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Tissue-specific regulation of acetyl-CoA carboxylase gene expression by dietary soya protein isolate in rats

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We have recently reported that intake of soya protein isolate (SPI) inhibited the DNA-binding activities of hepatic thyroid hormone receptor (TR). The genes for acetyl-CoA carboxylase (ACC), a rate-limiting enzyme in fatty acid synthesis, contain the thyroid hormone response element in their promoters and are regulated by TR. The present study has examined the effect of long-term feeding of SPI and soya isoflavones (ISF) on the gene expression and protein phosphorylation of different ACC isoforms in different tissues and plasma triacylglycerol (TAG) levels in rats. Sprague-Dawley female rats were fed diets containing 20 % casein or alcohol-washed SPI with or without supplemental ISF for 70, 190 and 310 d. SPI intake significantly reduced plasma TAG concentrations compared with casein, whereas supplemental ISF had no effect. Hepatic ACCα and ACCβ mRNA abundance and protein content were markedly lower in the rats fed SPI than in those fed casein. The protein contents of ACCα in the kidney and ACCβ, the predominant isoform in the heart and kidney, were unchanged by dietary SPI. The ratios of phospho-ACCα/ACCα and phospho-ACCβ/ACCβ were not different among dietary groups in all tissues measured. The present study demonstrates that ingestion of SPI decreases plasma TAG level and down-regulates ACCα and ACCβ gene expression in the liver but not in the heart and kidney. The results indicate that the effect of SPI is tissue-specific and that alteration of ACC gene expression rather than phosphorylation status may play a major role in the regulation of ACC activities by soya proteins.

Rats: Soya protein isolate: Acetyl-CoA carboxylase gene: Triacylglycerol

The blood triacylglycerol (TAG) level is one of the important risk factors for chronic diseases such as CVD, type 2 diabetes and atherosclerosis. In particular, high plasma TAG levels in women are better predictors of CVD risk than total or LDL-cholesterol (Cullen, 2000; Meagher, 2004).

Soya consumption has been associated with a decreased risk of CVD, type 2 diabetes and atherosclerosis (Anderson *et al.* 1995; Potter, 1998; Hermansen *et al.* 2001). These beneficial effects of soya appear to be, at least partially, due to its hypotriacylglycerolaemic actions. Soya diets decreased the blood TAG level in both animals (Lin *et al.* 2004; Moriyama *et al.* 2004; Ascencio *et al.* 2004) and hyperlipidaemic human subjects (Wang *et al.* 2004). The hypotriacylglycerolaemic action of soya components is believed to be a consequence of decreased biosynthesis and enhanced degradation of fatty acids.

Acetyl-CoA carboxylase (ACC) is a rate-limiting enzyme that catalyses the carboxylation of acetyl-CoA to form malonyl-CoA, the first step in the pathway of long-chain fatty acid biosynthesis (Wakil *et al.* 1983). Two isoforms of ACC have been identified. ACCα is a cytosolic protein and highly expressed in liver and adipose tissue, whereas ACCβ is located in mitochondria and is predominantly expressed in heart and muscle, and to a lesser extent in liver. ACCβ plays a pivotal role in the regulation of mitochondrial fatty acid β-oxidation (Hillgartner *et al.* 1996) through feedback inhibition of carnitine palmitoyltransferase-1, an enzyme that controls the entry of long-chain fatty acid CoA esters into the mitochondria for degradation (Abu-Elheiga *et al.* 2000). Suppression of ACCβ increases carnitine palmitoyltransferase-1 activity and enhances fatty acid β-oxidation. ACC activity in different tissues is regulated differently. For instance, ACCβ activity in skeletal muscle is mainly regulated by phosphorylation and dephosphorylation (Dyck *et al.* 1999; Minokoshi *et al.* 2002), whereas the hepatic ACCβ activity is regulated at the transcriptional level (Harwood *et al.* 2003; Oh *et al.* 2003).

Thyroid hormones play an important role in the regulation of lipid metabolism. Our previous studies showed that

Abbreviations: ACC, acetyl-CoA carboxylase; ISF, isoflavones; pACC, phospho-ACC; SPI, soya protein isolate; TAG, triacylglycerol; TR, thyroid hormone receptor.

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consumption of soya protein isolate (SPI) increased hepatic thyroid hormone receptor (TR) β1 protein content and inhibited the binding activities of hepatic nuclear TR to its target genes in rats (Xiao *et al.* 2004; Huang *et al.* 2005). These cellular responses of TR to dietary SPI are believed to affect thyroid hormone functions and alter the downstream gene expression. The ACCα gene contains thyroid hormone response elements in its promoter regions (Huang & Freake, 1998), and is regulated by thyroid hormones in a tissue-specific manner (Blennemann *et al.* 1995; Huang & Freake, 1998). Although soya protein intake was shown to suppress the enzymatic activity of ACC in rat liver (Iritani *et al.* 1986), the regulation of the gene expression for different ACC isoforms (α and β) in different tissues by dietary soya components are not fully understood.

The objective of the present study was to determine the effect of dietary soya proteins and soya-derived ISF on plasma TAG levels as well as ACCα and ACCβ phosphorylation and gene expression in different tissues of rats.

Materials and methods

Chemicals and reagents

Alcohol-washed SPI (Pro Fam 930) and Novasoy soya ISF concentrate were purchased from Archer Daniels Midland Company (Decatur, IL, USA). Casein protein (90 % total protein) was from ICN Biomedicals (Cleveland, OH, USA). Agarose, Tris and phenylmethylsulphonyl fluoride were from Sigma Chemical (St. Louis, MO, USA). ECL Western blotting detection kit was obtained from Amersham (Arlington Heights, IL, USA). Nitrocellulose membranes, acrylamide, N, N′-methylene-bis-acrylamide, ammonium persulphate, dithiothreitol, glycine, goat anti-rabbit IgG (H + L)–horseradish peroxidase conjugated antibody, goat anti-mouse IgG (H + L)–horseradish peroxidase conjugated antibody and Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories (Hercules, CA, USA). X-ray film was from MJS Biolynx (Brockville, ON, Canada). Rabbit polyclonal anti-rat phospho-ACC (pACC; Ser79) and anti-human ACCα and ACCβ antibodies that cross-react with rat proteins were purchased from Upstate (Lake Placid, NY, USA). TRIzol reagent and M-MLV RT were from Invitrogen Canada Inc. (Burlington, ON, Canada). Taq DNA polymerase was purchased from New England Biolabs (Beverly, MA, USA). QuantumRNA 18S internal standards were purchased from Ambion (Austin, TX, USA). Reagents for TAG quantitation in plasma were from Wako Chemicals (Richmond, VA, USA). Mouse leptin ELISA kits were from Crystal Chem (Downers Grove, IL, USA) and ultrasensitive rat insulin ELISA kits were purchased from ALPCO Diagnostics (Windham, NH, USA).

Animals and diets

Animal experimental protocols were approved by the Health Canada Animal Care Committee and all animal handling and care followed the guidelines of the Canadian Council for Animal Care. Pubertal Sprague-Dawley rats (Charles River, St. Constant, PQ, Canada) were pair housed in stainless steel cages and randomly allotted into three groups (thirty-five females per group), and kept in an environmentally controlled room with a 12 h light–dark cycle. After acclimation, starting at 50 d of age, rats were given free access to one of the three diets (Table 1) and tap water before being killed. All diets were formulated according to the specifications of AIN93G (Reeves *et al.* 1993) except that in diets 2 and 3, casein was replaced by alcohol-washed SPI (Pro Fam 930). In addition, diet 3 was supplemented with ISF (250 mg/kg diet; Novasoy ISF concentrate). Body weights and food consumption were recorded weekly. The actual content of ISF including genistein, daidzein and glycitein was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm (Wang & Murphy, 1994) and is shown in Table 1. After being fed for 70, 190 and 310 d, ten rats from each diet group were killed by exsanguination through cardiac puncture under general anaesthesia with isoflurane, and the tissues were collected, immediately frozen in liquid N and stored at −80°C until analysis.

Plasma triacylglycerol, insulin and leptin

Plasma TAG concentrations were measured using a microplate enzymatic colorimetric method (Wako Chemicals). Briefly, plasma (5 μl) was added to microtitre plate wells and incubated with 80 μl Enzyme Color A for 5 min to decompose free glycerol. Subsequently, 40 μl Enzyme Color B was added and incubated for 5 min. The absorbance was read at 595 nm in a microplate reader (Model 3550-UV; Bio-Rad). The TAG concentrations were calculated using the standard curves. Plasma insulin and leptin concentrations were determined using double-antibody sandwich enzyme immunoassay according to the manufacturer's instructions.

Table 1. Composition of experimental diets (g/kg diet)

Ingredient	Casein	SPI	SPI + ISF
Casein*	222·2	–	–
Soya protein†	–	222·2	222·2
Sucrose	100·0	100·0	100·0
Maizestarch	375·3	375·3	374·5
Dextronized Maizestarch	132·0	132·0	132·0
Soyabean oil	70·0	70·0	70·0
Cellulose	50·0	50·0	50·0
Mineral mix‡	35·0	35·0	35·0
Vitamin mix‡	10·0	10·0	10·0
Choline bitartrate	2·5	2·5	2·5
L-Cystine	3·0	–	–
L-Methionine	–	3·0	3·0
tert-Butylhydroquinone	0·014	0·014	0·014
Novasoya†	–	–	0·833
Total isoflavones (mg/kg diet)§	0·0	31·7	235·6
Genistein (mg/kg diet)§	ND	18·6	124·4
Daidzein (mg/kg diet)§	ND	10·5	90·9
Glycitein (mg/kg diet)§	ND	2·6	20·5

ISF, isoflavones; ND, not detectable; SPI, soya protein isolate.
* Casein from ICN Biomedicals contains 90 % crude protein.
† Alcohol-washed SPI contains 90 % crude protein, and Novasoy ISF concentrate contains 30 % total ISF.
‡ AIN-93G Mineral mix and AIN-93G Vitamin mix were from ICN Biomedicals.
§ The actual content of ISF (genistein, daidzein and glycitein) was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm.

Protein extraction and Western blot analysis of acetyl-CoA carboxylase and phospho-acetyl-CoA carboxylase

Total protein preparation and ACC analyses were carried out as recommended by Upstate with minor modifications. Briefly, rat tissues were homogenized in lysis buffer with a glass homogenizer and then sonicated three times for 30 s on ice. The homogenate was centrifuged to separate the cell debris, and the protein content of the extracts was determined. Total proteins (80 μg) were mixed with loading buffer and incubated at 37°C for 30 min before they were separated in 6 % SDS–PAGE. The resolved proteins were electrotransferred (30 V, overnight) on to nitrocellulose membranes. The blots were immunostained with ACC or pACC antibodies and the immunoreactivity was detected using the ECL kit. The intensities of ACCα, ACCβ, pACCα and pACCβ protein bands were measured densitometrically using Scion Image (Scion Corporation, Frederick, MD, USA), and normalized by the respective Ponceau-stained total protein (Xiao *et al.* 2003). The ratios of pACCα:ACCα and pACCβ:ACCβ were calculated. The specificities of these antibodies were confirmed using their respective blocking peptides.

Quantitation of hepatic acetyl-CoA carboxylase α and acetyl-CoA carboxylase β mRNA abundances

Total RNA was isolated from rat liver samples with TRIzol reagent according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed for cDNA synthesis. One-tenth of the cDNA synthesized was then amplified with the following primers: rat ACCα [forward: 5′-CTTTCACATGAGGTCCAGCA-3′ (213–232), reverse: 5′-ACTCGAAGACCACTGCCACT-3′ (797–778)]; rat ACCβ [forward: 5′-CCTGTAGATGCCAGTCAGCA-3′ (232–251), reverse: 5′- GACATGCTGGGCCTCATAGT-3′ (653–634)] and universal 18S rRNA primers and competimers (Ambion) in a ratio of 4:6 for ACCα or 3·5:6·5 for ACCβ. PCR cycle conditions were 94°C for 5 min, 94°C for 45 s, 60°C for 45 s and 72°C for 45 s, 72°C for 10 min. Amplification cycle numbers were twenty-six for ACCα and thirty-two for ACCβ. Samples were resolved on 2 % agarose gels and visualized with SYBR Green. ACCα and ACCβ mRNA levels were normalized against their respective 18S rRNA content.

Statistical analyses

Results are expressed as means with their standard errors. Effects of treatment on plasma insulin, leptin, TAG concentrations and mRNA or protein contents of ACCα and ACCβ, as well as the ratios of pACCα:ACCα and pACCβ:ACCβ were analysed using two-way ANOVA, which included the main effects of diet and feeding period as well as the interactions of diet × feeding period. The dietary effects on food consumption, body and liver weights were analysed by one-way ANOVA. Differences between individual means were determined by Fisher's least significant difference test. Dietary effects on renal or cardiac ACCα and ACCβ protein contents and the ratios of pACCα:ACCα or pACCβ:ACCβ were assessed by *t* test. A probability of $P < 0{\cdot}05$ was considered to be statistically significant. Data were analysed using Statistica version 7.1 (StatSoft, Tulsa, OK, USA).

Results

Food consumption, body and liver weights

Food consumption, body and liver weights did not differ among the dietary groups ($P > 0{\cdot}05$, data not shown), demonstrating the nutritional adequacy of the test diets.

Plasma triacylglycerol concentration

Plasma TAG concentrations in the rats fed the casein diet increased with age, whereas the rats fed the diets containing SPI for 70, 190 and 310 d had significantly lower TAG levels than those fed the casein-based diet. However, the TAG levels in rats fed SPI or SPI + ISF were not different (Fig. 1).

Hepatic acetyl-CoA carboxylase α and acetyl-CoA carboxylase β mRNA steady-state level and protein content. Hepatic ACCα and ACCβ mRNA abundances and protein contents in the rats fed the diet containing SPI for 70, 190 and 310 d were significantly lower than those fed casein-based diet (Figs 2 and 3(a)). Supplemental ISF had no additional effect on either ACCα or ACCβ protein content (Fig. 3(a)).

Tissue-specific regulation of acetyl-CoA carboxylase α and acetyl-CoA carboxylase β expression

ACCβ protein was detected in all the tissues measured, and its expression was higher in the liver and heart but was very low in the kidney. ACCα protein was detectable by Western blot only in the liver and kidney but not in the heart. Dietary SPI significantly decreased both ACCα and ACCβ protein contents in the liver, but had no effect on ACCα and ACCβ in the heart or kidney (Fig. 4(a)).

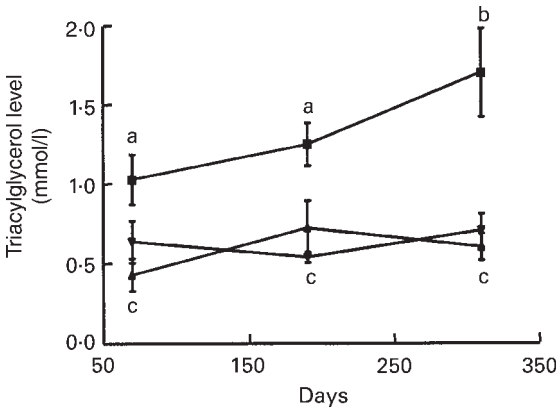

Fig. 1. Plasma triacylglycerol concentrations in rats fed diets containing either casein (■) or alcohol-washed soya protein isolate in the absence (▲) or presence (▼) of supplemental isoflavones (250 mg/kg diet) for 70, 190 and 310 d. For details of diets and procedures, see Table 1 and p. 1049. Values are means with their standard errors depicted by vertical bars (*n* 6). [a,b,c] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$).

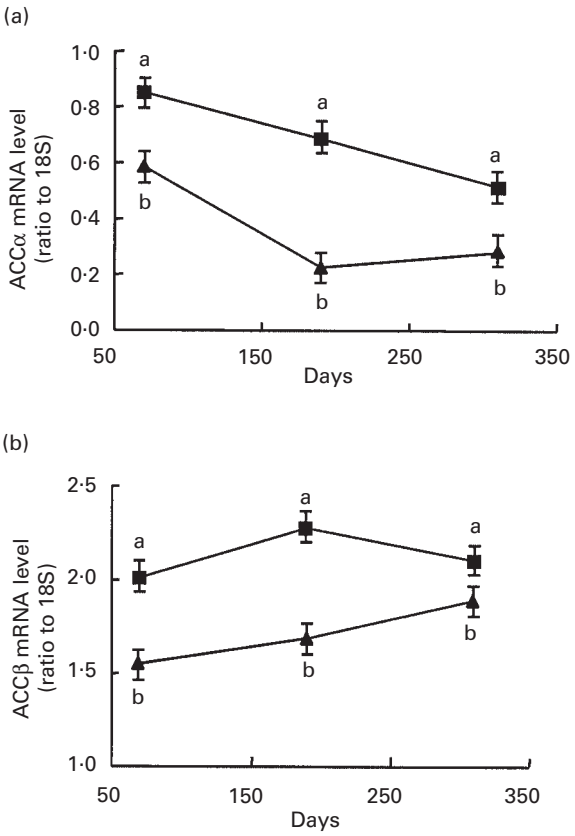

Fig. 2. Hepatic acetyl-CoA carboxylase (ACC) α and ACCβ mRNA abundances in the rats fed diets containing casein (■) or alcohol-washed soya protein isolate (▲) for 70, 190 and 310 d. For details of diets and procedures, see Table 1 and p. 1049. Values are means with their standard errors depicted by vertical bars (n 6). [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

Acetyl-CoA carboxylase α and acetyl-CoA carboxylase β phosphorylation

Both hepatic pACCα and pACCβ contents in the rats fed SPI-based diets were significantly lower than those fed the casein diet, and showed the same pattern as their total ACCα and ACCβ proteins (Fig. 3(b)). However, the ratios of pACCα:ACCα and pACCβ:ACCβ in the liver (Fig. 3(b)), heart and kidney (Fig. 4(b)) were not different among dietary groups except that supplemental ISF increased the pACCα:ACCα ratio in the rats fed for 70 d (Fig. 3(b)).

Plasma insulin and leptin concentrations

Dietary effects on plasma insulin concentration were not significant at all three time-points examined. However, the insulin levels in the rats fed SPI diet alone for 190 or 310 d were markedly higher than those fed the same diet for 70 d (Table 2). The rats fed diets containing casein or SPI alone for 310 d had significantly higher plasma leptin concentrations than those fed the same diets for 70 or 190 d ($P<0·01$). However, the dietary effect was not significant (Table 2).

Discussion

The tissue samples analysed in the present study actually were from a part of the multigeneration study as described previously (Xiao *et al.* 2004). The TR protein content and plasma thyroid hormone (triiodothyronine and thyroxine) levels of these animals were measured and reported (Xiao *et al.* 2004). In the present study, we have shown that ingestion of soya proteins remarkably decreased plasma TAG concentrations in female rats compared with a casein diet, regardless of the presence of added ISF. This is consistent with previous studies showing that soya proteins rather than ISF play major roles in lowering blood TAG levels (Greaves *et al.* 1999; Gardner *et al.* 2001; Fukui *et al.* 2004b). This concept is further supported by recent studies in healthy post-menopausal women in which soya ISF were shown to have no effect on serum TAG level (Cheng *et al.* 2004; Colacurci *et al.* 2005). Although the components responsible for hypotriglycerolaemic actions of soyabeans have not been confirmed, increasing evidence suggests that β-conglycinin, the major 7S soyabean globulin, may contain the bioactive fraction since intake of β-conglycinin reduced serum TAG level in mice (Moriyama *et al.* 2004), rats (Aoyama *et al.* 2001; Duranti *et al.* 2004; Fukui *et al.* 2004*a*) and young women (Baba *et al.* 2004). Furthermore, the α′ subunit of β-conglycinin was shown to be hypotriacylglycerolaemic and as effective as the whole proteins (Duranti *et al.* 2004).

ACC is a rate-limiting enzyme in the long-chain fatty acid biosynthesis. The results of the present study have demonstrated that both ACCα and ACCβ mRNA and protein expression in the liver were significantly reduced by dietary SPI, regardless of the presence of supplemental ISF. However, dietary SPI had no effect on ACCα and ACCβ protein contents in both kidney and heart, suggesting tissue specificity of the soya effect. This appears to be consistent with our recent observations that dietary SPI elevated TR protein content and suppressed its DNA binding activity in rat livers, but not in the other tissues (Xiao *et al.* 2004; Huang *et al.* 2005). Since the liver is a key organ for fatty acid synthesis, and plays pivotal roles in the regulation of blood TAG levels, down-regulation of hepatic ACCα and ACCβ expression may contribute, at least in part, to the hypotriacylglycerolaemic actions of SPI.

It has been suggested that modulation of insulin levels by soya components might play a role in the regulation of lipogenic gene expression because ingestion of soya proteins in the presence of ISF (410–456 mg/kg diet) (Tovar *et al.* 2002; Ascencio *et al.* 2004) or soyabean β-conglycinin (Moriyama *et al.* 2004) decreased serum insulin levels compared with casein. Administration of insulin up-regulated ACCα mRNA expression and stimulated ACC enzymatic activity in rat liver (Katsurada *et al.* 1990). However, discrepancies exist in this regard. For example, a recent human study showed that soya ISF markedly reduced blood glucose and insulin levels, but had no effect on serum TAG levels (Cheng *et al.* 2004). In addition, although plasma and liver TAG levels were significantly lowered by soya proteins in both lean and fatty rats, plasma insulin concentrations were either unaffected in lean rats or even markedly increased in fatty rats (Iritani *et al.* 1996). In the present study, plasma

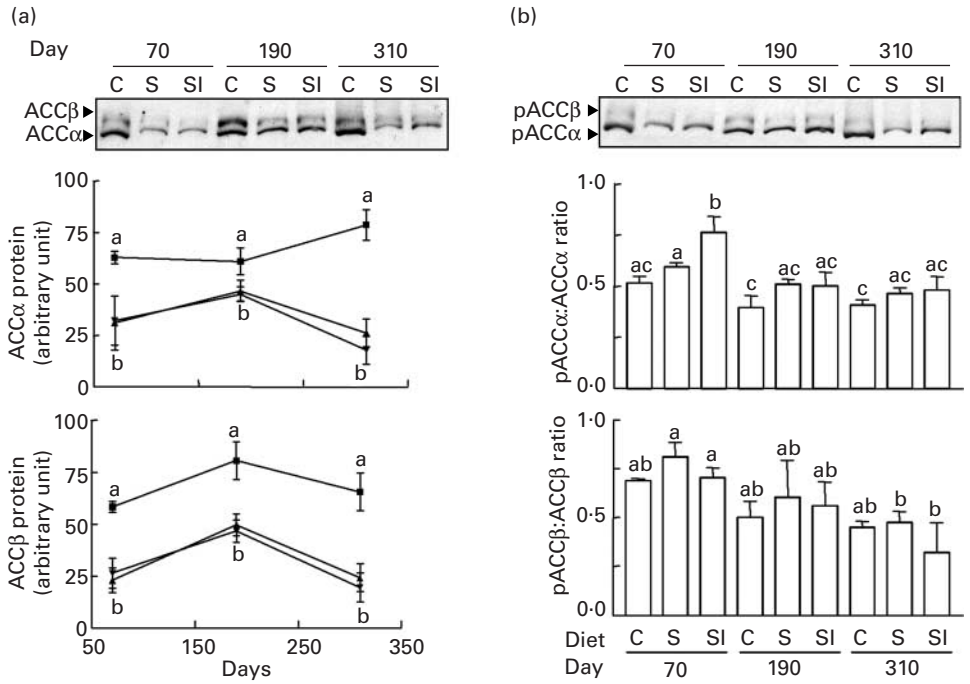

Fig. 3. Acetyl-CoA carboxylase (ACC)α and ACCβ protein contents (a) and the ratios of phospho-ACCα (pACCα):ACCα and phospho-ACCβ (pACCβ):ACCβ (b) in the livers of rats fed diets containing casein (C, ■) or alcohol-washed soya protein isolate in the absence (S, ▲) or presence (SI, ▼) of supplemental isoflavones (250 mg/kg diet) for 70, 190 and 310 d. The images shown are representatives of three replicates. For details of diets and procedures, see Table 1 and p. 1049. Values are means with their standard errors depicted by vertical bars (n 3). [a,b,c] Mean values with unlike letters at the same time-point were significantly different ($P<0{\cdot}05$).

insulin levels were not affected by either SPI or added ISF, which is supported by a recent study in fatty rats (Tovar *et al.* 2005). Taken together, all these findings suggest that decreased insulin levels might not be the only mechanism involved in the hypotriacylglycerolaemic actions of soya proteins.

We have recently suggested that modulation of hepatic TR may play important roles in the mediation of hypolipidaemic actions of soya through alteration of gene expression of key enzymes involved in lipogenesis. We demonstrated that dietary SPI remarkably inhibited the binding ability of hepatic nuclear TR to their target genes (Huang *et al.* 2005), which would include such lipogenic enzymes as malic enzyme (Petty *et al.* 1990), fatty acid synthase (Xiong *et al.* 1998) and ACC (Zhang *et al.* 2001) since these genes possess thyroid hormone response elements in their promoter regions. Furthermore, triiodothyronine stimulates ACCα mRNA expression and elevates ACC enzymatic activity in rat livers (Katsurada *et al.* 1990). Although thyroid hormone (triiodothyronine and thyroxine) levels from these animals were unchanged by dietary SPI as reported previously (Xiao *et al.* 2004), inhibition of TR function could block the stimulatory effect of thyroid hormones on downstream gene expression such as for ACC.

Intake of soya proteins was reported to reduce ACC enzymatic activity in rat liver (Iritani *et al.* 1986). It is known that ACC activity is mainly regulated by its phosphorylation status rather than altered enzyme content in certain tissues such as skeletal muscle (Dyck *et al.* 1999; Minokoshi *et al.* 2002). Whether dietary SPI and ISF influence ACC phosphorylation is unclear. We showed that the phosphorylation and dephosphorylation rates at Ser79 of both ACCα and ACCβ proteins were not different among dietary groups in all tissues measured, indicating that dietary SPI may regulate ACC mainly through modulation of its protein content rather than phosphorylation or dephosphorylation.

Leptin is a protein secreted by adipose tissues and stimulates oxidation of fatty acids by suppressing ACC activity (Minokoshi *et al.* 2002). Ingestion of soyabean β-conglycinin reduced the leptin levels in obese mice (Moriyama *et al.* 2004). However, the plasma leptin concentration was not affected by dietary SPI or ISF in the present study, which is consistent with the results obtained from previous studies in rats (Ascencio *et al.* 2004) and in pre- and post-menopausal women (Phipps *et al.* 2001). This excludes the possible involvement of leptin in the modulation of TAG levels and ACC expression by dietary SPI in the present study.

In summary, the present results show for the first time that consumption of 20 % alcohol-washed SPI suppressed hepatic ACCβ gene expression. In addition, dietary SPI lowered the plasma TAG level and reduced ACCα mRNA and protein contents in liver, but had no effect in both kidney and heart compared with a casein diet. Furthermore, supplemental ISF had no effect. The ratios of pACCα:ACCα and pACCβ:ACCβ were unchanged by SPI. This suggests that regulation of ACC by dietary SPI is mainly through alteration of its gene expression rather than phosphorylation or dephosphorylation. The potential involvement of TR function modification by SPI in the regulation of ACC gene expression and TAG levels is under investigation.

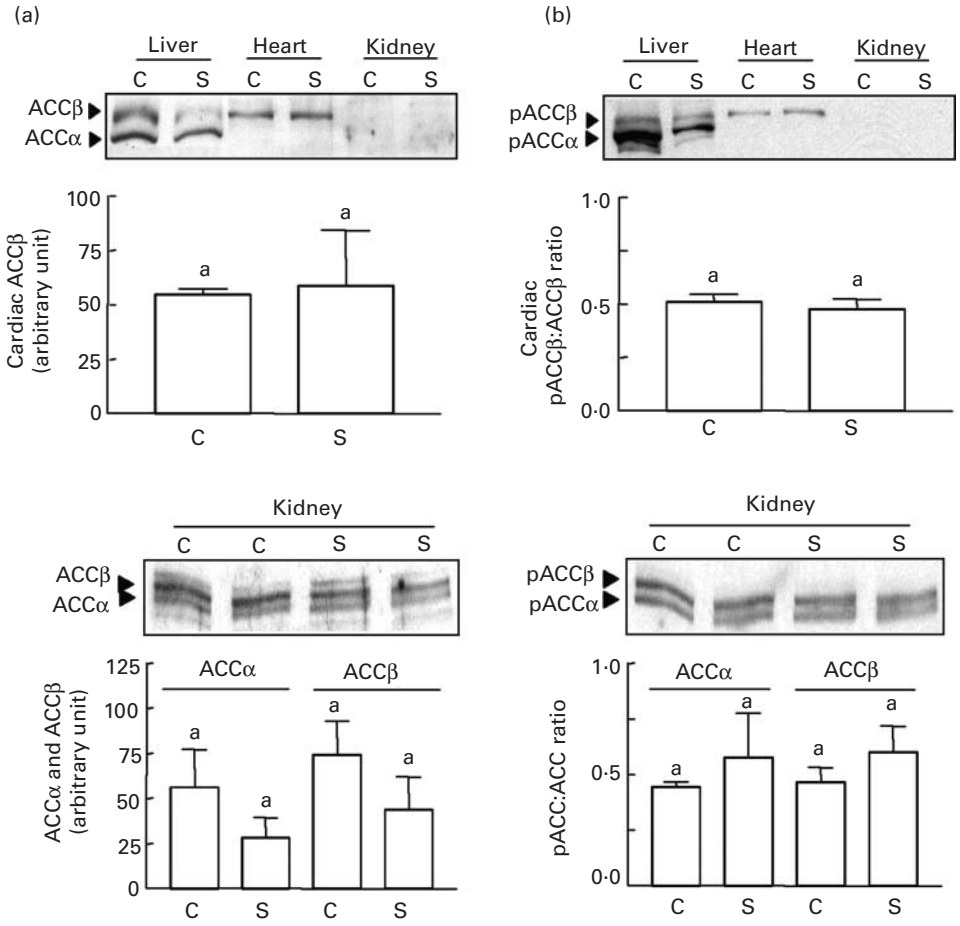

Fig. 4. Acetyl-CoA carboxylase (ACC) α and ACCβ protein contents and the ratios of phospho-ACCα (pACCα):ACCα and phospho-ACCβ (pACCβ):ACCβ in the heart (a) and kidney (b) of the rats fed diets containing casein (C) or alcohol-washed soya protein isolate (S) for 310 d. The images shown are representatives of three replicates. Expression of ACCα and ACCβ was extremely low in kidney, and the images shown were obtained after extended exposure of the film (b). For details of diets and procedures, see Table 1 and p. 1049. Values are means with their standard errors depicted by vertical bars (n 6). [a] Mean values with unlike letters were significantly different ($P<0·05$).

Table 2. Plasma insulin and leptin concentrations in rats fed diets containing either casein or alcohol-washed soya protein isolate (SPI) for different periods (pmol/l)*

(Mean values with their standard errors; n 6)

	Casein		SPI		SPI + ISF‡	
Days†	Mean	SEM	Mean	SEM	Mean	SEM
Insulin						
70	259·5[ab]	54·6	149·2[a]	42·1	270·3[ab]	56·7
190	325·4[b]	86·7	312·3[b]	42·4	303·0[b]	69·8
310	269·1[b]	47·0	353·6[b]	51·9	355·8[b]	92·0
Leptin						
70	174·8[a]	42·0	115·6[a]	42·3	137·2[a]	19·4
190	197·0[a]	26·6	216·8[a]	18·5	194·8[a]	31·0
310	316·0[b]	57·2	277·2[b]	56·0	241·5[ab]	35·0

ISF, isoflavones.
[a,b] Mean values within a row or column with unlike superscript letters were significantly different ($P<0·05$).
* For details of procedures, see p. 1049.
† Days of feeding the experimental diets.
‡ Alcohol-washed SPI supplemented with 250 mg ISF/kg diet.

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