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Effects of soy protein and isoflavone on hepatic fatty acid synthesis and oxidation and mRNA expression of uncoupling proteins and peroxisome proliferator-activated receptor γ in adipose tissues of rats☆

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Abstract

Soy protein rich in isoflavones profoundly affects lipid metabolism in experimental animals. To distinguish the roles of the protein and isoflavone components of a soy protein preparation in regulating lipid metabolism, we compared the effects of diets containing methanol-washed soy protein low in isoflavone supplemented with a 0-, 0.5- and 4-g/kg isoflavone preparation on hepatic fatty acid metabolism and adipose tissue gene expression in rats. Diets containing soy protein irrespective of the isoflavone levels decreased the activities and mRNA expression of enzymes involved in hepatic fatty acid synthesis to similar levels. Methanol-washed soy protein compared to casein increased the mRNA expression of peroxisome proliferator-activated receptor (PPAR) α, and supplementing the soy protein diet with isoflavone further increased this parameter dose-dependently. However, methanol-washed soy protein compared to casein was totally ineffective in altering the activities and mRNA levels of enzymes involved in fatty acid oxidation. Supplementation of soy protein diets with isoflavone slightly increased these parameters. The mRNA level of uncoupling protein (UCP) 1 in brown adipose tissue was significantly increased and mRNA levels of UCP2 and 3, and PPARγ2 tended to be higher in rats fed methanol-washed soy protein not supplemented with isoflavone than in the animals fed casein. Adding isoflavone to the soy protein diets dose-dependently increased these parameters. These results suggested that the protein rather than isoflavone component is primarily responsible for the physiological activity of soy protein rich in isoflavones in reducing hepatic lipogenesis. However, isoflavones may have a role in regulating heptic fatty acid oxidation and adipose tissue gene expression.

Keywords: Adipose tissue; Gene expression; Isoflavone; Liver; Serum lipids; Soy protein

1. Introduction

It has been demonstrated that soy protein compared to animal proteins lowers serum lipid levels in experimental animals and humans (reviewed in [1]). With regard to the mechanism underlying this hypolipidemic effect, studies have shown that soy protein compared to casein decreases cholesterol absorption but increases cholesterol degradation to form bile acid [1]. In addition, soy protein increases low-density lipoprotein (LDL) receptor activity [1,2] but decreases hepatic secretion of triacylglycerol-rich lipoprotein [3]. In relation to the physiological activity of soy protein in reducing lipoprotein production, studies have indicated that this dietary protein lowers hepatic lipogenesis through a sterol regulatory element-binding protein (SREBP)-1-dependent mechanism [4,5]. In addition, Tovar et al. [5] showed that soy protein compared to casein increased mRNA levels of hepatic peroxisome proliferator-activated receptor (PPAR) α. Therefore, alterations in hepatic lipogenesis and fatty acid oxidation may be responsible for soy protein-dependent decreases in lipoprotein production.

The soy protein preparations employed for previous human and animal studies usually contained considerable

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low-density lipoprotein; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; UCP, uncoupling protein.

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amounts of isoflavone [6]. The observations that soy isoflavones including genistein and daidzein lower serum and liver lipid levels in rodents [6] raise the possibility that the isoflavone, not protein, component of a soy protein preparation is responsible for the hypolipidemic propensity. In this context, studies have indicated that soy protein low in isoflavone had a weaker lowering effect on serum and liver lipid levels in rats [7] and mice [8]. However, reports exist that the elimination of isoflavones did not attenuate the lipid-lowering effect of soy protein in rats [9]. Therefore, the role of isoflavones in soy protein preparations affecting lipid metabolism is still controversial and needs to be clarified.

In the present study, we compared physiological effects of a diet containing soy protein low in isoflavone prepared by methanol-washing and diets supplemented with isoflavone on lipid metabolism in rats. A group of rats fed a diet containing casein free of isoflavone was also included for comparative purposes. As alterations in hepatic lipogenesis [4,5] and fatty acid oxidation [5] appear to be crucial to the lipid-lowering efficacy of soy protein rich in isoflavone, we examined the effects of various diets on the activity and mRNA level of enzymes involved in hepatic fatty acid synthesis and oxidation. Since some studies indicated that soy protein reduced body fat mass [1,10], we also analyzed the mRNA expression of genes involved in adipocyte differentiation (PPARγ) and energy expenditure [uncoupling proteins (UCPs)] in white and brown adipose tissues.

2. Materials and methods

2.1. Materials

Soy protein isolate (Fujipro) was donated by Fuji Oil, Osaka, Japan. This preparation contained 1.93 g/kg of isoflavone and was extracted twice with 10 volumes of hot 80% methanol to eliminate isoflavones and air-dried. The methanol-washed soy protein still contained small amounts of isoflavones (0.125 g/kg). Casein was purchased from Wako Pure Chemical Industries (Osaka, Japan). Analysis revealed that this protein source was free from isoflavones. A preparation rich in soy isoflavone (Soyaflavone HG) was also donated by Fuji Oil. This preparation was made by extracting soy germ with aqueous ethanol and subsequent treatment with a nonpolar resin column [11] and contained the following (in g/kg): isoflavones (mainly in the form of glycosides), 470; saponins, 91, moisture, 24; protein, 122; ash, 34; fat, less than 1; and others, 259.

2.2. Animals and experimental diets

Male Sprague–Dawley rats purchased from Charles River Japan (Kanagawa, Japan) at 4 weeks of age were housed individually in a room with controlled temperature (20–22°C), humidity (55–65%) and lighting (from 0700 to 1900 h) and fed a commercial nonpurified diet (Type NMF; Oriental Yeast, Tokyo, Japan). After 7 days of acclimatization to the conditions, the rats were randomly divided into four groups consisting of 7–8 animals each with similar mean body weights (133–135 g) and assigned experimental diets. One group of rats were fed a diet containing 200 g/kg of casein, and the other three groups were fed diets containing 200 g/kg of methanol-washed soy protein and 0, 1.06 or 8.51 g/kg of the soy isoflavone preparation. These amounts supplied 0, 0.5 or 4 g/kg in the diet with soy isoflavone. The compositions and isoflavone concentrations of the experimental diets are summarized in Table 1. The compositions of vitamin and mineral mixtures were the same as those descried by Reeves et al. [12]. Sucrose represented a major carbohydrate source and palm oil was employed as a dietary fat source in our experimental diets. The use of these constituents in diets was associated with a high rate of hepatic lipogenesis along with increased levels of serum and liver lipids [13,14]. Soy isoflavone was added to the experimental diets instead of sucrose. Animals had free access to diet and water during the experiment. After 14 days, the animals were killed by bleeding from the abdominal aorta

Table 1
Composition of experimental diets

Isoflavone added (g/kg)	Dietary protein			
	Casein	Soy protein		
	0	0	0.5	4
Ingredient (g/kg)				
Casein	200	–	–	–
Soy protein[a]	–	200	200	200
Sucrose	480	480	479	471
Cornstarch	150	150	150	150
Palm oil	100	100	100	100
Cellulose	20	20	20	20
Vitamin mixture	10	10	10	10
Mineral mixture	35	35	35	35
Choline bitartrate	2	2	2	2
L-Cystine	3	3	3	3
Isoflavone preparation[b]	0	0	1.1	8.5
Isoflavone species (g/kg)				
Daidzein	0	0.002	0.003	0.005
Glycitein	0	0.000	0.000	0.002
Genistein	0	0.006	0.006	0.006
Daidzin	0	0.003	0.172	1.355
Glycitin	0	0.000	0.082	0.656
Genistin	0	0.012	0.047	0.288
Malonyldaidzin	0	0.000	0.128	1.021
Malonylglycitin	0	0.000	0.048	0.387
Malonylgenistin	0	0.001	0.032	0.245
Acetyldaidzin	0	0.000	0.003	0.022
Acetylglycitin	0	0.000	0.004	0.032
Acetylgenistin	0	0.000	0.001	0.005
Aglycone equivalent (g/kg)				
Daidzein	0	0.004	0.174	1.361
Glycitein	0	0.000	0.081	0.646
Genistein	0	0.014	0.052	0.317
Total isoflavone	0	0.018	0.306	2.323

[a] Soy protein (Fujipro) was treated twice with hot 80% methanol.
[b] This preparation contained 470 g/kg of isoflavone mainly in the form of glycosides.

under diethyl ether anesthesia, and liver, epididymal and perirenal white adipose tissues and interscapular brown adipose tissue were quickly excised. Brown adipose tissue was carefully cleaned from the surrounding muscular tissue and white adipose tissue. This study was approved by the review board of animal ethics of our institute and we followed the institute's guidelines in the care and use of laboratory animals.

2.3. Analyses of lipids and glucose in serum and liver

Serum triacylglycerol, cholesterol, phospholipid, free fatty acid and glucose concentrations were analyzed using commercial enzyme kits (Wako Pure Chemical Industries). Hepatic lipids were extracted and purified [15]. Amounts of cholesterol in the liver lipid extracts were analyzed enzymatically [16], and amounts of triacylglycerol [17] and phospholipid [18], colorimetrically.

2.4. Analyses of enzyme activities

Approximately 1.5 g of each liver was homogenized with 10 ml of 0.25 M sucrose containing 1 mM EDTA and 3 mM Tris–HCl (pH 7.2). An aliquot of the homogenate (3.5 ml) was centrifuged at 200 000*g* for 30 min. The activities of enzymes involved in fatty acid synthesis were measured spectrophotometrically using the supernatant as an enzyme source. Fatty acid synthase activity was measured as malonyl-coenzyme A (CoA)-dependent oxidation of NADPH in the presence of acetyl-CoA [19]. Adenosine triphosphate (ATP)-citrate lyase activity represented the rate of CoA-dependent oxidation of NADH in the presence of citrate, ATP and malate dehydrogenase (Oriental Yeast) [20]. The rate of glucose 6-phosphate-dependent reduction of NADP in the presence of excess amounts of 6-phosphogluconate dehydrogenase (Oriental Yeast) represented glucose 6-phosphate dehydrogenase activity [21]. 6-Phosphogluconate dehydrogenase activity was measured as 6-phosphogluconate-dependent reduction of NADP [22]. The rate of reduction of NADP following the addition of malic acid was analyzed to measure malic enzyme activity [23]. The activities of enzymes involved in fatty acid oxidation were analyzed using the whole liver homogenate as an enzyme source. Acyl-CoA oxidase activity as the rate of palmitoyl-CoA-dependent formation of hydrogen peroxide was measured according to the methods of Osumi and Hashimoto [24]. The carnitine-dependent release of CoA from palmitoyl-CoA was analyzed to measure the activity of carnitine palmitoyltransferase [25]. The rate of crotonyl-CoA hydration following the addition of the enzyme source as measured by the decrease in optical density at 280 nm represented enoyl-CoA hydratase activity [26]. The rate of acetoacetyl-CoA-dependent oxidation of NADH was taken as the activity of 3-hydroxyacyl-CoA dehydrogenase [27]. 3-Keotacyl-CoA thiolase activity represented the rate of CoA-dependent cleavage of acetoacetyl-CoA [28].

2.5. Analysis of RNA

Total RNA was extracted from liver and adipose tissues, and mRNA levels were analyzed by real-time polymerase chain reaction (PCR) using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as detailed elsewhere [29]. Total RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems) to generate cDNA. The real-time PCR was conducted in a mixture (final volume, 20 μl) containing 20 ng of cDNA, 900 nmol/L each of the forward and reverse primers, 250 nmol/L of the probe labeled with FAM and TAMRA and 10 μl of 2×TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR profile was 50°C for 2 min and 95°C for 10 min and a subsequent 45 cycles of 95°C for 15 s and 60°C for 1 min. mRNA abundance was calculated as a ratio to the value for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each cDNA sample and expressed as a percentage assigning the value in rats fed a casein diet as 100. The nucleotide sequences of primers and probes used to detect each mRNA were designed using Primer Express Software (Applied Biosystems) according to sequences available in the GenBank database. The nucleotide sequences of primers and probes used to detect mRNAs for fatty acid synthase, acetyl-CoA carboxylase, ATP-citrate lyase, glucose 6-phosphate dehydrogenase, mitochondrial glycerol 3-phosphate acyltransferase, SREBP-1c, carnitine palmitoyltransferase II, trifunctional enzyme subunit α and β, acyl-CoA oxidase and bifunctional enzyme were the same as reported elsewhere [30]. Those for GAPDH, malic enzyme, Δ^6-desaturase, PPARα, PPARγ2 and UCP1, UCP2 and UCP3 are listed in Table 2.

2.6. Analysis of isoflavones in serum

Serum (1 ml) was added to 100 μl of 0.58 M acetic acid and 50 μl of a β-glucuronidase preparation (Type HP2, Sigma, St. Louis, MO, USA) and incubated at 37°C for 4 h. After the enzymatic hydrolysis, each sample was spiked with 6 μg of fluorescein dissolved in 50 μl of methanol as an internal standard and diluted with 4 ml of 70 mM sodium dihydrogenphosphate. Isoflavones in the samples were loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), and the cartridge was washed successively with 10 ml of 70 mM sodium dihydrogenphosphate and 2 ml of water. The isoflavones were then eluted with 6 ml of methanol and evaporated dry under nitrogen gas. The extract was dissolved in 1 ml of methanol and analyzed by high-performance liquid chromatography using a reversed-phase Capcell Pak AG120 C_{18} column (Shiseido, Tokyo, Japan) at a flow rate of 1.5 ml/min. The injection volume was 10 μl. The mobile phase consisted of 0.5% phospholic acid in water and acetonitrile (81:19, v/v). Isoflavones were detected with a Coulochem III electrochemical detector (ESA, Bedford, MA, USA) equipped with a 5021 guard cell (ESA) and a 5011 high-sensitivity analytical cell (ESA). The voltages for guard and analytical cells were set

Table 2
Nucleotide sequences of primers and probes for real-time PCR of mRNAs

Genes	Sense primer	Antisense primer	Probe	Length of PCR products (bp)	GenBank accession no.
Δ^6-desaturase	5′-ACCGCTGCTCATCCCTATGT-3′	5′-CACCCAGTCTCTGCGTCTGA-3′	5′-TTCCAGTACCAGATCATCATGACCAT-3′	70	NM_031344
GAPDH	5′-GCCGAGGGCCCACTAAAG-3′	5′-TGCTGTTGAAGTCACAGGAGACA-3′	5′-CATCCTGGGCTACACTGAGGACCA-3′	70	BC059110
Malic enzyme	5′-GGAAGAGAGGCAACAGCTGAA-3′	5′-GGACCTGGATCTCCTGGTTGA-3′	5′-TCATGGCTTGTTGCCACCCTGC-3′	71	NM_012600
PPARα	5′-AACGGCGTTGAAAACAAGGA-3′	5′-GACGGTCTCCACGGACACATG-3′	5′-AGGTCCGATTCTTCCACTGCTGCCA-3′	71	NM_013196
PPARγ2	5′-ACTCTGGGAGATCCTCCTGTTG-3′	5′-GAAGTGCTCATAGGCAGTGCAT-3′	5′-CCCAGAGCATGGTGCCTTCGCT-3′	68	Y12882
UCP1	5′-AGGGTTTGCGCCTTCTTTTC-3′	5′-CAGCTGTTCAAAGCACACAAACA-3′	5′-AGGGTTTGCGCCTTCTTTTC-3′	70	NM_012682
UCP2	5′-CCACAGCCACCGTGAAGTT-3′	5′-CGGACTTTGGCGGTGTCTA-3′	5′-CAGCAGCCTGTATTGCAGATCTCA-3′	88	NM_019354
UCP3	5′-TGAGTTTTGCCTCCATTCGAA-3′	5′-TGGAGTGGTCCGTTCCTTTG-3′	5′-TCTACGACTCTGTCAAGCAGTTCTACAC-3′	78	NM_013167

at 300 and 700 mV, respectively. Daidzein, glycitein and genistein purchased from Wako Pure Chemical Industries and equol from LC Laboratories (Woburn, MA, USA) were used as references to identify and quantify isoflavones.

2.7. Statistical analysis

The data were analyzed with a 1-way ANOVA, followed by a Tukey–Kramer post hoc analysis to detect significant differences of means at a level of $P<.05$.

3. Results

3.1. Animal growth and tissue weights

Body weight at the time of sacrifice and the growth of animals were significantly lower in rats fed soy protein diets containing varying amounts of isoflavone than in those fed a casein diet (Table 3). Food intake was also significantly lower in rats given soy protein than in the animals fed casein except in one instance (rats fed a casein diet versus the animals fed a soy protein diet without isoflavone supplementation). Supplementation with 4 g/kg of isoflavone slightly but significantly retarded growth and lowered body weight at the end of the experiment among rats given soy protein despite no significant differences in food intake among rats fed soy protein diets containing different amounts of isoflavone. Epididymal and perirenal white adipose tissue weights were significantly lower in rats given soy protein diets containing varying amounts of isoflavone than in the animals fed a casein diet except in one instance (perirenal depot in rats given a soy protein diet not supplemented with isoflavone preparation). Although the differences were not significant, supplementation of the soy protein diet with isoflavone dose-dependently decreased the weights of white adipose tissues. Interscapular brown adipose tissue weight was significantly higher in rats given a casein diet than in those fed a soy protein diet supplemented with 4 g/kg of isoflavone. No other differences were seen in this parameter among the groups. Type of dietary protein and amount of isoflavone did not affect the weight of the liver.

Table 4
Effects of dietary proteins and isoflavone on serum and liver lipid and serum glucose concentrations in rats

Isoflavone added (g/kg)	Dietary protein source			
	Casein	Soy protein		
	0	0	0.5	4
Serum components (mmol/L)				
Triacylglycerol	3.73±0.37^{c}	3.60±0.42^{bc}	2.71±0.20^{ab}	2.33±0.27^{a}
Cholesterol	2.02±0.22^{b}	1.28±0.09^{a}	1.29±0.06^{a}	1.26±0.08^{a}
Phospholipid	3.03±0.17^{b}	2.48±0.10^{a}	2.41±0.08^{a}	2.19±0.18^{a}
Free fatty acid	1.10±0.83	0.952±0.120	0.960±0.123	0.878±0.140
Glucose	10.1±0.3^{b}	8.83±0.20^{a}	8.98±0.14^{a}	8.74±0.31^{a}
Liver lipids (μmol/g)				
Triacylglycerol	55.7±9.6	66.3±12.0	55.8±8.2	57.3±11.3
Cholesterol	2.82±0.19	3.26±0.27	3.09±0.19	3.29±0.16
Phospholipids	38.8±0.7	37.5±1.1	39.3±0.6	38.9±1.2

Values represent the mean± S.E. for seven or eight rats.
Mean values within a row with different superscripts are significantly different ($P<.05$).

3.2. Serum and liver lipid levels

Serum triacylglycerol concentrations were comparable between rats given casein and those given methanol-washed soy protein low in isoflavone (Table 4). The supplementation of the soy protein diet with isoflavone dose-dependently decreased this parameter. Various soy protein diets compared to a casein diet significantly lowered serum cholesterol and phospholipid concentrations. However, isoflavone was totally ineffective in modulating these parameters among the groups of rats given soy protein. There were no significant differences in free fatty acid concentrations among the groups. Serum glucose concentrations were lower in groups of rats fed soy protein than in the animals fed casein. However, dietary isoflavone did not affect this value. Hepatic triacylglycerol, cholesterol and phospholipid concentrations were comparable among the groups (Table 4).

Table 3
Effects of dietary proteins and isoflavone on growth variables and tissue weights in rats

Isoflavone added (g/kg)	Dietary protein source			
	Casein	Soy protein		
	0	0	0.5	4
Body weight (g)	268±3^{c}	254±2^{b}	254±3^{b}	241±4^{a}
Body weight gain (g/14 d)	133±2^{c}	121±2^{b}	119±3^{b}	106±2^{a}
Food intake (g/d)	21.9±0.5^{b}	20.4±0.5^{ab}	20.1±0.2^{a}	18.9±0.3^{a}
Tissue weight (g/100 g body weight)				
Epididymal white adipose tissue	1.20±0.09^{b}	0.916±0.085^{a}	0.878±0.031^{a}	0.884±0.057^{a}
Perirenal white adipose tissue	1.29±0.13^{b}	1.01±0.12^{ab}	0.937±0.104^{a}	0.800±0.064^{a}
Interscapular brown adipose tissue	0.208±0.015^{b}	0.197±0.007^{ab}	0.188±0.010^{ab}	0.169±0.011^{a}
Liver	5.41±0.17	5.48±0.16	5.26±0.12	5.45±0.23

Values represent the mean±S.E. for seven or eight rats.
Mean values within a row with different superscripts are significantly different ($P<.05$).

3.3. Activities and mRNA levels of enzymes involved in lipogenesis and fatty acid desaturation

Various soy protein diets containing different amounts of isoflavone compared to a casein diet significantly lowered the activities of various enzymes involved in the regulation of fatty acid synthesis (fatty acid synthase, ATP-citrate lyase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme) (Table 5). Among rats fed various soy protein diets, the values were generally lower in those given the diets supplemented with 0.5 and 4 g/kg of isoflavone than in the animals given the diet not supplemented with isoflavone. However, a significant isoflavone-dependent change was observed only for ATP-citrate lyase, not for the other enzymes. The activity of ATP-citrate lyase was significantly lower in rats fed a soy protein diet supplemented with 4 g/kg of isoflavone than in those fed the diet without isoflavone.

mRNA levels of acetyl-CoA carboxylase, fatty acid synthase, glucose 6-phosphate dehydrogenase and malic enzyme were significantly lower in rats given various diets containing soy protein differing in isoflavone contents than in the animals fed a casein diet except in one instance (the acetyl-CoA carboxylase mRNA level in animals fed a soy protein diet supplemented with 4 g/kg of isoflavone) (Table 5). However, the extent of the reduction was considerably attenuated compared to the reduction in the enzyme's activity. In spite that various soy protein diets lowered the activity of ATP-citrate lyase to a level less than one half that in the animals given a casein diet, no significant protein-dependent changes were observed in the mRNA level of this enzyme. mRNA levels of an enzyme involved in the pathway of glycerolipid biosynthesis (mitochondrial glycerol-3-phosphate acyltransferase) were also lower in rats fed various soy protein diets containing different amounts of isoflavone than in the animals fed a casein diet (Table 5). Dietary isoflavone did not affect mRNA levels of these lipogenic enzymes among rats fed soy protein diets containing varying amounts of isoflavone. Soy protein compared to casein also lowered the mRNA expression of an enzyme involved in the desaturation of polyunsaturated fatty acid (Δ^6-desaturase). Again, dietary isoflavone did not affect this value among the groups of rats fed soy protein.

SREBP-1c is a transcription factor regulating the gene expression of enzymes involved in lipogenesis and fatty acid desaturation [31]. In spite that soy protein compared to casein lowered the activity and mRNA levels of many lipogenic enzymes, and mRNA level of Δ^6-desaturase, this protein source did not affect the mRNA expression of SREBP-1c (Table 5). Isoflavone at various dietary levels did not affect this parameter among the groups of rats fed soy protein.

3.4. Activities and mRNA levels of enzymes involved in fatty acid oxidation

The activities of enzymes involved in fatty acid oxidation were indistinguishable between rats fed a casein diet and those fed a methanol-washed soy protein diet not supplemented with isoflavone (Table 6). Isoflavone added to the soy protein diet at a level of 0.5 and 4 g/kg caused a significant 25% and 39% increase in the activity of acyl-CoA oxidase, respectively. The value was significantly higher in rats given a soy protein diet supplemented with 4 g/kg of isoflavone than in the animals fed a casein diet. Although the

Table 5
Effects of dietary proteins and isoflavone on the activities and mRNA levels of enzymes involved in lipogenesis and mRNA levels of Δ^6-desaturase and SREBP-1c in rat liver

Isoflavone added (g/kg)	Dietary protein source			
	Casein	Soy protein		
	0	0	0.5	4
Enzyme activity (nmol/min per mg protein)				
Fatty acid synthase	42.1±5.4^{b}	23.1±3.0^{a}	13.9±2.6^{a}	13.6±2.9^{a}
ATP-citrate lyase	95.1±6.5^{c}	47.6±5.4^{b}	42.5±2.4^{ab}	32.2±2.6^{a}
Glucose 6-phosphate dehydrogenase	158±20^{b}	46.5±5.1^{a}	42.5±2.0^{a}	39.2±3.3^{a}
6-Phosphogluconate dehydrogenase	97.7±6.5^{b}	66.4±2.7^{a}	60.5±4.1^{a}	60.5±2.3^{a}
Malic enzyme	112±13^{b}	39.6±5.6^{a}	38.2±2.1^{a}	33.7±1.6^{a}
mRNA level (%)				
Acetyl-CoA carboxylase	100±4^{b}	82.8±3.5^{a}	83.1±4.1^{a}	88.9±7.3^{ab}
Fatty acid synthase	100±7^{b}	76.7±2.8^{a}	69.6±7.9^{a}	68.9±10.8^{a}
ATP citrate lyase	100±5	81.6±6.1	84.9±7.4	89.4±12.9
Glucose 6-phosphate dehydrogenase	100±10^{b}	60.2±4.9^{a}	65.8±4.4^{a}	59.7±7.5^{a}
Malic enzyme	100±6^{b}	45.0±6.1^{a}	53.6±4.0^{a}	50.3±3.2^{a}
Mitochondrial glycerol 3-phosphate acyltransferase	100±7^{b}	59.0±3.7^{a}	63.6±2.1^{a}	66.8±7.5^{a}
Δ^6-Desaturase	100±6^{b}	65.4±5.9^{a}	74.2±2.0^{a}	65.4±3.2^{a}
SREBP-1c	100±3	94.5±7.6	110±14	97.4±12.7

Values represent the mean± S.E. for seven or eight rats.
Mean values within a row with different superscripts are significantly different, $P<05$.

Table 6
Effects of dietary proteins and isoflavone on the activities and mRNA levels of enzymes involved in fatty acid oxidation and PPARα in rat liver

Isoflavone added (g/kg)	Dietary protein source			
	Casein	Soy protein		
	0	0	0.5	4
Enzyme activity (nmol/min per mg protein)				
Acyl-CoA oxidase	1.01±0.06^{ab}	0.95±0.07^{a}	1.19±0.06b^{bc}	1.32±0.10^{c}
Carnitine palmitoyltransferase	3.39±0.18	3.33±0.12	3.35±0.11	3.70±0.26
Enoyl-CoA hydratase	5294±234^{a}	6114±234^{ab}	6510±348^{b}	6707±377^{b}
3-Hydroxyacyl-CoA dehydrogenase	198±7^{a}	232±12^{ab}	241±4^{b}	263±22^{b}
3-Ketoacyl-CoA thiolase	201±16	226±18	236±20	238±12
mRNA level (%)				
Acyl-CoA oxidase	100±3^{ab}	94.0±3.3^{a}	107±4.9^{bc}	115±4.3^{c}
Bifunctional enzyme	100±7^{a}	104±6^{a}	116±9^{ab}	127±6^{b}
Carnitine palmitoyltransferase II	100±2^{b}	96.1±5.2^{ab}	86.5±5.4^{a}	101±4^{b}
Trifunctional enzyme subunit α	100±5^{b}	87.9±3.1^{a}	101±4^{b}	108±3^{b}
Trifunctional enzyme subunit β	100±4^{b}	76.3±3.7^{a}	88.2±5.8^{ab}	93.2±5.4^{b}
PPARα	100±12^{a}	149±9^{b}	165±21^{bc}	200±20^{c}

Values represent the mean±S.E. for seven or eight rats.
Mean values within a row with different superscripts are significantly different ($P<.05$).

differences were not significant, supplementation of the soy protein diet with isoflavone also caused slight dose-dependent increases in the activities of various enzymes involved in fatty acid oxidation. As a result, the levels of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity were significantly higher in rats given soy protein diets supplemented with 0.5 and 4 g/kg of isoflavone than in the animals fed a casein diet. However, dietary isoflavone-dependent changes in the activities of carnitine palmitoyltransferase and 3-ketoacyl-CoA thiolase were not significant among the groups.

mRNA levels of enzymes involved in fatty acid oxidation in rats fed a soy protein diet not supplemented with isoflavone were comparable to (acyl-CoA oxidase, bifunctional enzyme, carnitine palmitoyltransferase II) or significantly lower (trifunctional enzyme subunit α and β) than the values in rats fed a casein diet (Table 6). Supplementation of a soy protein diet with isoflavone is generally associated with a dose-dependent increase in the mRNA expression of various enzymes involved in fatty acid oxidation. Accordingly, the values except for that of carnitine palmitoyltransferase II became significantly higher in rats fed a soy protein diet supplemented with 4 g/kg of isoflavone than in the animals fed the diet without isoflavone. mRNA levels of PPARα were significantly higher in rats fed various soy protein diets than in the animals fed a casein diet. Among rats fed soy protein, isoflavone dose-dependently increased this parameter.

3.5. mRNA levels of PPARγ2 and UCPs in adipose tissues

We analyzed mRNA levels of PPARγ2 and various isoforms of UCP in interscapular brown adipose tissue. PPARγ is involved in the differentiation of preadipocytes into mature adipocytes [32]. There are two isoforms for this transcription factor, termed PPARγ1 and PPARγ2. PPARγ2, not PPARγ1, is mainly involved in the differentiation [32]. UCPs are inner mitochondrial membrane proteins, which dissipate proton gradients and, hence, release stored energy as heat [33]. The mRNA abundance of UCP1 was significantly higher in rats fed a soy protein diet without isoflavone than in the animals fed a casein diet (Table 7). The values for UCP 2 and 3 and PPARγ2 were also higher in the former than in the latter; however, the differences were not significant. Supplementation of a soy protein diet with isoflavone is generally associated with an increase in mRNA levels of various UCPs and PPARγ2. The mRNA level of UCP2 was significantly higher in rats fed a soy protein diet supplemented with 4 g/kg of isoflavone than in the animals fed the diet without isoflavone. However, isoflavone-dependent changes were not statistically significant for the other parameters. We also analyzed mRNA levels of PPARγ2 and UCP2 and 3 in epididymal white adipose

Table 7
Effects of dietary proteins and isoflavone on mRNA levels of UCP and PPARγ2 in rat adipose tissues

Isoflavone added (g/kg)	Dietary protein source			
	Casein	Soy protein		
	0	0	0.5	4
mRNA level (%)				
Interscapular brown adipose tissue				
UCP1	100±6^{a}	134±5^{b}	163±18^{b}	155±11^{b}
UCP2	100±14^{a}	128±12^{a}	156±19^{ab}	193±30^{b}
UCP3	100±7^{a}	132±13^{ab}	158±13^{b}	159±19^{b}
PPARγ2	100±17^{a}	149±24^{ab}	170±23^{b}	165±20^{b}
Epididymal white adipose tissue				
UCP2	100±15	110±12	106±14	96.0±13
UCP3	100±14	84.4±3.8	87.8±10.1	86.5±11.9
PPARγ2	100±24	118±13	100±11	73.7±9.4

Values represent the mean±S.E. for seven or eight rats.
Mean values within a row with different superscripts are significantly different ($P<.05$).

Table 8
Effects of dietary proteins and isoflavone on serum isoflavone concentrations in rats

Serum isoflavones (μmol/L)	Dietary protein source			
	Casein	Soy protein		
Isoflavone added (g/kg)	0	0	0.5	4
Daidzein	0.0±0.0[a]	0.307±0.017[a]	1.99±0.22[b]	8.53±0.75[c]
Glycitein	0.0±0.0[a]	0.0548±0.0150[a]	0.604±0.063[b]	1.44±0.14[c]
Genistein	0.0±0.0[a]	0.315±0.014[b]	0.573±0.059[c]	1.01±0.12[d]
Equol	0.0±0.0[a]	0.374±0.072[ab]	4.25±0.68[b]	8.43±2.83[c]

Values represent the mean±S.E. for seven or eight rats.
Mean values within a row with different superscripts are significantly different ($P<05$).

tissue. However, significant differences were not observed among the groups (Table 7).

3.6. Serum isoflavone concentrations

To confirm the bioavailability of isoflavone, we analyzed concentrations of isoflavones in serum (Table 8). Isoflavones were detected in the serum of animals fed the diets containing soy protein differing in isoflavone content but not in the animals fed casein. Considerable amounts were detected even in rats fed a methanol-washed soy protein diet not supplemented with isoflavone. Although serum concentrations of isoflavone increased as the dietary levels of the flavonoid increased, the extent of the increase did not necessarily parallel that of the dietary level. For instance, the levels of daidzein, glycitein and genistein aglycons were 7.8, 8.0 and 6.1 times higher on a molar basis in the soy protein diet supplemented with 4 g/kg of isoflavone than 0.5 g/kg of isoflavone, respectively (Table 1). However, serum concentrations of these isoflavones were merely 4.3, 2.4 and 1.8 times higher in rats fed the former than in those fed the latter, respectively. These changes were exaggerated when the serum levels of isoflavones in rats fed soy protein diets supplemented with 0.5 and 4 g/kg of isoflavone were compared with those in the animals fed the diet not supplemented with isoflavone. In addition, large amounts of equol, a metabolite of daidzein produced by intestinal bacteria, were detected among rats fed soy protein diets. The concentrations of equol were comparable to or even greater than those of daidzein among rats fed soy protein diets. Supplementation of the diet with isoflavone dose-dependently increased this parameter, but the extent of the increase was again attenuated compared to the changes in the dietary levels.

4. Discussion

Studies [1,4,5] have demonstrated that soy protein rich in isoflavones compared to casein reduces hepatic lipogenesis. There is a possibility that the isoflavone rather than protein component of a soy protein preparation is responsible for this. However, this has not been examined previously. We observed that a methanol-washed soy protein low in isoflavone, compared to casein, reduced the activity and mRNA level of various lipogenic enzymes. However, supplementation with isoflavone of diets containing this protein preparation at a level approximating the amount that would be supplied by the original unwashed protein (0.5 g/kg) or even at a much higher level (4 g/kg) was rather ineffective in modulating the indices of hepatic lipogenesis. Therefore, it is unequivocal that isoflavone has little of a role in the physiological activity of soy protein preparations containing this flavonoid in reducing hepatic lipogenesis. As soy protein-dependent decreases in the activity of lipogenic enzymes were stronger than those observed for mRNA levels, it is possible that this protein affects not only the rate of transcription of lipogenic enzyme genes or the mRNA stability but also the translation or turnover of the enzyme molecules.

SREBP-1 is involved in the regulation of lipogenic enzymes [31]. Δ^6-desaturase, involved in the metabolism of polyunsaturated fatty acids, is also reported to be under the control of this transcription factor [31]. The observation in the present study that soy protein compared to casein decreased mRNA levels not only of lipogenic enzymes but also of Δ^6-desaturase supports the notion that soy protein decreases hepatic lipogenesis through a SREBP-1-dependent mechanism. In relation to this, several studies reported that soy protein compared to casein decreased mRNA levels of SREBP-1 [4,5]. There are two isoforms for this transcription factor, termed SREBP-1a and SREBP-1c, and the latter, compared to the former, predominates in tissues of rodents and humans [31] and is mainly involved in the regulation of lipogenesis. Previous studies [4,5] have not distinguished between the mRNAs of the two isoforms. In the present study, using the real-time PCR technique with specific primers and probes for SREBP-1c mRNA, we observed that soy protein did not affect the mRNA level of this transcription factor. However, this does not exclude the possibility that SREBP-1c is involved in soy protein-dependent changes in hepatic lipogenesis. In this context, measurements of the protein level of SREBP-1 by Western blotting using antibody to react with both SREBP-1a and SREBP-1c isoforms indicated that the mRNA level does not necessarily parallel the protein level of the mature form of SREBP-1 located in the nucleus [34,35]. Therefore, it is necessary to determine the protein level of the mature form of SREBP-1c to clarify the role of this transcription factor in soy protein-dependent changes in hepatic lipogenesis. Also, there is the possibility that transcription factors other than SREBP-1c are involved in soy protein-dependent changes in hepatic lipogenesis [36].

PPARα is a transcription factor involved in the regulation of the gene expression of enzymes contributing to hepatic fatty acid oxidation [32]. Upon the binding of ligands, PPARα heterodimerizes with the 9-*cis*-retinoic acid receptor, and binds to specific *cis*-acting DNA response elements termed *peroxisome proliferator response elements* and

activates the gene. It is also possible that an increase in the expression of PPARα is associated with the up-regulation of the expression of enzymes involved in hepatic fatty acid oxidation [32]. Isoflavones not only act as activators of PPARα but also increase its gene expression [7,8,37]. In relation to the physiological activity of soy protein affecting hepatic fatty acid oxidation, Akahoshi et al. [38] reported that soy protein compared to casein increased carnitine palmitoyltransferase activity in rat liver. Also, Tovar et al. [5] observed that soy protein increases the mRNA expression of PPARα in rats. As they employed a soy protein preparation containing isoflavones, it is possible that the isoflavone rather than protein component of the preparation is responsible for their findings. In the present study, we observed that methanol-washed soy protein low in isoflavone, compared to casein, increased the mRNA expression of PPARα without enhancing the activity and mRNA levels of various enzymes involved in hepatic fatty acid oxidation. This observation implies that the protein component of the soy protein preparation per se has the physiological activity to increase PPARα mRNA expression but may have little of a role in the regulation of hepatic fatty acid oxidation.

The results of the present study indicated that isoflavone has the activity to increase hepatic fatty acid oxidation despite that the effect was much smaller than that expected from its ability to activate PPARα [7,37]. Our study indicated that the protein component of a soy protein preparation is not effective in altering hepatic fatty acid oxidation. Therefore, the increase in the activity of enzymes involved in hepatic fatty acid oxidation in rats fed a soy protein preparation containing isoflavones observed previously [37] may represent the physiological activity of the isoflavone not protein component. Although the isoflavone-supplemented soy protein diet increased the mRNA expression of PPARα dose-dependently, a clear-cut relationship was not observed between the mRNA level of this transcription factor and activity and mRNA levels of enzymes involved in fatty acid oxidation among rats fed casein and soy protein. Therefore, it is not clear whether the alterations in the mRNA level of PPARα are involved in the isoflavone-dependent changes in the activity and mRNA levels of enzymes involved in hepatic fatty acid oxidation observed here.

Studies have indicated that soy protein rich in isoflavones reduces serum cholesterol levels in rats [1]. This was confirmed even in rats fed methanol-washed soy protein low in isoflavone, and supplementing the diet with isoflavone was not effective in altering this parameter. Therefore, it is suggested that the protein component, not isoflavone, is involved in the serum cholesterol-lowering effect of soy protein rich in isoflavone. Several studies also demonstrated that soy protein rich in isoflavones reduces serum triacylglycerol concentrations [1]. However, in the present study, methanol-washed soy protein low in isoflavone compared to casein failed to lower this parameter. In contrast, the supplementation of soy protein diets with isoflavone dose-dependently lowered this value. Therefore, it is reasonable to suggest that the serum triacylglycerol-lowering effect of the soy protein preparation containing isoflavones observed previously [1] is mainly ascribable to the isoflavone, not protein, component of the preparation. An isoflavone-dependent increase in hepatic fatty acid oxidation may play a role in this phenomenon. It is reasonable to consider that the alterations in hepatic fatty acid synthesis and oxidation modulate not only the serum but also the liver lipid level. However, liver lipid concentrations were comparable among the groups despite the considerable differences in the activity and mRNA levels of the enzymes involved in these metabolic pathways. Available information indicated that the alterations in hepatic lipid levels by dietary factors are not necessarily accounted solely by the changes in hepatic fatty acid oxidation and/or synthesis [39,40]. It should be stated that metabolic pathways other than fatty acid oxidation and synthesis may also be involved in regulating hepatic lipid levels. It has been indicated that soy protein increases LDL receptor activity [1,2] but decreases secretion of triacylglycerol-rich lipoprotein [3]. Also, soy isoflavone increases the activity and mRNA levels of the LDL receptor but decreases secretion of apolipoprotein B and the activity and mRNA levels of microsomal triacylglycerol transfer protein in HepG2 cells [41]. These changes may be associated with the reduction in serum lipid levels but may cause the increase in hepatic lipid levels. Also, other metabolic changes such as alterations in the mobilization of fatty acid from adipose tissue [42] and its uptake by liver [43] are factors modifying liver lipid levels despite that information on the effect of soy protein and/or isoflavone on these metabolic pathways is not available. Therefore, clarification of the soy protein- and isoflavone-dependent changes in metabolic pathways other than hepatic fatty acid oxidation and synthesis is necessary to account for the present observations regarding hepatic lipid levels.

It has been reported that soy protein rich in isoflavones reduces fat pad mass in rats [38,44]. In contrast, several studies indicated that soy protein low in isoflavones actually increases adiposity or adipose tissue weight in Zucker rats [10,45]. However, this consequence has not been confirmed in Sprague–Dawley rats [11]. We observed, in the present study using Sprague–Dawley rats, that epididymal and perirenal white adipose tissue weights were lower in rats given various soy protein diets than in the animals fed casein. In addition, adding isoflavone at a level of 0.5 or 4 g/kg to soy protein diets low in this flavonoid exaggerated this difference despite that the decreases were not significant among rats fed various soy protein diets. Although the methanol-washed soy protein employed in the present study still contained a small amount of isoflavone, it is plausible that both the protein and isoflavone components are responsible for the antiobesitic effect of soy protein rich in isoflavones observed previously [38,44]. Moreover, we found that reductions of fat pad mass caused by soy protein and isoflavone are associated with an increase in mRNA

levels of various UCPs in brown adipose tissue. Therefore, it is possible that an increase in energy expenditure through the up-regulation of UCPs is involved in the antiobesitic effect of soy protein and isoflavone. It has been reported that PPARγ stimulated the differentiation of brown adipocytes [46]. In addition, a PPARγ-specific agonist induced the mRNA expression of UCP1, 2 and 3 in brown adipose tissue of rats [47]. In relation to these observations, we observed that changes in mRNA levels of PPARγ2 paralleled those of various UCPs.

Inasmuch as the isoflavone preparation employed in the present study contained considerable amounts of saponins and unidentified compounds, it is possible that compounds other than isoflavones are responsible for the physiological activity of the preparation. The information obtained indicated that soy saponins influence lipid metabolism in rats despite that their effects on hepatic fatty acid metabolism and adipose tissue gene expression have not been elucidated. Potter et al. [48] demonstrated that soy saponins lowered blood levels of cholesterol but not triacylglycerol in rats presumably through an increase in fecal bile acid excretion. The current observation that the preparation rich in isoflavones lowered the serum level of triacylglycerol but not cholesterol does not support the notion that saponins, not isoflavones, are primarily responsible for the physiological activity of the isoflavone preparation employed in the present study. Also, it should be stated that the dietary levels of saponins (0.100–0.774 g/kg) in the current study expected given their concentration in the isoflavone preparation were much lower than the level (10.0 g/kg) employed by Potter et al. However, there is still the possibility that some unidentified compound(s) or a synergistic effect by the various bioactive compounds in the isoflavone preparation is responsible for the changes in indices of lipid metabolism observed here.

The inclusion in experimental diets of soy protein and isoflavone was generally associated with slight reductions in the intake of food by rats in the present study. Therefore, there is a possibility that the alterations in various metabolic parameters observed in the present study represent a consequence of changes in food intake, not the physiological activity, of soy protein or isoflavones. It is well documented that food deprivation causes strong reductions in hepatic activity and mRNA level of hepatic lipogenic enzymes [49,50], indicating that energy intake is crucial in regulating this metabolic pathway. However, no apparent relationship between food intake and the parameters of lipogenesis existed in the present study. Food deprivation increases hepatic fatty acid oxidation [50,51] primarily through a reduction in the malonyl-CoA concentration, not an increase in the activity of enzymes involved in the oxidation [52]. With regard to the effect of food consumption on the gene expression of UCPs in brown adipose tissue, studies have indicated that 2 days of fasting [53] or food restriction [54] was not associated with an up-regulation of the expression of various UCPs in this tissue. Therefore, it is difficult to ascribe the changes in various metabolic parameters observed in rats fed soy protein and isoflavone to alterations in food intake.

Serum concentrations of isoflavone may represent the bioavailability of the compound and, hence, are crucial when considering the physiological activity of dietary isoflavone. It has been reported that blood genistein and daidzein concentrations increased linearly as dietary levels of these compounds increased when they were administered in a free form in rats [55]. In the present study, where isoflavones (daidzein, glycitein and genistein) were added to the diet predominantly in the form of glycosides, the increases in dietary levels of these isoflavones were not associated with a parallel increase in the concentrations of these compounds in serum. It has been reported that isoflavone is less bioavailable in the form of a glycoside than in the form of an aglycon [56]. Therefore, it is suggested that large amounts of isoflavones in the form of glycosides cannot be handled by intestinal digestive enzymes and, hence, do not cause an increase in the serum concentration paralleling dietary levels.

As daidzein is the most abundant isoflavone in the preparation employed to supplement the experimental diets, serum concentrations of this compound much exceed those of glycitein and genistein in rats fed soy protein diets supplemented with 0.5 and 4 g/kg of isoflavone. However, the proportions in serum of daidzein among these three compounds (63% and 78% for animals fed soy protein diets supplemented with 0.5 and 4 g/kg of isoflavone, respectively) were considerably higher than those in the diets (59% and 61% on a molar basis, respectively). In addition, the corresponding value for daidzein was 45% in animals fed a soy protein diet not supplemented with isoflavone despite that daidzein comprises merely 25% of this experimental diet. In this context, King [57] reported that daidzein conjugates compared to genistein conjugates are more bioavailable in rats, probably because of the differences in the rate of absorption between these compounds. In addition, considerable amounts of equol, a metabolite of daidzein produced by intestinal bacteria, were detected in serum. As a result, equol and daidzein were the major isoflavones in serum. Notably, equol is reported to have stronger estrogenic and antioxidative activities than daidzein [58]. Therefore, it is possible that the physiological changes caused by dietary isoflavones, in the present study, are mainly ascribable to equol and daidzein. Various compounds other than equol are also produced by intestinal bacteria [59]. However, these compounds are barely detected in serum of rats fed a diet containing soy protein rich in isoflavones [59] and are weaker than genistein, daidzein and equol in terms of binding affinity for estrogen receptors [58]. Therefore, the significance of the isoflavone metabolites other than equol in regulating lipid metabolism is not apparent at present.

In conclusion, our study indicated that the protein, not isoflavone, component is responsible for the physiological activity of soy protein rich in isoflavones in reducing hepatic lipogenesis as observed previously [1]. Also, isoflavones,

not soy protein per se, may have weak but significant activity to stimulate hepatic fatty acid oxidation. In addition, our observations suggested that an up-regulation of the expression of UCPs in brown adipose tissue through a PPARγ-dependent mechanism is responsible for the antiobese effect of soy protein-rich isoflavones observed previously [38,44]. Both the protein and isoflavone components may participate in this.

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