3-Methylhistidine Production and Muscle Proteinase Activity in Growing Pigs

- Protein breakdown as a tool for growth modulation -

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3-Methylhistidine Production and Muscle Proteinase Activity in Growing Pigs

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Proefschrift

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STELLINGEN

1. Zolang de *in vivo* proteinase activiteit in spiercellen technisch niet bepaald kan worden, kan er geen directe relatie aangetoond worden tussen deze activiteit en de 3-methylhistidine produktie.
Dit proefschrift

2. Het verstrekken van een eiwitvrij voer, gemaakt door een isocalorische uitwisseling tegen koolhydraten, veroorzaakt effecten die te wijten zijn aan een gebrek van voedingseiwitten.
Dit proefschrift

3. Groeiende varkens passen binnen enkele dagen hun groeisnelheid en voerefficiëntie aan aan een eiwitvrij voerregime en herstellen, indien voedingseiwitten weer beschikbaar zijn, eveneens binnen enkele dagen. Het herstel is echter niet volledig binnen dit tijdstraject.
Dit proefschrift

4. Voor de produktie van 3-methylhistidine bij groeiende varkens is de bijdrage van het maagdarmkanaal zelf verwaarloosbaar klein.
Dit proefschrift

5. Veranderingen in proteinase activiteiten zijn van vergelijkbare orde van grootte in verschillende skeletspieren als gevolg van het voeren van verschillende voeders.
Dit proefschrift

6. De verhoogde aandacht voor het welzijn van dieren is omgekeerd evenredig met de aandacht voor het welzijn van mensen in de maatschappij.

7. De werknemer zou meer op de hoogte moeten zijn van de eisen die de wetgever oplegt aan de werkgever om zodoende de solidariteit binnen het bedrijf of instelling te verhogen en het ziekteverzuim terug te dringen.

8. Het weren van importprodukten op de Nederlandse tuinbouwveilingen getuigt van struisvogelpolitiek.

9. De benamingen 'proefschrift' en 'assistent-in-opleiding' bagatelliseren de werkelijkheid.

10. De vrijwilligheid bij de vrijwillige brandweer houdt op bij de aanmelding.

11. Een goede instelling is belangrijker dan een goede stelling.

Stellingen behorende bij proefschrift,
'3-Methylhistidine Production and Muscle Proteinase Activity in Growing Pigs: Protein breakdown as a tool for growth modulation'.
Henriëtte N.A. van den Hemel-Grooten, 18 juni 1996.

"Our greatest glory is not in never falling,
but in rising everytime we fall"

CONFUCIUS

Contents

1

Introduction

Nowadays, it is well established that accretion of body proteins is the resultant of both protein synthesis and breakdown. This was firstly shown by Schoenheimer and Rittenberg (1940). The simultaneous occurrence of protein synthesis and breakdown is always present, also in situations without protein deposition in the body. This dynamic process of synthesis and breakdown of proteins is also known as protein turnover. In FIGURE 1.1 a simplified model of protein metabolism is presented. Data which are presented are relevant to a growing pig of 50 kg.

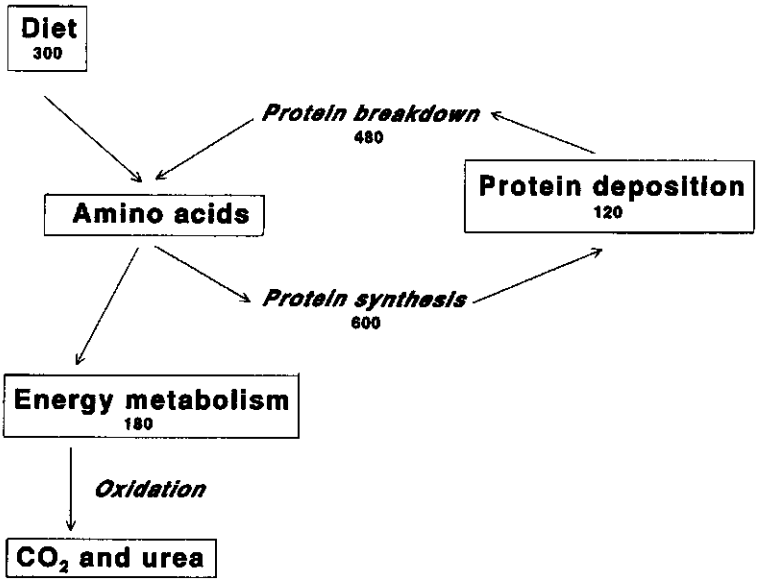

FIGURE 1.1 Metabolic processes underlying whole body protein and amino acid metabolism. Data represent grams/day for a growing pig of 50 kg (Reeds, 1989).

Free amino acids, either derived from diet (exogenous) or from protein breakdown (endogenous) can as such not be stored in the body. Therefore,

amino acids have to be polymerised in protein or they can be used for energy metabolism. In the latter case, as amino acids are oxidized, they are lost for protein metabolism. Based on this model, protein efficiency can be defined in two different ways. Firstly, that proportion of dietary protein that appears as net deposition (*i.e.*, 120/300 = 0.40) and secondly, that proportion of protein synthesis that appears as net deposition (*i.e.*, 120/600 = 0.20).

Although it is known for several decades that protein accretion is dependent on both synthesis and breakdown of protein, most of the scientific attention has been addressed to elucidating mechanisms and regulation of protein synthesis. Therefore, our knowledge on mechanisms and regulation of protein breakdown is limited, but this process is potentially as important as synthesis in the control of protein mass. Mechanisms of protein synthesis and breakdown are distinct (Reeds, 1989). Because of this, both pathways may be influenced independently. Theoretically, this would indicate that protein accretion can be increased by diminishing rate of protein breakdown, provided that protein synthesis remains the same (see TABLE 1.1).

TABLE 1.1 Theoretical calculation for improving growth rate in young pigs by decreasing fractional breakdown rate (FBR) by 10%.

	Normal conditions	Decreased FBR (-10%)
Body weight, kg	50	
Muscle protein, kg[a]	4	
Growth rate, kg/d	0.75	
FBR, %/d	3.5	3.15
FSR, %/d[b]	5.0	5.0
FAR, %/d[c]	1.5	1.85

For normal conditions: total amount of muscle protein breakdown under normal conditions is 3.5%/d of 4 kg = 0.14 kg/d. Growth rate of 0.75 kg/d implies a muscle protein accretion rate of 8% of 0.75 = 0.06 kg/d or 1.5%/d. Therefore, protein synthesis of muscle protein must have been 0.14 + 0.06 = 0.20 kg/d or 5%/d.

If FBR is decreased by 0.35%/d (=10% of 3.5%/d) to 3.15%/d, then improvement of growth rate is: (1.5 + 0.35)/1.5 x 100% = 123%

[a] Assumption that 8% of BW consists of muscle protein (Mulvaney *et al.*, 1985).
[b] FSR = fractional synthesis rate.
[c] FAR = fractional accretion rate.

One of the important goals of animal production is the production of lean meat. This can be translated as a more efficient production of muscle protein. Skeletal muscle contains about 50% of body proteins and is therefore one of the substantial tissues in protein metabolism (Simon, 1989). Skeletal muscle is composed of muscle bundles, which in turn consist of long, contractile fibres, the actual muscle cells. Each fibre is composed of myofibrils. The structure of the myofibril consists of sarcomeres, the smallest contractile unit, in which the contractile myofilaments are arranged in a regular way. Different myofilaments are present: thick filaments (mainly composed of myosin molecules), thin filaments (mainly composed of actin molecules) and a network of longitudinal and transverse filaments (the cytoskeletal network).

Turnover of muscle proteins comprise an important part of whole body protein turnover. Whole body protein turnover of the growing pig is supposed to require 15-22% of whole body energy expenditure. This may suggest that a lot of energy is used for an apparently 'futile' cycle. The possibility to decrease protein breakdown would not only favour net protein synthesis, but also the level of protein turnover and thus the energy costs of protein metabolism. Protein turnover may be seen as a futile cycle in a quantitative sense. It has a clear physiological significance in a qualitative sense. A very important function of protein turnover is that it gives an animal the ability to adapt to metabolic and physiological changes. In addition, protein breakdown is necessary to remove proteins which originate from mistakes made during the process of protein synthesis, mutations and/or ageing. Moreover, protein breakdown is needed during circumstances when there is a shortage of amino acids (*e.g.*, starvation) or nitrogen-containing components (*e.g.*, nucleic acids).

Several factors influence both protein synthesis and breakdown rates, among which age, nutrition, and hormones (Simon, 1989). Rates of both protein synthesis and breakdown in whole body or in individual tissues decline with age. Protein accretion is most efficient in immature animals, and differs between species. Highest efficiency (proportion of protein synthesis that appears as net deposition) is found in chickens, followed by pigs, sheep, and cattle.

Measurement of muscle protein breakdown

Methods for measurement of protein breakdown of skeletal muscles *in vivo* are limited. Two methods will be discussed here, since they are still in used. Firstly, an indirect method will be discussed, based on the fact that protein

accretion is the resultant of both protein synthesis and breakdown. This method was originally used for measurements of whole body protein synthesis, but can also be applied for measurements in skeletal muscle. Secondly, a direct method is discussed which make use of production of a marker amino acid, *e.g.*, 3-methylhistidine (3MH), which is released after muscle protein breakdown.

The indirect method

This method calculates fractional breakdown rate (FBR) from difference between fractional accretion rate (FAR) and synthesis rate (FSR):

$$FBR = FSR - FAR$$

Disadvantage of this indirect method is that 2 different groups of animals are needed to measure protein accretion rates and protein synthesis rate. Moreover, the time scale of both measurements is very different: protein accretion is measured over several days, whereas protein synthesis is usually measured over a period of minutes or hours. Thus, diurnal variation can cause errors in measurement of protein synthesis rate. Errors which occur in measurements of rates of either accretion or synthesis result in an over- or underestimation of fractional breakdown rate.

The direct method

This method measures the urinary excretion of 3-methylhistidine (3MH). 3-Methylhistidine is a specific constituent of myofibrillar proteins, actin and myosin heavy chain. The formation of 3MH occurs posttranslationally by methylation of one histidine residue per actin molecule and one histidine molecule per heavy chain myosin (Pearson and Young, 1989). Upon degradation, 3MH can not be reutilized for protein synthesis since there is no specific tRNA present for 3MH (Young *et al.*, 1972). 3-Methylhistidine can not be metabolized and is therefore excreted quantitatively in urine in most species. This means that the 3MH produced can be measured quantitatively in the urine. This is illustrated in FIGURE 1.2.

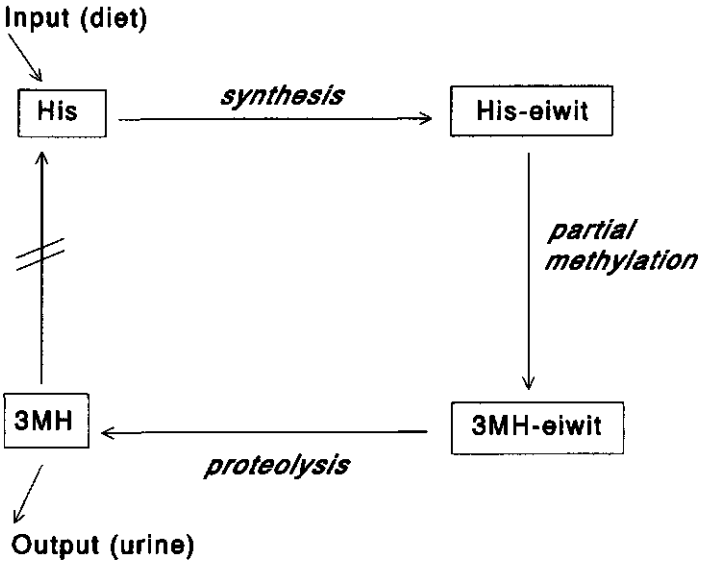

FIGURE 1.2 Posttranslational methylation of histidine (His) in myofibrillar proteins and excretion of 3-methylhistidine (3MH) upon proteolysis via urine.

However, in pigs and sheep, 3MH is not excreted quantitatively but largely stored in muscles as a dipeptide called balenine (ß-alanine-3-methylhistidine) (Harris and Milne, 1980; 1981). To measure production of 3MH rather than excretion in sheep and pigs, Rathmacher *et al.* (1993, 1996) have developed an alternative approach. The disappearance rate of an injected tracer of 3MH can be described by a kinetic compartmental model (see FIGURE 1.3 for pig model) and allows for the estimation of 3MH production.

As already mentioned, limitation of measuring 3MH production is that this amino acid is not representative for total muscle protein breakdown. Proteins in skeletal muscle can be divided into: myofibrillar (50 to 55%), sarcoplasmic (30 to 35%), and stroma proteins (15 to 20%) (Goll *et al.*, 1989). It is suggested from studies in rats, that myofibrillar and non-myofibrillar protein breakdown are regulated differently (Goodman, 1987; Kadowaki *et al.*, 1989). Thus, production of 3MH only provides an estimate for myofibrillar protein breakdown. Another limitation of the 3MH method is that 3MH can also be derived from smooth muscles like in gastro-intestinal tract and skin. Therefore these tissues

may also contribute to 3MH production and even in a manner that is disproportionate to their size. This latter will be the case if turnover rates of these tissues is higher than for skeletal muscle.

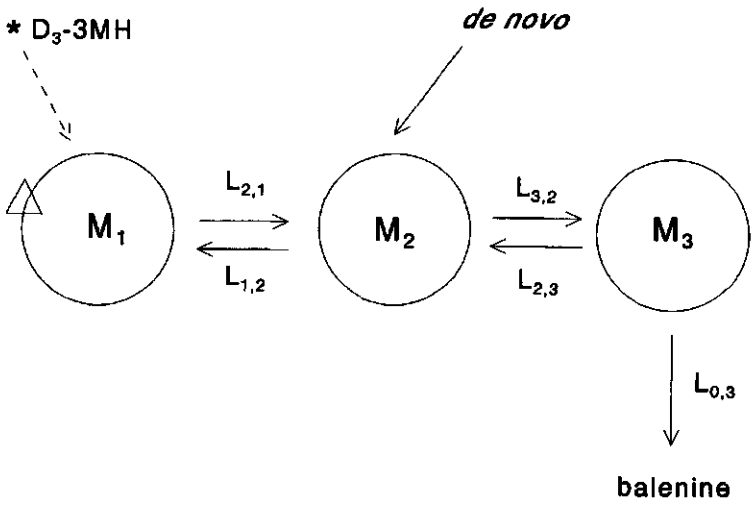

FIGURE 1.3 Schematic presentation of a three-compartmental model used to analyze the kinetics of distribution, metabolism, and de novo production of 3MH in pigs. M_1, M_2, and M_3 represent the mass of 3MH in compartments 1, 2, and 3, respectively. $L_{2,1}$, $L_{1,2}$, $L_{0,3}$, $L_{2,3}$, and $L_{3,2}$ are fractional transfer rate coefficients of 3MH within the system. The tracer, 3-[$^2H_3$-methyl]methylhistidine (D_3-3MH) was injected into compartment 1. Sampling was performed from compartment 1 (Δ). De novo production of 3MH was into compartment 2. Figure from Rathmacher *et al.* (1996).

Proteolytic enzyme systems in muscle

Studying the involvement of proteolytic enzymes is a first step in elucidating mechanisms and regulation of muscle protein breakdown *in vivo*, because they are responsible for the breakdown of muscle proteins. At present, the rate-limiting step in the cascade of events of underlying muscle protein degradation is unknown. If one of the proteolytic enzyme systems is responsible for this rate-limiting step, this would open up the way for intervention studies to

improve muscle protein deposition.

At least three different proteolytic systems are thought to be involved in breakdown of muscle proteins. These systems can be divided into cytoplasmic proteases and lysosomal proteases. To the first category belong the calpain system and multicatalytic proteinase (MCP; proteasome). Lysosomal proteases consist of the cathepsin family, which are inhibited by cystatins, situated in cytoplasm.

The calpain system consists of the proteolytic isoenzymes μ- and m-calpain (requiring micromolar and millimolar Ca^{2+}-concentration for activity, respectively), and their natural inhibitor calpastatin (Suzuki *et al.*, 1987). Both isoenzymes are heterodimers composed of a unique catalytic 80 kDa and an identical regulatory 30 kDa subunit. Both μ- and m-calpain are regulated by Ca^{2+}-binding, autoproteolytic modification and their natural inhibitor calpastatin (Croall and DeMartino, 1991). It has been discussed by Goll *et al.* from *in vitro* studies (1989; 1992) that calpains are involved in the first step of myofibrillar protein breakdown. The precise mechanism is unknown, but to date the calpains are the only candidates for the disassembly of myofibrils into filaments by specific cleavages that release thick filaments (mainly consisting of myosin monomers) and thin filaments (mainly consisting of actin monomers) from the myofibril (Goll *et al.*, 1992). Only recently, a new large-subunit calpain has been found, which unlike the distribution of μ- and m-calpain in various tissues, has only been found in skeletal muscle (Sorimachi *et al.*, 1989). This calpain has been designated skeletal muscle calpain. Soon after translation, skeletal muscle calpain is degraded by autolysis, but a small part probably enters into the nucleus, where it might regulate short-lived proteins (*e.g.*, transcription factors) (Sorimachi *et al.*, 1993).

After filaments are released during incubation with calpains, further breakdown is performed by other cytosolic proteases and/or lysosomal cathepsins (Goll *et al.*, 1992). Because of their size, it is unlikely that whole filaments are taken up by lysosomes, unless they are at least partly degraded by cytosolic proteases. The presence of MCP in skeletal muscles prompted the suggestion that this proteolytic system may play a major role in cytosolic protein breakdown (Rivett, 1989; Orlowski, 1990). The MCP is a high-molecular weight enzyme (650 to 700 kDa), composed of many different types of subunits (varying between 23 and 34 kDa) arranged in a hollow cylindrical structure. At least five distinct proteolytic activities have been identified and it has been suggested that the MCP contains the system of enzymes necessary for intracellular protein turnover and processing (Orlowski, 1993). Another

cytosolic multicomponent complex (1500 kDa) has been identified, which is capable of degrading ubiquitin-conjugated proteins. Ubiquitin is a small peptide and its function is supposed to be tagging proteins for breakdown. For this larger enzyme complex, ATP seems to be required. It has been suggested that MCP is a part of this larger complex (Driscoll and Goldberg, 1989). The lysosomal system seems to be responsible for the increased intracellular protein breakdown that occurs during shortage of nutrients, but also seems to play a role under basal conditions (Mayer and Doherty, 1986). Lysosomes are able to hydrolyse either proteins entering the cell by endocytosis or intracellular proteins and organelles.

If activities of one or more of these proteolytic enzyme systems are changed in parallel with changes of protein breakdown, this would identify the rate-limiting step in the cascade of muscle protein breakdown. Consequently, this would open possibilities for interventions to increase efficiency of protein deposition in meat animal production.

Aim of this thesis

The purpose of this thesis is to gain more insight into involvement of several muscle proteolytic enzymes systems during muscle protein breakdown *in vivo* in order to increase protein accretion by lowering muscle protein breakdown. As a model, rate of muscle protein breakdown in growing pigs was altered by dietary manipulation (*i.e.*, feeding a protein-free diet for 14 days). Estimation of myofibrillar protein breakdown *in vivo* was performed by measuring production of 3-methylhistidine (3MH) using the method of Rathmacher *et al.* (1996). In parallel, different proteolytic enzyme systems were studied in skeletal muscles of different metabolic type: the calpain system, multicatalytic proteinase (MCP), and cathepsins with their inhibitors cystatins.

Scope of this thesis

CHAPTER 2 describes 3MH production and proteinase activity and their relation in different skeletal muscles of growing pigs after a protein-free feeding period of 14 days.

In CHAPTER 3 the same experimental design was used to exclude that effects of feeding a protein-free diet were caused by an increase in dietary

carbohydrates. This was done by introducing another group of animals, which received a protein-free diet, made by isocaloric exchange of dietary protein by dietary fat. Moreover, half of the animals at the end of the protein-free feeding period were realimentated again for another 7 days to study the role of myofibrillar protein breakdown and proteinase activity during compensatory growth.

In CHAPTER 4, effects of these dietary treatments (of both the protein-free feeding period and the realimentation period) on chemical body composition (carcass, liver, and both small and large intestines) were studied.

In CHAPTER 5, the extent of the possibility was investigated that production of 3MH was overestimated in case part of 3MH originated from rapid-turning over sources, *i.e.*, smooth muscles in gastro-intestinal tract and skin. Especially during a situation of feeding a protein-free diet, 3MH production of these sources might be elevated resulting in apparent myofibrillar protein breakdown.

CHAPTER 6 relates to the problems of measuring proteinase and inhibitor activities in skeletal muscle, since it is not possible to measure enzyme activity *in situ* and in general measurements are performed under *in vitro* conditions. Therefore, regulation of the components of the calpain system were studied at the transcriptional level by measuring mRNA levels in skeletal muscle on several days during the protein-free feeding period.

In the general discussion (CHAPTER 7), the findings of the relation between 3MH production and muscle proteinase activity during a protein-free feeding period are discussed. Suggestions for future research are given.

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2

Comparison between 3-methylhistidine production and proteinase activity as measures of skeletal muscle breakdown in protein deficient growing barrows

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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; 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, multicatalytic 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 ($P < 0.05$). 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 compared with 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 ($P < 0.05$). 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.

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 labelled 3MH, methyl-$^2H_3$-N^τ-methylhistidine (d_3-3MH), to measure de novo production rather than excretion of 3MH (Rathmacher *et al.*, 1996).

Several proteinase systems are thought to be involved in skeletal muscle protein breakdown. These 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; Orlowski, 1990). However, MCP does not degrade myofibrillar proteins (Koohmaraie, 1992). Cathepsins seem to play a major role under conditions of nutritional deprivation (Kettelhut *et al.*, 1989). Ballard *et al.* (1988) found no relationship between the calpain system and growth rates. 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.

Materials and Methods

Experimental design

The study was carried out at the Roman L. Hruska U.S. MARC, Clay Center, NE, USA. 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 studied. On d -16, at an age of approximately 11 wk, barrows were moved to individual pens (1.22 m x 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 restricted-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 2.1. 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.

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 0.66 mm i.d. and 0.95 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.

TABLE 2.1 Composition of the experimental diets.

	Control	Protein-free
Ingredients, g/100 g		
Corn	69.0	-
Soybean meal	25.4	-
Corn starch	-	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	0.3
Fat, g/100 g	4.8	4.8
Carbohydrates, g/100 g	56.3	85.1
Crude fibre, 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

[a] Constant components are as follows: dicalcium phosphate, 2,0 g; limestone, 0.6 g; iodized salt, 0.4 g (supplied 0.28 mg of iodine per kilogram of diet); vitamin premix, 0.2 g; trace minerals, 0.2 g; and choline chloride 0.2 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, 0.35 mg; riboflavin, 0.53 mg; d-pantothenic acid, 2.12 mg; niacin, 2.82 mg; vitamin B_{12}, 2.64 μg; thiamine, 0.22 μg; biotin, 22.0 μg; and folic acid 88.2 μg. Composition of the 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.

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

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.* (1996). Briefly, a standard amount of d_3-3MH (0.1371 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 x *g* and plasma was stored at -70°C for later analysis of d_3-3MH 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·kilogram^{-1}·day^{-1}) was calculated as described by Rathmacher *et al.* (1992). The fractional breakdown rate (FBR) of proteins 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 skeletal muscle is 3.8742 μmol/g of muscle protein (Rathmacher *et al.*, 1996).

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 ß-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 x *g*, the supernatant was filtered through four layers of cheesecloth and then glass wool, and loaded onto a 1.5-cm x 20-cm column of DEAE-Sephacel, which had been equilibrated with elution buffer (40 mM Tris, 0.5 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} 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, 0.5 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 0.3 ml of 1% SDS and 1 ml of 0.1 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, cathepsins B, and B+L assay

The activity of cystatins, 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) utilizing 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 $MgCl_2$, 0.5 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 x *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 0.1 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 ± 2%, $P > 0.1$ for the M muscle; 84 ± 2%, $P > 0.5$ for the L muscle).

Statistical analyses

Data were analyzed by analysis of variance (ANOVA) followed by 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. Differences were considered statistically different when P-value was less then 0.05.

Results

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

Data in TABLE 2.2 show that during the experimental period body weight increased for both the control and 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 restricted groups from d 5 onward. Consequently, growth rates were different between all treatments ($P < 0.05$).

TABLE 2.2 Performance and feed intake characteristics of pigs under different dietary treatments[a]

Trait	Dietary treatment		
	Control	Intake-restricted	Protein-free
Body weight, kg			
d -3	36.1 (1.1)	36.7 (1.1)	36.3 (1.0)
d 0	38.3 (0.9)	38.8 (0.9)	35.5 (1.1)
d 5	43.4 (1.0)[b]	40.3 (0.8)[b]	35.4 (1.2)[c]
d 10	46.4 (1.2)[b]	42.3 (1.1)[c]	35.4 (1.0)[d]
d 14	48.6 (1.2)[b]	43.9 (0.8)[c]	36.0 (1.2)[d]
Growth rate, kg/d			
d 0 to d 14	0.73 (0.07)[b]	0.37 (0.05)[c]	0.03 (0.04)[d]
Feed intake, kg/d			
d 0 to d 14	2.08 (0.07)[b]	1.27 (0.05)[c]	1.34 (0.07)[c]
Gain/feed, kg/kg			
d 0 to d 14	0.35 (0.02)[b]	0.29 (0.04)[b]	0.01 (0.03)[c]

[a] Data are expressed as mean (SEM) 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 ($P < 0.05$).

The carbohydrate content in the protein-free diet was higher than 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 2.3. 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.

TABLE 2.3 Liver and muscle weights of pigs under different dietary treatments[a]

Trait	Dietary treatment		
	Control	Intake-restricted	Protein-free
Wet weight, g			
Liver	1024.4 (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 (0.9)[c]
Wet weight, % of BW			
Liver	2.11 (0.06)	2.11 (0.03)	2.22 (0.10)
Longissimus	3.59 (0.09)[b]	3.74 (0.05)[b]	3.21 (0.06)[c]
Masseter	0.18 (0.01)	0.19 (0.01)	0.19 (0.01)

[a] See legend to TABLE 2.2.
[b,c] Means within a row lacking a common superscript letter differ ($P < 0.05$).

Production rate of 3MH (micromoles·kilogram^{-1}·day^{-1}) and FBR (percentage/day) was highest for the protein-free group and significantly different from both other groups (FIGURE 2.1). Production rate of 3MH and FBR for the control and intake-restricted groups were not different.

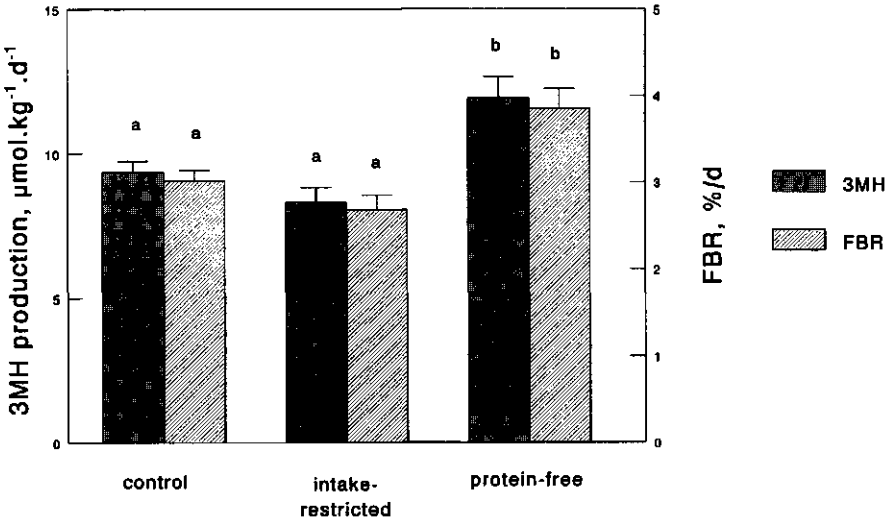

FIGURE 2.1 3-Methylhistidine production rate (3MH, micromoles·kilogram^{-1}·day^{-1}) and fractional breakdown rate (FBR, percentage/day) for different treatments. Means within group parameters (3MH or FBR) having a different letter differ ($P < 0.05$). Group means are 3.02, 2.68, and 3.85%/d for FBR and 9.4, 8.3, and 11.9 μmol.kg^{-1}.d^{-1} for 3MH production for control, intake-restricted, and protein-free group, respectively.

FIGURE 2.2 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.

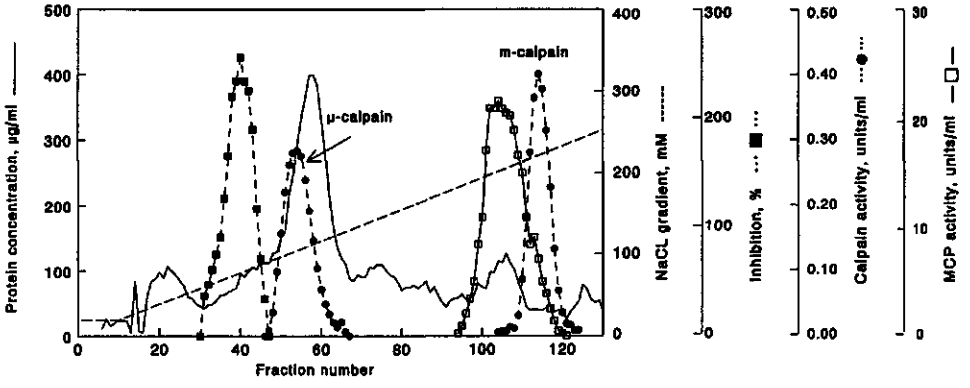

FIGURE 2.2 Chromatographic separation of calpastatin (··■··), μ- and m-calpain (··●··), and MCP (-□-) using DEAE-Sephacel with a linear NaCl gradient (---). Protein concentration in the different fractions is also shown (———) . See text for detailed information about elution conditions.

Proteinase activities in both skeletal muscles are presented in TABLE 2.4. 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 ($P < 0.05$). This was also true for their natural inhibitors, the cystatins. Between muscles, values for cathepsin B activity were similar; however, activities of cathepsins B+L and cystatins were significantly higher for L muscle than for M muscle.

TABLE 2.4 Proteinase activities in porcine skeletal muscles under different dietary treatments[a]

Trait	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)	9.83 (1.31)	11.51 (0.67)
	(P=0.007)[h]	*(P=0.016)*	*(P=0.179)*
μ-calpain[c]			
Masseter	7.79 (0.83)	8.02 (0.93)	8.10 (1.03)
Longissimus	4.05 (0.38)	3.43 (0.27)	3.80 (0.56)
	(P=0.012)	*(P=0.002)*	*(P=0.012)*
m-calpain[d]			
Masseter	10.27 (0.72)	9.24 (0.47)	8.34 (0.66)
Longissimus	3.63 (0.34)	2.71 (0.24)	2.96 (0.39)
	(P=0.012)	*(P=0.002)*	*(P=0.012)*
MCP[e]			
Masseter	6.09 (0.22)[i]	5.41 (0.22)[i]	4.27 (0.29)[j]
Longissimus	4.24 (0.14)	3.99 (0.14)	3.62 (0.22)
	(P=0.001)	*(P=0.001)*	*(P=0.013)*
Cathepsin B[f]			
Masseter	465.1 (64.2)	499.6 (61.6)	340.3 (37.7)
Longissimus	532.2 (183.9)	438.4 (59.5)	299.2 (21.0)
	(P=0.711)	*(P=0.416)*	*(P=0.188)*
Cathepsins B+L[f]			
Masseter	817.0 (58.0)	925.8 (76.9)	685.7 (59.1)
Longissimus	1437.2 (161.3)	1394.7 (95.1)	1174.8 (56.9)
	(P=0.007)	*(P=0.009)*	*(P=0.001)*
Cystatins activity[g]			
Masseter	7.13 (0.78)	9.39 (1.41)	9.20 (0.90)
Longissimus	19.73 (2.02)	18.19 (2.02)	24.89 (2.75)
	(P=0.001)	*(P=0.001)*	*(P=0.001)*

[a] See legend to TABLE 2.2.
[b] Inhibition of casein hydrolysis by m-calpain·grams of muscle protein^{-1}.
[c] Low Ca^{2+}-requiring calpain protease total activity·grams of muscle protein^{-1} (caseinolytic activity).
[d] High Ca^{2+}-requiring calpain protease total activity·grams of muscle protein^{-1} (caseinolytic activity).
[e] Total μM pNA released·hour^{-1}·grams of muscle protein^{-1}.
[f] Activity expressed as nmoles of NMec released·minutes^{-1}·grams of muscle protein^{-1}.
[g] Measured as the ratio of B+L activity after to before cystatin removal by affinity chromatography.
[h] P-value < 0.05 denotes difference between masseter and longissimus muscle.
[i,j] Means within a row lacking a common superscript letter differ (P < 0.05).

TABLE 2.5 Protein, DNA, and RNA content in porcine skeletal muscles under different dietary treatments[a]

Trait	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)0
	(P=0.001)[c]	*(P=0.001)*	*(P=0.001)*
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)
	(P=0.001)	*(P=0.001)*	*(P=0.001)*
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)
	(P=0.202)	*(P=0.001)*	*(P=0.001)*
DNA, μg/g of muscle			
Masseter	1646.1 (68.6)	1602.1 (57.0)	1680.1 (43.9)
Longissimus	877.1 (80.4)	972.9 (53.4)	1047.3 (54.6)
	(P=0.001)	*(P=0.001)*	*(P=0.001)*
RNA, μg/g of muscle			
Masseter	842.6 (21.0)[c]	814.7 (14.2)[c]	620.4 (17.3)[d]
Longissimus	664.7 (40.4)[c]	642.2 (18.1)[c]	526.3 (11.7)[d]
	(P=0.003)	*(P=0.001)*	*(P=0.001)*
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)
	(P=0.001)	*(P=0.001)*	*(P=0.001)*
RNA/DNA			
Masseter	0.52 (0.02)[d]	0.52 (0.03)[d]	0.37 (0.01)[e]
Longissimus	0.81 (0.09)[d]	0.68 (0.05)[d,e]	0.51 (0.03)[e]
	(P=0.006)	*(P=0.012)*	*(P=0.001)*
RNA/Protein[f]			
Masseter	5.63 (0.17)[d]	5.78 (0.08)[d]	4.36 (0.18)[e]
Longissimus	4.39 (0.22)[d]	4.28 (0.09)[d]	3.46 (0.15)[e]
	(P=0.001)	*(P=0.001)*	*(P=0.001)*

[a] See legend to TABLE 2.2.
[b] Protein amount as measured according to Lowry *et al.* (1951).
[c] P-value < 0.05 denotes difference between masseter and longissimus.
[d,e] Means within a row lacking a common superscript letter differ (P < 0.05).
[f] Protein amount as measured by using the biuret procedure.

Total, soluble, and myofibrillar protein contents were not significantly different between treatments for either muscle (TABLE 2.5). 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 2.5). 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 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 fibre 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 ($P < 0.05$). 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%/d 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 ($P > 0.10$). 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 accreting 12.47 g of muscle/d, whereas the protein-free group was losing 4.82 g of muscle/d. This corresponds to muscle accretion rates of +1.90 and -0.73%/d over the entire period for the control and protein-free groups, respectively. As a percentage of body weight, the L muscle percentage was decreasing in the protein-free group. For liver, the increase in weight over the entire period was 2.0 and 0.2%/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 -0.1%/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. Therefore, 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) in 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 fibre typing, there was no difference in calpastatin activity, but μ-calpain activity was significantly lower and m-calpain was significantly higher in M muscle compared with 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 fibre types.

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: $r = -0.267$, $P = 0.170$; for L muscle: $r = -0.179$, $P = 0.382$). 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 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 were 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. 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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3

Effects of a protein-free feeding period and a subsequent realimentation period on protein metabolism in growing pigs 1. Whole body 3-methylhistidine production and proteinase activities in different skeletal muscles

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Abstract

Whole body 3-methylhistidine (3MH) production rate and proteinase activities in porcine skeletal muscles were studied during a protein-free feeding period and subsequent realimentation. Fifty-four barrows (initial weight of 35 kg at d 0) were randomly divided over 6 treatments. Six animals were slaughtered at d 0. During the 14-d protein-free feeding period, 3 isocaloric diets were provided restricted: control diet (C), protein-free/carbohydrate-rich (PF/CH) or protein-free/fat-rich (PF/FAT). At the end of this period, 8 animal per treatment were slaughtered. During the 7-d realimentation period, all remaining animals received the control diet and formed the other 3 treatments: C-C, PF/CH-C, and PF/FAT-C. At d 21, these animals were slaughtered. Measurement of 3MH production rate was performed during the last 3 d of each period. At slaughter, samples were taken from *m. masseter* and *m. longissimus*. During the protein-free feeding period, growth rate and 3MH production rate of C group were significantly higher than for both PF groups. Transcriptional efficiency and capacity for protein synthesis in both muscles were significantly decreased in both PF groups compared to C group. Proteinase and inhibitor activities in both muscles were not clearly different between treatments. During the subsequent realimentation period, growth rate and feed efficiency were only significantly higher for both PF-C groups than their age controls during the first 3 d of this period. Whole body 3MH production rate was not different between treatments. Transcriptional efficiency, capacity for protein synthesis, and proteinase and inhibitor activities in both muscles were not different between treatments. These data suggest that some compensatory growth has occurred during the first 3 days of the realimentation period, but animals did not fully compensate.

Introduction

Understanding the underlying mechanisms and regulation of protein metabolism is a prerequisite for improving growth efficiency in farm animals. It is well established that proteins are continuously synthesised and degraded during growth and maturity (Reeds, 1989). Numerous research studies in the pig have focused on the underlying mechanisms of protein synthesis, but insight in the mechanism of protein degradation of skeletal muscle is, however, limited. This is probably partly due to the lack of methods to measure muscle protein breakdown *in vivo*. Although the use of urinary excretion rate of 3-methylhistidine (3MH) is a valid index of muscle protein breakdown in most species, this is not valid for pigs because 3MH is not excreted but stored in muscles as balenine (Harris and Milne, 1981). However, a recently developed technique enables a direct estimate of 3MH production *in vivo* in pigs: production of 3MH is calculated from 3MH kinetics in serial plasma samples using a compartmental computer model (Rathmacher *et al.* 1996).

To understand the role of proteinases in muscle protein breakdown is an important objective in research, which is focused on elucidating mechanisms and regulation of skeletal muscle protein breakdown. There is convincing evidence that the calpain system (μ- and m-calpain and their natural inhibitor calpastatin) is involved in the initial step of myofibrillar protein breakdown by weakening of the Z-disks and thereby releasing filaments (Goll *et al.* 1989; Koohmaraie, 1992). The multicatalytic proteinase (MCP) is also thought to be involved in the cascade of protein breakdown of skeletal muscle and it is suggested that the MCP can be seen as the counterpart of the ribosome (Orlowski, 1993). Even though the mechanisms by which muscle protein degradation is regulated are not clearly understood, it is apparently a tightly regulated process. The process of protein breakdown of skeletal muscle reacts both on acute and chronically to factors like nutritional state, hormones, and workload (Jepson *et al.* 1988). After a period of nutritional restriction, compensatory or catch up growth can occur if animals are realimentated. Compensatory growth is defined as the greater than normal growth rate compared to age controls (Ryan, 1990; Samuels & Baracos, 1995). Several mechanisms are proposed to explain this increase in growth rate (Ryan, 1990). A higher growth rate can be caused by a decreased rate of protein breakdown and or by an increased rate of protein synthesis.

The objective of the present study was threefold. Firstly, to determine breakdown rate of skeletal muscle protein during a period of feeding a protein-free diet and a subsequent period of protein realimentation. Secondly, to determine whether effects of a protein-free diet were influenced by energy source, *e.g.*, fat or starch. Finally, to determine the activity of several proteinases in different skeletal muscles during both experimental periods.

Materials and methods

Experimental design

Fifty-four crossbred barrows, Large White x (Dutch Landrace x Large White), were used at an initial weight of approximately 25 kg. On d -14, barrows were housed individually in a pen (1.5 x 2.0 m) at a constant temperature (22 ± 1°C). A starter diet (Cavo Latuco, Utrecht, The Netherlands) was available on an *ad libitum* basis during the pre-experimental period. On d 0, barrows were randomly assigned to one of the six treatments so that their group mean body weight was similar between treatments. On d 0, 6 animals were slaughtered and this group formed the initial group, who served as a reference group. The average body weight of all animals in the groups on d 0 was 35.3 ± .5 kg. The experimental design consisted of two periods: a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21). During the first period, 3 different, isocaloric diets were offered to the 3 treatments: a control diet (C group), a protein-free/carbohydrate-rich diet (PF/CH group) and a protein-free/fat-rich diet (PF/FAT group). The composition of the diets is shown in TABLE 3.1. At the end of the protein-free feeding period, 8 animals per treatment were slaughtered. During the realimentation period, all remaining animals received the control diet and these animals formed the other 3 treatments in this experiment (C-C group, PF/CH-C group and PF/FAT-C group). On d 21, the remaining animals were slaughtered. All animals received the experimental diets restricted (2.5 times energy for maintenance for control animals) throughout the experiment (2 times a day). Water was available on an *ad libitum* basis throughout the experiment. Feed intake was recorded daily and body weights on d 0, 5, 10, 14, 17, and 21.

Cannulation of the ear vein

On d 7 of the protein-free feeding period or on d 14 (start of the realimentation period), a catheter was surgically inserted into the jugular vein through the ear vein as described by Freriksen *et al.* (1996).

TABLE 3.1 Composition of the experimental diets.

	Control (C)	Protein-free Carbohydrate-rich (PF/CH)	Protein-free Fat-rich (PF/FAT)
Ingredients, g/kg			
Corn	690	-	-
Soybean meal	254	-	-
Corn starch	-	783	656
Soybean oil	20	54	161
Arbosel	-	121	136
Constant components[a]	36	42	47
Calculated, g/kg			
Protein	173	3	4
Fat	48	55	161
Carbohydrates	544	828	706
Crude fibre	34	39	44
Analyzed, g/kg			
Protein	192.4	4.3	3.2
Fat	47.4	53.3	160.5
Dry matter	870.0	884.2	898.2
Ash	48.8	28.8	32.8

[a] Constant components consisted of the following: dicalcium phosphate, 20 g; limestone, 6 g; iodized salt, 4 g; mineral premix, 2 g; vitamin premix, 2 g; choline chloride, 2 g. The composition of the mineral premix was as follows: $CoSO_4.7H_2O$, 2.5 mg; $Na_2SeO_3.5H_2O$, 0.2 mg; KI, 0.5 mg; $FeSO_4.7H_2O$, 400 mg; $CuSO_2.5H_2O$, 40 mg; MnO_2, 70 mg and $ZnSO_4.H_2O$, 300 mg. Composition of vitamin premix is described elsewhere (Bikker *et al.* 1994).

3-Methylhistidine production and calculation of myofibrillar fractional breakdown rate

During the final 3 d of each period 3MH production rate was measured according to the method of Rathmacher *et al.* (1996) with some minor modifications. Briefly, a standard amount of labelled 3-methylhistidine, L-N^{τ}-methyl-d_3-histidine (d_3-3MH; 0.1371 mg/kg BW), was dissolved in 10 ml of sterile saline, injected into the ear vein, and flushed with 2 ml of saline. This was followed by serial blood sampling (10 ml) at 1, 3, 5, 15, 30, 60, 90, 180, 360, 540, 720, 1440, 2160, 2880, 3600, and 4320 minutes. Each syringe contained 15 mg of EDTA as the anticoagulant. Additional blood samples were

collected before the injection of d_3-3MH to correct for background enrichment from the experimental samples. Blood samples were centrifuged for 15 min at 1500 x g and plasma was transferred to sampling tubes and stored at -20°C for later analysis for d_3-3MH and 3MH by gas chromatography / mass spectrometry as already described (Rathmacher *et al.* 1992). With the use of a three compartmental computer model, production rate of 3MH can be calculated from 3MH kinetics (Rathmacher *et al.*, 1996).

For the calculation of fractional breakdown rate (FBR) from 3MH production rate the total body pool of 3MH has to be known. Therefore, the following assumptions were made: 8% of body weight consists of muscle protein and the 3MH concentration in skeletal muscle is 3.8742 μmol/g of muscle protein as was used in our previous experiment (Van den Hemel-Grooten *et al.* 1995).

Sampling and analysis of muscle

After taking the last blood sample in each period, 8 barrows in each treatment were killed by using a captive bolt followed by exsanguination. Immediately after exsanguination a sample of approximately 150 to 200 g was taken from the longissimus muscle (L; between the third and fourth rib; left side of the carcass) followed by dissection of both masseter (M) muscles, total weight of each muscle was recorded, and an aliquot of 10 g of both L and M muscles was immediately used for analysis of proteolytic enzyme activity as described below. An aliquot of both L and M muscles was immediately frozen into liquid nitrogen and stored at -80°C for analysis of other components. Liver and both small and large intestines (after being flushed with water) were removed. The right side of the carcass, liver, and both intestines were stored at -20°C for later chemical analysis (Van den Hemel-Grooten *et al.* 1996). In order to obtain the total weight of the L muscle, it was dissected from the right side of the carcass before chemical analysis.

Within 10 minutes after exsanguination, masseter and longissimus muscles (except for muscles from the initial slaughter group) were prepared for analysis of calpastatin, μ-and m-calpain activity according to Koohmaraie (1990) and MCP according to Arbona & Koohmaraie (1993), both with some minor modifications. Briefly, 10 g of muscle was homogenised in 6 volumes of extraction buffer (50 mM Tris, 10 mM EDTA, 10 mM ß-mercaptoethanol (MCE); pH 8.3). After centrifugation at 2 h at 37,000 x g, the supernatant was filtered, and loaded onto a 1.5-cm x 20-cm column of DEAE-Sephacel, which had been equilibrated with elution buffer (40 mM Tris, 0.5 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 mM to 350 mM NaCl in elution buffer (230 ml of each). One unit of calpain activity is defined as the amount of proteinase that caused an increase of 1.0 absorbance unit at A_{278} nm in 60 min at 25°C using casein as a substrate. One unit of calpastatin activity is defined as the amount of inhibitor that inhibits one unit of m-calpain activity. One unit of MCP activity is defined as the amount of proteinase required to release 1 μmoles of p-nitroanaline (pNA) from the synthetic substrate, N-CBZ-Gly-Gly-Leu-pNA, in 60 min at 55°C.

Both muscles were also assayed for protein, DNA, and RNA concentration. Protein concentration was measured using the biuret reagent (Gornall *et al.* 1949). Concentration of DNA was determined using Hoechst 33258 reagent (bisbenzimidazole; Sigma Chemical Company, St. Louis, MO, USA) according to Labarca & Paigen (1980). Concentration of RNA was determined using the method of Munro & Fleck (1969).

Statistical analysis

Within each combination of treatments (C, PF/CH, and PF/FAT or C-C, PF/CH-C, and PF/FAT-C) data were analyzed by analysis of variance (ANOVA; oneway) followed by Tukey-B-test using the SPSS-PC$^+$, V3.0 program, in which the treatment was used as an independent factor (SPSS Inc., 1988). For comparison between periods, data of the control groups (Initial, C and C-C groups) were analyzed by a oneway followed by Scheffe-test, in which day of the experiment was the independent factor. This was done because different animals were used for the different control groups. For testing whether compensatory growth has occurred, growth rate of PF/CH-C or PF/FAT-C groups from d 15 to d 17 with either age controls (*i.e.*, C-C group from d 15 to d 17) or weight controls (C group from d 11 to d 14) were tested using an independent-t-test (1-sided). Results were considered statistically different when P-value was less than 0.05.

Results

Results of body weights are presented in FIGURE 3.1. There was no adaptation period to the experimental diets prior to the experimental period and feed intake of all pigs was reduced during the first 5 days. Since the first measurement of body weight was on d 5, only the period from d 6 was taken into account for the protein-free feeding period. Results of growth rate (TABLE

3.2) show that feeding either one of the protein-free diets for 14 d reduced growth rate significantly. Body weight at d 14 was significantly higher for the C group than for both PF groups. During the subsequent realimentation period, growth rate was increased for both PF groups (PF/CH-C and PF/FAT-C) to a level similar as for the control group (C-C), *i.e.*, age controls. Growth rate of both PF-C groups from d 15 to d 17 was significantly higher than that of the C group (d 11 to d 14, *i.e.*, weight controls).

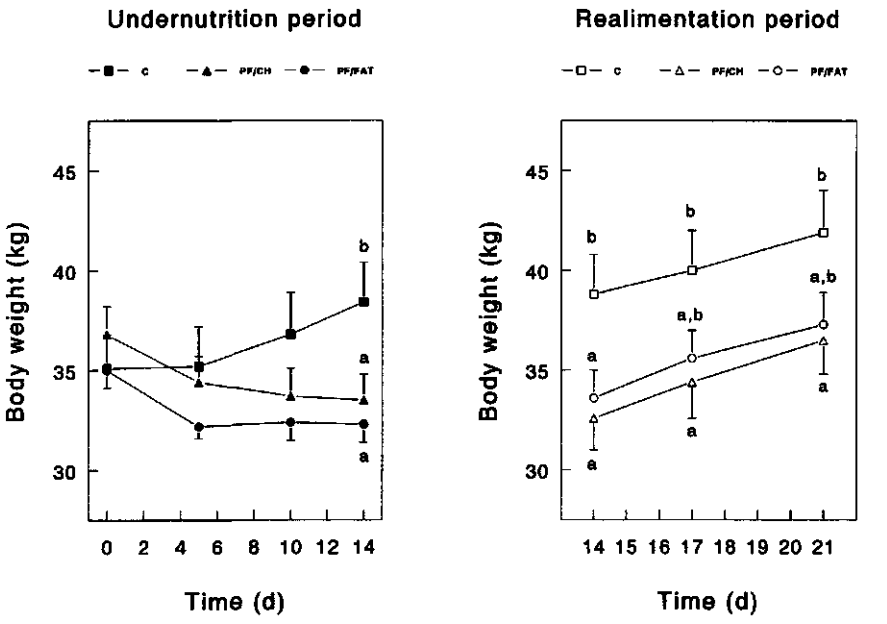

FIGURE 3.1 Body weights (mean ± SEM, for n=8 per treatment) of animals during the protein-free feeding period (d 0 to d 14) and the subsequent realimentation period (d 15 to d 21). The protein-free treatments were: C = control diet for 14 d; PF/CH = protein-free/carbohydrate-rich diet for 14 d; PF/FAT = protein-free/fat-rich diet for 14 d. The realimentation treatments were: C-C = C followed by C for 7 d; PF/CH-C = PF/CH followed by C for 7 d and PF/FAT-C = PF/FAT followed by C for 7 d. Body weight at a certain time point without the same letter differ ($P < 0.05$).

TABLE 3.2 Growth rate and feed efficiency during a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[a,b]

Trait	Protein-free treatments			Realimentation treatments		
	C	PF/CH	PF/FAT	C-C	PF/CH-C	PF/FAT-C
Growth rate, kg/d						
d 6 to d 10	0.32 (0.04)[d]	-0.13 (0.09)[c]	0.04 (0.10)[c]	0.55 (0.04)[e]	-0.07 (0.05)[c]	0.06 (0.03)[d]
d 11 to d 14	0.41 (0.05)[d]	-0.05 (0.06)[c]	-0.02 (0.05)[c]	0.38 (0.05)[d]	0.01 (0.04)[c]	-0.01 (0.04)[c]
d 15 to d 17				0.43 (0.06)	0.63 (0.08)[y,z]	0.65 (0.10)[y,z]
d 18 to d 21				0.47 (0.04)	0.51 (0.05)	0.43 (0.09)
Feed efficiency, g/MJ ME						
d 6 to d 10	23.4 (2.7)[d]	-11.9 (7.0)[c]	1.9 (6.1)[c]	39.0 (7.1)[d]	-5.6 (4.4)[c]	4.7 (2.7)[c]
d 11 to d 14	25.0 (3.5)[d]	-2.7 (2.9)[c]	-1.1 (2.8)[c]	22.6 (3.1)[d]	0.0 (3.1)[c]	-1.0 (3.6)[c]
d 15 to d 17				28.4 (3.7)	49.5 (4.5)[y,z]	51.3 (7.7)[y,z]
d 18 to d 21				25.7 (1.9)	32.5 (3.5)	27.8 (5.0)

[a] Data are expressed as MEAN (SEM) for 8 pigs per treatment.

[b] C = control diet for 14 d; PF/CH = protein-free/carbohydrate-rich diet for 14 d; PF/FAT = protein-free/fat-rich diet for 14 d; C-C = C followed by C for 7 d; PF/CH-C = PF/CH followed by C for 7 d and PF/FAT-C = PF/FAT followed by C for 7 d.

[c,d,e] Means within a combination of treatments lacking a common superscript letter differ ($P < 0.05$).

[y] Significantly different from weight controls (= C-group; d 11 to d 14), tested one-sided at $P < 0.05$.

[z] Significantly different from age controls (= C-C group; d 15 to d 17), tested one-sided at $P < 0.05$.

Data of feed efficiency in TABLE 3.2 showed that feed efficiency (g/MJ) of both PF groups (PF/CH and PF/FAT) was significantly lower than for the C group during the protein-free feeding period. However, during the first 3 d of realimentation, feed efficiency was significantly higher for both PF groups compared to both age and weight controls. During the final 4 d of the realimentation period, feed efficiencies for both the PF groups were similar to the C-C group.

Data of muscle weights and proteinase activities are shown in TABLE 3.3. Wet weight of both muscles increased significantly with age in the different control groups (Initial, C and C-C group). Wet weight of L was significantly lower for either PF groups compared to the C group in both the protein-free feeding and the realimentation period. The wet weight of M muscle did not change as a consequence of either dietary treatment or period. There was no effect of age on both muscles for the control groups when expressed as a percentage of BW. During the protein-free feeding period, relative weights in both L and M muscles were not different between treatments. However, during the realimentation period, relative L weight was significantly higher for C-C group than for both the PF/CH-C and PF/FAT-C group. Relative weight of M muscle was similar between treatments in the realimentation period.

No complete resolution between calpastatin and μ-calpain in M muscle could be reached. Therefore, activities of calpastatin and μ-calpain in M muscle are not presented. Activities of the calpain system in L muscle (calpastatin, μ- and m-calpain) were not influenced by either dietary treatment or experimental period except for calpastatin during the protein-free feeding period. Feeding a PF/FAT diet caused a lower calpastatin activity than for both C and PF/CH groups.

There were no significant differences in MCP activity in L muscle as a consequence of dietary treatment or experimental period. The MCP activity was higher in M muscle for PF/CH group than for PF/FAT group in both experimental periods; C and C-C group were intermediate.

TABLE 3.4 shows the results of protein and nucleic acid concentration of M and L muscles. Protein concentration in M muscle and DNA concentration in both muscles were significantly influenced by age. Ratios of protein:DNA and RNA:DNA in L muscle were significantly increased by age. Protein concentration of both muscles was similar between dietary treatments and experimental periods. RNA concentration in M muscle was significantly lower for both PF groups than for C group during the protein-free feeding period. This difference in RNA concentration of M muscle between protein-free treatments was not seen

during the subsequent realimentation period. For L muscle, RNA concentration did not change as a consequence of dietary treatment or experimental period. DNA concentration in either muscle was not significantly changed by dietary treatment or experimental period. During the protein-free feeding period a different dietary treatment (PF/CH or PF/FAT) did not effect the protein:DNA ratio. However, at the end of the realimentation period, the PF/FAT-C group had a significant higher protein:DNA ratio in L muscle than the PF/CH-C group; the C-C group was intermediate. In M muscle, no effects of treatment were found in protein:DNA ratio in either period. During the protein-free feeding period, both PF groups showed a significantly lower RNA:DNA ratio in M and L muscles than the C group. However, after realimentation no differences were observed in RNA:DNA ratio between treatments for both muscles. As a consequence of feeding either one of the protein-free diets, the RNA:protein ratio in both muscles was significantly lower compared to the C group. After the realimentation period differences in RNA:protein ratio between treatments in both muscles were no longer found.

TABLE 3.3 Muscle weights and proteinase activities in porcine skeletal muscles during a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[abcd]

Trait	Initial	Protein-free treatments			Realimentation treatments		
		C	PF/CH	PF/FAT	C-C	PF/CH-C	PF/FAT-C
Wet weight, g							
Longissimus[e]	696 (14)	776 (38)[d]	667 (25)[c]	599 (23)[c]	896 (49)[d]	690 (43)[c]	708 (36)[c]
Masseter[e]	29.7 (1.0)	38.9 (3.2)	33.2 (2.9)	32.0 (1.7)	40.5 (2.5)	36.4 (2.0)	37.4 (1.0)
Wet weight, % of BW							
Longissimus	4.02 (0.06)	4.06 (0.13)	4.01 (0.15)	3.71 (0.07)	4.28 (0.10)[d]	3.78 (0.16)[c]	3.80 (0.13)[c]
Masseter	0.17 (0.01)	0.20 (0.01)	0.20 (0.01)	0.20 (0.01)	0.19 (0.02)	0.20 (0.01)	0.20 (0.01)
Calpastatin, units/g of protein							
Longissimus		17.4 (1.1)[d]	17.6 (0.7)[d]	12.7 (1.1)[c]	11.7 (1.3)	13.7 (1.7)	12.1 (1.4)
μ-calpain, units/g of protein							
Longissimus		4.54 (0.46)	4.84 (0.80)	4.33 (0.59)	3.09 (0.63)	4.94 (0.83)	3.34 (0.25)
m-calpain, units/g of protein							
Longissimus		6.27 (0.48)	6.37 (1.12)	6.47 (1.21)	5.26 (0.40)	6.49 (0.76)	4.90 (1.20)
Masseter		11.40 (1.31)	12.93 (0.87)	11.98 (1.31)	12.69 (1.33)	14.31 (0.49)	11.32 (1.19)
MCP, units/μg of protein							
Longissimus		6.14 (0.55)	6.09 (1.07)	4.34 (0.38)	5.63 (0.49)	6.53 (0.43)	5.35 (0.68)
Masseter		4.67 (0.55)[c,d]	6.16 (0.61)[d]	4.19 (0.36)[c]	5.44 (0.42)[c,d]	6.48 (0.46)[d]	4.81 (0.30)[c]

[abcd] See Legend TABLE 3.2.

[e] Means of control groups (Initial, C, and C-C groups) differ ($P < 0.05$).

TABLE 3.4 Protein and nucleic acids concentrations in porcine skeletal muscles during a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[abcde]

Trait	Initial	Protein-free treatments			Realimentation treatments		
		C	PF/CH	PF/FAT	C-C	PF/CH-C	PF/FAT-C
Protein, mg/g of muscle							
Longissimus	205.6 (8.4)	184.7 (5.9)	188.3 (13.4)	202.8 (7.2)	206.4 (11.2)	190.0 (11.7)	220.9 (12.7)
Masseter[e]	230.6 (6.0)	207.0 (8.4)	195.8 (8.4)	208.1 (5.0)	194.9 (5.5)	186.4 (5.8)	202.6 (4.7)
RNA, μg/g of muscle							
Longissimus	397.5 (25.7)	392.1 (25.0)	344.8 (25.2)	367.7 (19.2)	441.5 (33.5)	392.7 (31.7)	425.5 (36.1)
Masseter	757.3 (48.3)	679.0 (43.2)[d]	557.5 (35.9)[c]	542.0 (20.0)[c]	665.2 (21.4)	687.1 (31.6)	647.6 (51.6)
DNA, μg/g of muscle							
Longissimus[e]	632.3 (21.7)	518.5 (14.6)	556.6 (35.1)	584.7 (18.3)	560.9 (34.3)	563.2 (40.2)	569.7 (32.9)
Masseter[e]	994.1 (30.4)	868.1 (34.2)	912.6 (55.8)	918.3 (46.8)	846.4 (23.1)	864.5 (27.5)	808.0 (29.3)
Protein/DNA							
Longissimus[e]	0.33 (0.01)	0.36 (0.01)	0.34 (0.01)	0.35 (0.01)	0.37 (0.01)[c,d]	0.34 (0.02)[c]	0.39 (0.02)[d]
Masseter	0.23 (0.01)	0.24 (0.01)	0.22 (0.01)	0.23 (0.01)	0.23 (0.01)	0.22 (0.02)	0.25 (0.03)
RNA/DNA							
Longissimus[e]	0.63 (0.04)	0.75 (0.03)[d]	0.62 (0.02)[c]	0.63 (0.02)[c]	0.79 (0.04)	0.70 (0.05)	0.75 (0.05)
Masseter	0.76 (0.05)	0.78 (0.02)[d]	0.61 (0.01)[c]	0.59 (0.02)[c]	0.79 (0.03)	0.80 (0.04)	0.80 (0.04)
RNA/Protein							
Longissimus	1.93 (0.09)	2.11 (0.08)[c]	1.84 (0.08)[d]	1.81 (0.07)[d]	2.13 (0.10)	2.07 (0.11)	1.91 (0.08)
Masseter	3.29 (0.20)	3.28 (0.16)[c]	2.84 (0.09)[d]	2.61 (0.08)[d]	3.43 (0.13)	3.69 (0.12)	3.19 (0.23)

[abcde] See legend TABLE 3.3.

FIGURE 3.2 presents data of 3MH production rate (μmol.kg^{-1}.d^{-1}) and fractional breakdown rate (%/d) for both the protein-free feeding and realimentation periods. Between treatments during the protein-free feeding period, 3MH production rate and FBR were significantly higher for both PF-groups than for the C group. During the subsequent realimentation period, no differences in 3MH production rate and FBR were observed between the treatments.

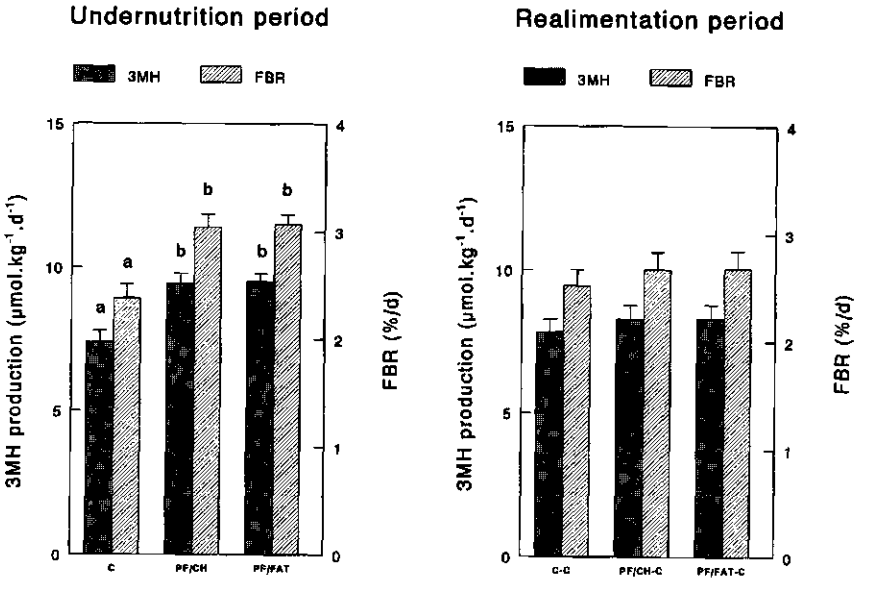

FIGURE 3.2 Mean 3-methylhistidine production rate (3MH, μmol.kg^{-1}.d^{-1}) and myofibrillar fractional breakdown rate (FBR, percentage/d) during the protein-free feeding period (d 0 to d 14) and the subsequent realimentation period (d 15 to d 21). The protein-free treatments were: C = control diet for 14 d; PF/CH = protein-free/carbohydrate-rich diet for 14 d; PF/FAT = protein-free/fat-rich diet for 14 d. The realimentation treatments were: C-C = C followed by C for 7 d; PF/CH-C = PF/CH followed by C for 7 d and PF/FAT-C = PF/FAT followed by C for 7 d. Means within group parameters (3MH or FBR) in each experimental period having a different letter differ ($P < 0.05$).

Discussion

The present experiment was undertaken to investigate 3-methylhistidine production and proteinase activity in porcine skeletal muscle under a protein-free feeding period and a subsequent realimentation period. A protein-free feeding period was realised by providing 2 different protein-free diet for 14 d. During the realimentation period, all pigs were given the control diet for another 7 d. We expected compensatory growth to occur during the realimentation period and because our interest was in the involvement of several muscle proteinases in myofibrillar protein degradation, short term effects were studied. In the next article, whole body composition and blood parameters as indicators for total protein metabolism under these circumstances are discussed (Van den Hemel-Grooten *et al.* 1996).

Compensatory growth

Data of growth rate (TABLE 3.2) show that feeding a protein-free diet stopped growth, which is in agreement with our previous finding (Van den Hemel-Grooten *et al.* 1995). After realimentation for 7 d, growth rate of both PF-groups increased again to values similar to age controls, but animals do not completely compensate at the end of the 7 d realimentation period (FIGURE 3.1). This suggests that compensatory growth, defined as the greater than normal growth compared to age controls, has not occurred (Ryan, 1990; Samuels & Baracos, 1995). However, if only the first 3 d of the realimentation period are considered, growth rate of both PF-groups was significantly higher than in age controls, which suggests that compensation only occurred during the first few days of realimentation. Besides comparison of growth rate with those of age controls, it can also be done with weight controls. Since the same body weight range for weight controls is not available, two options are present: d 6 to d 10 or d 11 to d 14. However, we can not rule out the possibility that some compensatory growth has occurred also in the C group, because of the decreased feed intake during the first few days of the experiment (adjustment to the feed). Therefore, the best comparison should be made with growth rate between d 11 and d 14. Statistical analysis shows that growth rate of both PF-groups during first 3 d of realimentation was significantly increased compared to C group form d 11 to d 14.

Differences between protein-free diets

In order to rule out the possibility that differences between C and PF/CH group could be due to increased amount of carbohydrates in the diet and not a lack of dietary protein, another protein-free diet was also used, in which dietary protein was isocalorically exchanged against dietary fat. Results show that most of the parameters measured in this study reveal similar effects for the two

protein-free diets. However, for proteinase activities in both muscles there was a difference in both PF-diets. Calpastatin in L muscle during protein-free feeding period and MCP activity in M muscle in both periods were significantly higher for PF/CH treatment than for PF/FAT treatment. Growth rate, feed efficiency and FBR were not different between both PF diets. This means that changes for these parameters between controls and PF groups were indeed caused by a lack of dietary protein. However, feeding either one of the protein-free diets did change some of the proteinase or inhibitor activities. This means that another component than the lack of dietary protein is responsible for these effects.

Myofibrillar protein degradation rate during both periods

Results in FIGURE 3.2 show that feeding either one of the protein-free diets caused significantly higher rates of FBR than C group. This increase is in agreement with our previous findings (Van den Hemel-Grooten *et al.* 1995) and others (Kadowaki *et al.* 1989). The FBR rates, however, were higher in the previous experiment than in the present experiment, *e.g.*, 3.02%/d compared to 2.38%/d for controls (Van den Hemel-Grooten *et al.* 1995), but the increase of 27% of C group to both PF groups was similar between both experiments. This difference in FBR rates can be explained by the different selection lines that were used. A disadvantage of using 3MH as a marker for myofibrillar protein degradation is that no distinction can be made between production form smooth muscle (gastro-intestinal tract and skin) and skeletal muscle. Therefore, it is also possible that the contribution of 3MH from smooth muscles to the total 3MH production was increased during the protein-free feeding period.

During the realimentation period, no significant differences in FBR were observed between treatments. Samuels & Baracos (1995) have shown in their study with weanling rats that compensatory growth after infection occurs within a couple of days by reducing fractional breakdown rates of skeletal muscle proteins. So, the reason why we did not find any difference in FBR between treatments may be that measurements should have been performed sooner. Goodman & Del Pilar Gomez (1987) have shown that fasting for 1 d increased and refeeding decreased 3MH excretion to control values again using a perfused hindquarter of growing rats. Li & Wassner (1984) state that an increase in muscle mass of rats during refeeding is due to a deceleration of the rate of protein degradation rather than an above normal rate of protein synthesis.

Activities of muscle proteinases during both experimental periods

During the protein-free feeding period, calpastatin activity in L muscle of PF/FAT group was significantly lower than in both C and PF/CH groups. In our previous experiment, we also did not find differences in calpastatin activity between C and PF/CH groups (Van den Hemel-Grooten *et al.* 1995). A lower

calpastatin activity of the PF/FAT group would suggest an increased rate of muscle protein degradation if calpain activities are not increased. This could explain a lower relative wet weight, although this difference was not statistically different. For the other proteinases of the calpain system, no effects were seen by dietary treatment or experimental period. This is in agreement with our previous finding (Van den Hemel-Grooten *et al.* 1995) and it was suggested that the calpain system is probably not influenced by dietary treatments as performed in our studies.

The activity of MCP in L muscle in the present experiment was not affected by either dietary treatment or experimental period. This is not in agreement with our previous experiment, in which MCP activity was decreased as a consequence of feeding a protein-free diet. We have no explanation for this. The MCP activity in M muscle was only decreased by feeding a PF/FAT diet and this effect was still present after 1 week of realimentation. This means that MCP activity is altered by a PF/FAT diet, but this change is not restored during a realimentation period of 7 d.

Although measured enzyme activities were not different between treatments it could have been that mRNA levels were increased during the protein-free feeding period as is suggested by Ilian and Forsberg (1992, 1994). They showed in their studies with 8-d fasted rabbits that mRNA levels of μ- and m-calpain and calpastatin were increased 3- to 4-fold compared to controls without a difference in enzyme activities between treatments. They suggested that turnover rates of calpains and calpastatin were increased. In addition, it should be kept in mind that proteinase and inhibitor activities were measured *in vitro*, which means that maximal capacity in both muscle was measured, which may not represent their physiological activity.

Underlying mechanism responsible for compensatory growth

Mechanisms responsible for increased growth rate after a period of nutritional limitation are not well known. Feed efficiency of both PF-groups was significantly increased during the first 3 d of realimentation compared to C-C group. During the last 4 d of the realimentation period feed efficiency became similar for all treatments, which suggests that this adaptation has stopped. An increase in feed efficiency can cause the increased growth rate, but the underlying mechanism for this is unknown. It is suggested that a higher feed intake is responsible for compensatory growth (Ryan, 1990). But data of the present study do not indicate this, because feed intake was similar between treatments (2.5 times energy for maintenance for control animals). This is supported by other observations after protein restriction (Zimmerman & Khajarern, 1973) and feed restriction (Prince *et al.* 1983) in pigs. Data of DNA, RNA, and protein concentration in both muscles can also give information about growth processes. The RNA:protein ratio is indicative for capacity for protein

synthesis (Sugden & Fuller, 1991). Data of the present experiment show that this capacity is decreased in both muscles during a period of feeding a protein-free diet, which is also in agreement with our previous findings (Van den Hemel-Grooten *et al.* 1995). However, during realimentation there were no differences in both muscles in RNA:protein ratio between treatments. This suggests that an adaptation has indeed occurred within the period of realimentation. A similar pattern is observed for the RNA:DNA ratio in either muscle, which is indicative for the transcriptional efficiency of the muscle cell.

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4

Effects of a protein-free feeding period and a subsequent realimentation period on protein metabolism in growing pigs 1. Chemical composition of carcass and organs and plasma parameters as indicators for whole body protein metabolism

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Abstract

This experiment was conducted to study changes in fat and protein concentration in body compartments during a protein-free feeding period of 14 d and a subsequent realimentation period of 7 d. Six crossbred barrows (35 kg on d 0) were slaughtered on d 0 to serve as s reference group and 48 barrows were randomly divided over 6 treatments (8 animals each). During the protein-free feeding period of 14 d three different isocaloric diets were offered: C diet (control diet; C group), PF/CH diet (protein-free/carbohydrate-rich diet; PF/CH group), PF/FAT diet (protein-free/fat-rich diet; PF/FAT group). During the realimentation period, all animals received the control diet and formed the other 3 groups (C-C, PF/CH-C, and PF/FAT-C). All diets were provided at 2.5 times energy for maintenance and water was available *ad libitum*. On d 14 and d 21 animals were slaughtered and right side of the carcass, liver, and both large and small intestines were weighed and analyzed for ash, fat, dry matter, and nitrogen content. Feeding either one of the protein-free diets caused fattening of carcass but not liver, whereas protein concentration of carcass, liver, and intestines decreased significantly. This impaired protein status was also reflected in decreased urea and creatine plasma levels. After realimentation for 7 d, protein concentration of carcass was not fully restored, which was also seen in creatine levels of plasma. Protein concentration in both liver and intestines was similar between treatments. Results of present study with growing pigs

suggest that protein metabolism in liver and intestines is responding rapidly to dietary changes, whereas adaptation in carcass is more slowly.

Introduction

Studies on changes of body composition during different physiological and nutritional circumstances can provide important information on fat and protein deposition and shifts of different components between organs and tissues. Conflicting results have been reported on adaptations of growing pigs to a diet with a protein concentration lower than recommended. Feeding a low protein diet to growing pigs decreased growth rate, feed efficiency and protein deposition in carcass, thereby increasing carcass fat concentration (10% crude protein, Zimmerman and Khajarern, 1973; 14 and 16% crude protein, Tyler *et al.* 1983; 13 and 17% crude protein, Adeola *et al.* 1993). However, Wahlstrom and Libal (1983) did not observe a change in carcass characteristics in growing pigs after dietary protein restriction (12 and 14% crude protein). These apparently conflicting reports can not be compared because of differences in experimental design other than dietary protein level, like sex, feeding level, age, and duration of restriction. These concerns are also valid for effects seen on chemical body composition during compensatory responses after a period of dietary protein restriction (Zimmerman and Khajarern, 1973; Hogberg and Zimmerman, 1978; Wahlstrom and Libal, 1983).

Literature on chemical composition of carcass and/or organs of growing pigs after feeding a protein-free diet is scarce. Interest in effects of feeding protein-free diets to young pigs on chemical composition of body compartments originates from research which is focused on investigating underlying mechanisms and regulation of protein degradation of skeletal muscle. Therefore, we also studied several proteolytic enzyme systems in skeletal muscles and myofibrillar protein degradation under these circumstances (Van den Hemel-Grooten *et al.* 1996).

Besides chemical composition, blood parameters can give information on protein status of the body (albumin, urea) and on muscle mass in the body (creatinine). Plasma urea levels are highly and inversely correlated with the net protein utilization of a diet and have been used as a predictive indicator of amino acid nutrition status of an animal (Cai *et al.* 1994). Creatine is produced in liver from glycine, arginine, and methionine and absorbed from blood by muscle, which contains 98% of total creatine. In muscles, creatine is converted

into creatinine, which is excreted by the urine. Therefore, plasma levels express the quantity produced, if renal filtration is not impaired (Hanset and Michaux, 1986).

The objective of the present study was to examine changes in protein and fat concentrations in body compartments (carcass, liver, and intestines) during a protein-free feeding and a subsequent realimentation period. Furthermore, to determine whether energy source, *e.g.*, fat or starch, influences effects of feeding a protein-free diet.

Materials and methods

Experimental design

Fifty-four crossbred barrows, Large White x (Dutch Landrace x Large White) were housed individually. After an adaptation period to the housing system of 10 d, the experiment started. Six pigs were slaughtered at the start of the experiment and served as a reference group. The remaining 48 barrows were then randomly divided over 6 treatments, in a way that their mean body weights were similar. The experiment consisted of 2 periods: a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21). During the first period 3 different isocaloric diets were offered: control diet (21% of ME consisted of dietary protein; control group), a protein-free/carbohydrate-rich diet (protein was exchanged against carbohydrates; PF/CH group) and a protein-free/fat-rich diet (protein was exchanged against fat; PF/FAT group). On d 14, 8 animals in each treatment were slaughtered and all remaining animals received the control diet and they formed the other 3 treatments: C-C group, PF/CH-C group, and PF/FAT-C group. These animals were slaughtered on d 21. From d 0 of the experiment, animals received their diets at 2.5 times energy for maintenance for controls (twice a day) and water was available *ad libitum*. A more detailed description of the experimental design and the composition of the diets have been presented elsewhere (Van den Hemel-Grooten *et al.* 1996).

Slaughter procedures and chemical analyses

After overnight fast, animals were killed by using a captive bolt followed by exsanguination. After being removed form the body weights of liver, and both small and large intestines (after being flushed with water) were recorded. Both sides of the carcass were also weighed (hot carcass weight). The right side of

the carcass, and liver, and intestines were stored separately in a plastic bag at -20 °C for later chemical analysis. The frozen carcass, liver, and intestines were homogenised separately in a 45 l (carcass) or 15 l (intestines or liver) cutter. Each fraction was subsampled and frozen at -20°C until further analysis of dry matter, ash, fat, and nitrogen. Dry matter content of the carcass was determined after drying samples in a vacuum oven at 50°C and a vacuum of 10 kPa using anhydrous calcium chloride as the drying agent according to ISO 6496 (1983). After 16 h, the vacuum was changed to 2 kPa and the samples were weighed every 2 h until constant weight. Dry matter content of liver and intestines were obtained after 36 h of freeze drying. Ash content in all samples was analyzed by burning oven-dried samples in a muffle furnace at 550°C according to ISO 5984 (1978). Lipid content in all samples was assessed by extraction of freeze-dried samples with petroleum ether and drying the extract at 105°C to a constant weight according to ISO 6492 (1985). Nitrogen content in all samples was assayed in the fresh samples by Kjeldahl analysis according to ISO 5983 (1979). Protein content of the samples was calculated by multiplying nitrogen concentration by 6.25.

Measurement of plasma parameters

Plasma was prepared from blood samples taken at 0, 12, 24, 36, 48, 60, and 72 h before the end of each experimental period from a catheter which was placed into the ear vein as described elsewhere (Van den Hemel-Grooten *et al.* 1996). Creatine and creatinine were determined using a kit for creatinine (creatinine-PAP; Boehringer, Mannheim, Germany) according to the method of Siedel *et al.* (1984). Urea content was determined according to Wybenga *et al.* (1971) and albumin content was determined according to Doumas *et al.* (1971).

Calculations and statistical analysis

Deposition of chemical components during the protein-free feeding period was calculated from the difference between measured composition on d 14 and the corrected chemical composition on d 0. Corrected chemical composition on d 0 for C, PF/CH, and PF/FAT groups was calculated by correcting for a difference in body weight on d 0. Deposition of chemical components during the realimentation period was calculated from the difference between measured composition on d 21 and the corrected chemical composition on d 14. A similar correction was used as for protein-free feeding period: corrected chemical composition of C-C, PF/CH-C, and PF/FAT-C groups at d 14 were calculated by correcting chemical composition of C, PF/CH, and PF/FAT groups for a

difference in body weight on d 14.

Data of plasma parameters were averaged per animal, because there were no significant differences between times of sampling.

Within each combination of treatments (C, PF/CH, and PF/FAT or C-C, PF/CH-C, and PF/FAT-C) data were analyzed by analysis of variance (ANOVA; one-way) followed by Tukey-B-test, in which the treatment was the independent factor using SPSS-PC$^+$, V 3.0 program (SPSS, 1988). Results were considered statistically different when P-value was less than 0.05.

Results

Results of carcass, liver, and intestines weights are presented in TABLE 4.1. Carcass weight was not different between treatments in both the protein-free feeding period and the realimentation period. However, expressed as a percentage of body weight, carcass weight of both PF groups was significantly higher than that of C group. After realimentation, no differences were observed between treatments. During the protein-free feeding period, liver weight was significantly lower for PF/FAT group than for C group; PF/CH group was intermediate. After 7 d of realimentation, no differences in liver weights were observed between treatments. Expressed as a percentage of body weight, liver weights were unaffected by dietary treatments in the protein-free feeding period, but after realimentation liver weight of PF/FAT group was significantly higher than for both C-C and PF/CH-C groups. For intestines, results show that providing either one of the PF diets caused a significant decrease in both absolute and relative weights. After 7 d of realimentation, no differences were observed any more in weights of intestines.

TABLE 4.1 Body weights and weights of carcass, liver, and intestines of growing barrows after a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[a,b]

Trait	Initial	Protein-free treatments			Realimentation treatments		
		C	PF/CH	PF/FAT	C-C	PF/CH-C	PF/FAT-C
Body weight, kg							
day 0		35.1 (1.7)	36.8 (1.4)	35.0 (0.9)	34.8 (2.1)	35.4 (1.4)	35.5 (1.6)
at slaughter	34.7 (0.7)	38.4 (2.1)[d]	33.5 (1.3)[c]	32.3 (0.9)[c]	41.9 (2.1)[d]	36.5 (1.7)[c]	37.3 (1.6)[c,d]
Carcass weight							
kg	26.9 (0.4)	29.4 (1.6)	26.0 (1.0)	25.4 (0.7)	32.6 (1.7)	28.0 (1.5)	28.4 (1.1)
% of BW	77.8 (0.5)	76.4 (0.4)[c]	77.8 (0.4)[d]	78.5 (0.4)[d]	77.7 (0.4)	76.8 (0.5)	76.2 (0.5)
Liver weight							
kg	0.75 (0.05)	0.66 (0.04)[d]	0.57 (0.03)[c,d]	0.53 (0.02)[c]	0.69 (0.03)	0.60 (0.02)	0.73 (0.05)
% of BW	2.16 (0.13)	1.71 (0.05)	1.70 (0.06)	1.64 (0.05)	1.64 (0.04)[c]	1.67 (0.07)[c]	1.95 (0.09)[d]
Intestines weight							
kg	1.59 (0.05)	1.71 (0.05)[d]	1.36 (0.06)[c]	1.31 (0.03)[c]	1.79 (0.08)	1.69 (0.05)	1.74 (0.10)
% of BW	4.57 (0.10)	4.52 (0.17)[d]	4.07 (0.13)[c]	4.07 (0.07)[c]	4.29 (0.16)	4.68 (0.15)	4.66 (0.19)

[a] Data are expressed as MEAN (SEM) for 6 pigs for initial group and 8 pigs for other treatments.

[b] C = control diet for 14 d; PF/CH = protein-free/carbohydrate-rich diet for 14 d; PF/FAT = protein-free/fat-rich diet for 14 d; C-C = C followed by C diet for 7 d; PF/CH-C = PF/CH followed by C diet for 7 d and PF/FAT-C = PF/FAT followed by C diet for 7 d.

[c,d] Means within a combination of treatments lacking a common superscript letter differ ($P < 0.05$).

Data of chemical composition (gram/kilogram body weight) of carcass, liver, and intestines after a protein-free feeding period and a subsequent realimentation period are shown in TABLE 4.2. After the protein-free feeding period, ash concentration of carcass was significantly higher for both PF groups than for C group. Carcass of PF/FAT group contained significantly more fat and less protein than C group; PF/CH group was intermediate. Dry matter concentration of carcass was not different between treatments. For liver, fat and protein concentration was significantly lower for both PF groups than for C group. Ash concentration in liver of PF/FAT group was significantly lower than for C group; PF/CH group was intermediate. Dry matter concentration of liver was not different between treatments. For intestines, both ash and protein concentration were significantly lower for both PF groups than for C group. Fat and dry matter concentration in intestines did not differ between treatments. After realimentation, protein concentration in carcass was significantly lower for both PF groups than for C-C group. Ash, fat, and dry matter concentration in carcass were not different between treatment. For liver, differences were only observed in ash concentration: PF/FAT-C group contained significantly more ash than PF/FAT group. No differences in chemical composition of intestines were observed between treatments.

TABLE 4.3 presents data of deposition (grams/day) of ash, fat, dry matter, and protein during both experimental periods in carcass, liver, and intestines. During the protein-free feeding period, protein deposition in carcass was significantly lower for both PF groups than for C group. Deposition of ash, fat or dry matter in carcass did not differ between treatments. For liver, deposition of all components was significantly lower for both PF groups than for C group. For intestines, ash and protein deposition were significantly higher for C group than for both PF groups. Dry matter deposition in intestines was significantly higher for C group than for PF/CH group; PF/FAT group was intermediate. Fat deposition in intestines did not differ between treatments. During realimentation, deposition of all components in carcass was not different between treatments. For liver, deposition of ash, fat, and protein was significantly higher for both PF groups than for C-C group. Dry matter deposition in liver was significantly higher for PF/FAT-C group than for both C-C and PF/CH-C groups. For intestines, ash deposition was significantly higher for both PF groups than for C-C group.

TABLE 4.2 Chemical composition (grams/kilogram body weight) of carcass, liver and intestines of growing barrows after a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[a,b]

Trait	Initial	Protein-free treatments			Realimentation treatments		
		C	PF/CH	PF/FAT	C-C	PF/CH-C	PF/FAT-C
Carcass, g/kg BW							
ash	25.9 (0.4)	26.2 (0.4)[c]	30.2 (0.4)[d]	29.8 (0.6)[d]	26.3 (0.6)	28.5 (0.6)	28.4 (0.4)
fat	127.4 (2.4)	123.4 (6.5)[c]	145.3 (12.8)[c,d]	168.7 (5.6)[d]	134.3 (7.8)	144.2 (12.0)	151.7 (4.6)
dry matter	295.1 (2.2)	290.8 (6.6)	316.1 (11.0)	340.3 (6.1)	303.9 (7.2)	307.6 (9.7)	314.3 (3.7)
protein	133.3 (1.2)	135.6 (1.3)[d]	134.5 (3.0)[c,d]	132.1 (2.2)[c]	137.3 (1.9)[d]	127.8 (2.9)[c]	127.5 (1.8)[c]
Liver, g/kg BW							
ash	0.30 (0.01)	0.26 (0.01)[d]	0.25 (0.01)[c,d]	0.24 (0.01)[c]	0.25 (0.01)[c,d]	0.25 (0.01)[c]	0.29 (0.01)[d]
fat	0.60 (0.02)	0.46 (0.03)[d]	0.32 (0.01)[c]	0.35 (0.02)[c]	0.43 (0.03)	0.44 (0.03)	0.50 (0.03)
dry matter	6.64 (0.40)	5.07 (0.13)	4.91 (0.18)	4.71 (0.14)	4.84 (0.09)	4.90 (0.20)	5.54 (0.25)
protein	4.14 (0.15)	3.80 (0.11)[d]	3.20 (0.07)[c]	3.27 (0.13)[c]	3.68 (0.10)	3.58 (0.12)	3.96 (0.16)
Intestines, g/kg BW							
ash	0.48 (0.01)	0.46 (0.02)[d]	0.38 (0.02)[c]	0.37 (0.01)[c]	0.42 (0.02)	0.44 (0.01)	0.47 (0.02)
fat	2.23 (0.15)	2.26 (0.23)	2.91 (0.33)	3.25 (0.31)	2.73 (0.31)	3.19 (0.35)	3.07 (0.25)
dry matter	9.10 (0.28)	8.96 (0.42)	8.76 (0.53)	9.00 (0.38)	9.04 (0.41)	9.75 (0.38)	9.84 (0.41)
protein	6.04 (0.16)	5.90 (0.24)[d]	5.19 (0.21)[c]	5.00 (0.13)[c]	5.49 (0.22)	5.72 (0.11)	5.91 (0.15)

[abcd] See legend to TABLE 4.1.

TABLE 4.3 Ash, fat, dry matter, and protein deposition (grams/day) in carcass, liver, and intestines of growing barrows during a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[a,b]

	Protein-free treatments						Realimentation treatments					
Trait	C		PF/CH		PF/FAT		C-C		PF/CH-C		PF/FAT-C	
Carcass, g/d												
ash	6.95	(2.06)	4.18	(1.38)	3.88	(1.96)	11.95	(3.49)	7.18	(3.49)	8.15	(1.50)
fat	20.76	(21.26)	12.79	(35.37)	71.50	(17.46)	122.10	(50.92)	86.82	(68.73)	-0.77	(30.41)
dry matter	61.39	(29.24)	-19.41	(35.53)	48.55	(23.19)	209.24	(45.60)	142.28	(58.03)	40.33	(29.87)
protein	38.56	(9.61)[d]	-29.10	(5.35)[c]	-28.22	(4.68)[c]	71.18	(15.65)	40.64	(14.10)	44.85	(8.12)
Liver, g/d												
ash	-0.04	(0.03)[d]	-0.21	(0.03)[c]	-0.20	(0.02)[c]	0.04	(0.04)[c]	0.15	(0.04)[d]	0.36	(0.08)[d]
fat	-0.25	(0.09)[d]	-0.82	(0.05)[c]	-0.70	(0.05)[c]	0.05	(0.16)[c]	0.79	(0.15)[d]	1.01	(0.17)[d]
dry matter	-2.77	(0.60)[d]	-5.77	(0.68)[c]	-5.75	(0.40)[c]	0.87	(0.66)[c]	2.53	(1.00)[c]	6.91	(1.51)[d]
protein	0.08	(0.48)[d]	-3.27	(0.32)[c]	-2.82	(0.32)[c]	0.82	(0.64)[c]	3.70	(0.54)[d]	5.41	(0.97)[d]
Intestines, g/d												
ash	0.04	(0.05)[d]	-0.35	(0.05)[c]	-0.35	(0.05)[c]	0.01	(0.13)[c]	0.53	(0.06)[d]	0.73	(0.12)[d]
fat	0.48	(0.67)	1.06	(0.90)	1.83	(0.71)	4.30	(2.16)	3.18	(1.83)	0.93	(0.46)
dry matter	1.44	(1.20)[d]	-3.11	(1.53)[c]	-2.12	(1.02)[c,d]	5.04	(2.36)	10.15	(2.07)	9.48	(2.54)
protein	0.86	(0.71)[d]	-3.54	(0.60)[c]	-3.59	(0.40)[c]	0.34	(1.30)[c]	5.64	(0.51)[d]	7.53	(0.99)[d]

[abcd] See legend to TABLE 4.1.

TABLE 4.4 Plasma parameter levels of young growing barrows during a protein-free feeding period (d 0 to d 14) and a subsequent realimentation period (d 15 to d 21)[abcd]

	Protein-free treatments			Realimentation treatments		
Trait	C	PF/CH	PF/FAT	C-C	PF/CH-C	PF/FAT-C
Creatinine, mmol/l	0.84 (0.04)[c]	1.11 (0.05)[d]	1.08 (0.03)[d]	0.79 (0.03)	0.75 (0.05)	0.81 (0.03)
Creatine, mmol/l	1.47 (0.12)[d]	0.69 (0.15)[c]	0.90 (0.19)[c]	1.94 (0.22)[d]	1.04 (0.10)[c]	1.21 (0.12)[c]
Urea, mmol/l	5.69 (0.37)[d]	1.94 (0.18)[c]	1.79 (0.27)[c]	5.57 (0.32)	6.48 (0.29)	5.53 (0.35)
Albumin, mg/100 ml	28.85 (1.29)	27.64 (1.70)	27.54 (1.84)	32.27 (0.70)[d]	26.15 (1.57)[c]	28.26 (1.38)[c,d]

[abcd] See legend to TABLE 4.1.

During the protein-free feeding period, plasma creatine and urea concentrations were significantly lower for both PF groups than for C group (TABLE 4.4). Creatinine was significantly higher for both PF groups than for C group. Albumin concentration was not influenced by either one of the protein-free diets. During the realimentation period, creatinine and urea concentrations were similar for all treatments, but creatine was still lower for both PF groups than for C-C group. Albumin was significantly lower in PF/CH-C group compared to C-C group.

Discussion

Purpose of this study was to determine changes in chemical composition of carcass, liver, and intestines of growing pigs during a protein-free feeding period and a subsequent realimentation period. It was concluded that some compensation occurred during the first 3 d of the realimentation period based on growth rate and feed efficiency data (growth rate: 0.43, 0.63, and 0.65 kg/d and feed efficiency: 28.4, 49.5, and 51.3 g/MJ ME for C-C, PF/CH-C, and PF/FAT-C groups, respectively) (Van den Hemel-Grooten *et al.* 1996). Although limited compensatory response was seen in body weight, compensation in specific body components could also provide important information.

Chemical changes during the protein-free feeding period

Feeding either one of the protein-free diets caused reduced body weight gain (Van den Hemel-Grooten *et al.* 1996). This reduction in weight was mainly due to a decreased growth rate of liver and intestines, because those weights were even less than for the initial slaughter group; carcass weight remained similar during the 14-d feeding trial. However, it should be noted that liver weight of C group was also lower than initial group. This was probably due to *ad libitum* feeding during rearing. Apparently, liver weight is more dependent on feeding level than carcass or intestines.

Data of chemical composition show that the carcass of PF/FAT group contained more fat than of C group; PF/CH group was intermediate. This is similar to studies in which dietary protein level was reduced to minimal 12 g/100 g (Zimmerman and Khajarern, 1973, Tyler *et al.* 1983, Adeola *et al.* 1993). Because of a lack of essential amino acids protein deposition is impaired and thus the energy in the diet can only be deposited as body fat. However, fat deposition decreased in liver for both PF groups and in intestines it did not differ

between treatments. Calculation of fat deposition (g/d) in all body compartments analyzed in this study showed that fat deposition is not significantly different between treatments. This indicates that there was a shift in body fat between tissues. Protein concentration was decreased in all compartments of both PF groups compared to C group, which was also indicated by data of protein deposition. Decreased urea levels in plasma of both PF groups also reflect decreased protein status of these animals. Plasma albumin concentrations are also used to determine protein status of the body. Results of present study indicate that feeding either one of the PF diets for 14 d did not change albumin concentration in plasma. This is not in agreement with Pond *et al.* (1980), who found reduced levels in young pigs during dietary protein restriction at a level of 12 g/100 g crude protein for 8 weeks. This suggests that albumin is more indicative for prolonged protein deficiency. Since 1 to 2% of creatine is converted into creatinine each day, creatinine levels in plasma is indicative for the amount of skeletal muscle if renal filtration is not impaired (Hanset and Michaux, 1986). Feeding either one of the PF diets increased level of creatinine compared to C group, which would suggest a higher amount of muscle for both PF groups. However, this is not very likely, since chemical composition of carcass indicated that protein concentration was highest for C group, followed by PF/CH and then PF/FAT group, suggesting that amount of skeletal muscles has decreased. This is also in agreement with the finding that myofibrillar fractional breakdown rates of both PF groups was increased compared to C group (Van den Hemel-Grooten *et al.* 1996). Therefore, the increased creatinine levels for both PF groups can be due to increased breakdown of muscle proteins which also releases creatinine. Creatine levels of both PF groups were significantly lower than for C groups, which can be explained by the lack of the essential amino acid methionine.

Changes during the realimentation period

Body weight gain was only significantly different during the first 3 d of the realimentation period, suggesting limited compensatory growth response of both PF-C groups as discussed before (Van den Hemel-Grooten *et al.* 1996). Weights of carcass, liver, and intestines were also not significantly different between treatments. However, liver weight as a percentage of body weight was significantly higher for PF/FAT-C than for C-C and PF/CH-C group. Data of chemical composition of liver do not indicate that there was a change in composition, except for increased ash concentration. Deposition rates also give no explanation for this increased relative liver weight of PF/FAT-C group.

At the end of the realimentation period, protein concentration of carcass is not fully restored again, but liver and intestines have similar protein concentrations between treatments. This means that protein compensation has occurred in liver and intestines, but in carcass compensation was not complete. Data of protein deposition rate in liver and intestines confirm this by significantly higher rates for both PF-C groups compared to C-C group. It is suggested that an increased protein efficiency during realimentation can explain the phenomenon of compensatory growth (Ryan, 1990). Protein efficiency was similar between treatments, *e.g.*, 27.8, 25.3 and 30.3% for C-C, PF/CH-C, and PF/FAT-C group, respectively. However, protein efficiency is calculated over complete realimentation period, and compensation has probably only occurred during the first 3 d of this period as discussed before.

Plasma urea levels suggest that protein status of both PF-C groups was improved again. Creatinine levels in plasma, reflecting skeletal muscle mass, were increased again in both PF-C groups to C-C levels. Myofibrillar protein degradation was similar between treatments at the end of the realimentation period (Van den Hemel-Grooten *et al*, 1986), which points the same direction as creatinine levels in plasma. Creatine levels were still lower for both PF-C groups, which suggest that production may be still impaired under the present circumstances. Although albumin levels were not different between treatments during the protein-free feeding period they were significantly higher for C-C group than for PF/CH-C during realimentation. We have no explanation for this.

Taken together, after the protein-free feeding for 14 d protein deposition all body compartments studied is significantly lower for both PF groups than for C group. After subsequent realimentation for 7 d, protein concentration in both liver and intestines is fully restored and in carcass only partly. This suggests that liver and intestines are more rapidly depleted and restored than carcass. These effects are similar for both PF diets, which indicates that observed effects are indeed caused by lack of dietary protein and rule out the possibility that effects were influenced by energy source.

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5

Contribution of gastro-intestinal tract to whole-body 3-methylhistidine production in growing pigs

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Abstract

The aim of the present study was to estimate the contribution of the gastrointestinal (GI) tract in relation to whole-body 3-methylhistidine (3MH) production in growing pigs. Secondly, whether this contribution changes during a protein-free feeding period in pigs. Seven crossbred barrows with an initial weight of approximately 32 kg, were used in a cross-over design. The experiment consisted of two dietary treatments (control diet or protein-free diet) and two experimental periods, which were 14 d in duration. The pigs were surgically cannulated with an indwelling catheter placed in the portal vein (P) and carotid artery (A). Diets were offered at a feeding level of 2.5 times energy for maintenance in four portions at 6 h interval each day. Data were analyzed with analysis of variance with dietary treatment and experimental period as main effects. Experimental period had no effect on any of the measured parameters. Difference in 3MH production rate as measured from P and A sampling and calculated from 3MH kinetics using a published compartmental computer model did not differ significantly between treatments. The contribution of GI tract to whole-body 3MH production was less than 6%. and was not altered by the dietary treatments. Fractional breakdown rate (FBR, %/d) was significantly higher for protein-free group than for control group (2.56 and 2.16%/d, respectively). These results indicate that contribution of GI tract to whole-body 3MH production is not substantially and this contribution is not

influenced by a protein-free feeding period. Therefore it is concluded that increased 3MH production during a protein-free feeding period does not originates from an increased protein degradation in GI tract but mainly from increased breakdown of skeletal muscle.

Introduction

The use of urinary 3-methylhistidine (3MH) excretion as a measure for protein degradation of skeletal muscle was first proposed by Asatoor and Armstrong (1967). 3-Methylhistidine originates from the degradation of myofibrillar proteins, actin and myosin, in which specific histidine residues are posttranslationally methylated. Upon protein degradation, 3MH is released and not reutilized for protein synthesis because of a lack of a specific tRNA (Young *et al.*, 1972). Moreover, 3MH is not metabolized and therefore quantitatively excreted in urine in most species. However, urinary excretion of 3MH produced is not quantitatively in sheep and pigs, since part of it is stored in muscle as a dipeptide balenine (Harris and Milne, 1980; 1981). An approach to measure 3MH production was developed by Rathmacher *et al* (1996) to circumvent this problem. In this approach, disappearance of labelled 3MH, given as a bolus injection, is measured via serial blood sampling. Production rate of 3MH is calculated from 3MH kinetics using a compartmental computer model.

Over the years, there has been much discussion about the validity of using 3MH production as a marker for skeletal muscle protein catabolism. Muscle proteins can be divided into: sarcoplasmic (30-35%), myofibrillar (50-55%), and stroma proteins (15-20%) (Goll *et al.*, 1989). It is known from studies in rats that turnover of sarcoplasmic and myofibrillar proteins is regulated differently (Kadowaki *et al.*, 1989). Therefore, production of 3MH represents only myofibrillar protein degradation.

Another problem is that 3MH is also present in contractile proteins of smooth muscle, like in GI tract and skin. Although more than 90% of 3MH is supposed to be present in skeletal muscle (Nishizawa *et al.*, 1977; 1979), contribution of non-skeletal-muscle sources is also dependent on degree of turnover. Rennie and Millward (1983) stated that protein turnover in intestines is 20 times faster (at 29%/d) than in skeletal muscle, so even though intestines contain only 2% of the whole body pool of 3MH, it could account for over 20% of the excretion. Several studies in rats suggest that the contribution of 3MH from non-skeletal-muscle sources is between 17% (Nishizawa *et al.*, 1977) and even 75%

(Millward *et al.*, 1980). However, others state that smooth muscles determine not more than 10% of whole-body 3MH production (Haverberg *et al.*, 1975; Harris, 1981).

To our knowledge, no estimates have been reported on the contribution of GI tract to whole-body 3MH production in pigs. In order to estimate this, two indwelling catheters were surgically placed into the carotid artery (A) and portal vein (P). Determination of differences in 3MH production as measured from P and A provides information about the contribution of GI tract to whole-body 3MH production using the method of Rathmacher *et al.* (1996).

Objective of the present study was to investigate the magnitude of the contribution of GI tract to whole-body 3MH production. Moreover, to investigate if this contribution changes as a consequence of feeding a protein-free diet to growing barrows.

Materials and methods

Experimental design

This experiment was carried out at the TNO Toxicology and Nutrition Institute, Department of Animal Nutrition and Physiology (ILOB) in Wageningen, The Netherlands. The experimental design was approved by the TNO Committee for Animal Welfare.

Out of a group of ten crossbred barrows, Great Yorkshire x (Finnish Landrace x Dutch Landrace), seven animals with an initial weight of approximately 32 kg were used, since in these animals both catheters remained patent. The experimental design consisted of two dietary treatments (control or protein-free diet) and two periods of 14 d in a cross-over design. The control diet consisted of 173 g/kg crude protein (of non-animal origin), 48 g/kg fat, and 544 g/kg carbohydrates (14.0 MJ ME/kg). The protein-free diet was made by isocaloric exchange of dietary protein by carbohydrates. Composition of both experimental diets is described elsewhere (Van den Hemel-Grooten *et al.*, 1996a).

Animals were housed individually in metabolism cages. During the adaptation period of at least 14 d, all animals were fed the control diet. Throughout the experiment, daily diets were provided at 2.5 times energy for maintenance (460 kJ ME x metabolic weight) in four portions (2.00, 8.00, 14.00, and 20.00 h). Water was offered at a level of two times dry feed intake. After about 7 d in the adaptation period, two indwelling catheters were surgically introduced into carotid artery and portal vein according to the method of Van Leeuwen *et al.*

(1995).

After a recovery period of 7 d, the experimental period started. Animals were randomly divided over both treatments in a way that their mean body weight did not differ. The protein-free diet was offered gradually, *i.e.*, 75% and 25% on d 0, 50% and 50% on d 1, 25% control diet and 75% protein-free diet on d 2, respectively. On d 3, 100% protein-free diet was offered to the animals. On d 14, the second period started and animals received the other dietary treatment. Again, the change to the protein-free diet was gradually as described above. Body weights were recorded on d 0, 14, and 28.

Measurement of 3-methylhistidine (3MH) production rate and calculation of fractional breakdown rate (FBR)

During the last 3 days of each period, serial blood samples were taken from both catheters almost simultaneously in order to calculate 3MH production rate according to the method of Rathmacher *et al.* (1996). Briefly, a bolus injection of labelled 3-methylhistidine, L-N$^{\tau}$-methyl-d_3-histidine (d_3-3MH, 0.1371 mg/kg BW; MSD Isotopes, Montreal, Canada), was dissolved in 10 ml of sterile saline, injected into the portal vein and flushed with 2 ml of saline just after pigs received their morning meal on d 11. Subsequently, serial blood samples (8 ml) were taken at 1, 3, 5, 15, 30, 60, 90, 180, 360, 540, 720, 1440, 2160, 2880, 3600, and 4320 minutes from both catheters. Portal vein was sampled first and within 1 minute a sample was also taken from carotid artery. Blood samples were centrifuged for 15 min at 1500 x g and plasma was collected and stored at -20°C for further analysis of d_3-3MH and 3MH by gas chromatography / mass spectrometry (Rathmacher *et al.*, 1992). Using a compartmental computer model, production rate of 3MH was estimated from the decay curve of d_3-3MH/3MH against time as previously described by Rathmacher *et al.* (1996).

For the calculation of fractional breakdown rate (FBR, %/d) from 3MH production rate, whole body 3MH pool has to be known. Therefore, the following assumptions were made: 8% of final body weight consists of muscle proteins and 3MH concentration in porcine muscle is 3.8742 μmol/g muscle protein, as was used in our previous studies (Van den Hemel-Grooten *et al.*, 1995; 1996a).

Contribution of GI tract to whole-body 3MH production

Ratio of d_3-3MH to 3MH in serial plasma samples of portal vein and carotid artery were modelled separately as described by Rathmacher *et al.* (1996) in

order to obtain production rates of 3MH at both places. Ratio between difference in 3MH production as measured from portal vein and carotid artery to 3MH production as measured in carotid artery represents contribution of GI tract to whole-body 3MH production.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the SPSS-PC$^+$, V3.0 program, with diet and experimental period as main effects (SPSS, 1988). Data were considered statistically different when P-value was less than 0.10.

Results and discussion

In TABLE 5.1 data are presented of body weights and growth rates for both dietary treatments and experimental periods. Although animals were divided randomly over both treatments in a way that their mean body weight did not differ, there was a non-significant, but still a numerical difference of 4 kg in initial body weight between control and protein-free group in period 1. This can be explained by the fact that 3 pigs were withdrawn from the experiment because of disfunctioning of one of the catheters. Body weights did not differ between treatments at the end of each experimental period. This was unexpected, but can be explained in period 1 by the same reason as discussed above and in period 2 by the large variation in body weights. However, as expected, growth rate was significantly lower for animals who received the protein-free diet than for those who received the control diet in both periods. These data are in agreement with our previous findings (Van den Hemel-Grooten *et al.*, 1996a), that feeding a protein-free diet for 14 d to young pigs does not allow them to grow.

TABLE 5.1 Body weights and growth rates of growing pigs during both a control and a protein-free feeding period in both experimental periods of 14 d[a]

	Period 1				Period 2				
Trait	Control		Protein-free		Control		Protein-free		Sign.[b]
Body weight, kg									
initial	29.5	(1.0)	33.5	(3.3)	35.5	(5.5)	37.1	(2.2)	
final	36.7	(1.6)	34.0	(3.5)	44.9	(7.6)	37.4	(3.0)	
Growth rate, kg/d	0.51	(0.06)	0.04	(0.07)	0.68	(0.15)	0.02	(0.05)	D
n	4		3		2		3		

[a] Data are expressed as MEAN (SEM).
[b] Analysis of variance: D, significant diet effect; P, significant period effect; D*P, significant interaction.

Data of 3MH production rate and fractional breakdown rate of myofibrillar proteins are presented in TABLE 5.2. For all parameters measured, both experimental periods gave similar values. Also no significant interaction between dietary treatment and experimental period was observed. It should be mentioned that considerable variation exists within treatments for results of 3MH production. There were no significant differences in 3MH production rate as measured from either portal vein or carotid artery between dietary treatments. Corrections were made for differences in final body weight and intestinal protein weight between treatments, but this had no effect on the finding that there was no difference between both dietary treatments. If this difference in 3MH production is expressed as a percentage of whole-body 3MH production, as measured in carotid artery, the contribution of GI tract in 3MH production can be calculated. Contribution of GI tract was less than 6% for both dietary treatments. These data indicate that GI tract does not contribute substantially to whole-body 3MH production. Moreover, dietary treatments did not change the contribution of GI tract to whole-body 3MH production.

TABLE 5.2 Production rate of 3-methylhistidine (3MH) as measured from portal vein (P) and carotid artery (A) and myofibrillar fractional breakdown rate (FBR, %/d) of control and protein-free group in both experimental periods[a]

	Period 1				Period 2				
Trait	Control		Protein-free		Control		Protein-free		Sign.[b]
3MH production, μmol.d^{-1}									
from P	234.9	(22.7)	291.9	(12.7)	329.4	(10.3)	284.8	(51.1)	
from A	226.5	(19.0)	280.1	(16.1)	313.1	(22.3)	284.4	(43.9)	
Difference P-A,									
μmol.d^{-1}	8.45	(4.82)	11.77	(4.36)	16.33	(12.0)	0.04	(9.33)	
μmol.kg BW^{-1}.d^{-1}	0.23	(0.14)	0.37	(0.15)	0.42	(0.24)	-0.01	(0.25)	
μmol.kg int^{-1}.d^{-1},[c]	39.65	(23.43)	71.86	(28.92)	71.32	(57.26)	-3.56	(50.09)	
% of total	3.43	(2.06)	4.37	(1.71)	5.51	(4.22)	-0.82	(3.81)	
FBR, %/d	2.02	(0.23)	2.68	(0.11)	2.29	(0.23)	2.43	(0.20)	D
n		4		3		2		3	

[a,b] See Legend to TABLE 5.1.

[c] Int = intestinal protein. Data for intestinal protein weights were calculated using previous results, *i.e.*, 5.90 g/kg BW and 5.19 g/kg BW for control and protein-free treatment, respectively (Van den Hemel-Grooten *et al.*, 1996b).

Results from 3MH production rate show that portal vein sampling reflects a slightly higher 3MH production rate than carotid artery for both dietary treatments. Sjölin *et al.* (1989) measured arteriovenous 3MH differences in infection in humans and concluded also that splanchnic 3MH release was low and there might even be an uptake of 3MH by splanchnic tissues in patients with a high FBR. The latter is not applicable to our findings, since we find a positive, not significant correlation between those parameters as is shown in FIGURE 5.1. The correlation between FBR and difference in 3MH production as measured from P and A is 0.69 for control group and 0.93 for protein-free group. TABLE 5.2 shows clearly that animals in protein-free group have higher FBR than animals in control group at any difference of 3MH production rate as measured from P and A. FIGURE 5.1 also shows that the slope of both lines is similar (0.025 and 0.021 for control and protein-free group, respectively). Moreover, protein-free group shows a higher level of FBR than control group.

This indicates that elevated FBR can not be explained by increased 3MH production from myofibrillar protein degradation in GI tract.

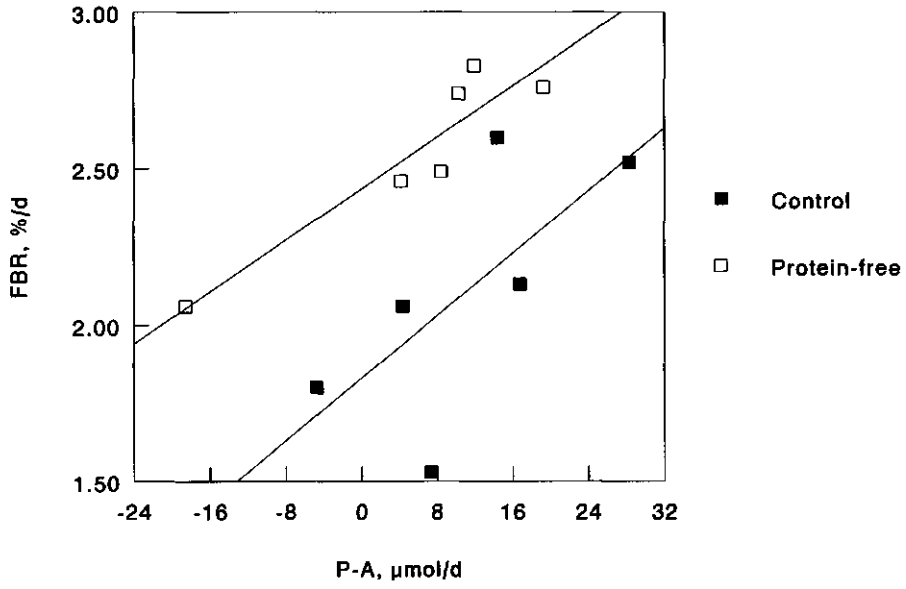

FIGURE 5.1 Fractional breakdown rate (FBR, %/d) and difference in 3-methylhistidine production as measured from portal vein and carotid artery (P-A, μmol/d) during both a control and a protein-free feeding period of 14 d.

Fractional breakdown rate was significantly higher for protein-free group than for control group, which is in agreement with our previous results (Van den Hemel-Grooten *et al.*, 1995; 1996a). In those experiments, only one catheter was used to measure 3MH production rate and the contribution of GI tract could not be measured. With the present experiment we can rule out the possibility that elevated FBR during a protein-free feeding period is caused by an increased 3MH production in GI tract. However, another non-skeletal-muscle source for 3MH production rate is smooth muscle present in skin. Wassner and Li (1982) showed in their study with rats that perfused hemicorpus with or without skin showed modest difference in 3MH release that were not statistically significant. In rats, skin contains 6.4% of whole-body 3MH and a FBR of 1.4%/d (Wassner and Li, 1982), it is not very likely that skin contributes

substantially to whole-body 3MH production.

Taken together, our results on pigs are in agreement with others, who state that GI tract does not contribute substantially to whole-body 3MH production and that 3MH production mainly originates from skeletal muscle (Haverberg *et al.*, 1975; Harris, 1981). The increase of 3MH production during a protein-free feeding period is not caused by an increased myofibrillar protein degradation in GI tract. Therefore, we can conclude that increased FBR during a protein-free feeding period is caused by increased degradation of myofibrillar protein breakdown in skeletal muscle.

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6

mRNA levels of the calpain system in longissimus muscle of young pigs during a 15-day protein-free feeding trial

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Abstract

This experiment was conducted to investigate the effects of feeding a protein-free diet for 15 d on mRNA levels of the calpain system in skeletal muscle of growing pigs. Twenty crossbred barrows (initial weight of 38.3 kg) were randomly divided over two dietary treatments: control or protein-free diet. Daily diets were provided at 2.5 times energy for maintenance (twice a day). On d 0, 3, and 14 biopsies were taken from longissimus muscle between 3rd and 4th rib (d 0 and d 3) and between 4th and 5th rib (d 14) using a shotgun. On d 15, animals were slaughtered and longissimus muscles were dissected and analyzed for calpastatin, μ- and m-calpain activity. From biopsies, mRNA level of skeletal muscle calpain, μ- and m-calpain, and calpastatin was measured using reversed transcription PCR. Subsequently, PCR products were quantified using ELISA. Feeding the protein-free diet lowered growth rate to almost zero. Only total level of mRNA of μ-calpain on d 14 was influenced by dietary treatments, being lower for protein-free group than for control group ($P < 0.05$). However, proteolytic activities were not different between treatments. Total RNA concentration in longissimus muscle decreased during the experiment for both treatments, but on d 14 this was more pronounced for protein-free group than for control group ($P < 0.05$). If mRNA levels were corrected for this change, specific mRNA level on d 14 of both skeletal muscle calpain and μ-calpain were

lower ($P < 0.05$) for protein-free group than for control group. These data suggest that activity of the components of the calpain system are differentially regulated.

Introduction

The calpain system is thought to be involved in one of the first steps of degradation of myofibrillar protein by weakening of the Z-disc (Goll *et al.*, 1989). The calpain system consists of the heterodimers μ- and m-calpain and their natural inhibitor, calpastatin. Besides these components, a novel member of the calpain large-subunit family has only been found in skeletal muscle and designated as skeletal muscle calpain (Sorimachi *et al.*, 1989) and is expressed at a 10-fold higher level than those of μ- and m-calpain (Sorimachi *et al.*, 1993).

Regulation of enzyme activity can occur at many metabolic stages, including transcriptional, translational, and post-translational levels. A disadvantage of investigating enzyme activity at post-translational stages is that at present it is not possible to determine *in situ* activity. Therefore, additional information may be obtained if other stages of regulation are also studied to improve our insight into involvement of the calpain system during myofibrillar protein degradation *in vivo*.

Investigating mRNA levels has the advantage that it does not require much sample and several biopsies can be obtained from the same animal during the experiment. In this way, adaptation of regulation at transcriptional level to different experimental conditions can be investigated.

The objective of the present study was to study mRNA levels of the components of the calpain system in longissimus muscle at several days during a 15-day control and protein-free feeding trial in growing pigs.

Materials and methods

Experimental Design

The Institutional Care and Use Committee approved all procedures involving animal handling. Twenty crossbred barrows, Large White x (Dutch Landrace x Large White), were used at an age of approximately 13 weeks in a 15-day feeding trial with two dietary treatments, *i.e.*, control or protein-free diet. The composition of both experimental diets is shown in TABLE 6.1. On d -5, barrows

were randomly divided over both treatments in a way that their mean body weight was similar (38.3 kg). Barrows were housed individually in a pen (1.5 x 1.5 m) at a constant temperature (22 ± 1°C). Water was available at all times. During an adaptation period of 5 d, animals received a mixture of a commercial starter diet (Cavo Latuco, Utrecht, The Netherlands) and the control diet on a 50% - 50% basis. Throughout the experiment, daily diets were offered at 2.5 times energy for maintenance in two portions at 7.30 am and 3.00 pm. On d 0, the experimental period started by providing experimental diets to the animals. The protein-free diet was provided gradually, *i.e.*, 75% and 25% on d 0, 50% and 50% on d 1, and 75% protein-free diet and 25% control diet on d 2, respectively. From d 3, 100% protein-free diet was offered. Body weights were recorded on d -5, 0, 7, and 15.

TABLE 6.1 Composition of the experimental diets.

	Control	Protein-free
Ingredients, g/kg		
Corn	690	-
Soybean meal	254	-
Corn starch	-	783
Soybean oil	20	54
Arbosel	-	121
Constant components[a]	36	42
Calculated, g/kg		
Protein	173	3
Fat	48	55
Carbohydrates	544	828
Crude fibre	34	39
Analyzed, g/kg		
Protein	180.1	3.8
Fat	50.6	41.0
Dry matter	879.4	881.3
Ash	48.3	25.0

[a] Constant components consisted of the following: dicalcium phosphate, 20 g; limestone, 6 g; iodized salt, 4 g; mineral premix, 2 g; vitamin premix, 2 g; choline chloride, 2 g. The composition of the mineral premix is described previously by Van den Hemel-Grooten *et al.* (1996a) and composition of the vitamin premix is described elsewhere (Bikker *et al.*, 1994).

Taking of muscle biopsies

On d 0, 3, and 14 biopsies were taken from longissimus muscle using a shotgun. Animals were restraint in a weighing box in order to take the biopsy at standardized place, *i.e.*, between 3rd and 4th rib on d 0 and d 3 from right side and left side, respectively. On d 14, biopsy was taken from the right side between 4th and 5th rib. Biopsies were taken 30 min prior to the afternoon meal. Biopsy needle (i.d. 7 mm, length 3.5 cm) was disinfected with 70% alcohol before use. No (local) anaesthesia or antibiotics were used. The complete biopsy sample was put into a small tube, frozen into liquid nitrogen, and stored at -80°C until further analysis.

Slaughter procedure and muscle sampling

On d 15, animals were killed using a captive bolt followed by exsanguination. Immediately after slaughter, a sample of approximately 100 to 150 g was taken from longissimus muscle from the left side of the carcass, between 4th and 5th rib for measurement of calpastatin, μ- and m-calpain activities as described previously (Van den Hemel-Grooten *et al.*, 1995). Both longissimus muscles were dissected, weighted, and checked for any visible damages to the tissue due to the previous biopsies.

RNA extraction and development of PCR conditions

From the frozen biopsies, skin and connective tissue were removed. From approximately 100 mg of muscle tissue total RNA was extracted essentially as described by Chomczynski and Sacchi (1987). RNA was quantified spectrophotometrically.

GeneAmp RNA PCR kit (Perkin Elmer, Branchburg NJ, USA) was used for the reverse transcription of 1 μg RNA into cDNA and subsequent amplification in polymerase chain reaction (PCR). The cDNA in PCR is equivalent to .15 μg RNA. For each gene studied in the present experiment, final volume of PCR reaction was 30 μl, of which 10 μl was used for ELISA and 18 μl was separated by 5%, 2%, 1%, and 1% agarose gel electrophoresis for skeletal muscle calpain, μ- and m-calpain, and calpastatin, respectively. Number of cycles run in PCR were within the quantitative range for each gene.

For porcine skeletal muscle calpain, PCR primers and reaction conditions were published in accession number of EBI database: U23954 (Briley *et al.*, unpublished). Length of PCR product is 152 bp. Number of PCR cycles used were 19 and 21.

For μ-calpain and m-calpain both cDNA and primer sequences were reported

for pigs by Sun *et al.* (1993); accession numbers U01180 and U01181, respectively. Cycling conditions for PCR of μ-calpain were 94°C for 30 s, 58°C for 45 s and 72°C for 1 min for 21 and 23 cycles. Cycling conditions for PCR of m-calpain were 94°C for 30 s, 55°C for 45 s and 72°C for 1 min for 24 and 26 cycles. Length of PCR product of μ-calpain is 289 bp and of m-calpain is 629 bp.

For porcine calpastatin, only cDNA sequence was published by Asada *et al.* (1989); accession number D50827. The primer sequences were 5'-GAAGTGTGGTGAGGATGATG-3' and 5'-GTCTGTATTCAGGTGGGATAG-3' for forward and reverse primer, respectively. Conditions for PCR were firstly a hot start at 80°C for 5 min and 25°C for 1 min. Cycling conditions were 94°C for 30 s, 57°C for 45 s, and 72°C for 1 min for 24 and 26 cycles. Length of PCR product is 878 bp.

Quantification of PCR products

Quantification of PCR products was performed using a PCR ELISA kit (Boehringer, Mannheim, Germany). Briefly, after optimalization of PCR conditions for all 4 components of calpain system, PCR products were labelled with digoxigenin (DIG)-dUTP during the amplification process. The labelled PCR products were analyzed by solution hybridization to a specific capture probe that is complementary to the inner part of the amplification product. Sequences of capture probe were as follows: 5'-GTCTGCAGCACCAGGTCACT-3' for skeletal muscle calpain, 5'-ACCATCCTCAACAGGATCAT-3' for μ-calpain, 5'-GAGCGATCGGACACCTTCAT-3' for m-calpain, and for calpastatin 5'-TCCTCCTGATTATAGATTAG-3'. Capture probes were end-labelled with biotin to allow immobilization of the hybrid to a streptavidin-coated microtiterplate surface. The bound hybrid can be detected and colorimetrically quantified at 405 nm (A_{405}) by an anti-DIG-peroxidase conjugate using ABTS^R (Boehringer, Mannheim, Germany) as a substrate. Each microtiterplate contained the following controls: standard sample (sample of control group), negative PCR product, H_2O, and ABTS^R. For each gene, three microtiterplates were used, which contained both number of cycles used and a four times dilution of each number of cycle of the final PCR product. In order to decide which data of both PCR cycles should be used, the ratio of absorption values for undiluted and diluted samples should be close to 2. For each gene, corrections were made for variation between microtiterplates by proportionally multiplying values of controls and samples by the difference for the standard sample between plates. Absorption data were also corrected for background, as measured with H_2O and

$ABTS^R$.

Different dilutions of anti-DIG-peroxidase were needed for the different genes. The following dilutions were used from stock solution (50 units/ml): 5000x, 250x, 50x, and 25x for skeletal muscle calpain, μ-calpain, m-calpain, and calpastatin, respectively. Values of negative PCR and both H_2O and $ABTS^R$ were similar for all three calpains. However, negative PCR of calpastatin was 1.6 times higher than for both H_2O and $ABTS^R$, although no visible PCR product was found on agarose electrophoresis. Therefore, values were corrected for negative PCR.

Statistical analysis

Data of body weight, growth rate, calpain and calpastatin activities were analyzed by Student's t-test using SPSS-PC$^+$, V3.0 program (SPSS, 1988). Effects of day of sampling on total RNA concentration and total and specific mRNA level of each gene were analyzed using paired t-test. Dietary treatment effects total on RNA concentration and total and specific mRNA level of each gene were tested using Student's t-test. Results were considered statistically different when P-value was less then 0.05.

Results

Influence of feeding strategy on growth rate

Feeding a protein-free diet for 15 d to growing barrows stopped growth as is shown in TABLE 6.2. After 7 d, there was no significant difference in body weight between control group and protein-free group, but at the end of the feeding trial of 15 d, body weight of control group was significantly higher than for protein-free group. This is also reflected in significantly higher growth rate for control group than for protein-free group.

Weight of longissimus muscle of protein-free group was significantly lower than for control group (TABLE 6.2). Expressed as a percentage of total body weight, no significant differences were observed in muscle weight between control and protein-free group.

TABLE 6.2 Body weight, growth rate and weight of longissimus muscle of growing pigs during a 15-day control and protein-free feeding trial[a]

Trait	Dietary treatments	
	Control	Protein-free
Body weight, kg		
d -5	38.3 (1.4)	38.3 (1.2)
d 0	39.5 (1.3)	38.7 (1.2)
d 7	42.9 (1.5)	38.9 (1.3)
d 15	47.5 (1.7)[c]	39.4 (1.3)[b]
Growth rate, kg/d		
d 0 to d 15	0.54 (0.03)[c]	0.05 (0.02)[b]
Longissimus weight		
kg	1.84 (0.68)[c]	1.46 (0.76)[b]
% of BW	3.89 (0.08)	3.69 (0.10)

[a] Data are expressed as MEAN (SEM) for 10 animals per treatment.
[b,c] Means within a row lacking the same superscript letter differ ($P < 0.05$).

Influence of feeding strategy on activity and mRNA levels of the calpain system

Activities of calpastatin, μ- and m-calpain in longissimus muscle were not different between treatments (data not shown).

TABLE 6.3 presents results of total RNA concentration and total mRNA levels of all four components of the calpain system. On d 14, total RNA concentration was significantly lower for protein-free group than for control group. Total RNA concentration was significantly lower on d 3 and 14 than on d 0 for control group. For protein-free group, total RNA concentration decreased during the 15-d feeding trial. Total level of mRNA of skeletal muscle calpain was not influenced by dietary treatments. For both control group and protein-free group, total mRNA level was significantly higher on d 3 than on d 0 and was lowest on d 14. Feeding a protein-free diet for 14 d caused a significantly lower total mRNA level of μ-calpain than for control group. Total level of mRNA of μ-calpain of the control group had increased after 3 d and was significantly higher on d 14 compared to d 0. Total level of mRNA of μ-calpain of the protein-free group was significantly higher on d 3 than on d 14; total mRNA level on d 0 was intermediate. Dietary treatments had no significant effect on total mRNA levels of m-calpain. No differences were observed in total mRNA levels for control

group during the experiment. Level of total mRNA of protein-free group was significantly lower on d 14 than on both other days. Total levels of mRNA of calpastatin were not influenced by dietary treatments. For both control and protein-free group, total mRNA level was significantly higher on d 3 than on both other days of sampling.

TABLE 6.3 Total RNA concentration and total mRNA level of skeletal muscle calpain, μ- and m-calpain, and calpastatin in longissimus muscle at 3 time points during a control and protein-free feeding period[a]

Trait Dietary treatment	Day 0	Day 3	Day 14
Total RNA concentration, μg/g tissue			
Control	576.6 (27.6)[c]	501.7 (24.9)[b]	499.4 (18.5)[b]
Protein-free	575.6 (41.2)[d]	459.0 (22.1)[c]	396.6 (21.4)[b]
	(P=0.984)[e]	*(P=0.216)*	*(P=0.002)*
Skeletal muscle calpain			
Control	1.32 (0.03)[c]	1.39 (0.04)[d]	1.21 (0.05)[b]
Protein-free	1.33 (0.03)[c]	1.46 (0.02)[d]	1.20 (0.03)[b]
	(P=0.913)	*(P=0.117)*	*(P=0.792)*
μ-Calpain			
Control	0.25 (0.04)[b]	0.35 (0.03)[b,c]	0.37 (0.02)[c]
Protein-free	0.28 (0.04)[b,c]	0.39 (0.04)[c]	0.26 (0.03)[b]
	(P=0.582)	*(P=0.337)*	*(P=0.002)*
m-Calpain			
Control	0.26 (0.05)	0.17 (0.04)	0.23 (0.06)
Protein-free	0.41 (0.12)[c]	0.30 (0.06)[c]	0.15 (0.03)[b]
	(P=0.301)	*(P=0.090)*	*(P=0.245)*
Calpastatin			
Control	0.41 (0.09)[b]	1.00 (0.11)[c]	0.24 (0.14)[b]
Protein-free	0.53 (0.10)[b]	1.17 (0.07)[c]	0.22 (0.15)[b]
	(P=0.358)	*(P=0.225)*	*(P=0.950)*

[a] Data are expressed as MEAN (SEM) for 10 animals per treatment.
[b,c,d] Data within a row lacking the same superscript letter differ ($P < 0.05$).
[e] P-value less than 0.05 is indicative for difference between dietary treatments.

TABLE 6.4 Specific mRNA levels for skeletal muscle calpain, μ- and m-calpain, and calpastatin in longissimus at 3 time points during a control and protein-free feeding period[abcde]

Trait Dietary treatment	Day 0	Day 3	Day 14
Skeletal muscle calpain			
Control	0.76 (0.03)[d]	0.70 (0.04)[c]	0.60 (0.03)[b]
Protein-free	0.76 (0.05)[c]	0.68 (0.04)[c]	0.48 (0.03)[b]
	(P=0.997)[e]	*(P=0.701)*	*(P=0.007)*
μ-Calpain			
Control	0.14 (0.03)	0.18 (0.02)	0.19 (0.01)
Protein-free	0.17 (0.03)[c]	0.18 (0.02)[c]	0.10 (0.01)[b]
	(P=0.564)	*(P=0.839)*	*(P=0.001)*
m-Calpain			
Control	0.15 (0.03)[c]	0.08 (0.02)[b]	0.11 (0.03)[b,c]
Protein-free	0.25 (0.09)	0.14 (0.03)	0.06 (0.01)
	(P=0.330)	*(P=0.141)*	*(P=0.126)*
Calpastatin			
Control	0.23 (0.06)[b]	0.50 (0.06)[c]	0.11 (0.07)[b]
Protein-free	0.32 (0.08)[c]	0.54 (0.05)[d]	0.10 (0.07)[b]
	(P=0.367)	*(P=0.600)*	*(P=0.862)*

[abcde] See legend to TABLE 6.3.

TABLE 6.4 presents specific mRNA levels, *i.e.*, total mRNA levels which have been corrected for the observed changes in total RNA concentrations. Thus, these data represent mRNA levels per g of muscle tissue. On d 14, specific mRNA level of skeletal muscle calpain of protein-free group was significantly lower than for control group. Specific level of mRNA decreased significantly during the 15-d feeding trial for control group. For protein-free group, specific mRNA level on d 14 was significantly lower than on both other days. Specific level of mRNA of μ-calpain on d 14 was significantly lower for protein-free group than for control group. Specific level of mRNA of control group was similar for all three time points. Protein-free group had a significantly lower specific mRNA level on d 14 than on both d 0 and d 3. Specific level of mRNA of m-calpain was not influenced by dietary treatments. For control group, specific mRNA level was significantly higher on d 0 than on d 3; specific mRNA level on d 14 was intermediate. Dietary treatments did not cause a change in specific mRNA level of calpastatin. For control group, specific mRNA level on

d 3 was significantly higher than on both other days. For protein-free group, specific mRNA level was significantly different between all time points, being highest on d 3 and lowest on d 14.

Discussion

The present experiment was performed to investigate mRNA levels of several components of the calpain system during a protein-free feeding period in growing pigs. In previous experiments, we have shown that production of 3-methylhistidine, a marker for myofibrillar protein breakdown, was increased after a period of feeding a protein-free diet to growing pigs (Van den Hemel-Grooten *et al.*, 1995, 1996a). However, activities of calpastatin, μ- and m-calpain did not differ significantly between dietary treatments. This discrepancy can be explained by several factors. Firstly, 3-methylhistidine production may be increased from sources other than skeletal muscle, like smooth muscle in gastro-intestinal tract or skin during a lack of dietary protein. Especially the contribution of gastro-intestinal tract can play an important role since protein turnover rate is much higher than in skeletal muscle (Rennie and Millward, 1983). However, this possibility can be ruled out, because we found that the contribution of 3-methylhistidine from gastro-intestinal tract to whole body was less than 5%. Moreover, feeding a protein-free diet to growing pigs for 14 d did not change this contribution (Van den Hemel-Grooten *et al.*, 1996b). Secondly, the rate-limiting step in the cascade of myofibrillar protein degradation is unknown. Thus, other proteolytic enzymes than those of the calpain system can be responsible for this step. Goll *et al.* (1989, 1992) discussed that μ- and m-calpain are very likely candidates for initiating myofibrillar protein degradation. To our knowledge, no other proteolytic enzymes have been proposed to be involved in one of the first steps of myofibrillar protein degradation. Thirdly, measurement of proteolytic activity is performed *in vitro*, which means that optimal conditions are used to express the proteolytic activity. However, this activity does not necessarily represent physiological activity, but more likely its maximal level present in the tissue examined. At present, no technique is available to measure physiological activity *in situ*.

The above discussion necessitates an alternative approach to investigate regulation and mechanisms of myofibrillar protein degradation *in vivo* by proteolytic enzyme systems. Regulation of enzyme activity probably occurs at

different stages: transcriptional, translational, and post-translational levels. Expression at the transcriptional level of the calpain system is studied in the present experiment in order to gain more insight into the possible involvement of the calpain system during myofibrillar protein degradation.

In agreement with our previous findings, feeding a protein-free diet to growing pigs reduced growth rate to almost zero (Van den Hemel-Grooten *et al.*, 1995; 1996a). After the 15-d feeding trial, weight of longissimus muscle of protein-free group was 80% of that of the control group.

Total RNA concentration decreased significantly during the experiment in both control and protein-free group, but decreased more for protein-free group than for control group. This indicates that protein synthetic capacity at the end of the experiment is lower for protein-free group than for control group. This is similar to our previous findings (Van den Hemel-Grooten *et al.*, 1995, 1996a). Ilian and Forsberg (1992, 1994) also observed significant lower RNA concentrations in skeletal muscle of rabbits after fasting for 8 d.

In order to perform the ELISA, different concentrations of anti-DIG-peroxidase were needed for each gene. This resulted in increased absorption levels for negative PCR, H_2O and $ABTS^R$. For all three calpains, absorption values of these controls were similar and remained within background ranges (0.04, 0.06 and 0.16 for skeletal muscle calpain, μ- and m-calpain, respectively). However, for calpastatin 1.6 times higher values were obtained for negative PCR than for both H_2O and $ABTS^R$ (the latter controls being 0.29), although no visible PCR product was seen on agarose gel electrophoresis. Therefore, absorption values for mRNA levels of calpastatin were corrected for values obtained from negative PCR. An explanation for the need to increase the anti-DIG-peroxidase concentration may be due to size of PCR product. Size of PCR product is 152, 289, 629, and 878 bp for skeletal muscle, μ- and m-calpain, and calpastatin, respectively. A larger PCR product may cause more easily a secondary structure and this may influence the availability of incorporated DIG to its antibody by steric hindrance.

Results of mRNA levels for all genes are presented in two different ways. TABLE 6.3 presents data of total mRNA levels which originates from the same standard amount of total RNA for all samples. TABLE 6.4 presents data of specific mRNA levels, which are corrected for the observed changes of total RNA concentration in muscle tissue. These data are indicative for mRNA level per g of muscle. Only the level of mRNA of skeletal muscle calpain gives different results when expressed per unit of total RNA or per g of muscle tissue: for protein-free group, mRNA levels per unit of total RNA were not different

between dietary treatments. However, per g of muscle tissue, mRNA levels of skeletal muscle calpain were significantly lower for protein-free group than for control group on d 14. Ji *et al.* (1992) found that feeding a low and control protein diet (10 and 18% crude protein, respectively) for 24 d did not alter the level of mRNA of skeletal muscle calpain in finishing pigs. The physiological role of skeletal muscle calpain is unknown. It is suggested by Sorimachi *et al.* (1993) that soon after translation skeletal muscle calpain moves to the nuclear membrane, where it is subjected to autolysis. A small percentage is imported into the nucleus where it regulates the levels of short-lived regulatory proteins (such as transcription factors). Our present data would then suggest that proteolysis of transcription factors is decreased, since mRNA levels of skeletal muscle calpain of protein-free group is decreased on d 14 compared to control group. Higher levels of transcription factors would indicate that muscle tissue is trying to keep transcription at the same level despite loss of total RNA.

Both total and specific level of mRNA of μ-calpain was also influenced by dietary treatment. On d 14, total and specific mRNA level were significantly lower for protein-free group than for control group. However, activity of μ-calpain was not influenced by dietary treatments (Van den Hemel-Grooten *et al.*, 1995; 1996a). These results indicate that total and specific mRNA levels of μ-calpain were decreased in protein-free group compared to control group, but this had no effect on its activity. Thus between gene transcription and its ultimate activity, adaptation has occurred in a way that proteolytic activity was not altered. This adaptation may have been by increased stability of either mRNA or μ-calpain or by the possibility that altered mRNA levels are not yet reflected by a change in enzyme activity. Ilian and Forsberg (1992, 1994) also did not find a close relation between activity and mRNA levels of components of calpain system in their studies with fed and fasted rabbits. However, they found that mRNA level of μ- and m-calpain were increased as a result of fasting. Besides species differences, fasting for 8 d may have more pronounced effects on regulation at mRNA levels than our experimental conditions. Ilian and Forsberg (1992) conclude that higher levels of mRNA of μ- and m-calpain are needed to ensure continued synthesis and concentration of proteinases, which would allow continued mobilization of amino acids from muscle protein reserves. This explanation may support the above discussion about a possible function of skeletal muscle calpain during protein-free feeding conditions.

For m-calpain and calpastatin, no effects were observed for total and specific mRNA levels between dietary treatments, which indicates that feeding a protein-free diet did not cause a change in mRNA levels at the transcriptional

level. This correlates with data for their activity of present and previous studies (Van den Hemel-Grooten *et al.*, 1995; 1996a).

Day of sampling revealed differences in mRNA level for all components of the calpain system for both control and protein-free group. This would indicate that there has been an adaptation of these genes to the experimental conditions. However, for both control and protein-free group changes for mRNA levels per g of muscle point into the same direction, especially for skeletal muscle calpain and calpastatin. Thus, this is not an adaptation to the different diets in this experiment. An explanation can be that the animals were not yet used to the feeding strategy, which may also explain the reduction in total RNA concentration of the control group on d 3 and d 14. Another possibility can be that taking the biopsies itself caused these changes, *e.g.*, injury or even appearance of inflammation, because most changes are seen on d 3. We have no data to support the possibility that the calpain system plays a role during injury. The other possibility is not very likely, because after taken the 40 biopsies on d 0 and d 3 of the experiment, only 4 animals showed a light inflammation.

Implications

Data of the present experiment reveal that feeding a protein-free diet to growing pigs caused decreased total and specific levels of mRNA of skeletal muscle calpain and specific mRNA of μ-calpain. For μ-calpain, proteolytic activity remained similar, which indicates that regulation of its activity also occurs at a different level than at transcription. Dietary treatments did not influence total or specific mRNA levels of calpastatin and m-calpain, which is similar to measurement of their activities. Thus, different levels of regulation determine μ-calpain activity, whereas mRNA levels of m-calpain and calpastatin correlate with their post-translational activities.

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7

General discussion

The main reason for starting this project was the hypothesis that net protein accretion could be elevated by decreasing the rate of protein degradation in skeletal muscle. However, this can only be brought about under the assumption that rate of protein synthesis remains at the same level. This assumption is not only dependent on the availability of amino acids, precursors for protein synthesis, which are derived from exogenous sources (diet) or endogenous (degradation of tissue proteins), but also on losses of precursors by oxidation (see FIGURE 1.1).

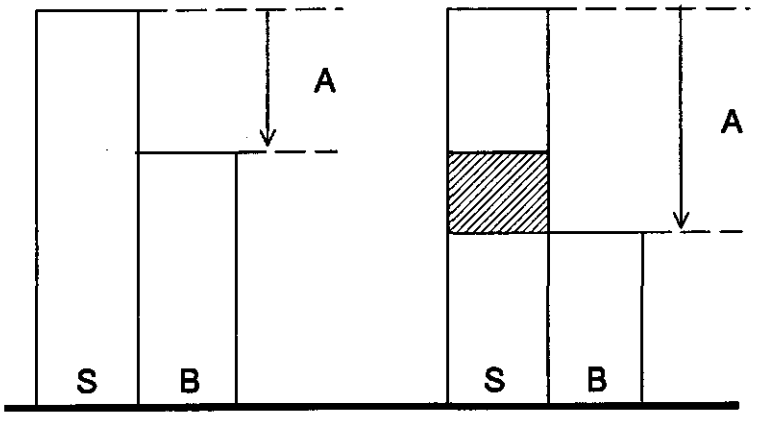

FIGURE 7.1 Situation I: normal situation in a growing animal where protein synthesis (S) rate is higher than protein breakdown (B) rate, resulting in a accretion (A) of protein in the body. Situation II: theoretically altered situation in a growing animal where B is decreased, but S is at similar level as in situation I. To maintain this capacity of S, supply of precursors (shaded part) should originate from oxidation of amino acids and/or dietary protein intake.

Therefore, by decreasing the rate of protein degradation, the amount of precursors need to be elevated by decreased level of oxidation and/or by increased dietary intake to maintain similar level of protein synthesis (FIGURE 7.1). In the former case, this would lead to the possibility that less dietary protein is needed to deposit similar amounts of body proteins in skeletal muscle (and thus meat) as in present situation. In the latter case, waste of nitrogen into the environment would be reduced, which is of great benefit for animal production nowadays. In addition to improving protein deposition, decreasing the rate of protein breakdown would also favour the level of protein turnover and thus the energy costs for protein metabolism.

In order to be able to manipulate degree of protein breakdown of skeletal muscle, underlying mechanisms and regulation need to be known. Up till now, most of the scientific attention has been addressed to elucidate mechanisms and regulation of protein synthesis. This is probably due to the difficulty of measuring protein breakdown *in vivo*. In this thesis, production of 3-methylhistidine is used as an index for measuring myofibrillar protein degradation *in vivo* as described by Rathmacher *et al.* (1996). 3-Methylhistidine is a specific constituent of the myofibrillar proteins, actin and myosin heavy chain, and upon degradation it can not be reutilized for protein synthesis. Since proteinases are responsible for the breakdown of muscle proteins into amino acids, the approach of this thesis was to investigate the relation between activity of several proteolytic enzyme systems and myofibrillar protein breakdown. As a model for studying this relation, rate of myofibrillar protein breakdown was manipulated by dietary restriction, *i.e.*, feeding a protein-free diet for 14 days to growing pigs. Goll *et al.* (1992) have presented a tentative model in which the involvement of the different muscle proteinases during turnover of myofibrillar proteins is shown (FIGURE 7.2).

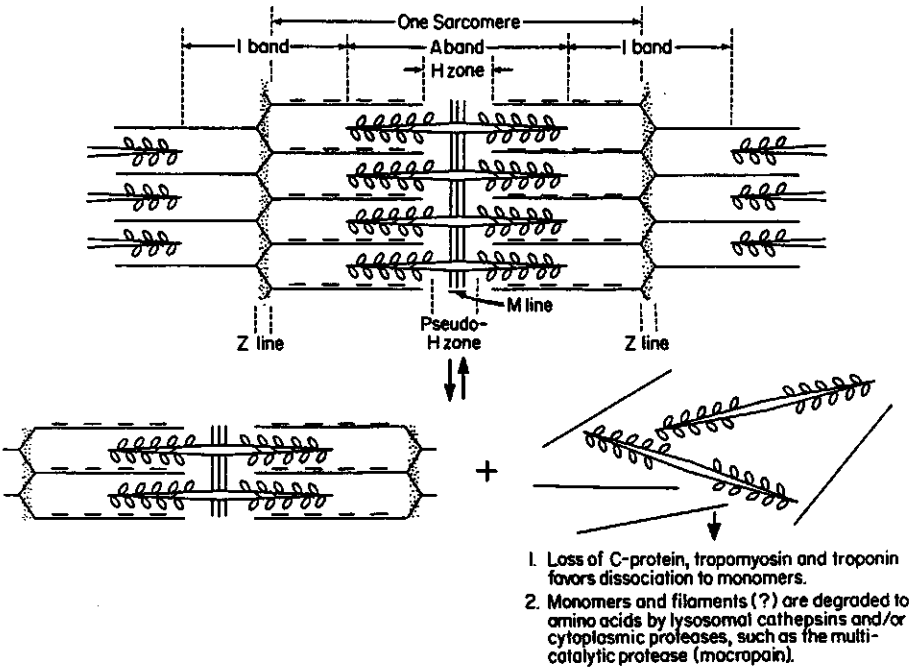

FIGURE 7.2 Schematic model showing how the calpains could initiate turnover of myofibrillar proteins. After incubation with the calpains, the resulting myofibril is narrower by two thick (mainly composed of myosin monomers) and two thin (mainly composed of actin monomers) filaments but is otherwise unchanged. The filaments released may either reassociate with the myofibril or be degraded by cytosolic proteases, possible the multicatalytic proteinase (MCP). Figure from Goll *et al.* (1992).

Production of 3-methylhistidine

Feeding a protein-free diet for 14 days increased production rate of 3-methylhistidine, and thus most probably myofibrillar protein degradation (CHAPTER 2, 3, AND 5). A limitation of using 3MH as an index for myofibrillar protein degradation is that other sources than skeletal muscle, like gastro-intestinal tract, also contribute to 3MH production. Based on data from chemical composition of small and large intestines (CHAPTER 4), intestines of animals in

the protein-free group contained less protein than the animals in the initial group. This indicates that 3MH production from intestines may have been increased during a protein-free feeding period. However, this possibility is ruled out, because contribution of gastro-intestinal tract to whole body 3MH production is low and not elevated after feeding a protein-free diet for 14 days to growing pigs (CHAPTER 5).

In all of our experiments, the formula which is used to calculate fractional breakdown rate makes use of a constant 3MH pool in skeletal muscle for all dietary treatments. We assumed, 8% of total body weight to be muscle protein. This value is derived from the study of Mulvaney *et al.* (1985) using 22 and 45 kg pigs. The 3MH content in porcine muscle is 3.87 μmol/g of muscle protein (Rathmacher *et al.*, 1996). It can be expected that feeding a protein-free diet to growing pigs would reduce the amount of muscle protein in the body, and then we would have even underestimated fractional breakdown rate.

From the data in CHAPTER 2, we can calculate accretion rates of myofibrillar proteins (FAR). Together with our measurements on fractional breakdown rate (FBR), an indirect calculation of synthesis rate (FSR) can be made, since FAR = FSR - FBR (TABLE 7.1). Unfortunately, no data are available from animals at the start of the experiment. Therefore, the following assumptions were made: myofibrillar protein concentrations are constant and thus similar to measured values, *i.e.*, 110 mg/g of muscle, since no differences were observed at slaughter between dietary treatments. Based on similar relative weights of longissimus muscle at the start and end of 14-d feeding trial in experiment described in CHAPTER 3, weight of longissimus muscle is assumed to be 3.7% of body weight on d -3. Furthermore, the intake-restricted group in CHAPTER 2 is comparable with control groups in all other experiments based on feed intake of 2.5 times energy for maintenance. These data indicate that synthesis rate of myofibrillar proteins has decreased during the feeding trial for the protein-free group and that myofibrillar proteins are lost from the longissimus muscle. Therefore, net myofibrillar protein degradation can be explained by the need for amino acids and nitrogen containing components, which are needed for other, more vital tissues than skeletal muscle.

TABLE 7.1 Fractional rates of breakdown (FBR), accretion (FAR), and synthesis (FSR) of myofibrillar proteins in longissimus muscle during a 14 d protein-free feeding trial of growing pigs

	Control	Protein-free
Longissimus weight, kg		
d -3[a]	1.36	1.34
d 14	1.64	1.15
Myofibrillar protein, g[b]		
d -3	149.6	147.4
d 14	180.4	126.5
Myofibrillar accretion		
g	30.8	-20.9
g/d	1.81	-1.23
%/d	1.21	-0.83
Myofibrillar FBR, %/d	2.68	3.85
Myofibrillar FSR, %/d[c]	3.89	3.02

[a] Based on the assumption that relative longissimus weight at start is similar to relative weight at slaughter, *i.e.*, 3.7% of BW consists of longissimus.
[b] Based on the assumption that protein composition of muscle is similar at start and at slaughter between treatments, *i.e.*, 110 mg myofibrillar proteins/g of muscle.
[c] Calculated indirectly using formula: FSR - FBR = FAR.

The finding that myofibrillar and sarcoplasmic protein concentrations are similar in muscles between dietary treatments (CHAPTER 2) indicates that a constant tissue composition is still maintained under our experimental conditions. Thus, fractional accretion rates (FAR) of the different protein fractions need to be similar. However, the fractional synthesis and breakdown rates of both the myofibrillar and sarcoplasmic protein fractions may respond differently to changes in nutritional conditions. This is found in several studies with rats (Bates and Millward, 1983; Bates *et al.*, 1983; Goodman, 1987; Kadowaki *et al.*, 1989).

Muscle proteinase activities

Three proteolytic enzyme systems were studied in this thesis: calpain system (μ- and m-calpain, and their inhibitor calpastatin) (CHAPTER 2 AND 3), multicatalytic proteinase (MCP) (CHAPTER 2 AND 3) and lysosomal cathepsins

with their inhibitors the cystatins (CHAPTER 2). These activities were measured under *in vitro* conditions, which may not represent *in situ* activities. In CHAPTER 6, levels of mRNA of components of the calpain system (including skeletal muscle calpain) were also investigated in order to obtain additional information about regulation of these enzymes. However, only for μ-calpain different effects were observed between enzyme activity and level of mRNA in longissimus muscle. Against our expectation, feeding a protein-free diet for 14 days caused a lower mRNA level of μ-calpain than feeding a control diet, whereas enzyme activities were not different between treatments. Level of mRNA of skeletal muscle calpain was influenced in a similar manner as μ-calpain, but since function of this enzyme is suggested to be associated with regulation of short-lived proteins such as transcription factors (Sorimachi *et al.*, 1993), its action may trigger a complete other mechanism than both other calpains do.

In general, activities of the three proteolytic enzyme systems in skeletal muscle as measured *in vitro* were not influenced by feeding a protein-free diet for 14 days. One possibility is already mentioned above, that *in vitro* measurements do not reveal *in situ* activity. Another possibility may be that under our experimental conditions, these enzyme systems are not involved in the rate-limiting step in myofibrillar protein degradation. It should be taken into consideration that 3MH is an end product of the degradation of the myofibril and calpains are not able to degrade actin and myosin. Only if calpains are involved in the rate-limiting step, a relation can be expected between their activities and production of 3MH. Our results suggest that calpains are not involved in the rate-limiting step during myofibrillar protein degradation.

Millward (1980) proposed a model for myofibrillar turnover, which is shown in FIGURE 7.3. The main difference between this model and that proposed by Goll *et al.* (1992) is that in the former model myofilament subunits (myosin and actin) are in a dynamic equilibrium with the intact myofibril. These subunits can reversibly interact with the degrading system. There are two possibilities for steps to be rate-limiting step in the breakdown process. Firstly, the rate of dissociation of subunits from the peripheral myofilaments. The degrading systems is then dependent on the release of these subunits. Secondly, the overall rate of degradation might be a function of the capacity of the enzymatic degrading system.

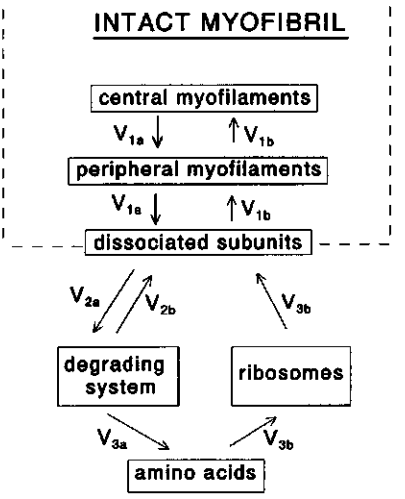

FIGURE 7.3 Hypothetical model for myofibrillar protein turnover. Two possibilities are given for the rate-limiting step in myofibrillar protein turnover, *i.e.*, the dissociation of the subunits from the myofilaments itself or the interaction of the dissociated subunits with the degrading system. Figure from Millward (1980).

Directions for future research

Since our knowledge about mechanisms and regulation of muscle protein degradation is still very limited, research on elucidating these processes should focus on:

* Fundamental approach in order to elucidate the precise mechanisms which are responsible for the degradation of the myofibril into amino acids. For this type of research *in vitro* set-ups are preferred, although it is very difficult to obtain an anabolic state. Major questions are:
  - what is the rate-limiting step in degradation of myofibril into amino acids?
  - what proteolytic enzymes systems, and thus including their inhibitors and activators, are responsible for the degradation of myofibrils into amino acids?

* Multidisciplinary approach in order to investigate relation between protein degradation of skeletal muscle and muscle proteinases.
  - investigating hormonal influences
  - studying other models than dietary manipulation, *e.g.*, use of different selection lines, use of repartitioning agents?

* The underlying assumption in this thesis for improving protein deposition by lowering protein breakdown of skeletal muscle is that protein synthesis capacity and rates are maintained. This assumption should be emphasized:
  - investigate the consequences on protein synthesis rates in skeletal muscle, because they might largely be dependent on amount and quality of protein degradation
  - investigate the extent of nitrogen losses into the environment.

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Summary

Improving efficiency of protein deposition is one of the important goals in animal meat production. Theoretically as far as meat is involved, this efficiency can be increased by diminishing rate of muscle protein breakdown, provided that the rate of protein synthesis remains the same. In that case, the precursors needed for protein synthesis have to be made available by either decreasing the rate of oxidation of amino acids and/or by an increased intake of dietary protein. This implicates that waste of nitrogen into the environment would be reduced. In addition, less dietary protein is needed to deposit similar amounts of proteins in skeletal muscle (and thus meat) as in present situation. Moreover, by reduction of rate of protein breakdown, rate of protein turnover decreases, which reduces whole body energy requirement.

In order to manipulate the rate of muscle protein breakdown for interventions to increase efficiency of protein accretion, mechanisms and regulation of this process have to be known. However, the knowledge in this field is limited. The first approach of this thesis has been to study the relation between muscle protein breakdown *in vivo* and activity of proteolytic enzyme systems in skeletal muscle. The rate-limiting step in the cascade of muscle protein breakdown would be identified if activity of one of the proteolytic enzyme systems would change in parallel with a change in muscle protein breakdown. Thus, this would then open possibilities to manipulate efficiency of protein accretion.

The experiments presented in this thesis are carried out to investigate the relation between proteolytic enzyme systems and production of 3-methylhistidine (3MH). 3-Methylhistidine is a specific constituent of the myofibrillar protein, actin and myosin heavy chain. In most species, urinary excretion of 3MH can be used as an index for myofibrillar protein degradation, because this excretion is representative for the production of 3MH. In pigs, however, 3MH is not excreted in the urine, but is stored in skeletal muscle as a dipeptide called balenine (ß-alanine-3-methylhistidine). In pigs, production rate of 3MH, rather than excretion rate, can be estimated using a compartmental model analysis. At the same time, three different proteolytic enzyme systems were studied in this thesis: the calpain system (μ- and m-calpain and their inhibitor calpastatin), multicatalytical proteinase and the lysosomal cathepsins, inhibited by the cystatins. As an experimental model, we have manipulated myofibrillar protein degradation by feeding a protein-free diet for 14 days to growing pigs.

In the first experiment (CHAPTER 2), relation between 3MH production and proteinase activities in different skeletal muscles was studied. Feeding a protein-

free diet reduced growth rate to almost zero. The impaired muscle growth was also reflected by a reduced DNA transcription and translation. Production of 3MH was increased in the animals fed the protein-free diet, indicating that myofibrillar protein degradation was increased. However, no change was found in activities of one of the proteolytic enzyme systems in different skeletal muscles between treatments. This discrepancy can be explained in several ways. Firstly, production of 3MH may have been elevated form sources other than skeletal muscle. Secondly, other proteases are responsible for the rate-limiting step in the breakdown of myofibrillar proteins. Thirdly, proteinase assays performed *in vitro* may not represent physiological activity.

In the second experiment (CHAPTER 3), the findings of the first experiment were repeated (except for the lysosomal system). In addition, we examined the possibility that the observed findings by feeding a protein-free diet could be due to an increase in dietary carbohydrates. Therefore, a second protein-free diet was made by isocaloric exchange of dietary protein by fat. Half of the animals in each group were also realimentated after the protein-free feeding period for another 7 d, to investigate the relation between proteolytic enzyme systems and 3MH production during compensatory growth. No differences were found between treatments for 3MH production and for proteinase activities. Some compensation has only occurred during the first 3 d, based on data of growth rate and feed efficiency.

In CHAPTER 4, chemical composition of carcass, liver, and both large and small intestines were analyzed of the animals of the second experiment in order to study changes in fat and protein concentrations. Feeding either of the protein-free diets caused fattening of the carcass while protein concentration of carcass, liver, and intestines were decreased. After realimentation, protein concentrations were restored in liver and intestines, but not in the carcass. This suggests that protein metabolism in liver and intestines responds more rapidly to dietary changes than carcass.

In the third experiment (CHAPTER 5), the possibility that 3MH production was increased by sources other than skeletal muscle was investigated. The 3MH contribution from the gastro-intestinal tract was investigated by using two catheters, *i.e.*, placed in carotid artery and in portal vein. Results show that the 3MH contribution of gastro-intestinal tract to whole body 3MH production was not substantial and stayed below 5%. Moreover, feeding a protein-free diet did not change this contribution. Thus, increased 3MH production after feeding a protein-free diet originates mainly form increased breakdown of skeletal muscle.

In the fourth experiment (CHAPTER 6), an alternative approach was used to obtain more insight into the involvement of the calpain system during

myofibrillar protein breakdown, since proteolytic activity is measured *in vitro* which may not reveal physiological activity. In this study, the calpain system was investigated at the transcriptional level by measuring mRNA levels. Results indicated that for m-calpain and calpastatin, mRNA levels correspond to proteolytic activity. For μ-calpain, the mRNA level was reduced on d 14 for animals in the protein-free group, though proteolytic activity was not different between dietary treatments. These data suggest that the calpain system is not involved in the rate-limiting step of myofibrillar protein degradation during a protein-free feeding period.

The general discussion (CHAPTER 7) describes the relation between 3-methylhistidine and proteolytic enzyme systems during a protein-free feeding period. The proteolytic enzyme systems examined do not seem to be responsible for the rate-limiting step during increased myofibrillar protein degradation as a consequence of feeding a protein-free diet. Therefore, other proteolytic enzyme systems must be responsible for this step. A model is discussed in which the degrading system does not necessarily contains the rate-limiting step. The rate-limiting step may be related to the dissociation of the myofibrillar structure itself.

Samenvatting

Eén van de belangrijkste doelen in de vleesproducerende industrie is het verbeteren van de efficiëntie van eiwitaanzet. De mate van eiwitaanzet wordt bepaald door de eiwitturnover, gedefinieerd als het gelijktijdig optreden van zowel eiwitaanmaak als van -afbraak. Theoretisch gezien kan dus, wat betreft de vleesproduktie, de efficiëntie van de eiwitaanzet verhoogd worden door het verlagen van de snelheid van spiereiwitafbraak, mits de snelheid van spiereiwitaanmaak niet veranderd wordt. De voorwaarde voor een gelijkblijvende eiwitaanmaak is dat er voldoende precursors (de aminozuren) voorhanden zijn voor de eiwitaanmaak. Aangezien via een verminderde eiwitafbraak de hoeveelheid aminozuren eveneens verlaagd wordt, moet via andere processen de beschikbaarheid van aminozuren verhoogd worden. Dit kan gebeuren door middel van een verminderde oxydatie van aminozuren en/of door een verhoogde opname van voedingseiwit via het voer. Dit impliceert in het eerste geval dat het stikstofverlies naar het milieu zou verminderen. In het tweede geval zou met een verminderde hoeveelheid voer eenzelfde hoeveelheid eiwitaanzet (en dus vlees) gerealiseerd worden. Bovendien heeft een verminderde eiwitafbraak een verlaging van de eiwitturnover tot gevolg, waardoor de energiebehoefte van het gehele lichaam gereduceerd is.

Om het proces van de spiereiwitafbraak te kunnen beïnvloeden om zodoende de efficiëntie van de eiwitaanzet te kunnen verbeteren, moeten de onderliggende mechanismen en regulatie van dit proces bekend zijn. Echter, in tegenstelling tot de kennis over de spiereiwitaanmaak is er weinig bekend over de spiereiwitafbraak. Onze eerste benadering was om de relatie te bestuderen tussen de mate van spiereiwitafbraak *in vivo* en de activiteit van verschillende enzymsystemen, die verantwoordelijk zijn voor de afbraak van eiwitten van de skeletspier. Voor de volledige afbraak van spiereiwitten tot aminozuren is een reeks aan enzymen verantwoordelijk. De snelheidsbepalende stap in deze reeks is nog onbekend en kan geïdentificeerd worden als de activiteit van één van de enzymsystemen zou veranderen tegelijkertijd met de verandering in de afbraak van spiereiwitten. Dit zou dan dus mogelijkheden kunnen bieden voor manipulaties van de efficiënties van spiereiwitaanzet.

De onderzoeken die gepresenteerd worden in dit proefschrift zijn uitgevoerd om de relatie aan te tonen tussen proteolytische (=eiwitsplitsende) enzymsystemen en de produktie van 3-methylhistidine (3MH). 3-Methylhistidine is een specifiek bestanddeel van de myofibrillaire eiwitten, actine en myosine (zware keten). Het eiwitbestanddeel van de spier bestaat voor ruim 55% uit

myofibrillaire eiwitten, die het bestanddeel vormen van de myofibril in de skeletspier. In de meeste diersoorten, is de uitscheiding van 3MH in de urine representatief voor de produktie van 3MH en kan als zodanig dus gebruikt worden als maat voor de myofibrillaire eiwitafbraak. Echter, bij varkens wordt 3MH niet uitgescheiden, maar opgeslagen in de spieren in de vorm van een dipeptide genaamd balenine (ß-alanine-3-methylhistidine). De produktiesnelheid van 3MH in plaats van de uitscheidingssnelheid kan geschat worden door middel van een compartimenten model analyse. Naast deze bepaling werden er drie verschillende enzymsystemen bestudeerd: het calpaïne systeem (μ- en m-calpaïne met hun natuurlijke remmer calpastatine), het multicatalytische proteinase en de lysosomale cathepsinen met hun natuurlijke remmers, de cystatinen. Als een experimenteel model, is de myofibrillaire eiwitafbraak gemanipuleerd door het verstrekken van een eiwitvrij voer gedurende 14 dagen aan groeiende varkens.

In het eerste experiment (HOOFDSTUK 2) is de relatie tussen 3MH produktie en proteolytische enzymactiviteiten in verschillende skeletspieren bestudeerd. Het verstrekken van een eiwitvrij voer verlaagde de groeisnelheid tot ongeveer nul. De verminderde spiergroei was ook zichtbaar door een verminderde DNA transcriptie en translatie, dus via de aanmaak van spiereiwitten. De 3MH produktie was verhoogd bij de dieren die een eiwitvrij voer kregen, wat aangeeft dat de myofibrillaire eiwitafbraak verhoogd was. Echter, er waren geen verschillen zichtbaar in de activiteiten van één van de verschillende enzymsystemen in de verschillende skeletspieren tussen de behandelingen. Deze tegenstrijdigheid kan verklaard worden door verschillende mogelijkheden. Ten eerste, de 3MH produktie kan ook verhoogd zijn geweest door andere bronnen dan de skeletspieren (namelijk de gladde spieren). Ten tweede, andere proteolytische enzymen kunnen verantwoordelijk zijn voor de snelheids-bepalende stap in de afbraak van myofibrillaire eiwitten. Ten derde, het vaststellen van enzymactiviteit gebeurt onder *in vitro* omstandigheden en dit weerspiegelt wellicht niet de fysiologische oftewel de *in situ* activiteit.

In het tweede experiment (HOOFDSTUK 3) zijn de bevindingen van het eerste experiment herhaald (op het lysosomale enzymsysteem na). Bovendien is de mogelijkheid uitgesloten dat de waargenomen effecten van het verstrekken van een eiwitvrij voer te wijten zijn aan een verhoging van de hoeveelheid koolhydraten in het voer. Daartoe was een tweede eiwitvrij voer gemaakt, waarbij eiwit isocalorisch is vervangen door vet. De helft van de dieren in elke behandelingsgroep werden gedurende zeven dagen hervoed na de 14 dagen ondervoedingsperiode om zodoende de relatie te bestuderen tussen de 3MH produktie en enzymactiviteit in spieren tijdens compensatoire groei. Er werden

geen verschillen gevonden in 3MH produktie en enzymactiviteit tussen de verschillende behandelingen in beide perioden. Gebaseerd op de resultaten van de groeisnelheid en voerefficiëntie kan geconcludeerd worden dat slechts enige compensatie heeft plaats gevonden tijdens de eerste drie dagen van de hervoedingsperiode.

In HOOFDSTUK 4 zijn de resultaten van de chemische samenstelling van het karkas, de lever en zowel dikke en dunne darmen van de dieren uit het tweede experiment beschreven om veranderingen in vet- en eiwitconcentraties te bestuderen. Het verstrekken van één van beide eiwitvrije voeders veroorzaakte een vervetting van het karkas, terwijl de eiwitconcentratie in het karkas, de lever en darmen afnam. Na hervoedering herstelde de eiwitconcentratie in zowel de lever als de darmen, maar dit gebeurde niet in het karkas. Deze resultaten suggereren dat het eiwitmetabolisme in de lever en darmen sneller reageert op veranderde voedingsomstandigheden dan het karkas.

In het derde experiment (HOOFDSTUK 5) is de mogelijkheid bestudeerd dat de 3MH produktie verhoogd kan zijn geweest vanuit andere bronnen dan de skeletspier. De bijdrage van het maagdarmkanaal is bestudeerd door gebruik te maken van twee catheters, namelijk in de *a. carotis* en in de poortader. De resultaten geven aan dat de bijdrage van het maagdarmkanaal aan de totale 3MH produktie marginaal is (lager dan 5%). Bovendien veroorzaakt het verstrekken van een eiwitvrij voer geen verhoging van deze bijdrage. Dus, de verhoogde 3MH produktie na het voeren van een eiwitvrij voer is voornamelijk afkomstig van de verhoogde afbraak van skeletspieren.

In het vierde en laatste experiment (HOOFDSTUK 6) is voor een alternatieve benadering gekozen om meer inzicht te verkrijgen in de betrokkenheid van het calpaïne systeem tijdens de myofibrillaire eiwitafbraak. Naast het meten van de *in vitro* activiteit, die wellicht niet de fysiologische activiteit weerspiegelt, is het calpaïne systeem bestudeerd op transcriptie-niveau door het meten van de mRNA niveaus. Het mRNA, oftewel 'messenger' of boodschapper RNA, bevat de code voor de aanmaak van het desbetreffende eiwit. Resultaten laten zien dat het mRNA niveau van zowel m-calpaïne als calpastatine overeenkomen met hun proteolytische enzymactiviteiten. Het mRNA voor μ-calpaïne was verlaagd op dag 14 van het experiment van de dieren in de eiwitvrije groep, terwijl de proteolytische enzymactiviteit niet verschillend was tussen de behandelingen. De resultaten suggereren dat het calpaïne systeem niet betrokken is bij de snelheidsbeperkende stap van myofibrillaire eiwitafbraak tijdens een eiwitvrije voerperiode.

In de algemene discussie (HOOFDSTUK 7) is de relatie tussen de 3MH produktie en proteolytische enzymactiviteit tijdens een eiwitvrije voerperiode

bediscussieerd. De bestudeerde enzymsystemen lijken niet verantwoordelijk te zijn voor de snelheidsbepalende stap tijdens verhoogde myofibrillaire eiwitafbraak als gevolg van het verstrekken van een eiwitvrij voer. Dus, andere enzymsystemen moeten hiervoor verantwoordelijk zijn. Een mogelijk model wordt bediscussieerd waarin het proteolytische enzymsysteem niet noodzakelijkerwijs de snelheidsbepalende stap hoeft te bevatten. De snelheidsbepalende stap zou namelijk ook gerelateerd kunnen zijn aan de dissociatie van de myofibrillaire eiwitstructuur zelf.

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Curriculum vitae

Henriëtte Nicole Adèle Grooten werd geboren op 5 december 1966 te Utrecht. Zij behaalde het gymnasium-ß diploma aan de Scholengemeenschap St. Ursula te Horn (L.) in 1985. In datzelfde jaar begin zij haar studie Voeding van de Mens aan de toenmalige Landbouw Hogeschool te Wageningen. In januari 1990 werd de opleiding gevolgd tot functionaris ex artikel 9 van de Wet op de Dierproeven aan de Rijksuniversiteit Utrecht. In juni 1991 studeerde zij af aan de Landbouwuniversiteit Wageningen met als hoofdvakken Voedingsleer, Toxicologie en Fysiologie van Mens en Dier. Vanaf 1 augustus 1991 was zij tijdelijk aangesteld als redactioneel assistent van prof. dr ir A.C. Beynen, vakgroep Proefdierkunde van de Rijksuniversiteit Utrecht voor het verwerken van onderzoeksresultaten tot (concept)publikaties. Vanaf 1 november 1991 was zij aangesteld als Assistent in Opleiding (AIO) bij de vakgroep Veevoeding, Landbouwuniversiteit Wageningen, en gedetacheerd bij het voormalige DLO-Instituut voor Veeteeltkundig Onderzoek (IVO-DLO) te Zeist. Tijdens deze AIO-aanstelling werd van mei tot en met november 1992 onderzoek verricht bij het Roman L. Hruska U.S. Meat Animal Research Center (USDA-ARS), Clay Center, Nebraska, VS, onder leiding van dr M. Koohmaraie.

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