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Dietary soya protein intake and exercise training have an additive effect on skeletal muscle fatty acid oxidation enzyme activities and mRNA levels in rats

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Exercise training and regular physical activity increase oxidation of fat. Enhanced oxidation of fat is important for preventing lifestyle diseases such as hypertension and obesity. The aim of the present study in rats was to determine whether intake of dietary soya protein and exercise training have an additive effect on the activity and mRNA expression of enzymes involved in skeletal muscle fatty acid oxidation. Male Sprague–Dawley rats (*n* 32) were assigned randomly into four groups (eight rats per group) and then divided further into sedentary or exercise-trained groups fed either casein or soya protein diets. Rats in the exercise groups were trained for 2 weeks by swimming for 120 min/d, 6 d/week. Exercise training decreased hepatic triacylglycerol levels and retroperitoneal adipose tissue weight and increased skeletal muscle carnitine palmitoyltransferase 1 (CPT1) activity and mRNA expression of CPT1, β-hydroxyacyl-CoA dehydrogenase (HAD), acyl-CoA oxidase, PPARγ coactivator 1α (PGC1α) and PPARα. Soya protein significantly decreased hepatic triacylglycerol levels and epididymal adipose tissue weight and increased skeletal muscle CPT1 activity and CPT1, HAD, acyl-CoA oxidase, medium-chain acyl-CoA dehydrogenase, PGC1α and PPARα mRNA levels. Furthermore, skeletal muscle HAD activity was the highest in exercise-trained rats fed soya protein. We conclude that exercise training and soya protein intake have an important additive role on induction of PPAR pathways, leading to increased activity and mRNA expression of enzymes involved in fatty acid oxidation in skeletal muscle and reduced accumulation of body fat.

Soya protein: Fatty acid oxidation enzymes: Adipose tissue weight: Skeletal muscle: Exercise-trained rats

Exercise training and regular physical activity are known to increase fat oxidation in both healthy (Friedlander *et al.* 1998, 1999) and obese (Pritzlaff-Roy *et al.* 2002) individuals. It is likely that several benefits of regular exercise, such as decreased insulin resistance, lower blood pressure and reduced levels of plasma LDL, are related to enhanced oxidation of fat (Toth *et al.* 1995; Dengel *et al.* 1998). Obtaining a better understanding of the factors that influence the rate of fat oxidation at rest and during exercise is therefore important. It is also well established that low-intensity prolonged exercise training simultaneously increases the activity of skeletal muscle mitochondrial enzymes involved in the tricarboxylic acid cycle and fatty acid β-oxidation (Holloszy *et al.* 1970; Grinton *et al.* 1992; Carter *et al.* 2001). Previous studies demonstrated that PPARγ coactivator 1 (PGC1) is expressed in several tissues, including skeletal muscle and brown adipose tissue. It has been reported to increase mitochondria biogenesis and fatty acid oxidative metabolism (Wu *et al.* 1999; Vega *et al.* 2000; Hara *et al.* 2002). In rats, PGC1 mRNA and protein levels are increased after a single bout of exercise as well as after several days of training (Goto *et al.* 2000). In man, Pilegaard *et al.* (2003) observed a transient increase in PGC1 mRNA levels after a single bout of exercise.

Many studies have shown that consumption of soya protein is associated with a reduction in cardiovascular risk (Meeker & Kesten, 1940; Sugano & Koba, 1993; Anderson *et al.* 1995). This effect is attributed to the finding that soya protein reduced plasma cholesterol in animal models whereas casein and other animal proteins had no such effect. The effect of soya protein on blood cholesterol concentrations was first reported in rabbits in the 1940s (Meeker & Kesten, 1940). Results in male human subjects with mild hypercholesterolaemia found that soya protein caused significant lowering of total and LDL cholesterol whilst it maintained HDL cholesterol concentrations (Anderson *et al.* 1995). Soya protein has also been shown to reduce hepatic triacylglycerol (Morifuji & Aoyama, 2002), some lipogenic enzymes (Iritani *et al.* 1986) and sterol regulatory element binding protein-1 mRNA expression (Tovar *et al.* 2002).

The components of soya include protein, lipids, fibre and phytochemicals including isoflavones. Considerable research effort has focused on isoflavones as the main hypolipidaemic agent in soya protein (Mezei *et al.* 2003; Song *et al.* 2003; Wu

Abbreviations: CPT1, carnitine palmitoyltransferase 1; FAT/CD36, fatty acid translocase; HAD, β-hydroxyacyl-CoA dehydrogenase; PGC1, PPARγ coactivator 1; PGC1α, PPARγ coactivator 1α.

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et al. 2004; Kim *et al.* 2005). Previous evidence from a study in obese Zucker rats showed that ingestion of a high-isoflavone soya protein diet improved insulin resistance, suggesting that some of the beneficial effects may have been mediated by PPAR (Mezei *et al.* 2003). It is therefore possible that isoflavones may also have beneficial effects on lipid metabolism. However, the role of isoflavones in the regulation of lipid metabolism remains unclear.

Although it is known that the expression of many fatty acid-metabolizing enzymes is regulated by PPAR at the transcriptional level, it is not clear from animal models whether intake of soya protein stimulates fatty acid oxidation in skeletal muscle. This led us to speculate whether the combination of exercise training and intake of soya protein may have an additive effect of enhancing fatty acid oxidation in skeletal muscle. The aim of the present study was to determine whether dietary soya protein influenced the activity and mRNA expression of enzymes involved in fatty acid oxidation in skeletal muscle of exercise-trained rats.

Materials and methods

Animals

Male Sprague–Dawley rats (CLEA Japan Inc., Tokyo, Japan) were used in the present study. All the rats were housed individually in temperature-controlled rooms (22°C), with light from 08.00 to 20.00 hours and dark from 20.00 to 08.00 hours. The study was approved by the Animal Committee of Meiji Seika Kaisha Ltd, Food & Health R&D Laboratories, with the animals receiving care under the guidelines laid down by this committee.

Diets

The design of the experimental diets followed the AIN-93 protocol (Reeves *et al.* 1993) with the composition of the diets shown in Table 1. Casein and soya protein were used as the source of dietary protein. The protein content, calculated as nitrogen concentration × 6·38 (casein), or × 5·8 (soya protein), was measured using the Kjeldahl method. Casein (87·7 g crude protein/100 g) and soya protein (79·3 g crude protein/100 g) were added as 200 g protein/kg to the diets. The difference in the protein content between the two diets was compensated for by the addition of maize starch. Soya protein contained 19·72 mg isoflavones/g protein, mainly as genistein (17·43 mg).

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

	Casein	Soya
Casein*	228	–
Soya protein†	–	254
Vitamin mixture‡	10	10
Choline bitartrate§	2·5	2·5
Mineral mixture‡	35	35
Maize oil‖	70	70
Maize starch¶	504·5	478·5
Sucrose**	100	100
Cellulose††	50	50

* Oriental Yeast Co. Ltd, Tokyo, Japan.
† Fuji Oil Co. Ltd, Osaka, Japan.
‡ AIN-93 diet, Nosan Corporation, Kanagawa, Japan.
§ Wako Pure Chemical Industries Ltd, Osaka, Japan.
‖ Ajinomoto Co., Inc., Tokyo, Japan.
¶ Taiyo Kagaku Co. Ltd, Mie, Japan.
** Nippon Beet Sugar Manufacturing Co. Ltd, Tokyo, Japan.
†† Asahi Kasei Corporation, Tokyo, Japan.

Experimental protocol

Thirty-two male Sprague–Dawley rats (eight per group) with body weight of approximately 120 g were allowed free access to food and water for 2 weeks. Rats were assigned randomly into four groups and then divided further into sedentary or exercise-trained groups fed either casein or soya protein diets. Rats in the exercise-trained groups swam simultaneously without a load for 6 d/week at 120 min/d in a barrel filled with water maintained at 35°C to a depth of 50 cm so that the average surface area available to each animal was 170 cm^2. At the end of the 2-week training period, all the rats were rested for 24 h after the end of the last training session. The rats were killed between 09.00 and 11.00 hours under the non-fasting condition. Postcaval blood samples were collected from all the animals under ether anaesthesia, centrifuged at 3000 ***g*** for 15 min and the serum stored at − 80°C. After blood collection, the abdominal cavity was opened and the liver, triceps muscle, retroperitoneal adipose tissue and epididymal adipose tissue were quickly excised, washed, weighed and frozen at − 80°C until assay. The rat triceps muscle was used in the study as it is used extensively during swimming exercises, as evidenced by glycogen depletion and an adaptive increase in GLUT4 content and citrate synthase activity (Host *et al.* 1998).

Serum analyses

Serum triacylglycerol, cholesterol, phospholipids, NEFA and glucose were measured by enzymatic methods using a commercial kit (Wako Pure Chemical Industries Ltd, Osaka, Japan). Serum ketone bodies were analysed using a commercial kit (Sanwa Kagaku Kenkyusho Co. Ltd, Tokyo, Japan). Serum insulin concentration was determined using an ELISA kit obtained from Shibayagi Co. Ltd (Gunma, Japan).

Liver triacylglycerol content

The total concentration of lipids extracted and purified from the liver was measured according to the method of Folch *et al.* (1957). Liver triacylglycerol was determined using the same technique.

Liver and skeletal muscle enzyme activities

Aliquots of skeletal muscle were homogenized in 0·1 M-Tris-HCl buffer (pH 7·4) using a glass type homogenizer. The homogenate was centrifuged for 15 min at 900 ***g*** at 4°C and this fraction was used immediately to assay the activity of the following mitochondrial enzymes. β-Hydroxyacyl-CoA dehydrogenase (HAD; *EC* 1.1.1.35) was assayed using the method of Bass *et al.* (1968) and carnitine palmitoyltransferase 1 activity (CPT1; *EC* 2.3.1.21) was measured according to the method described by Markwell *et al.* (1973). The total protein concentration of the tissue homogenate supernatant was

measured using bicinchoninic acid with bovine serum albumin as the standard (Smith *et al.* 1985).

Total RNA isolation and cDNA

Total RNA was isolated from skeletal muscle by the guanidine thiocyanate method of Chmczynki and Sacchi (1987) using Isogen solution (Nippon Gene Co. Ltd, Tokyo, Japan). The RNA extracted was then dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at a wavelength of 260 nm. Reverse transcription was used to produce cDNA from RNA using a first standard cDNA synthesis kit (Fermentas Inc., Hanover, MD, USA). The cDNA was stored at −80°C for subsequent analysis.

Quantitative real-time RT–PCR analysis

Real-time PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Primers and probes (TaqMan® Assays-on-Demand™ Gene Expression Products) were designed at Applied Biosystems from gene sequences obtained from Gene Bank (CPT1: NM_012930; HAD: NM_057186; medium chain acyl-CoA dehydrogenase: NM_016986; acyl-CoA oxidase: NM_145770; PPAR coactivator 1α (PGC1α): NM_031347; PPARα: NM_013196; fatty acid translocase (FAT/CD36): NM_031561; 18S rRNA: X03205). DNA amplification was carried out in 12·5 μl Taqman Universal PCR Master Mix, 1·25 μl primer and probes, 2·5 μl cDNA and 8·75 μl RNase and DNase free water in a final volume of 25 μl/well. The samples were loaded in a MicroAmp ninety-six-well reaction plate and then run using the ABI sequence detection system. After 2 min at 50°C and 10 min at 95°C, the plates were co-amplified by fifty repeated cycles, with each cycle consisting of a 30 s denaturing step at 95°C and a 1 min annealing/extending step at 59°C. Data were analysed by ABI software using the cycle threshold (C_T), a value calculated as the time, measured as cycle number, at which the reporter fluorescent emission increased beyond a threshold level, defined as the background number at which cDNA amplification was first detected. Fluorescent emission data were captured and mRNA levels quantified for each gene using the C_T value. The ΔC_T was calculated by subtracting the C_T for 18S rRNA from the C_T for the gene of interest. The relative expression of the gene of interest was then calculated using the expression $2^{-\Delta\Delta C_T}$, with the results being expressed as arbitrary units.

Statistics

Data were analysed using two-way ANOVA with *post hoc* analyses being carried out by Tukey's test. Differences between groups were considered to be significant at $P<0·05$.

Results

Initial body weight, food intake and body weight gain

Food intake did not differ between the groups. Body weight gain is lower in exercise-trained groups than in sedentary groups. Furthermore, the soya protein diet significantly decreased body weight gain compared to the casein diet (Table 2).

Liver triacylglycerol and adipose tissue weight

Exercise training decreased both hepatic triacylglycerol concentration and retroperitoneal adipose tissue weight, but had no effect on the weight of epididymal adipose tissue. The soya protein diet lowered liver triacylglycerol levels significantly compared to the casein diet. Epididymal adipose tissue weight in the soya protein groups was lower than in the casein groups, whereas the type of dietary protein had no effect on retroperitoneal adipose tissue weight (Fig. 1).

Serum parameters

Exercise training for 2 weeks significantly decreased serum insulin levels. Furthermore, significant decreases in serum triacylglycerol, phospholipids, NEFA and glucose levels were observed in rats fed soya protein compared to those fed casein. Serum ketone bodies were higher in the soya protein groups than in the casein groups. Serum cholesterol levels were highest in sedentary rats fed the casein diet (Table 3).

Fatty acid oxidation enzyme activities

Skeletal muscle CPT1 activity was increased by exercise training. Skeletal muscle CPT1 activity was increased significantly in rats fed soya protein compared to animals fed casein. Skeletal muscle HAD activity was the highest in exercise-trained rats fed soya protein (Fig. 2).

Skeletal muscle mRNA levels of transcriptional factor, fatty acid translocase and enzymes involved in fatty acid oxidation

Exercise training for 2 weeks significantly increased skeletal muscle CPT1, HAD, acyl-CoA oxidase, PGC1α, PPARα and FAT/CD36 mRNA expressions. Skeletal muscle CPT1, HAD, acyl-CoA oxidase, medium-chain acyl-CoA dehydrogenase, PGC1α, PPARα and FAT/CD36 mRNA levels were increased significantly in rats fed soya protein compared to animals fed casein (Table 4).

Table 2. Initial body weight, food intake and body weight gain in sedentary or exercise-trained rats fed the casein or soya protein diet*

(Mean values with their standard errors)

	Initial body weight (g)		Food intake (g/14 d)		Body weight gain (g/14 d)	
Group	Mean	SE	Mean	SE	Mean	SE
Sedentary – casein	118	1	269	3	106	2
Sedentary – soya	119	1	261	5	101	4
Exercised – casein	123	3	272	5	100	3
Exercised – soya	122	3	265	6	95·3	3·8
Two-way ANOVA						
Diet	0·855		0·139		0·020	
Exercise	0·080		0·532		0·008	
Diet × Exercise	0·701		0·913		0·278	

* For details of procedures, see this page.

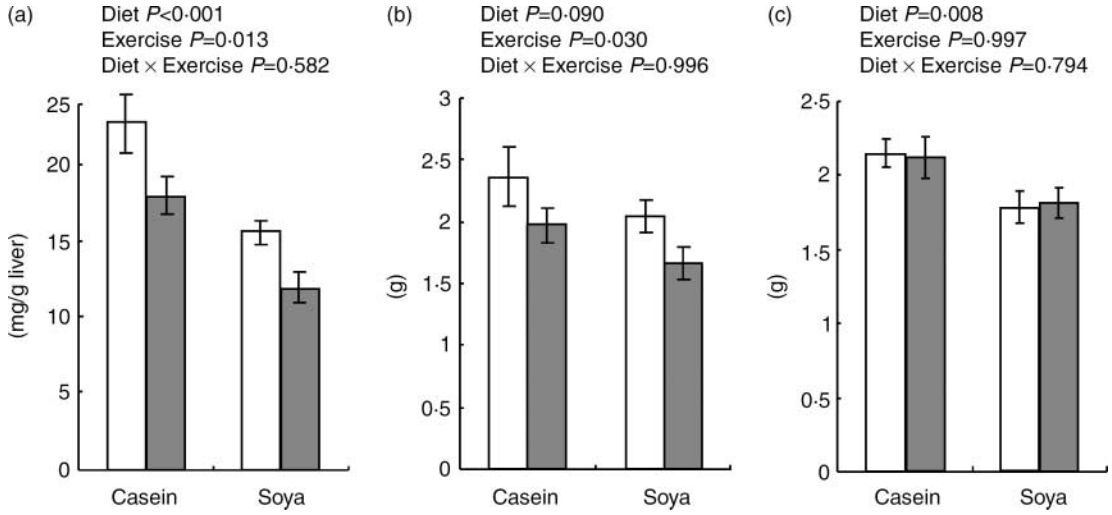

Fig. 1. Effect of dietary protein on liver triacylglycerol levels (a), retroperitoneal adipose tissue weight (b) and epididymal adipose tissue weight (c) in sedentary (□) or exercise-trained (■) rats. For details of procedures, see p. 471. Values are means with their standard errors depicted by vertical bars.

Discussion

The present study showed that rats fed a soya protein diet had increased skeletal muscle fatty acid oxidation enzyme activities and mRNA levels compared to rats fed a casein diet. The soya protein diet also lowered liver triacylglycerol levels and epididymal adipose tissue weight significantly compared to the casein diet. We also demonstrated that exercise training decreased hepatic triacylglycerol levels and retroperitoneal adipose tissue weight. The present results indicated that a combination of exercise training and soya protein intake had an additive effect of increasing the activity of enzymes in skeletal muscle involved in fatty acid oxidation, thereby decreasing hepatic triacylglycerol levels and body fat mass.

It is well established that exercise training and regular physical activity reduce body fat by increasing fat oxidation (Friedlander *et al.* 1998, 1999). Low-intensity prolonged exercise training increases the activity of skeletal muscle mitochondrial enzymes involved in fatty acid β-oxidation (Holloszy *et al.* 1970; Grinton *et al.* 1992; Carter *et al.* 2001). In the present study, the activity and mRNA expression of fatty acid oxidation enzymes were increased in skeletal muscles of exercise-trained rats. We also found increased mRNA expression for skeletal muscle PGC1α and PPARα mRNA in the exercise-trained groups. The present findings are consistent with reports that skeletal muscle PGC1 mRNA and protein levels are increased after a single bout of exercise as well as after several days of training in rats (Goto *et al.* 2000; Baar *et al.* 2002; Terada *et al.* 2002) and man (Russell *et al.* 2003). An increase in PGC1α in skeletal muscles may enhance mitochondrial biogenesis and fatty acid oxidation, including PPARα target genes (Wu *et al.* 1999; Vega *et al.* 2000; Miura *et al.* 2003). Moreover, PPARα controls the transcription of many genes involved in lipid catabolism including FAT/CD36. The effect of endurance training on skeletal muscle PPARα has, however, not been evaluated in either animal or human studies. Horowitz *et al.* (2000) and Russell *et al.* (2003) reported that endurance exercise training increased PPARα mRNA levels in human skeletal muscle. The results of the present study in animals are consistent with the results from the previous studies in human subjects. Taken together, the results indicate that an increase in muscle PGC1α mRNA and increased fatty acid

Table 3. Serum parameters in sedentary or exercise-trained rats fed the casein or soya protein diet*
(Mean values with their standard errors)

	Triacylglycerol (mmol/l)		Phospholipids (mmol/l)		Cholesterol (mmol/l)		NEFA (μEq/l)		Ketone body (μmol/l)		Glucose (mmol/l)		Insulin (μg/l)	
Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Sedentary – casein	3·19	0·41	2·96	0·08	2·74[b]	0·08	461	28	120	11	11·0	0·2	4·55	0·65
Sedentary – soya	1·84	0·08	2·07	0·05	2·09[a]	0·09	402	33	179	20	9·97	0·24	3·96	0·97
Exercised – casein	3·63	0·47	2·67	0·11	2·22[a]	0·08	532	46	154	15	10·7	0·4	3·35	0·87
Exercised – soya	2·07	0·22	2·19	0·09	2·22[a]	0·09	387	32	202	21	10·5	0·2	1·68	0·41
Two-way ANOVA														
Diet	<0·001		<0·001		<0·001		0·008		0·004		0·026		0·074	
Exercise	0·319		0·364		<0·026		0·442		0·107		0·621		0·008	
Diet × Exercise	0·763		0·278		<0·001		0·235		0·726		0·179		0·380	

[a,b] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).
* For details of procedures, see p. 471.

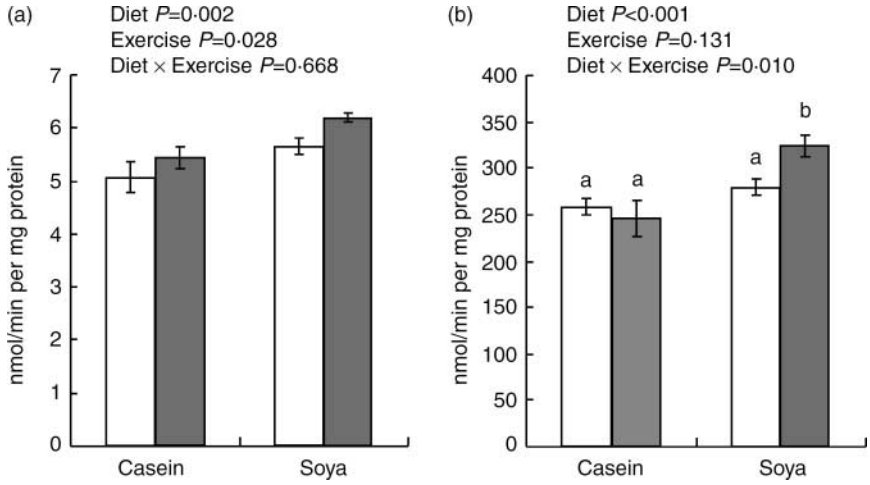

Fig. 2. Effect of dietary protein on skeletal muscle carnitine palmitoyltransferase 1 (a) and β-hydroxyacyl-CoA dehydrogenase (b) activities in sedentary (□) or exercise-trained (■) rats. For details of procedures, see p. 471. Values are means with their standard errors depicted by vertical bars.

metabolism resulting from exercise training are both important factors in enhancing muscle fatty acid oxidative capacity.

Although it is generally accepted that intake of soya protein improves lipid metabolism in both animals and man, little attention has been paid to the effect of soya protein on fatty acid oxidation in skeletal muscle. We showed that soya protein, relative to casein, significantly increased the activities and mRNA levels of skeletal muscle fatty acid oxidation enzymes, PGC1α and PPARα mRNA expression.

Some researchers have focused on isoflavones, namely genistein, daidzein and glycitein, as the important bioactive components of soya protein. Soya isoflavones bind and activate both PPARα and PPARγ (Mezei *et al.* 2003). Genistein supplementation (2 g/kg diet) caused a significant increase in PPARα and PPARγ mRNA expression in a mouse model of obesity (Kim *et al.* 2005). Therefore, the increase in fatty acid oxidation enzyme activities in the soya groups may be related to isoflavones that stimulate fatty acid oxidation via PPAR pathways. The soya protein diet used in the present study contained 5·0 g isoflavones/kg diet. In previous reports, combined intervention of soya isoflavones (1·6 g/kg diet) and moderate exercise prevented increases in body fat in ovariectomized mice (Wu *et al.* 2004). Similarly, a soya protein diet containing high isoflavones (1·16 g/kg diet) was reported to cause a significant decrease in triacylglycerol and cholesterol levels in the liver of obese Zucker rats compared to either a casein or low isoflavone soya protein diet (< 0·009 g/kg) (Mezei *et al.* 2003). Furthermore, supplementation of daidzein (0·33 g/kg) to the casein diet resulted in a significant decrease in plasma triacylglycerol and cholesterol levels in female hamsters (Song *et al.* 2003). As these earlier studies showed that only small amounts of isoflavones were required to improve lipid metabolism in animals, the quantity of isoflavones (5·0 g/kg diet) used in the present study may have been sufficient to affect fatty acid oxidation enzyme activity and mRNA levels in skeletal muscle.

On the other hand, there is evidence that soya protein downregulates lipogenic enzymes thereby reducing the availability of long-chain fatty acids required for triacylglycerol synthesis in the liver (Iritani *et al.* 1986, 1992). A study also showed that intake of soya protein decreased sterol regulatory element binding protein-1c and hepatic lipogenic enzyme activities and mRNA expression (Ascencio *et al.* 2004). Iritani *et al.* (1986) demonstrated that when dietary protein was replaced with an

Table 4. Skeletal muscle mRNA levels of transcriptional factor, fatty acid translocase (FAT/CD36) and enzymes involved in fatty acid oxidation in sedentary or exercise-trained rats fed the casein or soya protein diet*

(Mean values with their standard errors)

	CPT1		HAD		MCAD		ACO		FAT/CD36		PGC1α		PPARα	
Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Sedentary – casein	100	10	100	7	100	14	100	9	100	10	100	12	100	7
Sedentary – soya	138	14	149	12	147	12	143	13	142	11	136	24	128	9
Exercised – casein	139	19	138	21	123	18	143	13	164	32	205	13	129	13
Exercised – soya	184	22	216	36	207	38	206	27	254	48	269	22	169	20
Two-way ANOVA														
Diet	0·045		0·012		0·014		0·008		0·048		0·020		0·046	
Exercise	0·046		0·034		0·112		0·008		0·032		< 0·001		0·042	
Diet × Exercise	0·886		0·533		0·469		0·599		0·469		0·489		0·725	

ACO, acyl-CoA oxidase; CPT1, carnitine palmitoyltransferase 1; HAD, β-hydroxyacyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; PGC1α, PPARγ coactivator 1α.

* Data are expressed in arbitrary units. For details of procedures, see p. 471.

amino acid mixture similar to either casein or soya protein this resulted in lower activities of malic enzyme and glucose 6-phosphate dehydrogenase in the group fed the soya-type diet compared to the group fed the casein-type diet. The present results suggest that differences in amino acid composition may decrease liver triacylglycerol levels and adipose tissue weights as a consequence of inhibiting lipogenesis in the liver. However, little is known about whether individual amino acids stimulate skeletal muscle fatty acid oxidation.

In summary, the present results demonstrated that a combination of exercise training and soya protein intake was more effective for decreasing liver triacylglycerol and epididymal adipose tissue weight than either treatment alone. The reduction in hepatic triacylglycerol levels and body fat mass may be associated with an increase in fatty acid oxidation in skeletal muscle induced by activation of PPAR pathways. We conclude that a combined intervention of soya protein supplementation and moderate exercise may offer an effective regimen for preventing lifestyle-related health problems such as obesity.

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