British Journal of Nutrition (2001), **85**, 447–457
DOI: 10.1079/BJN2000291

Activation of skeletal muscle protein breakdown following consumption of soyabean protein in pigs

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(Received 22 March 2000 – Revised 21 August 2000 – Accepted 10 November 2000)

Diets with protein of inferior quality may increase protein breakdown in skeletal muscle but the experimental results are inconsistent. To elucidate the relationship, pigs were fed isoenergetic and isonitrogenous diets based on soyabean-protein isolate or casein for 15 weeks, with four to six animals per group. A higher plasma level of urea (2·5-fold the casein group value, $P = 0{\cdot}01$), higher urinary N excretion (2·1-fold the casein group value, $P = 0{\cdot}01$), a postabsorptive rise in the plasma levels of urea, 3-methylhistidine and isoleucine in soyabean protein-fed pigs suggested recruitment of circulatory amino acids by protein breakdown in peripheral tissues. Significant differences between dietary groups were detected in lysosomal and ATP-dependent proteolytic activities in the *semimembranosus* muscle of food-deprived pigs. A higher concentration of cathepsin B protein was found, corresponding to a rise in the cathepsin B activity, in response to dietary soyabean protein. Muscle ATP-stimulated proteolytical activity was 1·6-fold the casein group value ($P = 0{\cdot}03$). A transient rise in the level of cortisol (2·9-times the casein group value, $P = 0{\cdot}02$) occurred in the postprandial phase only in the soyabean group. These data suggest that the inferior quality of dietary soyabean protein induces hormonally-mediated upregulation of muscle protein breakdown for recruitment of circulatory amino acids in a postabsorptive state.

Amino acid limitation: Protein degradation: Energy retention

An improvement of protein quality by supplementation with essential amino acids increased both protein synthesis and degradation in swine (Salter *et al.* 1990; Kerr & Easter, 1995). These reports contrast with studies that did not find significant changes in protein turnover by amino acid supplementation in pigs (Fuller *et al.* 1987) and rats (Garlick & Grant, 1988). The mechanism underlying the action of dietary proteins with inferior quality is incompletely understood, so that the biological background of the differing results remains to be clarified. Hormones such as insulin and glucocorticoids have been reported to be involved in the response to changes in the amino acid supply (Seve & Ponter, 1997). Recent studies have suggested that liver and muscle protein synthesis and degradation (Fereday *et al.* 1998; Patti *et al.* 1998) are the targets of the hormonally induced regulation to match changes in diet.

The conflicting results mentioned earlier may arise from different methods of measuring protein synthesis and degradation (Grizard *et al.* 1995; Smith *et al.* 1998). However, interactions among hormonal and dietary effects can also provide ambiguous results, since insulin-induced inhibition of proteolysis seems to be sensitive to amino acid supply (Fereday *et al.* 1998). Thus, the aim of the present study was a direct analysis of some proteolytic activities in skeletal muscle in relation to plasma levels of amino acids in response to diets based on soyabean protein or casein.

Materials and methods

Materials

Nutrients were purchased from Deutsches Milchkontor GmbH Hamburg, Interfood, Bad Homburg, and Schleicher and Schüll, Dassel, Germany. Catheters were from Cook, Mönchengladbach, Germany. Rabbit antisera to cathepsin B and cystatin C and cathepsin B standard were from Calbiochem-Novabiochem GmbH, Bad Soden, Germany; proteinase inhibitors from Alexis, Grünberg, Boehringer-Mannheim, and Serva, Heidelberg, Germany; the calpain substrate, *tert*-butoxylcarbonyl-L-leucyl-L-methionine-7-amino-4-choloromethylcoumarin; the cathapsin substrates,

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7-amino-4-methylcoumarin, benzyloxycarbonyl-L-arginyl-L-arginine amide, and 7-amino-4-chloromethylcoumarin, benzyloxycarbonyl-L-arginine amide from Molecular Probes, Eugene, OR, USA. Dimethyl sulfoxide, 1,4-dithiothreitol, 3-((3-cholamidopropyl)dimethylammonio)-1-propane-sulfonate (Chaps), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), EDTA and other chemicals and biochemicals were bought from Sigma, Deisenhofen, Germany.

Experimental procedure

Twelve male, castrated Landrace pigs (25 (SD 2) kg at the start) were randomly assigned to two dietary groups, each having six animals per group. The experiments began with an adaptation period (3 weeks) followed by the experimental period (12 weeks). The pigs were single-housed in pens (ambient temperature 23 ± 1°C, relative humidity 60–70 %). In week 2, they were accustomed to a respiration chamber. Then, they were again single-housed in pens. The daily ration was given once a day in the morning to measure postprandial and postabsorptive changes in plasma metabolites. The pigs had free access to tap water. In week 10, the animals were fitted with a silicone catheter in the *vena jugularis externa*, and allowed to recover from surgery for 10 d. Blood was then withdrawn and N balance and energy balance were analysed using four respiration chambers for a period of 4 d after 2 d for the animals to become accustomed to the chambers (Hoffman *et al.* 1993). Blood sampling was repeated the following week. The mean values of the data from both samples are indicated in the results (p. 449) when not otherwise stated. Blood samples (10 ml) were withdrawn after a morning meal every 15 min within the first hour, then every 1 h up to 5 h postprandially, next in 2 h intervals up to 19 h after the meal and then every 1 h up to 24 h. The 23 h sample is referred to as the fasting value in the text. The corresponding body weight was recorded weekly. The procedure was approved by the local Governmental Animal Care Advisory Committee. State of health was monitored by measuring the rectal temperature daily. Health problems occurred in both dietary groups after catheterization. Two animals in the soyabean-protein group died (week 15). Blood analyses were performed only in specimens from animals with a normal rectal temperature, having four to six animals per group as indicated in the results. Fasted animals were killed (week 15) and tissue specimens (liver and *m. semimembranosus*) were immediately frozen in liquid N and stored (−80°C) until analyses were performed. The body was minced and freeze-dried samples were analysed for DM, fat and N.

Diets

The pigs were given a semipurified, isoenergetic and isonitrogenous diet. The composition was (g/kg DM): maize starch 410, protein (either casein or soyabean-protein isolate) 90, sucrose 200, fat 150 (margarine 75, lard 75), cellulose 70, minerals and vitamins 80 (the composition has been previously described by Schmitz *et al.* 1991). Soyabean protein was commercially isolated (Interfood) by heat treatment under mild alkaline conditions. The diet (with casein or soyabean-protein isolate as the only independent variable) provided 2·5-times the maintenance requirement of metabolizable energy, i.e. 1850 kJ ME/kg body weight$^{0·62}$ per d, corresponding to approximatively 110 g DM/d and /kg body weight$^{0·62}$. The casein diet was supplemented with 11·5 g methionine, 5·8 g threonine and 4·6 g trytophan/kg dietary protein.

Energy and nitrogen balance

The energy balance was measured by indirect calorimetry based on the C and N balances. Measurements were performed in four open-circuit respiration chambers using four successive 24 h periods with daily collection of urine and faeces separately, stored at 0–3°C until analyses. The concentration of O_2, CO_2 and CH_4, the temperature, relative humidity and the rate of air flow were recorded on-line every 10 min. Standing and lying periods were monitored by a video camera. N and DM in feed, faeces and urine were determined by the Kjehldahl method and by oven-drying at 101°C for 48 h respectively. The energy contents of feed, freeze-dried faeces and urine were analysed by an adiabatic bomb calorimeter (C 400; Janke & Kunkel GmbH, Staufen, Germany) followed by gravimetric determination of CO_2 for calculation of the C content in feed and faeces as described by Hoffmann *et al.* (1993).

Analyses

Food amino acids were measured by automatic ion-exchange chromatography (Biochrom 20; Pharmacia-Biotech Europe GmbH, Freiburg, Germany) following acid hydrolysis (6 M-HCl, 134°C, 2 h) in an autoclave, cysteine and methionine after oxidation with peroxyformic acid, tryptophan after alkaline hydrolysis with NaOH. Acid-soluble amino acids in plasma or muscle were determined by ion-exchange chromatography after precipitation of protein (sulfosalicylic acid, 0·25 M final concentration, 20 min on ice) and centrifugation (4000 ***g***, 10 min, 4°C) using plasma or muscle extracts. The latter were prepared by homogenization (Ultra Turrax; Janke & Kunkel GmbH) of minced muscle (maximal speed, repeated homogenization for 2 min with 30 s intervals) on ice in five volumes 5 mM-PBS (pH 7·0)/g tissue and subsequent centrifugation (4000 ***g***, 10 min, 4°C). Glycogen was measured in the homogenate (omitting centrifugation) by α-amylase-catalysed release of glucose, detecting glucose before and after digestion. Standard methods were also used for the analysis of glucose (Sigma Diagnostics, catalogue no. 315), of aqueous-soluble protein (Bradford, 1976), of albumin (Sigma Diagnostics, catalogue no. 631) and of urea-N (Sigma Diagnostics, catalogue no. 535). Commercially available enzyme immunoassays were used for assaying cortisol (catalogue no. MDKC01, Milenia, Bad Nauheim, Germany), thyroxine and triiodothyronine (catalogue no. 3894 and 1615, DRG Diagnostica, Bad Nauheim, Germany) and insulin (no. 43914809, Wako Pure Chemical Industries, Bad Nauheim, Germany).

Western blot analysis

Muscle was homogenized as described earlier using a buffer consisting of: 20 mM-*N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (Hepes) pH 7·3, 10 mM-NaCl, 10 mM-KCl, 10 mM-3-((3-cholamidopropyl) dimethylammonio)-1-propane-sulfonate; 5 g Triton X100/l, 1 mg pepstatin A/l, 1 mg aprotinin/l, 1 mg leupeptin/l, 1 mM-phenylmethylsulfonyl fluoride. After ultracentrifugation (110 000 ***g***, 30 min, 4°C) the total protein concentration in the supernatant fraction was determined and the supernatant fraction was stored (−80°C). After thawing, the supernatant fraction was mixed with non-reducing electrophoresis loading buffer (1:2, v/v), incubated for 2 min at 90°C, and electrophoresis and blotting was performed as described by Löhrke *et al.* (1993, 1998) using 40 μg protein per lane. An enhanced chemiluminescence kit (Amersham, Braunschweig, Germany) and a chemiluminescence analyser (Fluor-S Multi Manager; BioRad, Hamburg, Germany) were used to visualize and to quantify the protein bands.

Proteolytic activities

Cathepsin B activity in muscle fibres was detected by flow cytometry (EPICS Elite, Coulter, Krefeld, Germany) as described by Löhrke *et al.* (1995). This technique enables a single cell fluorescence analysis of fibres separate from other cells arising from digestion of muscle tissue by collagenase (Löhrke *et al.* 1995, 2000). The cathepsin B activity was analysed in 20 mM-potassium phosphate (pH 6·5)-buffered saline (130 mM-NaCl), containing the fluorogenic substrate 7-amino-4-methylcoumarin, benzyloxycarbonyl-L-arginyl-L-arginine amide, selective for cathepsin B in cell systems (Assfalg-Machleidt *et al.* 1992; Inubushi *et al.* 1996), in several doses and 1 mM-4-(2-aminothyl) benzenesulfonylfluoride, 2 mM-EDTA, and 2 mM-1,4-dithiothreitol. The activity was measured in initial rate conditions, incubating the cells for 20 min at 37°C according to a preliminary experiment, indicating linearity in the fluorescence increase during the first 20 min of an incubation period using the substrate in approximately saturated concentration (30 μM). The specificity of the test was examined using the cell-permeable cathepsin B inhibitor *N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) (Towatari *et al.* 1991), dissolved in dimethylsulfoxide. The final concentrations were 20 μM CA-074 (a maximal-effective dose) and 1·4 mM-dimethylsulfoxide. Cathepsin H activity was assayed with 7-amino-4-chloromethylcoumarin-arginine in PBS, pH 7·0, using *N*-(L-3-trans-ethoxycarbonyl oxirane-2-carbonyl)-L-leucyl-3-methylbutylamide (E-64d) (100 μM) to inhibit the activity as described by Towatari *et al.* (1991). Calpain activities were determined with *tert*-butoxylcarbonyl-leucine-methionine-7-amino-4-chloromethylcoumarin as a cell-permeable fluorogenic substrate following a method described by Rosser *et al.* (1993). ATP-dependent proteolysis was measured in the cytosolic fraction of the muscle using azocasein as substrate according to a slightly modified method (Glickman *et al.* 1998). The homogenization buffer consisted of: 20 mM-*N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (pH 7·8), 20 mM-NaCl, 10 mM-KCl, 2 mM-$MgSO_4$, 0·5 mM-EDTA, 1 mM-1,4-dithiothreitol, 100 ml glycerol/l. After centrifugation (110 000 ***g***, 30 min, 4°C), the protein concentration of the supernatant fraction was determined with the Bradford reagent. The supernatant fraction was mixed (1:2, v/v) with azocasein (10 mg/ml) dissolved in homogenization buffer, then 0·01 volumes 5 mM-potassium phosphate (pH 6·5) or ATP (50, 100, 200 mM) dissolved in this buffer were added. After an incubation for 60 min at 37°C, the reaction was stopped on ice. Bovine serum albumin (5 mg/ml final concentration) and TCA (0·37 M final concentration) were added. After 15 min on ice, the precipitate was spun off (4000 ***g***, 10 min, 4°C), and the staining of the supernatant fraction was read at 405 nm. Controls (the same procedure as described earlier but with buffer instead of homogenate) were subtracted. Specificity of the ubiquitin-linked proteolytical pathway (Dick *et al.* 1996) was examined by *clasto*lactacystin β-lactone dissolved in 1,4-dithiothreitol with final concentrations of 2 μM-*clasto*-lactacystin β-lactone and 2 mM-1,4-dithiothreitol.

Statistical analysis

A computer-aided Jandel scientific statistical package (version 1.02 1994, Erkrath, Germany) was used. Variances were calculated by ANOVA (animals within diets) and orthogonal contrasts were used for the test of differences between dietary groups in serial data (time-dependent levels of amino acids). Differences between dietary groups were evaluated to be statistically significant by *t* test or as indicated by the tests for normality and equal variances. Paired *t* test was used to compare temporal differences within dietary groups.

Results

Nitrogen and energy balance

The soyabean-protein diet significantly reduced the N deposition (0·75-fold the casein group value) and increased the urinary N excretion ($P = 0{\cdot}01$) 2·1-fold the casein group value (Table 1). The energy balance data in Table 2 show that only the protein-energy retention markedly responded to the soyabean-protein diet with a 1·5-fold decrease ($P = 0{\cdot}01$ *v.* the casein value). The striking increase in urinary N excretion was associated with a strong elevation of the plasma urea level (Fig. 1) in similar magnitude (2·5-fold the casein group value, $P = 0{\cdot}001$). The postabsorptive urea level increased (paired *t* test, $P = 0{\cdot}02$) only in the soyabean group (Fig. 1). These data indicate an increase in net overall rate of protein breakdown and amino acid oxidation respectively (Grofte *et al.* 1998; Young *et al.* 2000).

Temporal course of circulatory amino acid levels

A dietary deficit in essential amino acids (Fig. 2(c)) caused a postprandial imbalance in circulatory amino acid levels (Fig. 2(a)) as expected. However, the postprandial differences

Table 1. Nitrogen balance in pigs fed soyabean protein- or casein-based diets*
(Mean values and standard deviations)

		Weight gain (g/d)		N intake (g N/d)		N excretion				N deposition (g N/d)	
						Urine (g N/d)		Faeces (g N/d)			
Protein	*n*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Casein	6	358	33	14·2	0·1	3·3	0·4	2·4	0·1	8·5	0·4
Soyabean	4†	337	17	15·4	0·3	6·9	0·6	2·2	0·2	6·4	0·3
P value‡		0·06		0·07		0·01		0·08		0·01	

* For details of diets and procedures, see p. 448.
† Two pigs were omitted due to health problems.
‡ *t* test, casein- *v.* soyabean protein-based diet.

in amino acids markedly deficient in soyabean protein not only disappeared postprandially but differences occurred in the circulatory concentration of other amino acids (Fig. 2(b)). Details of this postabsorptive response are illustrated in Fig. 3 for lysine and threonine, moderately deficient in soyabean-protein isolate (Fig. 2(c)), and isoleucine, which is supplied in similar concentrations by soyabean protein- and casein-based diets (Fig. 2(c)).

The compensatory postabsorptive response (Fig. 3) can be the consequence of reduced absorption through, or increased release of amino acids from, tissues into the circulation with the prerequisite of an increase in proteolytic activities. The latter hypothesis was examined because of the increase in net overall protein breakdown using soyabean-protein diet-induced rise in plasma urea level and in N excretion as indicators (Table 1 and Fig. 1).

Acid-soluble muscle amino acids

Skeletal muscle can recruit amino acids released by protein breakdown (Cannon *et al.* 1991; Mackenzie *et al.* 1993). In addition, amino acids by themselves affect proteolytic activities (Mortimore & Pösö, 1987). Hence, we looked in food-deprived pigs for the concentration of acid-soluble amino acids in the *semimembranosus* muscle representative for porcine hindleg muscles (Fig. 4). In contrast to other amino acids, the concentrations of lysine, threonine, and phenylalanine were found to be significantly lower (Fig. 4(a)) in soyabean protein-fed pigs ($P \leq 0{\cdot}03$ *v.* the casein group), raising the possibility that the muscle contributes to maintenance of the circulatory postabsorptive level of these amino acids. The muscle of soyabean protein-fed pigs contained histidine and its derivative, 3-methylhistidine, in higher concentration (Fig. 4(b)) than the muscle of the casein group ($P = 0{\cdot}04$ and $P = 0{\cdot}01$ respectively), suggesting myofibrillar protein breakdown contributed to the dietary response.

Proteolytic activities in the semimembranosus *muscle*

All three major proteolytic activities in the muscle, the lysosomal, Ca^{2+}- and ATP-dependent pathways, can degrade myofibrillar proteins as reported for calpains (Mortimore & Pösö, 1987), lysosomal cathepsin B (Hirao *et al.* 1984), and proteasomes (Attaix *et al.* 1998). ATP-dependent proteolysis of azocasein differed between the dietary groups (Fig. 5). In the presence of 2 mM-ATP, the rate of substrate hydrolysis was 1·6-fold higher in the muscle from the soyabean-protein group than from the casein group ($P = 0{\cdot}02$). The activity was significantly inhibited by *clasto*lactacystin β-lactone, a selective inhibitor of proteasome activities (Fenteany *et al.* 1995; Dick *et al.* 1996). In contrast to Ca^{2+}-dependent protolysis (data not reported), significant differences were also detected between the dietary groups in the activity of lysosomal cathepsins B and H (Fig. 6). The cell permeable inhibitor of cathepsin B (*N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074)) (Towatari *et al.* 1991), and of cathepsin H (*N*-(L-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl-3-methylbutylamide (E64d)) used in a dose of ≥ 100 μM (Towatari *et al.* 1991), significantly reduced the hydrolysis of the substrates for these cathepsins (Fig. 6), indicating that these enzymes contributed to a major portion of substrate hydrolysis.

Table 2. Energy balance in pigs fed soyabean protein- or casein-based diets*
(Mean values and standard deviations)

		Protein intake (g/kg BW$^{0.62}$ per d)		ER (kJ/kg BW$^{0.62}$ per d)		Protein ER (kJ/kg BW$^{0.62}$ per d)		Fat ER (kJ/kg BW$^{0.62}$ per d)		Heat (kJ/kg BW$^{0.62}$ per d)	
Protein	*n*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Casein	6	8·0	0·3	742	53	119	2	623	52	1074	57
Soyabean	4†	8·4	0·1	720	55	82	7	638	48	1108	33
P value‡		0·07		0·11		0·01		0·16		0·09	

ER, energy retention; BW, body weight.
* For details of diets and procedures, see p. 448.
† Two pigs were omitted due to health problems.
‡ *t* test, casein- *v.* soyabean protein-based diet.

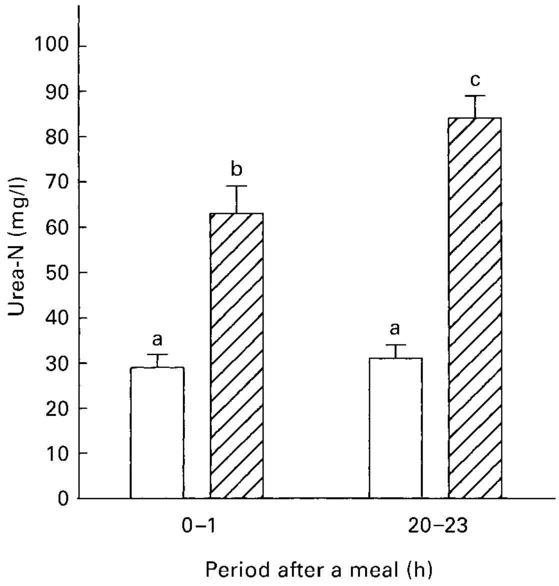

Fig. 1. Plasma urea nitrogen in response to soyabean protein- (▨) or casein- (☐) based diets. Values are means for six pigs per group with standard deviations shown by vertical bars. For details of diets and procedures, see p. 448. ^{a,b,c}Mean values with unlike superscript letters were significantly different (0–1 h *v.* 20–23 h within the soyabean-fed group, $P = 0{\cdot}02$; soyabean-fed group *v.* casein-fed group, $P \leq 0{\cdot}001$ (paired *t* test).

Expression of cathepsin B and cystatin C proteins

Immunoblot data demonstrating marked differences in cathepsin B forms between the dietary groups are shown in Fig. 7. The cathepsin B protein with relative molecular mass >30 000 (Fig. 7, line I) corresponds to the enzyme precursor, with relative molecular mass 27 000–30 000 to active dimers (Fig. 7, line II), and with relative molecular mass 22 000–25 000 (Fig. 7, line III) to one of the subunits of the enzyme (Baricos *et al.* 1988). In contrast, differences among the dietary groups in cystatin C, one of the major natural inhibitors of cathepsins (Barrett *et al.* 1986; Leonardi *et al.* 1996), were not found (Fig. 8).

Response of cortisol levels to the diets

Lysosomal cathepsins have been reported to be positively regulated by glucocorticoids (Hong & Forsberg, 1995; Inubishi *et al.* 1996). Hence, we looked for dietary effects on cortisol concentrations. A transiently prolonged postprandial rise in the cortisol level was evident in pigs fed the soyabean-protein diet (Fig. 9). The cortisol concentration following the soyabean-protein meal was 2·9-times the value of the casein group ($P = 0{\cdot}01$) at this time.

Discussion

The results demonstrate that dietary soyabean protein induced an increase in several muscle proteolytic activities

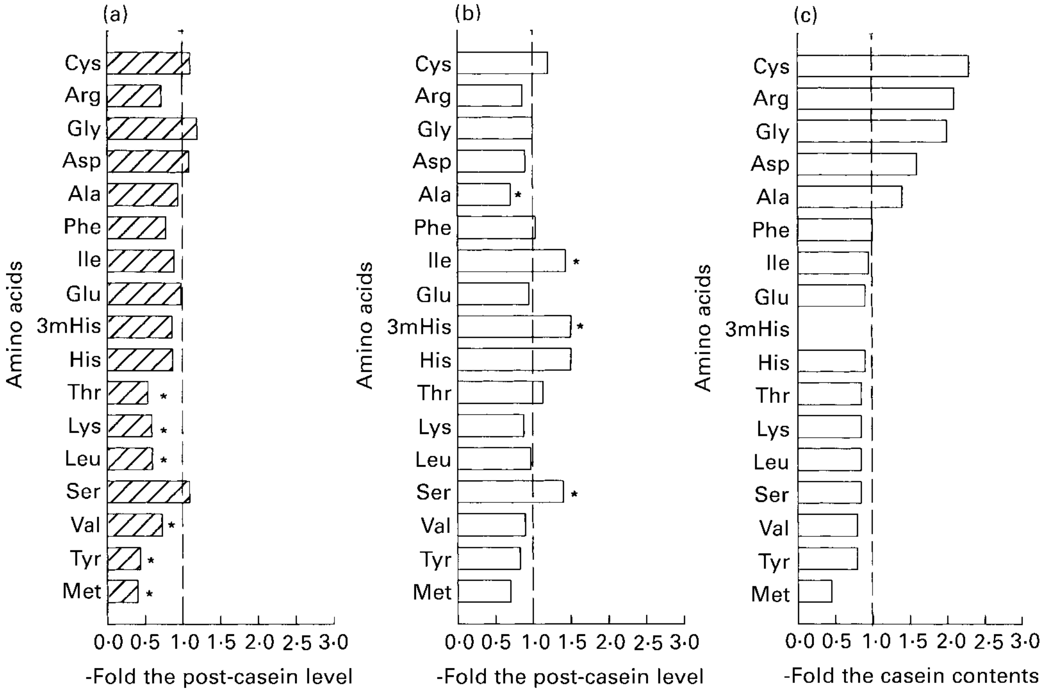

Fig. 2. Relative contents of amino acids in soyabean-protein isolate and in the plasma of soyabean-protein isolate-fed pigs. Values are ratios (soyabean:casein, mM/mM) of individual amino acids in plasma of (a) a postprandial period (0·25–2 h after a meal), (b) a postabsorptive period (20–23 h after a meal), (c) in dietary proteins (mmol/g DM). For details of diets and procedures, see p. 448. Cys, cysteine; Arg, arginine; Gly, glycine; Asp, aspartic acid; Ala, alanine; Phe, phenylalanine; Ile, isoleucine; Glu, glutamine; 3m His, 3-methylhistidine; His, histidine; Thr, threonine; Lys, lysine; Leu, leucine; Ser, serine; Val, valine; Tyr, tyrosine; Met, methionine. Mean values underlying the ratios were significantly different (paired *t* test): soyabean-fed (*n* 4) *v.* casein-fed (*n* 6), $^{*}P < 0{\cdot}05$.

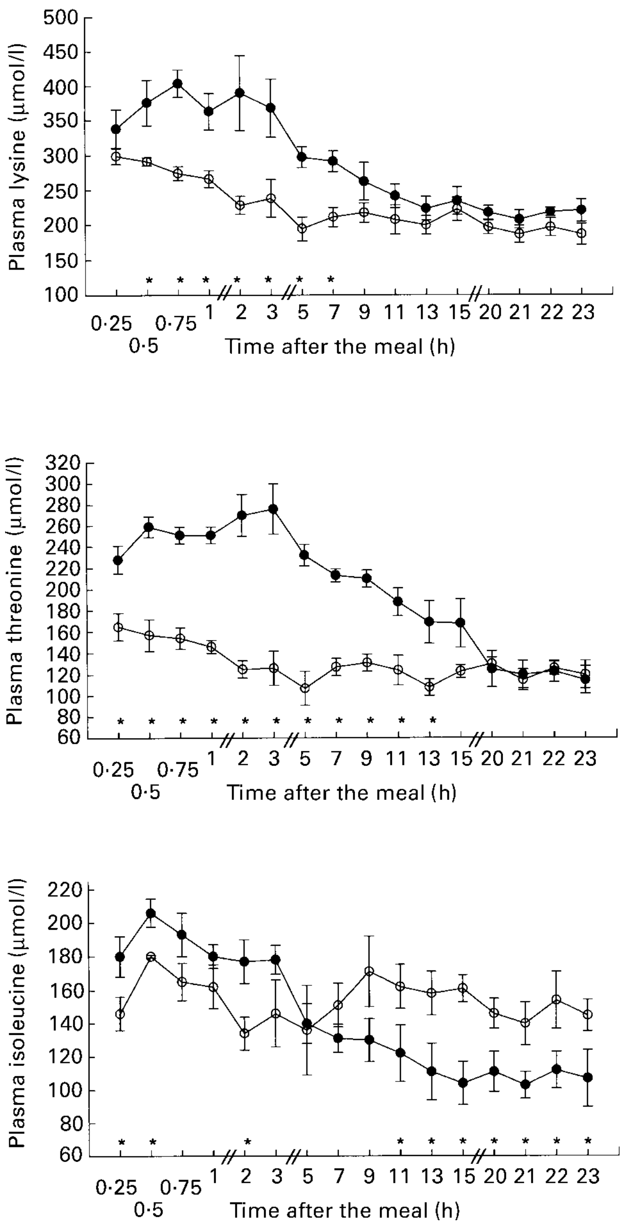

Fig. 3. Diurnal profile of essential amino acids in plasma from pigs fed soyabean protein- (○) or casein- (●) based diets. Values are means with standard errors of the means shown by vertical bars. For details of diets and procedures, see p. 448. Means values were significantly different: soyabean-fed (*n* 4) *v.* casein-fed (*n* 6), **P* < 0·05.

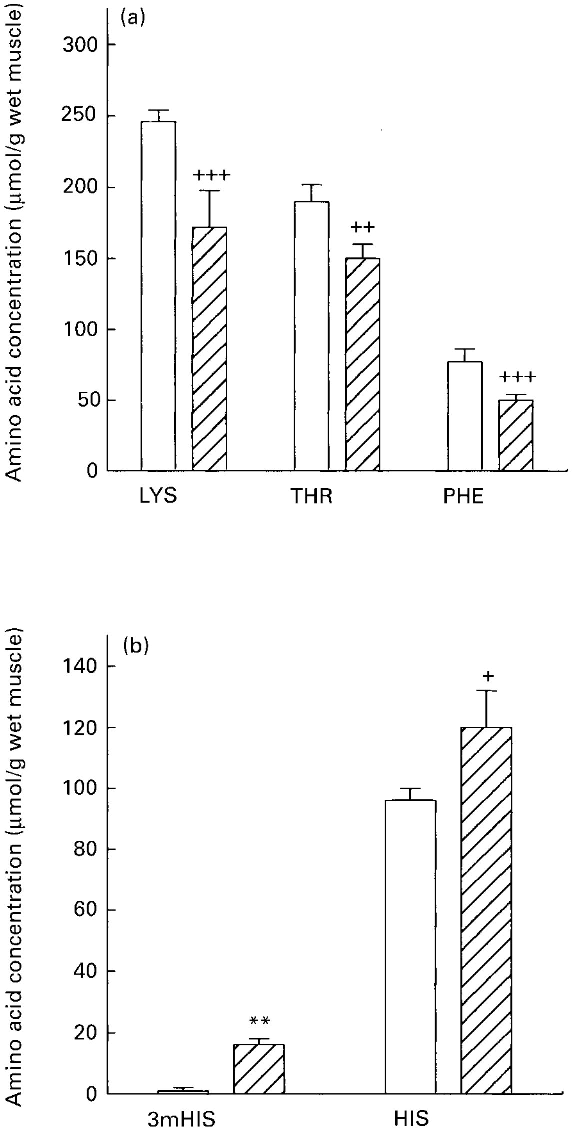

Fig. 4. Dietary response of amino acids in *semimembranosus* muscles of pigs fed soyabean protein- (□) or casein- (▨) based diets. Values are means with standard deviations shown by vertical bars. (a), acid-soluble essential amino acids; (b), 3-methylhistidine and histidine. For details of diets and procedures, see p. 448. Lys, lysine; THR, threonine; PHE, phenylalanine; 3m HIS, 3-methylhistidine HIS, histidine. Mean values were significantly different (*t* test): soyabean-fed (*n* 4) *v.* casein-fed (*n* 6), †*P* = 0·04, ††*P* = 0·03, †††*P* = 0·02, ***P* = 0·01.

and, as a corollary, a rise in muscle 3-methylhistidine. They also show an elevation of plasma urea and urinary N excretion and an altered pattern of diurnal plasma amino acids in response to the soyabean-protein diet. The postprandial differences between soyabean protein- and casein-fed pigs in the plasma concentrations of essential amino acids disappeared and the isoleucine level increased in the postabsorptive period. These results are indicative of a decrease in the utilization and/or of recruiting the levels of these amino acids by protein degradation. There are two ultimate sources of essential amino acids for cells, the external milieu and endogenous protein degradation. In contrast to leucine, valine, and most other essential amino acids, isoleucine is not markedly deficient in soyabean protein. The liver is not the major site for catabolism of branched-chain amino acids, whereas the skeletal muscle oxidizes significant amounts (Schneible *et al.* 1981). Thus, under isoenergetic and isonitrogenous conditions as well as lower N deposition but sufficient dietary supply of isoleucine, the postabsorptive rise in circulatory isoleucine seems to reflect excessive isoleucine arising from endogenous protein breakdown in protein-storing tissues, such as skin, gut and skeletal muscle. These tissues recruit, at least in part, their protein stores postprandially, as indicated

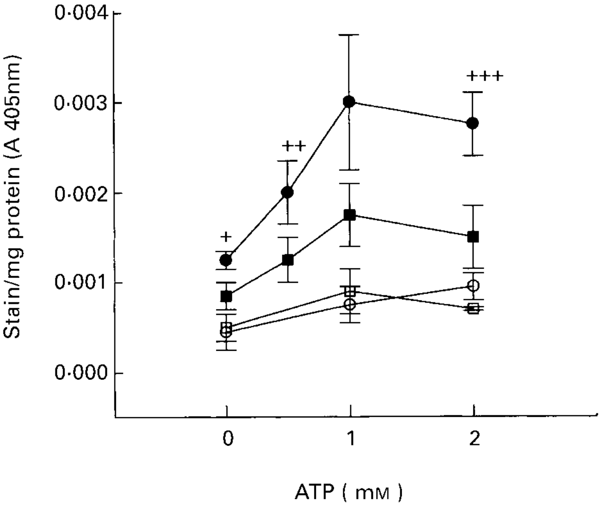

Fig. 5. ATP-dependent proteolytic activity in *semimembranosus* muscles of pigs fed soyabean protein- (●, ○) or casein- (■, □) based diets. Azocasein was used as substrate for ATP-responsive protein-degrading activity in muscle cytosolic fraction, inhibitable by 2 μM *clasto*lactacystin β-lactone (○, □), an inhibitor of proteasome activity. Acid-soluble stain (azogroups-containing digest) after incubation of azocasein with muscle cytosol was expressed as stain/mg cytosolic protein. A, absorption. Values are means with standard deviation shown by vertical bars. For details of diets, see p. 448. Mean values were significantly different (*t* test): soyabean-fed (*n* 4) *v.* casein-fed (*n* 6), †$P = 0{\cdot}04$, ††$P = 0{\cdot}03$, †††$P = 0{\cdot}02$.

by the postprandial course of the isoleucine level, which was similar to the other essential amino acids. The muscle was found to be one of the responsive tissues. A coordinate activation of the ATP-dependent and the lysosomal proteolysis was observed. The latter was indicated by an increase in the expression of cathepsin B and in the activities of cathepsins B and H. The soyabean-protein diet also induced myofibrillar protein degradation using muscle 3-methylhistidine level as an indicator. However, 3-methylhistidine is readily converted into dipeptides in porcine tissues (Harris & Milne, 1987). Therefore, the difference may also arise from different concentrations of constituents of these dipeptides such as β-alanine. Results from previous studies suggest that myofibrillar proteins require an initial attack by Ca^{2+}-dependent proteases in the cytosol in order to be degraded within autophagic vacuoles (Mortimore & Pösö, 1987). More recently, proteasome-mediated proteolysis has also been shown to be responsible for myofibrillar protein breakdown (Mansoor *et al.* 1996). Differences in the rate of cleaving a substrate by calpain action were not detected between the dietary groups. Thus, involvement of this pathway in the proteolytic response to the soyabean-protein-isolate diet remains to be clarified. In contrast, ATP-dependent and lysosomal proteolytic activities were found to be elevated by the soyabean diet. The data are consistent with results indicating stimulated protein turnover in the muscle of pigs following soyabean-protein infusion in comparison with a casein infusion (Deutz *et al.* 1998). In turn, these results contrast somewhat with the concept that skeletal muscle protein synthesis is depressed while initially skeletal muscle protein degradation rate remains unchanged, thereby resulting in a net catabolic state when feeding a poor-quality-protein diet (Fuller *et al.* 1987; Garlick *et al.* 1980; Garlick & Grant, 1988).

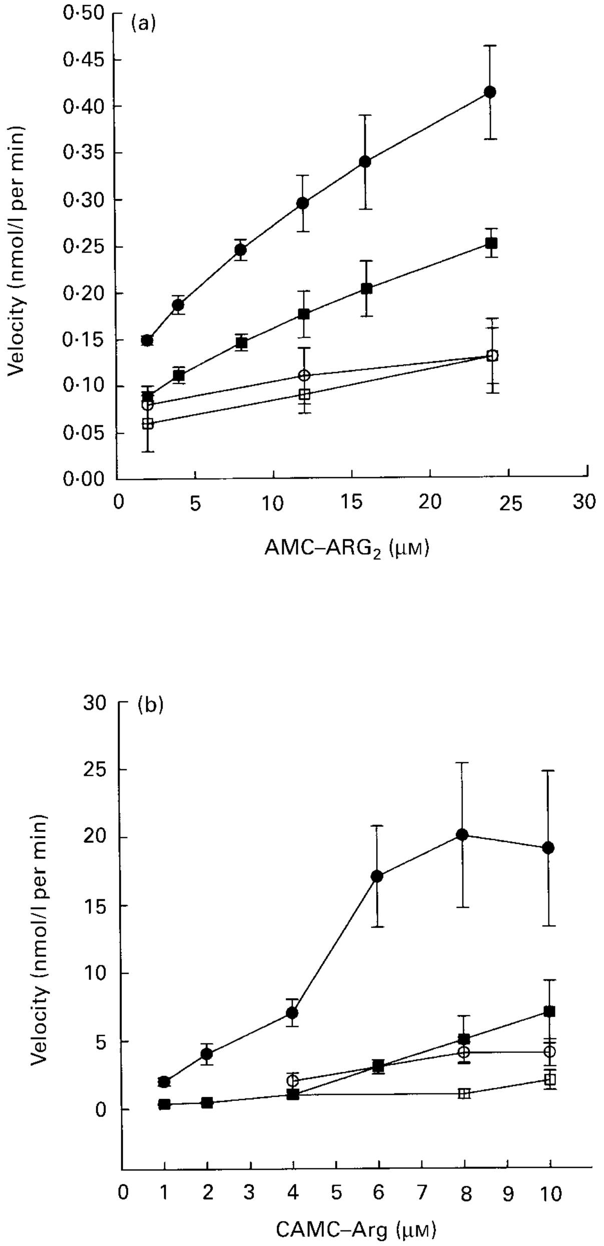

Fig. 6. Activity of (a), cathepsin B and (b), cathepsin H in muscle cells of pigs fed soyabean protein- (●, ○) casein- (■, □) based diets. Hydrolysis of fluorogenic substrates in concentrations indicated on the abscissa were measured in myofibres by flow cytometry. Fluorescence was converted into concentration by calibration with fluorescent standard leads. AMC-Arg$_2$, 7-amino-4-methylcoumarin, benzyloxycarbonyl-L-arginyl-L-arginine amide; CAMC-Arg, 7 amino-4-chloromethylcoumarin, arginine. Values are means with standard deviations shown by vertical bars. ○, □, Fluorescence production in the presence of inhibitors (20 μM-*N*-(L-3-trans-propyl carbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074), 100 μM-*N*-(L-3-trans-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl-3-methylbutylamide (E64d)). For details of diets, see p. 448. Mean values were significantly different for all reactions without inhibitors: soyabean-fed *v.* casein-fed, $P < 0{\cdot}05$).

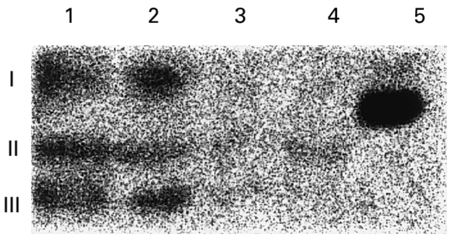

Fig. 7. Immunoblot analysis demonstrating soyabean protein isolate-based diet stimulates expression of cathepsin B protein in *semimembranosus* muscles of pigs. For details of diets and procedures, see p. 448. Lanes 1 and 2 show immunoreactive proteins from two soyabean protein-fed pigs, lanes 3 and 4 the corresponding proteins from casein-fed pigs, representative for each group. Lane 5 shows cathepsin B rat liver standard. I, II, and III indicate the molecular forms of relative molecular mass 40 000, 27 000–30 000, 22 000–25 000 respectively.

Soyabean-derived products may contain antinutritional factors, some of which are thought to suppress carcinogenesis (Hawrylewicz *et al.* 1995; Kennedy, 1995). The soyabean protein used in the diet was commercially isolated by heat treatment under mild alkaline conditions, destroying heat-labile factors, including trypsin inhibitor activity and lectins (Liener, 1994, 1995). Protease inhibitors exert their antinutritional effect by causing pancreatic hypertrophy and hyperplasia while lectins act on intestinal mucosa, inhibiting growth by interfering with the absorption of nutrients. In contrast, heated soyabean flour did not induce a pancreatic response (Nakai *et al.* 1992). Impaired absorption of nutrients is expected to induce differences in balance data among the dietary groups. Neither N intake, retention of energy and fat, nor heat production, a measure of metabolic rate, nor faecal N excretion differed between the groups. Hence, interference with absorption of nutrients is unlikely to cause the lower N deposition and the changes in plasma amino acids and urea as a consequence of feeding dietary soyabean protein.

Deficient essential amino acids, especially methionine, have been reported to act as antinutritional factors and methionine supplementation reversed some adverse effects of soyabean protein on metabolism (Moundras *et al.* 1995). Moreover, starvation or malnutrition, including reduced amino acid supply, can affect food intake, amino acid metabolism, and expression of glucocorticoid-responsive proteolytic enzymes by altering the function of the hypothalamic–pituitary–adrenal axis (Suemaru *et al.* 1986; Chua *et al.* 1991; Woodward *et al.* 1991). Meal intake is a strong stimulus for this axis, inducing glucocorticoid secretion (Hansen *et al.* 1997). Therefore, the altered cortisol response demonstrated here seems to be the consequence of hypothalamic sensitization due to transient and, in the case of methionine, permanent amino acid deficit. Apart from the effects of glucocorticoids, a decrease in amino acid concentration by itself is known to induce coordinate activation of proteolytic activities (Mortimore & Pösö, 1987; Belkhou *et al.* 1994; Fereday *et al.* 1998). Some amino acids can participate in the regulation of gene expression by interacting with amino acid response elements in promoters of responsive genes. Pleiotropic effects arise from a change in the expression of transcription factors, which regulate the expression of a cascade of genes. Amino acid limitation has been reported to affect the expression of a transcription factor, which interacts with C/enhancer binding protein, i.e. transcription factors of the group of CCAAT/enhancer binding proteins (Bruhat *et al.* 1997; Jousse *et al.* 1999). One of these C/enhancer binding protein, C/enhancer binding protein-α, plays a central role in the maintenance of energy homeostasis by regulating the expression of genes encoding leptin (He *et al.* 1995) and transporters of insulin-responsive glucose uptake (Kaestner *et al.* 1990). Glucose transport is energized by pathways also used in amino acid transport mediated by system A of the skeletal muscle (Bonadonna *et al.* 1993; Tsakiridis *et al.* 1995; Hajduch *et al.* 1998). System A transports both glucogenic and some essential amino acids, including methionine, which is deficient in soyabean protein. Thus, amino acid limitation predicts C/enhancer binding protein-α-regulated response which affects cellular transport processes and, as a corollary, the absorption of amino acids from circulative pool. Moreover, genes encoding some lysosomal enzymes,

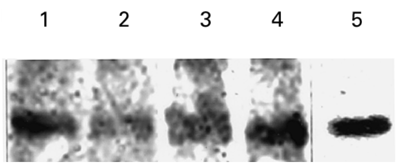

Fig. 8. Immunoblot analysis demonstrating expression of cystatin C protein in *semimembranosus* muscles is not changed by diet in pigs fed soyabean- or casein-based diets. For details of diets and procedures, see p. 448. Lanes 1 and 2 show immunoreactive proteins from two soyabean protein-fed pigs, lanes 3 and 4 the corresponding proteins from casein-fed pigs, representative for each group. Lane 5 shows rainbow molecular mass standard of relative molecular mass 30 000.

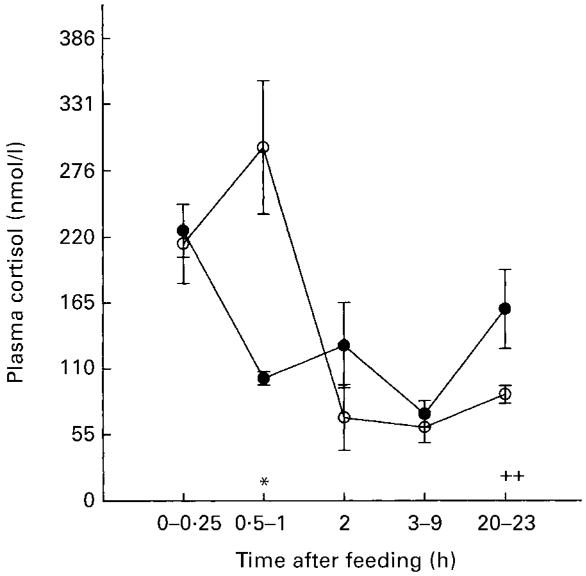

Fig. 9. Response of circulative cortisol concentrations in pigs fed soyabean protein- (○) or casein- (●) based diets. Values are means with standard errors of the means shown by vertical bars. For details of diets and procedures, see p. 448. Mean values were significantly different (*t* test): soyabean-fed (*n* 4) *v.* casein-fed (*n* 6), ††$P = 0{\cdot}03$, *$P = 0{\cdot}01$.

including cathepsins L and H, contain both C/enhancer binding protein and cyclic nucleotide response elements (Ishidoh *et al.* 1989). Therefore, these genes are targets of signals such as catecholamines and some proteohormones, whose effects are mediated by membrane receptors capable of signal transduction by altering intracellular levels of cyclic nucleotides (cAMP, cGMP). Glucocorticoids can increase the density and the activity of cAMP-producing receptors (Reynisdottir *et al.* 1993). Thus, the rise in muscle cathepsin H activity could be a consequence of the markedly elevated cortisol level in concert with changes in cyclic nucleotide-mediated signal transduction following a soyabean-protein diet.

Apart from Ca^{2+}-dependent and lysosomal proteolysis, the ATP-dependent proteasome proteolytic pathway is thought to be of major importance in the breakdown of skeletal muscle proteins (Attaix *et al.* 1998). Glucocorticoids have been reported either to exert no effects (Hong & Forsberg, 1995) or to activate proteasome-mediated proteolysis (Mansoor *et al.* 1996), while cytokines have been consistently reported to induce lysosomal (Cannon *et al.* 1991; Hall-Angeras *et al.* 1991; Hong & Forsberg, 1995), and proteasome-mediated protein breakdown in skeletal muscles (Attaix *et al.* 1998). Hyperinsulinaemia and hyperaminoacidaemia reduce the expression of ubiquitin mRNA in skeletal muscle (Larbaud *et al.* 1996). However, ubiquitination and proteasomal degradation are not obligatorily coupled. Some enzymes, and the model substrate casein, are degraded by proteasomes in ubiquitination-independent fashion (Driscoll & Goldberg, 1990; Murakami *et al.* 1992). Therefore, we used an assay that measured ATP-stimulated hydrolysis of azocasein, a reaction independent of ubiquitination, in the absence and presence of *clasto*lactacystin β-lactone, a compound known to selectively inhibit proteasome-mediated proteolysis (Fenteany *et al.* 1995; Dick *et al.* 1996). Despite a low cleavage rate, significant differences were detected between soyabean and casein groups, suggesting the proteasome pathway as a target of regulatory responses to amino acid limitation in soyabean-protein diet.

Collectively, the results indicate that dietary soyabean protein can induce an amino acid imbalance. Deficient amino acids are known to trigger regulatory responses, including an activation of muscle protein degradation, which could contribute to a recruitment of circulatory amino acids. Glucocorticoids are probably involved in the proteolytic stimulation. These responses are ultimately linked to higher urea production and urinary N excretion, indicators of an increase in net overall rate of protein breakdown occurring in soyabean protein-fed pigs.

Acknowledgements

The study was financially supported by the Deutsche Forschungsgemeinschaft. We appreciate the excellent technical assistance of R. Brose, G. Gratopp, G. Karwath, H. Pröhl, H. Schott and T. Viergutz in performing the experiments and analyses. The paper is dedicated to Professor Dr mult. Karl Rothe on the occasion of his 70th birthday.

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