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Effects of different dietary protein sources on expression of genes related to protein metabolism in growing rats

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Protein metabolism is known to be affected by dietary proteins, but the fundamental mechanisms that underlie the changes in protein metabolism are unclear. The aim of the present study was to test the effects of feeding growing rats with balanced diets containing soya protein isolate, zein and casein as the sole protein source on the expression of genes related to protein metabolism responses in skeletal muscle. The results showed that feeding a zein protein diet to the growing rats induced changes in protein anabolic and catabolic metabolism in their gastrocnemius muscles when compared with those fed either the reference protein casein diet or the soya protein isolate diet. The zein protein diet increased not only the mRNA levels and phosphorylation of mammalian target of rapamycin (mTOR), but also the mRNA expression of muscle atrophy F-box (MAFbx)/atrogin-1 and muscle ring finger 1 (MuRF1), as well as the forkhead box-O (FoxO) transcription factors involved in the induction of the E3 ligases. The amino acid profile of proteins seems to control signalling pathways leading to changes in protein synthesis and proteolysis.

Zein: Protein synthesis: Proteolysis: Muscle: Rats

According to the gastric emptying rate and digestive characteristics of proteins in the gut, 'fast' and 'slow' proteins can be defined[1,2]. 'Fast' and 'slow' proteins have different effects on the protein turnover of the whole body and various organs[3,4]. Recently, animal and human studies have reported a higher whole-body retention and protein synthesis efficiency after a 'slow' protein (i.e. casein) intake, leading to high dietary N incorporation in the peripheral area[4–7].

Being plant proteins, both soya protein and zein are always included in animal diet formulations in swine and poultry production[8,9]. Although abundant in natural products, soya protein and zein are known to have a lower protein utilisation for growth as compared with animal proteins[10]. Therefore, a large gap between supply and demand exists in the soya and maize markets of China[11]. Our previous work demonstrated that balanced diets containing soya protein isolate (SPI) or zein have different influences on protein turnover in liver and muscle compared with casein as a standard protein meal in growing rats and that stimulation of protein synthesis in one splanchnic organ is compensated for by a decrease in other peripheral organs, in agreement with the study by Combe *et al.*[12]. However, in each tissue, the mechanisms that underlie the changes in anabolic and catabolic metabolism are uncertain. Thus, the objective of the present study was to clarify whether growth responses were accompanied by gene expression related to protein metabolism in skeletal muscles caused by the type of protein in the diet.

Mammalian target of rapamycin (mTOR) is well known as a regulator playing a key role in the synthetic pathway which transduces signals to 4E-BP1 and/or P70S6K, resulting in changes in translation initiation[13]. Regulation of mTOR signalling is induced not only by an increase in insulin through the insulin–phosphatidylinositol-3-kinase (PI3K)–Akt pathway, but also by amino acids *per se in vitro* [13–18]. Therefore, being potential regulators, amino acids and insulin may regulate the enhanced stimulation of protein synthesis by feeding. Different concentrations of endocrine hormones and different amino acid profiles in plasma were observed after the ingestion of various dietary proteins[12,19,20]; however, whether and how protein sources change the synthetic response is unknown.

The ubiquitin–proteasome pathway (UPP) plays a very important role in degrading the majority of intracellular proteins (abnormal proteins, short- or long-lived proteins and proteins of the endoplasmic reticulum) via the protein kinase B (PKB)–forkhead box-O (FoxO) transcription factor signalling pathway[21]. The rate-limiting step in the UPP is the ubiquitin conjugation process, namely the recognition of the substrate protein by muscle-specific ubiquitin ligases, muscle ring finger 1 (MuRF1) and muscle atrophy F-box (MAFbx; atrogin-1)[22–27]. The responses of muscle-specific

Abbreviations: FoxO, forkhead box-O; MAFbx, muscle atrophy F-box; mTOR, mammalian target of rapamycin; MuRF1, muscle ring finger 1; SPI, soya protein isolate; UPP, ubiquitin–proteasome pathway.

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E3 ligases have also been shown to be triggered by a cascade of pro-catabolic signalling events in the malnourished state[28]. Nevertheless, whether transcription regulation of E3 ligase genes is altered by protein sources in the normal state is not clear.

Our hypothesis was that the supply of various protein sources could affect skeletal muscle protein anabolic and catabolic metabolism. The aim of the present study therefore was to investigate the effects of the quality of ingested protein on the expression of genes related to protein synthesis and proteolysis in the muscle of growing rats.

Materials and methods

Animals

The experimental procedures followed the actual law of animal protection that was approved by the Animal Care Advisory Committee of Sichuan Agricultural University. A total of forty male Sprague–Dawley rats (62·55 g) were divided into homogeneous groups of ten based on initial average body weight in a completely randomised design for 14 d. Rats were housed individually in stainless-steel metabolism cages (25 cm × 15 cm × 15 cm) in a temperature- and humidity-controlled room, maintained at 22 ± 1°C on a 12 h light–dark cycle starting at 07.00 hours. Animals were given free access to water and restricted-fed food. Body weights were recorded daily during the experimental period.

Diets and feeding

The experimental proteins tested included SPI and zein. Casein, the reference protein of the Animal Nutrition Research Council, was used as a control protein. Casein (Sigma, St Louis, MO, USA), SPI (ADM International, Inc., Chicago, IL, USA) and zein (Wako, Osaka, Japan) were purchased commercially. Isonitrogenous (168 g/kg DM) and isoenergetic (19 510 kJ) diets were formulated with sole protein sources following the recommendations of the American Institute of Nutrition (AIN)[29] to meet the nutritional requirements for growing rats. The composition of the diets is shown in Table 1.

All rats were offered casein, SPI and zein protein meals daily at 07.00 hours according to a pair-feeding procedure: the amount of restricted-fed diets (casein and SPI) was calculated after measuring the actual individual DM intake of the low-quality protein diet (zein) *ad libitum*-fed paired rat; this led to a 3 d delay between the group. Records of daily food consumption were kept before feeding each rat every morning. There was an acclimatisation period of 3 d before the formal feeding experiment.

Analysis and tissue preparations

Total N of the diets was determined by the Kjeldahl method and the protein content was calculated by multiplying the N content with a N:protein factor of 6·25. Protein was hydrolysed with 6 M-HCl at 110°C for 24 h to determine the essential amino acids except for tryptophan[30]. All amino acids were determined with an auto amino acid analyser (L-8800; Hitachi, Tokyo, Japan).

Table 1. Composition of the balanced diets

	Casein diet	Soya protein isolate diet	Zein diet
Ingredients (%)			
Casein	21·40	–	–
Soya protein isolate	–	20·44	–
Zein	–	–	24·50
Maize starch	39·90	40·87	36·80
Sucrose	10·00	10·00	10·00
Dextrinised maize starch	13·50	13·50	13·50
Soyabean oil	7·00	7·00	7·00
Fibre	5·00	5·00	5·00
Mineral mix*	2·94	2·93	2·94
Vitamin mix†	0·06	0·06	0·06
Choline bitartrate	0·20	0·20	0·20
Total	100·00	100·00	100·00
Analysed content			
Crude protein (%)	16·82	16·80	16·80
Total energy (MJ/kg)	19·51	19·52	19·51
Lysine (g/kg)	12·39	9·33	1·76
Methionine (g/kg)	4·62	1·62	3·74
Threonine (g/kg)	6·13	5·22	4·22
Phenylalanine (g/kg)	8·16	8·07	9·93
Valine (g/kg)	8·46	5·47	4·94
Leucine (g/kg)	14·22	11·16	24·84
Isoleucine (g/kg)	7·82	6·38	6·28

* Mineral mixture (per kg diet): 2·9 g $CaCO_3$; 13·1 g $CaHPO_4$; 1·3 g NaCl; 8·4 g K_2SO_4; 3·5 g $MgSO_4.H_2O$; 122·8 mg $FeSO_4.H_2O$; 21·1 mg $CuSO4.5H_2O$; 33·1 mg $MnSO_4.H_2O$; 36·6 mg $ZnSO_4.H_2O$; 3·9 mg KI; 15 mg Na_2SeO_3.

† Vitamin mixture (per kg diet): 8100 μg vitamin A; 150 μg vitamin D_3; 48 mg DL-α-tocopheryl acetate; 6 mg vitamin K_3; 6 mg vitamin B_1; 9 mg vitamin B_2; 12 mg vitamin B_6; 45 μg vitamin B_{12}; 255 μg D-biotin; 3 mg folic acid; 69 mg nicotinamide; 30 mg D-pantothenic acid.

At the end of experimental period, *in vivo* tissue protein synthesis rates were determined in the fed state (4 h after equal ingestion) by the flooding-dose method using L-[U-^{14}C]leucine according to the method of Garlick *et al.*[31]. On the day of determination, 1 ml of a solution of L-[U-^{14}C]leucine (100 μmol/ml) containing 1·12 MBq (30 μCi) was induced by intraperitoneal injection per 100 g body weight of each rat. General anaesthesia was induced by intraperitoneal injection of pentobarbital sodium (0·2 ml per 100 g body mass) 18 min after leucine injection. At 2 min later the rats were killed by cervical dislocation. Blood was collected into heparinised tubes for centrifugation and then the plasma was collected. Gastrocnemius muscles were quickly excised, blotted and chilled on ice-cold dishes to stop tracer incorporation. Tissue samples were weighed and then frozen in liquid N_2 and kept at −70°C until further analysis.

Samples for plasma amino acid concentration measurements were mixed with an equal volume of aqueous solution of methionine sulfone (internal standard) and centrifuged. The filtrate was lyophilised and the amino acids were analysed by reverse-phase HPLC for their phenylisothiocyanate derivatives (PicoTag; Waters, Woburn, MA, USA). Plasma insulin was measured by RIA with the Bi-Insulin RIA kit (ERIA Diagnostics Pasteur, Marne la Coquette, France). Protein was extracted with buffer[32] and protein concentration was measured with the bicinchoninic acid assay method[33]. The capacity for protein synthesis (Cs) was calculated by dividing the RNA concentration (mg) by the protein content (g) of gastrocnemius muscles.

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RNA isolation and real-time quantitative PCR

Total RNA was isolated from samples of gastrocnemius muscle using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration and purity were measured spectrophotometrically. Optical density at 260/280 nm > 1·9 in all RNase-free water-treated RNA samples was considered a very low degree of contamination[34]. The integrity of the RNA was checked by formaldehyde gel electrophoresis and visualisation of intact 18S and 28S ribosomal RNA bands under UV light.

Reverse transcription was performed using the Prime-Script™ RT reagent Kit (Takara, Shiga, Japan) with a 2 μg RNA sample according to the manufacturer's instructions. Expression levels of FoxO, MuRF1 and MAFbx in gastrocnemius muscles were analysed by real-time quantitative PCR with SYBR® Green PCR reagents (Takara) and performed by means of the Opticon DNA Engine (Bio-Rad, Hercules, CA, USA) using the following cycle parameters: 95°C for 10 s and forty cycles at 95°C for 5 s and 60°C for 25 s with a final extension at 72°C for 5 min. A melting curve analysis was generated following each real-time quantitative PCR assay to check and verify the specificity and purity of all PCR products, which were further checked for size and specificity by agarose gel electrophoresis. The primers used are given in Table 2.

Relative quantification of the target gene transcript with a chosen reference gene transcript (β-actin) was made following the relative standard curve method[32] with the Opticon DNA Engine Software (Bio-Rad). Each standard and sample were run simultaneously in duplicate on the same PCR plate and the average of each duplicate value expressed as numbers of copies was used for subsequent statistical analysis.

Protein immunoblot analysis for measurement of mammalian target of rapamycin phosphorylation

Proteins were extracted from muscle homogenates and were used for Western blot analysis as previously described[32]. The samples were subjected to separation on 6 % polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes for detection of mTOR[35,36]. The membrane was incubated with primary antibodies for total mTOR (rabbit polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphorylated (Ser2448) mTOR (Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was then washed with 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-buffered saline–Tween 20 solution and incubated with a secondary antibody. The blots were exposed to X-ray film and were scanned using a Microtek ScanMaker V scanner (Microtek International, Inc., Hsinchu, Taiwan). Images were obtained with Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA) and quantified using Scion Image software (Scion Corp.oration, Frederick, MD, USA).

Statistical analysis

A one-way ANOVA was performed with dietary treatment group as the independent variable. When a significant overall effect was detected, differences among individual means were assessed using Duncan's multiple-range test. The level of significance was set at $P<0·05$ for all statistical tests. Values are expressed as mean values with their standard errors.

Results

Growth performance and muscle characteristics

Growth rate was compared across dietary treatments (Table 3). Compared with the pair-fed casein control group, growth rate and food efficiency were significantly lower in the SPI- and zein-fed groups ($P<0·001$), with an 85 % lower growth rate in the zein-fed group than in the SPI-fed group ($P<0·01$).

Muscle tissue is composed of the main protein mass in the whole body. Wet weight (g/100 g body weight) of gastrocnemius muscle (Table 3) was significantly lower in the group given zein than in the other groups (1·04 (SEM 0·03) and 0·97 (SEM 0·03) g/100 g body weight in the casein control and SPI groups, respectively). Protein concentrations normalised by body weight in gastrocnemius muscle were dramatically increased in the groups given the casein and SPI diets than were those in the zein diet group ($P<0·05$). The fractional rates of protein synthesis (%/d) and ribosomal capacity (mg RNA/g protein) expressed as the ratio of RNA concentration to protein content in the muscle sample in the zein group were significantly higher than those in the

Table 2. Primers used for the real-time PCR analyses

Primer	Sequence 5′- to 3′	Product size (bp)	Accession number
mTOR forward	CACCCAAGCCTGGGACCTCTA	156	NM_019906
mTOR reverse	GGCTGGTTGGGGTCATATGTT		
FOXO1 forward	TACGGCCAATCCAGCAT	143	XM_342244
FOXO1 reverse	TGGGGAGGAGAGTCAGAAGT		
FOXO3 forward	CGGCTCACTTTGTCCCAGAT	163	NM_001106395
FOXO3 reverse	TCTTGCCAGTCCCTTCGTTC		
FOXO4 forward	GTGCTCGCATCTCCTACTGAAG	93	NM_001106943
FOXO4 reverse	CATGTCGCACTCCAGGTTCT		
Atrogin-1 forward	CCATCAGGAGAAGTGGATCTATGTT	73	AY059628
Atrogin-1 reverse	GCTTCCCCCAAAGTGCAGTA		
MuRF1 forward	TGTTCTGGTAGGTCGTTTCCG	69	AY059627
MuRF1 reverse	ATGCCGGTCCATGATCACTT		
β-Actin forward	TCGTACCACTGGCATTGTGAT	233	NM_031144
β-Actin reverse	CGAAGTCTAGGGCAACATAGCA		

mTOR, mammalian target of rapamycin; FOXO, forkhead box-O; MuRF1, muscle ring finger 1.

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Table 3. Growth rate and protein metabolism in gastrocnemius muscle
(Mean values with their standard errors for ten animals per treatment)

	Casein diet		Soya protein isolate diet		Zein diet	
	Mean	SEM	Mean	SEM	Mean	SEM
Initial weight (g)	62·83[a]	1·56	61·79[a]	1·64	62·68[a]	1·55
Growth rate (g/d)	7·45[a]	1·14	5·28[b]	0·90	0·79[c]	0·30
DM intake (g/d)	15·97[a]	1·96	15·07[a]	1·25	15·56[a]	1·10
Wet weight (g/100 g body weight)	1·04[a]	0·03	0·97[a,b]	0·03	0·93[b]	0·03
Protein content (mg/100 g body weight)	91·13[a]	8·23	99·30[a]	11·94	55·41[b]	6·75
RNA (mg/100 g body weight)	0·54[a]	0·06	0·47[a,b]	0·07	0·37[b]	0·05
Ribosomal capacity (mg RNA/g protein)	5·93[b]	0·54	4·73[b]	0·30	6·68[a]	0·71
FSR (%/d)	6·23[b]	0·68	6·52[a,b]	0·93	8·47[a]	0·94

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).
FSR, fractional rate of protein synthesis.

casein control group (8·47 (SEM 0·94) *v.* 6·23 (SEM 0·68) %/d; 6·68 (SEM 0·71) *v.* 5·93 (SEM 0·54) mg RNA/g protein, respectively; $P<0·05$).

Plasma parameters

As shown in Table 4, a significant difference was found for plasma urea N concentrations between the SPI- and zein-fed groups, with about 53 % higher in the zein group than in the SPI-fed group. No difference was observed for concentrations of insulin in plasma between the SPI and zein groups, but the values in the zein-fed group were lower than those in the casein-fed group ($P<0·05$).

Concentrations of amino acids in plasma were detected (Table 4). No differences were observed in the plasma concentrations of threonine, valine and isoleucine between the SPI- and zein-fed groups and the values of threonine and valine concentration were 47 and 61 % lower than those in the casein control group, respectively ($P<0·01$). Methionine concentration was significantly lower in the SPI-fed group than in the casein- and zein-fed groups ($P<0·01$). Nearly 2-fold greater levels were observed for leucine in zein-fed rats than in casein- and SPI-fed rats ($P<0·01$). Plasma concentrations of lysine were about 24 % and about 123 % higher in the SPI group than those in the casein and zein groups, respectively ($P<0·05$).

Muscle expression of genes related to protein synthesis and mammalian target of rapamycin phosphorylation

mTOR is regulated via mechanisms involving processes of mRNA translation initiation and the ubiquitin–proteasome proteolytic pathway. The mRNA levels of mTOR in gastrocnemius muscles were significantly increased in the zein-fed group ($P<0·01$) (Fig. 1). Also, the levels of mTOR phosphorylation on residue S2448 normalised for total mTOR content were about 2-fold higher in the zein meal group compared with the casein and SPI groups ($P<0·05$) (Fig. 2).

Muscle expression of genes related to proteolysis

In the present study, we investigated the expression of genes related to the ubiquitin–proteasome proteolytic pathway in gastrocnemius muscle, which is controlled by the expression of E3 ubiquitin ligases. The mRNA levels of atrogin-1 in the group fed zein meal increased about four-fold compared with the groups fed casein and SPI ($P<0·01$) (Fig. 3). The value of MuRF1 gene expression with zein meal was nearly 8-fold the value with casein and SPI meal ($P=0·003$) (Fig. 4). The main FoxO transcription factors consist of FoxO1A, FoxO3A and FoxO4A, which act as upstream signals of muscle-specific E3 ligase, atrogin-1 and MuRF1. The mRNA levels of FoxO1A, FoxO3A and

Table 4. Plasma parameters
(Mean values with their standard errors for ten animals per treatment)

	Casein diet		Soya protein isolate diet		Zein diet	
	Mean	SEM	Mean	SEM	Mean	SEM
Plasma urea N (mmol/l)	6·44[a]	1·65	6·07[a]	1·39	9·27[b]	2·89
Plasma insulin (μIU/ml)	19·85[a]	6·64	17·43[a]	6·02	13·90[b]	1·73
Threonine (μmol/l)	783·81[a]	45·76	417·05[b]	39·82	530·52[b]	59·28
Valine (μmol/l)	305·18[a]	28·32	150·84[b]	13·72	118·61[b,c]	21·38
Methionine (μmol/l)	94·48[a]	6·41	24·55[b]	2·28	60·14[c]	7·13
Isoleucine (μmol/l)	76·50[a]	4·14	72·56[a]	6·63	88·47[a]	11·11
Leucine (μmol/l)	237·81[b]	9·69	208·29[b]	25·35	395·76[a]	49·62
Phenylalanine (μmol/l)	82·37[a,b]	4·21	68·31[b]	10·14	90·07[a]	4·53
Lysine (μmol/l)	112·23[b]	7·95	148·24[a]	13·98	66·54[c]	8·60

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

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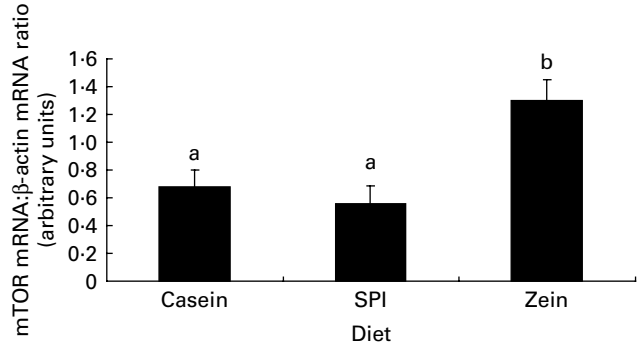

Fig. 1. Effect of dietary protein source (casein, soya protein isolate (SPI) or zein) on the gene expression of mammalian target of rapamycin (mTOR) in the gastrocnemius muscle of growing rats. Relative mRNA levels were analysed by real-time RT-PCR. mRNA levels were normalised using β-actin. Values are means for ten animals per treatment, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

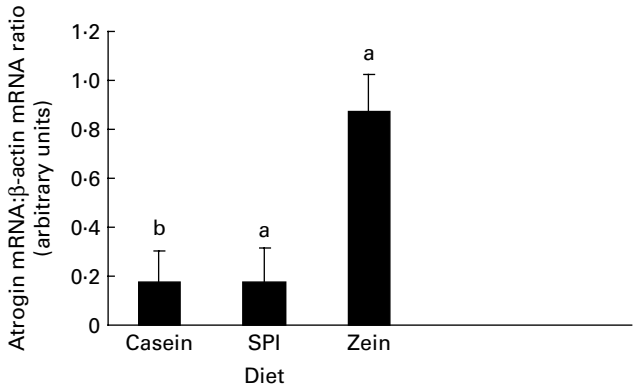

Fig. 3. Effect of dietary protein source (casein, soya protein isolate (SPI) or zein) on the gene expression of atrogin 1 in the gastrocnemius muscle of growing rats. Relative mRNA levels were analysed by real-time RT-PCR. mRNA levels were normalised using β-actin. Values are means for ten animals per treatment, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

FoxO4A displayed similar responses by various protein diet treatments. The values of FoxO1A, FoxO3A and FoxO4A in the zein group were significantly higher than those of the casein and SPI groups. Significantly higher values were observed in the zein group than in the SPI group (4·08-, 2·53- and 1·56-fold increases in FoxO1A, FoxO3A and FoxO4A, respectively, as compared with the SPI group) ($P<0·005$) (Fig. 5).

Discussion

In the present study, SPI and zein with a high concentration of true protein (about 90 % of total protein) were used in order to avoid additional effects of other nutrients (for example, carbohydrate) on metabolic responses. After feeding protein meals, concentrations of endocrine hormones and amino acids in plasma may act as potential regulators to control the growth rate and protein turnover of rats[20,37,38]. Since no different concentrations of insulin in the plasma were observed in rats fed the SPI and zein diets, intake of deficient essential amino acids including lysine, threonine and valine in the zein-fed group may cause reduced growth performance compared with that after the casein and SPI diets. Considering that DM intake remained at the same level among the dietary treatments, and that each diet had a sole protein source with no crystal amino acid supplementation, the profile of amino acids in each protein meal played a key role in the regulation of growth rate and protein metabolism. This implies that limited protein utilisation for the growth of the rats can be explained by the amino acid imbalance of zein protein.

In the present study, a flooding-dose method was used for the protein synthesis measurements. It was of importance to show that the method *per se* did not affect the rate of protein synthesis[31], and the insulin and glucose concentrations in plasma[39]. The validation of this method created by the flooding-dose technique was that a large dose of leucine (100 μmol/100 g body weight) was sufficiently large to flood all possible precursor pools to the same and constant specific radioactivity[40]. In our previous study, we demonstrated that the specific radioactivity of free leucine in plasma increased logarithmically ($P < 0·05$) to a plateau level, 99 % of which was reached 12 min post-injection and that the free pool tracer leucine radioactivity in plasma and various tissues and organs did not change beyond 12 min after injection (data not shown). In the present study, although

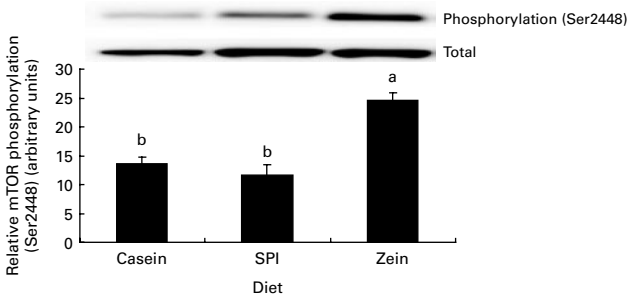

Fig. 2. Effect of dietary protein source (casein, soya protein isolate (SPI) or zein) on mammalian target of rapamycin (mTOR) phosphorylation in the gastrocnemius muscle of growing rats. Values for phosphorylated mTOR at Ser2448 were normalised for total mTOR content. Values are means for ten animals per treatment, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

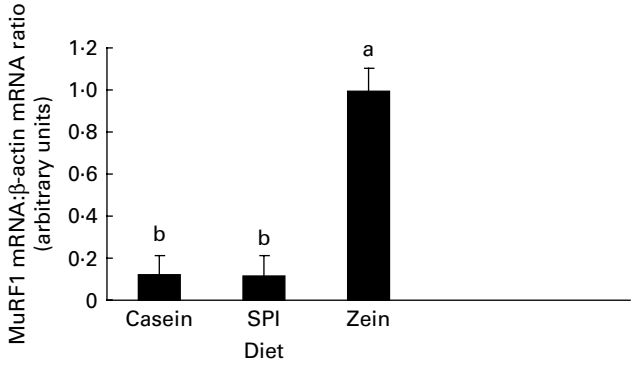

Fig. 4. Effect of dietary protein source (casein, soya protein isolate (SPI) or zein) on the gene expression of muscle ring finger 1 (MuRF1) in the gastrocnemius muscle of growing rats. Relative mRNA levels were analysed by real-time RT-PCR. mRNA levels were normalised using β-actin. Values are means for ten animals per treatment, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

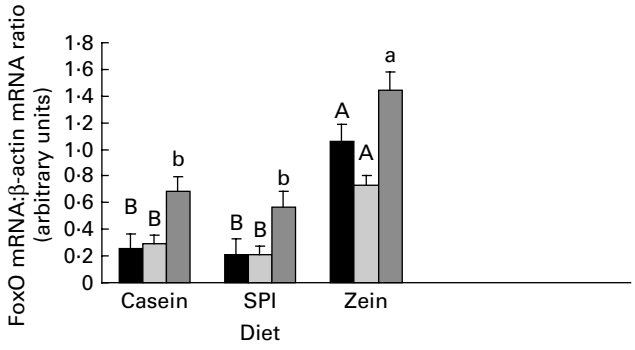

Fig. 5. Effect of dietary protein source (casein, soya protein isolate (SPI) or zein) on the gene expression of forkhead box-O (FoxO) 1A (■), FoxO3A (■) and FoxO4A (□) in the gastrocnemius muscle of growing rats. Relative mRNA levels were analysed by real-time RT-PCR. mRNA levels were normalised using β-actin. Values are means for ten animals per treatment, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·05$). [A,B] Mean values with unlike letters were significantly different ($P<0·01$).

both the fractional rates of protein synthesis (8·47 %/d) and protein degradation (4·87 %/d) were higher ($P<0·01$) in gastrocnemius with the zein diet than those with the casein and SPI diets, proteolysis could make a greater contribution than protein synthesis, as there is an amino acid imbalance (lysine deficiency) in zein protein, to the poor growth performance of the growing rats.

Different proteins have different metabolic responses no matter in peripheral or splanchnic tissues[4], and good correlations between the quality of dietary proteins and the ability of tissue protein synthesis have been observed[41]. Generally, the more balanced the amino acid composition of a diet is, the more improved the amino acid concentrations in the plasma and consequently in the liver and brain will be, which can induce the fractional rate of protein synthesis in these tissues[20,42]. However, previous work has indicated that the capacity for protein synthesis in the large intestine, liver and gastrocnemius was not affected by protein meals[12]. In a recent study, about 42 % higher capacity for protein synthesis (Cs) in gastrocnemius muscles of growing rats fed a zein diet was described. This discrepancy may be due to the nature of the protein components, the animal model, the experimental period and the assay methods. Because of the high true protein content in each protein diet without additional amino acid supplementation and very low levels of other non-N elements, as well as anti-nutritional factors contained in soya beans[43], kidney beans[44], peas[45] or lentils[12], the present results can better show the correlations between the quality of dietary proteins *per se* and the ability of protein synthesis in skeletal muscles. The higher protein and RNA contents in muscles corresponded to an increase in fractional rates of protein synthesis with the zein diet. Similar effects on muscle mass and protein synthesis rates have been described in cooked beans and cooked lentils[38].

In the past few years, studies on insulin and amino acid signalling pathways leading to translation initiation that regulate the changes in protein synthesis have been conducted. mTOR can be activated by amino acids as well as insulin stimulation[14]. In the present study, the SPI and zein diets did not increase circulating concentrations of insulin. Therefore, the changes in amino acid profile of dietary proteins reflected in plasma amino acid composition seem to be signal regulators to function on mTOR transcription level and mTOR phosphorylation at Ser2448, allowing the tentative explanation of why different dietary proteins have different effects on mTOR activation[19]. Concentrations of branched-chain amino acids in plasma, especially the leucine content, are considered able to enhance the protein anabolic response in skeletal muscle both in *in vivo* and *in vitro* studies[46–48]. Thus, mRNA translation initiation in muscle may be stimulated because of the about 2-fold higher concentrations of leucine in plasma with the zein diet as compared with the casein and SPI ddiets (Table 4). mTOR activates 4E-BP1 and P70 S6 kinase, both of which are translation initiation factors. Activation of S6K1 leads to the activation of ribosomal protein S6 which enhances the translation of specific mRNA, including those involved in the translational machinery. eIF4E can be released when 4E-BP1 is phosphorylated, and then binds to eIF4G to form an eIF4E–eIF4G complex to increase translation initiation[16,49,50]. Therefore, activation of mTOR in growing rats given zein meal indicates induced anabolic metabolism in gastrocnemius muscle, which agrees with previous findings that levels of mTOR phosphorylation were affected by meals containing soya and whey protein after exercise[51,52]. Other reasons for the high level of mTOR activation found in the zein diet may be the result of an adaptation to malnutrition during the experimental period in order to avoid acceleration of muscle losses.

The lysosomal pathway, the non-lysosomal Ca^{2+}-dependent proteolytic pathway and the UPP are considered the main substrates of different proteolytic pathways in mammalian cells[19]. The UPP has taken centre stage in the control of proteolysis especially via atrogin-1/MAFbx and MuRF1 signalling regulated by the FoxO family of transcription factors[53–55]. Activation of FoxO proteins by dephosphorylation are transferred from the cytoplasm to localise in the nucleus to promote transcription of atrogin-1/MAFbx and MuRF1[56–58]. Our data showed that the transcription of zatrogin-1/MAFbx, MuRF1 and FoxO followed the similar pattern across the dietary treatments. The transcriptional up-regulation of atrogin-1/MAFbx and MuRF1 noted in the restricted-fed growing rats in the zein group indicated an increased protein degradation rate in this group. FoxO protein levels were not demonstrated in the present study. Therefore it cannot be clarified the correlation between levels of FoxO activation and transcription of its downstream elements (atrogin-1/MAFbx and MuRF1)[59]. Atrogin-1/MAFbx and MuRF1 are modulated via mechanisms not only involving the protein kinase B (PKB)–FoxO transcription factors but also involving PKB–mTOR by amino acid availability *in vivo* [43,45]. Dietary amino acids, varying amounts of which are reflected in plasma concentration, are recognised as nutrient signal molecules acting on mTOR signalling involved in mRNA translation and proteolysis[60]. Despite the results *in vitro* that showed that mTOR inhibition by rapamycin abolished the amino acid-related decrease in atrogin expression, cross-talk between the activation of mTOR and the transcription of atrogin-1 cannot be defined *in vivo* [61]. Since changes in protein metabolism are caused by the amino acid profile of protein

diets, protein synthesis could be stimulated through the mTOR signalling pathway and proteolysis may be dependent on the control of the ubiquitin–proteasome proteolytic pathway which is induced by dietary protein sources. Based on the discrepancy between proteolysis and protein synthesis regulation that has been observed with zein protein diets in recent studies, we hypothesised that the 2-fold higher plasma leucine concentrations observed in the zein-fed group may be responsible for the possible protein synthesis stimulation. On the other hand, the increased proteolysis may be an adaptive response of skeletal muscles to some limited essential amino acids because of the protein synthesis stimulation and the amino acid imbalance of the zein diet (lysine deficiency).

In conclusion, the expression and activation of mTOR related to translation initiation and expression of atrogin-1/MAFbx and MuRF1 related to proteolysis are altered by various dietary protein sources, with more activation of mTOR and over-expression of atrogin-1/MAFbx and MuRF1 in muscles of growing rats fed zein meal. The amino acid profile of proteins may have a potential function in the signalling pathway leading to translation initiation and proteolysis. Further studies *in vitro* are needed to better understand the potential effects of amino acid profile in controlling the processes of mTOR-dependent mRNA translation and ubiquitin–proteasome-dependent proteolysis.

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