data were analyzed by analysis of variance (ANOVA) followed by the Scheffé-test using the SPSS-PC+ program, in which the treatment was used as an independent factor (SPSS, 1988). Differences between M and L muscle were analyzed using a paired *t*-test. Pearson's correlation coefficients were calculated between FBR derived from 3MH kinetics and proteinase activities in both muscles. Results were considered statistically different when the *P*-value was less than .05.

Results

During the surgery of the cannulation of the jugular vein, one pig died in the control group.

Data in Table 2 show that during the experimental period body weight increased for both the control and intake-restricted groups, but not for the protein-free group. The increase in body weight was significantly less for the intake-restricted group than for the control group. From d 10 onward, the intake-restricted group

had a significantly lower body weight than the control group. Body weight of the protein-free group remained constant over the entire experiment and was significantly lower than that of both the control and intake-restricted groups from d 5 onward. Consequently, growth rates were different between all treatments ($P < .05$).

The carbohydrate content in the protein-free diet was higher than that in the control diet. Therefore, the calculated energy density of the protein-free diet was also higher. However, the analyzed composition of the experimental diets showed that energy density was not very different between the diets. Voluntary feed intake was significantly lower for the protein-free group than for the control group. The amount of feed consumed by the intake-restricted and protein-free groups was 61 and 64% of that of the control group, respectively, which was approximately 2.5 times energy for maintenance. Feed conversion was similar between the control and the intake-restricted groups, whereas feed conversion for the protein-free group was significantly lower than for both other groups.

Tissue weights are presented in Table 3. Absolute wet weights of liver were significantly lower for the protein-free group than for the control group; the intake-restricted group was intermediate. Expressed as a percentage of body weight, no differences occurred in liver weights between treatments. Absolute wet weights of both L and M muscles were significantly lower for the protein-free group than for either the control or intake-restricted groups. Relative to body weight, only the L muscle of protein-free group was significantly reduced compared with the other groups.

Production rate of 3MH (micromoles per kilogram per day) and FBR (percentage/day) was highest for the protein-free group and significantly different from

Table 2. Performance and feed intake characteristics of pigs under different dietary treatments^a

Trait	Dietary treatment		
	Control	Intake-restricted	Protein-free
Body wt, kg			
Day -3	36.1 ± 1.1	36.7 ± 1.1	36.3 ± 1.0
Day 0	38.3 ± .9	38.8 ± .9	35.5 ± 1.1
Day 5	43.4 ± 1.0 ^b	40.3 ± .8 ^b	35.4 ± 1.2 ^c
Day 10	46.4 ± 1.2 ^b	42.3 ± 1.1 ^c	35.4 ± 1.0 ^d
Day 14	48.6 ± 1.2 ^b	43.9 ± .8 ^c	36.0 ± 1.2 ^d
Growth rate, kg/d			
Day 0 to 14	.73 ± .07 ^b	.37 ± .05 ^c	.03 ± .04 ^d
Feed intake, kg/d			
Day 0 to 14	2.08 ± .07 ^b	1.27 ± .05 ^c	1.34 ± .07 ^c
Gain/feed, kg/kg			
Day 0 to 14	.35 ± .02 ^b	.29 ± .04 ^b	.01 ± .03 ^c

^aData are expressed as mean ± SE for 9, 10, and 10 pigs for control, intake-restricted, and protein-free group, respectively.

^{b,c,d}Means within a row lacking a common superscript letter differ ($P < .05$).

Table 3. Liver and muscle weights of pigs under different dietary treatments^a

Trait	Dietary treatment		
	Control	Intake-restricted	Protein-free
Wet wt, g			
Liver	1,024.4 ± 39.0 ^b	924.9 ± 21.9 ^{b,c}	798.5 ± 45.4 ^c
Longissimus	869.1 ± 15.6 ^b	821.1 ± 17.8 ^b	575.1 ± 16.8 ^c
Masseter	43.9 ± 1.3 ^b	42.6 ± 1.4 ^b	34.4 ± .9 ^c
Wet wt, % BW			
Liver	2.11 ± .06	2.11 ± .03	2.22 ± .10
Longissimus	3.59 ± .09 ^b	3.74 ± .05 ^b	3.21 ± .06 ^c
Masseter	.18 ± .00	.19 ± .01	.19 ± .01

^aData are expressed as mean ± SE for 9, 10, and 10 pigs for control, intake-restricted, and protein-free group, respectively.

^{b,c}Means within a row lacking a common superscript letter differ ($P < .05$).

both other groups (Figure 1). Production rates of 3MH and FBR for the control and intake-restricted groups were not different.

Figure 2 shows the elution pattern of calpastatin, μ -calpain, m-calpain, and MCP after DEAE-Sephacel chromatography. The elution profile was similar for the different treatments and both muscles. Calpastatin eluted from DEAE-Sephacel between 75 and 120 mM NaCl, almost directly followed by μ -calpain (between 125 and 165 mM NaCl). The MCP eluted as a single peak from DEAE-Sephacel between 230 and 280 mM NaCl, partly co-eluting with m-calpain.

Proteinase activities in both skeletal muscles are presented in Table 4. For both M and L muscles, there were no significant differences between treatments for activity of any component of the calpain system (i.e.,

calpastatin, μ -calpain, and m-calpain). For both calpains, M muscle contained significantly higher activity than L muscle for all treatments. Calpastatin activity was also significantly higher for M than for L muscle in the control and intake-restricted groups. However, this difference was not observed for the protein-free group. The MCP activity in M muscle was significantly lower in the protein-free group than in the controls. For L muscle, MCP activity was not different between treatments. For all treatments, M muscle had significantly higher MCP activity than L muscle. Activities of cathepsins B and B+L did not differ between treatments in either muscle ($P < .05$). This was also true for their natural inhibitors, the cystatins. Between muscles, values for cathepsin B activity were similar; however, activities of cathepsins

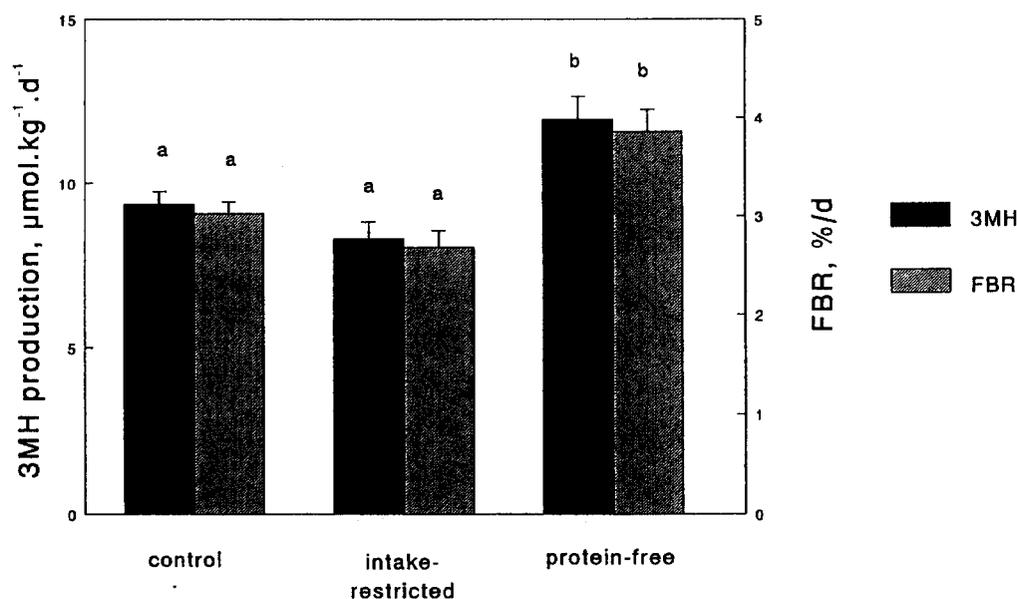


Figure 1. 3-Methylhistidine production rate (3MH, micromoles-kilogram⁻¹·day⁻¹) and fractional breakdown rate, (FBR, percentage/day) for different dietary treatments. Means within group parameters (3MH or FBR) with a different letter differ ($P < .05$). Group means are 3.02, 2.68, and 3.85%/d for FBR and 9.4, 8.3, and 11.9 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for 3MH production for control, intake-restricted, and protein-free group, respectively.

Table 4. Proteinase activities in porcine skeletal muscles under different dietary treatments^a

Trait and muscle	Dietary treatment		
	Control	Intake-restricted	Protein-free
Calpastatin^b			
Masseter	19.48 ± 2.26	16.34 ± 1.74	15.46 ± 1.87
Longissimus	9.42 ± 1.17 (<i>P</i> = .007) ^h	9.83 ± 1.31 (<i>P</i> = .016)	11.51 ± .67 (<i>P</i> = .179)
μ-Calpain^c			
Masseter	7.79 ± .83	8.02 ± .93	8.10 ± 1.03
Longissimus	4.05 ± .38 (<i>P</i> = .012)	3.43 ± .27 (<i>P</i> = .002)	3.80 ± .56 (<i>P</i> = .012)
m-Calpain^d			
Masseter	10.27 ± .72	9.24 ± .47	8.34 ± .66
Longissimus	3.63 ± .34 (<i>P</i> = .012)	2.71 ± .24 (<i>P</i> = .002)	2.96 ± .39 (<i>P</i> = .012)
MCP^e			
Masseter	6.09 ± .22 ⁱ	5.41 ± .22 ⁱ	4.27 ± .29 ^j
Longissimus	4.24 ± .14 (<i>P</i> = .001)	3.99 ± .14 (<i>P</i> = .001)	3.62 ± .22 (<i>P</i> = .013)
Cathepsin B^f			
Masseter	465.1 ± 64.2	499.6 ± 61.6	340.3 ± 37.7
Longissimus	532.2 ± 183.9 (<i>P</i> = .711)	438.4 ± 59.5 (<i>P</i> = .416)	299.2 ± 21.0 (<i>P</i> = .188)
Cathepsins B+L^f			
Masseter	817.0 ± 58.0	925.8 ± 76.9	685.7 ± 59.1
Longissimus	1,437.2 ± 161.3 (<i>P</i> = .007)	1,394.7 ± 95.1 (<i>P</i> = .009)	1,174.8 ± 56.9 (<i>P</i> = .001)
Cystatin activity^g			
Masseter	7.13 ± .78	9.39 ± 1.41	9.20 ± .90
Longissimus	19.73 ± 2.02 (<i>P</i> = .001)	18.19 ± 2.02 (<i>P</i> = .001)	24.89 ± 2.75 (<i>P</i> = .001)

^aData are expressed as mean ± SE for 9, 10, and 10 pigs for control, intake-restricted, and protein-free group, respectively.

^bInhibition of casein hydrolysis by m-calpain·gram of muscle protein⁻¹.

^cLow Ca²⁺-requiring calpain protease total activity·gram of muscle protein⁻¹ (caseinolytic activity).

^dHigh Ca²⁺-requiring calpain protease total activity·gram of muscle protein⁻¹ (caseinolytic activity).

^eTotal μmol pNA released·hour⁻¹·gram of muscle protein⁻¹.

^fActivity expressed as nmol of NMEC released·minute⁻¹·gram of muscle protein⁻¹.

^gMeasured as the ratio of B+L activity after to before cystatin removal by affinity chromatography.

^h*P*-value < .05 denotes difference between masseter and longissimus muscle.

^{i,j}Means within a row lacking a common superscript letter differ (*P* < .05).

B+L and cystatins were significantly higher for L muscle than for M muscle.

Total, soluble, and myofibrillar protein contents were not significantly different between treatments for either muscle (Table 5). The percentages of soluble and myofibrillar proteins in both M and L muscles were not influenced by the treatments. The contribution of soluble proteins to total protein was significantly higher in L muscle than in M muscle, and the contribution of myofibrillar protein to total protein was significantly lower in L muscle than in M muscle. The DNA concentrations in M and L muscles were not influenced by the dietary treatments (Table 5). The L muscle contained significantly less DNA than M muscle for all treatments. The RNA contents in both muscles were significantly lower for the protein-free group than for the other two groups. The RNA concentration was significantly higher in M muscle

than in L muscle. Protein:DNA ratio was not different between treatments for M and L muscles but was significantly higher for L muscle than for M muscle. The RNA:DNA ratio in both muscles was significantly lower for the protein-free group than for the other groups. The RNA:protein ratio in both muscles was significantly lower for the protein-free group than for both the control and intake-restricted groups. For all treatments, the L muscle contained a higher RNA:DNA ratio than the M muscle, whereas the M muscle contained a higher RNA:protein ratio than the L muscle.

Discussion

Skeletal muscles contain approximately 50% of total body protein (Simon, 1989). Changes in muscle

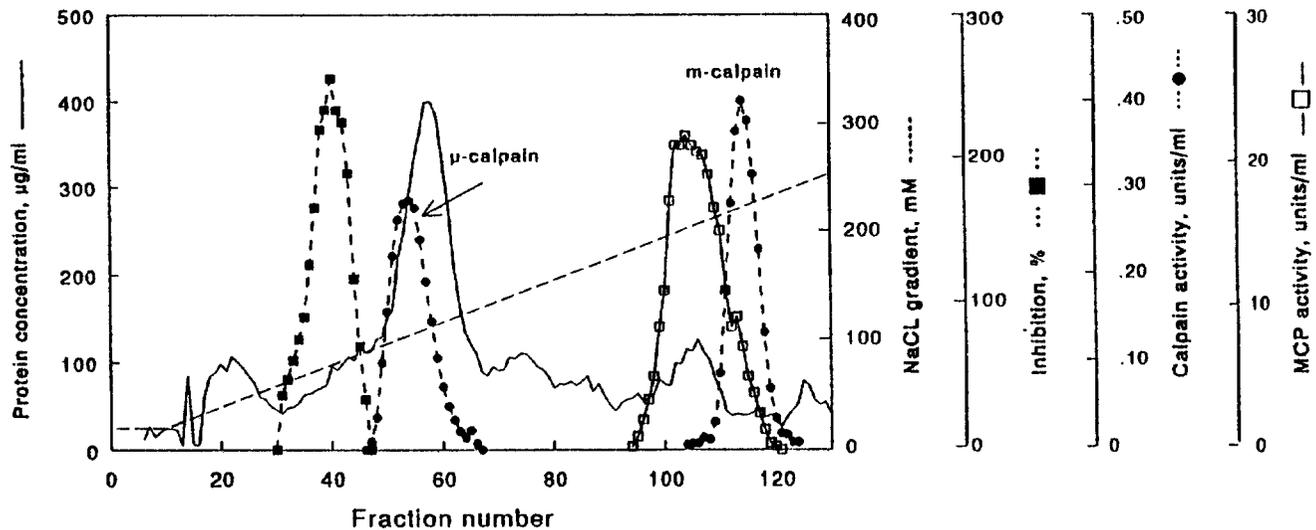


Figure 2. Chromatographic separation of calpastatin (■···), μ -calpain (●···), and m-calpain (○···), and MCP (—□—) using DEAE-Sephacel with a linear NaCl gradient (---). Protein concentration in the different fractions is also shown (—). See text for detailed information about elution conditions. The elution profile was similar for the different treatments and both muscles, and therefore only one representative elution profile is shown.

protein mass are the net result of the balance between synthesis and breakdown of protein. Thus, both processes determine the efficiency of protein gain. To optimize this efficiency, the mechanisms and regulation of both processes must be known. In the present experiment, a protein-free diet was offered to barrows to investigate changes in myofibrillar protein breakdown, as determined by 3MH production. In addition, we wished to establish whether changes in 3MH production were reflected by parallel changes in the activities of some muscle proteinases thought to be involved in muscle protein breakdown.

The L and M muscles were studied in this experiment because they are of different fiber types and have different metabolic characteristics. The L muscle is a type IIB muscle (fast-twitch, glycolytic), whereas the M muscle is a predominantly type I muscle (slow-twitch, oxidative) (Ouali and Talmant, 1990).

The dietary treatments caused different growth rates ($P < .05$). The final body weight of the control group was 26% greater than that of the protein-free group. The protein-free group maintained a constant body weight during the 14-d feeding trial.

Results of the present experiment suggested that feeding a protein-free diet caused an elevated FBR of myofibrillar proteins compared with both the control and the intake-restricted groups. To calculate FBR from 3MH production rate, it was assumed that 8% of total body weight consists of muscle protein. This is probably an overestimation for the protein-free group and consequently the FBR will then be underestimated. However, because the actual muscle protein

content is unknown, the value of 8% is still used in the calculations. Mulvaney et al. (1985) calculated FBRs from protein synthesis and protein accretion rates of 4.4 and 3.4%/d in intact male pigs of 25 and 45 kg, respectively, which is similar to our finding of 3.0% for the control group. The higher FBR for the protein-free group is consistent with results reported by Kadowaki et al. (1989), who concluded from their study with rats that myofibrillar protein breakdown was increased by 18% after 1 wk on a protein-free diet, although this was not significant ($P > .10$). This increase in FBR was similar in magnitude to our finding of 27%. However, other reports suggest that myofibrillar protein breakdown is diminished in adult rats fed a protein-free diet (Funabiki et al., 1976) or low-protein diets (Nishizawa et al., 1977b) as measured by urinary 3MH excretion. Differences in animal ages between these experiments may explain these conflicting results. Millward et al. (1976) described that during protein-free feeding both synthesis and breakdown rates of muscle proteins were decreased in rats. However, after a certain period (30 d), breakdown of muscle proteins was increased again. A decrease of both protein synthesis and breakdown rates in older animals is probably enough to increase the availability of amino acids for other purposes, but in younger animals muscle protein breakdown may have to increase to provide amino acids.

If we assume that the weight of both L muscles on d -3 was 3.6% of the body weight, then one L muscle weighed 657 g. Under this assumption, over the next 17 d the control animals were gaining 12.47 g of muscle/d, whereas the protein-free group was losing 4.82 g of muscle/d. This corresponds to muscle

Table 5. Protein, DNA, and RNA content in porcine skeletal muscles under different dietary treatments^a

Trait and muscle	Dietary treatment		
	Control	Intake-restricted	Protein-free
Total protein, mg/g of muscle ^b			
Masseter	183.4 ± 2.6	187.3 ± 2.3	179.7 ± 1.8
Longissimus	220.1 ± 2.2	219.5 ± 3.8	218.9 ± 2.4
	(<i>P</i> = .001) ^c	(<i>P</i> = .001)	(<i>P</i> = .001)
Soluble protein, mg/g of muscle			
Masseter	51.8 ± 1.9	51.0 ± 1.3	48.1 ± 2.0
Longissimus	77.3 ± 2.4	74.5 ± 1.2	72.1 ± 1.1
	(<i>P</i> = .001)	(<i>P</i> = .001)	(<i>P</i> = .001)
Myofibrillar protein, mg/g of muscle			
Masseter	103.8 ± 2.2	105.1 ± 2.0	102.4 ± 2.4
Longissimus	108.9 ± 2.0	111.0 ± 1.8	111.0 ± 1.8
	(<i>P</i> = .202)	(<i>P</i> = .001)	(<i>P</i> = .001)
DNA, μg/g of muscle			
Masseter	1,646.1 ± 68.6	1,602.1 ± 57.0	1,680.1 ± 43.9
Longissimus	877.1 ± 80.4	972.9 ± 53.4	1,047.3 ± 54.6
	(<i>P</i> = .001)	(<i>P</i> = .001)	(<i>P</i> = .001)
RNA, μg/g of muscle			
Masseter	842.6 ± 21.0 ^d	814.7 ± 14.2 ^d	620.4 ± 17.3 ^e
Longissimus	664.7 ± 40.4 ^d	642.2 ± 18.1 ^d	526.3 ± 11.7 ^e
	(<i>P</i> = .003)	(<i>P</i> = .001)	(<i>P</i> = .001)
Protein/DNA ^f			
Masseter	92.1 ± 2.8	89.2 ± 4.7	85.6 ± 4.0
Longissimus	184.0 ± 17.7	159.6 ± 9.5	148.8 ± 6.5
	(<i>P</i> = .001)	(<i>P</i> = .001)	(<i>P</i> = .001)
RNA/DNA			
Masseter	.52 ± .02 ^d	.52 ± .03 ^d	.37 ± .01 ^e
Longissimus	.81 ± .09 ^d	.68 ± .05 ^{d,e}	.51 ± .03 ^e
	(<i>P</i> = .006)	(<i>P</i> = .012)	(<i>P</i> = .001)
RNA/protein ^f			
Masseter	5.63 ± .17 ^d	5.78 ± .08 ^d	4.36 ± .18 ^e
Longissimus	4.39 ± .22 ^d	4.28 ± .09 ^d	3.46 ± .15 ^e
	(<i>P</i> = .001)	(<i>P</i> = .001)	(<i>P</i> = .001)

^aData are expressed as means ± SE for 9, 10, and 10 pigs for control, intake-restricted, and protein-free group, respectively.

^bProtein amount as measured according to Lowry et al. (1951).

^c*P*-value < .05 denotes difference between masseter and longissimus.

^{d,e}Means within a row lacking a common superscript letter differ (*P* < .05).

^fProtein amount as measured by using the biuret procedure.

accretion rates of +1.90 and −.73%/d over the entire period for the control and protein-free groups, respectively. As a percentage of body weight, the L muscle was decreasing in the protein-free group. For liver, the increase in weight over the entire period was 2.0 and .2%/d for the control and protein-free groups, respectively. This is similar to the change in body weight during those 17 d of 2.0 and −.1%/d for control and protein-free groups, respectively.

The method used in the present study for measuring 3MH production determines the breakdown rate of myofibrillar proteins, because 3MH is a specific constituent of actin and fast-twitch myosin (Sugden and Fuller, 1991). A limitation of the model used to calculate 3MH production rate, however, is that it does not correct for 3MH production from non-skeletal muscle sources such as skin and the gastrointestinal tract. The FBR in the protein-free group may also

have been increased because of elevated 3MH production rate by these other tissues. Contribution of these sources to total 3MH production varies between 17 and 40% in rats (Nishizawa et al., 1977a; Wassner and Li, 1982). However, it is not clear from the literature whether feeding a protein-free diet affects this contribution. Another possibility, which cannot be ruled out, is that balenine may have been broken down under protein-free conditions, increasing apparent fractional breakdown rate. However, no literature data are available to support this hypothesis.

Results of protein composition for both muscles indicate that there was no change in these components under the experimental conditions. Thus, elevated breakdown of myofibrillar proteins during protein deficiency must have been accompanied by an equivalent increase in the breakdown of sarcoplasmic proteins. However, this possibility is not in agreement

with results reported by Kadowaki et al. (1989), who found that in rats fed a protein-free diet the degradation of sarcoplasmic proteins was decreased by 84%. However, adaptations in protein metabolism during protein-free feeding probably change over time, as discussed above (Millward et al., 1976). Lowell et al. (1986) suggested that adaptations in protein metabolism during fasting occur in several phases. Thus, an increase in myofibrillar protein breakdown may not be directly reflected by the proportion of myofibrillar to sarcoplasmic proteins in the skeletal muscle.

In the present experiment, results for both μ - and m-calpain and calpastatin activity in both muscles suggest that the calpain system was not influenced by the dietary treatments. So, although FBR of myofibrillar proteins seemed to be increased, no effect on the activity of the calpain system was noted. Ballard et al. (1988) also found no relationship between calpain or calpastatin activity and different growth rates in growing chickens after 16 to 20 d of feeding diets differing in protein content (between 105 and 212 g of protein/kg). However, these results disagree with those of Morgan et al. (1993). Morgan et al. (1993) found a significant correlation between calpastatin activity and protein breakdown in steers. This suggests that the calpain system may not be influenced by dietary protein deficiency. The significantly higher activity of the proteinases of the calpain system in M muscle than in L muscle is in agreement with results of Whipple and Koohmaraie (1992) using ovine skeletal muscles. Ouali and Talmant (1990) also found higher calpastatin and m-calpain activity in M muscle than in L muscle in pigs. However, our results are not in agreement with Kim et al. (1993). They found that in porcine skeletal muscles, which differed in fiber typing, there was no difference in calpastatin activity, but μ -calpain activity was significantly lower and m-calpain was significantly higher in M muscle than in semimembranosus muscle (type IIB muscle). A difference in the technique used for separation of the proteinases of the calpain system could be an explanation for this discrepancy in calpastatin and μ -calpain activity between type I and IIB fibers.

The activity of MCP was decreased in only the M muscle of the protein-free group. Pearson's correlation coefficients between FBR and MCP activity did not reveal a linear relationship (for M muscle: $r = -0.267$, $P = .170$; for L muscle: $r = -0.179$, $P = .382$). In their *in vivo* study with rats on a protein-free diet Kadowaki et al. (1989) found that non-myofibrillar (sarcoplasmic) protein breakdown was decreased by 84%. Therefore, if MCP is also involved in breakdown of sarcoplasmic proteins, this could explain the decreased MCP activity in M muscle of the protein-free group. It is unclear why a similar result was not seen in L muscle.

Activities of cathepsins B and B+L and cystatins were not influenced by the dietary treatments. The experimental period might have been too short to detect a markedly increased activity of cathepsins and/or decreased activity of cystatins. From the literature, it is unclear whether the activity of

cathepsins and cystatins in skeletal muscle are influenced by dietary protein deficiency.

Summarizing, the activity of the proteinases in both M and L muscle was not influenced by the dietary treatments, except for MCP activity in M muscle. One potential explanation for not finding a relationship between elevated FBR and proteinase activity includes the possibility that other proteinases were involved and that these proteinases were responsible for the rate-limiting step in the process of myofibrillar protein breakdown. Additionally, the proteinases were assayed *in vitro*, so that their potential capacity was measured, which does not necessarily represent their physiological activity.

The observed difference in muscle growth rate in the present experiment may also be due to diminished protein synthetic rates. This is reflected by the DNA, RNA, and protein data. The protein:DNA ratio is indicative of average cell size, and this ratio increases with age (Powell and Aberle, 1975). In both muscles, protein:DNA ratio was not affected by the treatments, suggesting that dietary treatments did not influence cell size. Because most of the RNA in the cell is ribosomal, RNA:protein ratio gives an estimate of capacity for protein synthesis (Sugden and Fuller, 1991). The RNA:protein ratio was lower for the protein-free group than for the other groups. The RNA:DNA ratio provides an estimate for the transcriptional efficiency of DNA and was also reduced in both muscles of the protein-free group. Thus, the decrease in synthetic capacity was attributable to a fall in rRNA transcription in the protein-free group. These data suggest that the impaired muscle growth of the protein-free group reflected decreases in DNA transcription and translation, as well as an increase in muscle protein breakdown.

Implications

This experiment shows that proteinase activity in two different muscles is not influenced by a lack of dietary protein for 14 d, whereas 3-methylhistidine production rate was increased under these circumstances. Although it should be taken with caution, this suggests that other proteinases may be responsible for the rate-limiting step or that proteinase capacity is not a valid predictor of physiological proteinase activity. It is also possible that the production rate of 3MH was increased after protein deficiency due to a higher contribution from sources other than skeletal muscle or that balenine was broken down.

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