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Tofu (Soybean Curd) Lowers Serum Lipid Levels and Modulates Hepatic Gene Expression Involved in Lipogenesis Primarily through Its Protein, Not Isoflavone, Component in Rats

Yoko Takahashi*,† and Tomokazu Konishi‡

†National Food Research Institute, National Agriculture and Food Research Organization (NARO), 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

‡Department of Bioresource Sciences, Akita Prefectural University, Shimosinjyo Nakano, Akita 010-0195, Japan

S *Supporting Information*

ABSTRACT: Although soy foods are recommended to prevent hyperlipidemia and related diseases, it is unclear how their active ingredients exert their effects. Here, the effects of tofu (soybean curd) and its putative active components, protein and isoflavone, on lipid metabolism in male rats were compared. Tofu and soy protein significantly lowered serum triacylglycerol and cholesterol levels compared to casein and, through microarray analyses, were found to exclusively alter transcriptomes involved in fatty acid and/or steroid synthesis in the liver, where most of the serum lipids are synthesized. In contrast, isoflavone supplementation had little effect on serum lipid levels or gene expression and exerted no synergistic effects with soy protein or tofu. The importance of the proteinaceous components was further confirmed by the lower activity of enzymes involved in lipogenesis. From these findings it was concluded that the protein, not isoflavone, fraction of soy reduces lipogenesis in liver through gene expression and that this may result in lower serum lipid levels.

KEYWORDS: tofu (soybean curd), soy protein, isoflavone, lipid metabolism, liver, microarray, principal component analysis

INTRODUCTION

Hyperlipidemia is the condition of abnormally elevated levels of any lipids in the blood, and it is well established as one of the major causes of the development of atherosclerotic cardiovascular disease.[1] Many of the dietary strategies incorporating functional foods such as fruits, vegetables, and whole grains are proposed to optimize serum lipids in patients.[2] Among these foods, soybeans and soy foods have generated much interest as a result of evidence that populations consuming large amounts of soybean have a lower risk of heart disease.[3] Indeed, epidemiological studies demonstrated that an increase in the intake of soy products significantly correlated with a reduction in serum total cholesterol concentrations in Asian populations.[4,5] Intervention studies have also suggested that a soy-rich diet improves blood lipid profiles and reduces the risk of cardiovascular disease.[6,7]

Studies to identify the active components of soy and elucidate the mechanisms that ameliorate hyperlipidemia have been performed for decades.[8−10] Soy contains components such as soluble fiber, phytosterol, and polyunsaturated fatty acids that potentially influence lipid metabolism.[3,11] Two of these components, soy protein and isoflavones, have been examined extensively for their ability to lower serum lipid levels in animals and humans.[8−10,12] We have previously demonstrated that dietary soy protein was primarily responsible for the physiological activity in reducing hepatic lipogenesis, whereas high concentrations of isoflavones in diet may have a role in regulating hepatic fatty acid oxidation in rats.[13] The regulation of lipid metabolism has been observed at the level of gene expression of some transcription factors such as sterol regulatory element-binding proteins (SREBPs), liver X receptor (LXR), and peroxisome-proliferator activated receptors (PPARs), thereby changing the downstream gene expression involved in lipogenesis and lipolysis.[10]

Although extensive investigations of individual soy components suggested several putative mechanisms for each, an overall picture of how soy foods improve the lipid profile has not been provided. Because soy foods consist of various ingredients other than proteinaceous components and isoflavones, their functionality would differ from the ingestion of the purified protein and/or isoflavones. The various ingredients of soy foods may mutually interact to modulate the physiological functions involved in lipid metabolism in animals. Although combinations of dietary chemopreventive agents/whole foods can sometimes result in significant activities at concentrations where any single agent is inactive,[14] such synergistic effects of soy compounds have not been comprehensively studied. Of course, the combined impact of several food components cannot be predicted simply from the known functions of the individual components.

In the present study, the hypolipidemic effects of soy foods were examined in rats by comparison with known active components, soy proteins and isoflavones. To represent the effects of soy foods, rats were fed freeze-dried tofu, a protein-rich soy food made by freezing and drying soybean curd, which contains polyunsaturated fats, dietary fiber, minerals, and isoflavones.[15] As expected, the freeze-dried tofu diets reduced body weight gain and altered serum lipid contents in the present study. To find out

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Table 1. Composition of Experimental Diets

ingredient (g/kg diet)	diet					
	C	CI	S	SI	T10	T20
casein	225	225	0	0	113	0
soy protein isolate	0	0	220	220	0	0
freeze-dried tofu	0	0	0	0	199	398
soybean oil	150	150	150	150	81.6	13.2
corn starch	150	150	150	150	150	150
cellulose	20	20	20	20	17.3	14.4
vitamin mixture[a]	10	10	10	10	10	10
mineral mixture[b]	35	35	35	35	35	35
L-cystine	3	3	3	3	3	3
choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
isoflavone mixture[c]	0	0.12	0	0.10	0	0
sucrose	404.5	404.4	409.5	409.4	388.6	373.9
total energy (kJ/kg diet)[d]	18120	18120	18171	18171	17693	17605

[a] AIN-93 formula (Oriental Yeast Co., Tokyo, Japan).[17] [b] AIN-93G formula (Oriental Yeast Co., Tokyo, Japan).[17] [c] The ratio of genistein to daidzein (3:1, w/w) in the isoflavone mixture was determined on the basis of the composition of these compounds in the freeze-dried tofu. [d] Calculated according to the standards announced by the Ministry of Health, Labour and Welfare of Japan.[15]

how the hypolipidemic effects occurred, we further investigated the global changes in hepatic gene expression by using a microarray, because the liver is a major organ that serves as the first relay station for the processing of dietary information and contains all of the biochemical machinery for both glucose and lipid storage and disposal.[16]

MATERIALS AND METHODS

Materials. Powdered freeze-dried tofu was purchased from MISUZU-CO Co., Ltd. (Nagano, Japan). It contained 2193 kJ/100 g and the following components (in g/100 g): moisture, 7.4; protein, 50.3; fat, 34.4; carbohydrate, 1.9; dietary fiber, 1.4; and ash, 4.6. The tofu also contained isoflavones (in mg aglycone equiv/100 g): genistein, 21.9; daidzein, 7.65; and glycitein, 0.519. Soy protein isolate (FUJIPRO) was donated by Fuji Oil Co. Ltd., Osaka, Japan. The soy protein was treated with hot aqueous methanol, and the isoflavone contents of the isoflavone-eliminated soy protein were determined as described previously [13] (in mg aglycone equiv/100 g): genistein, 6.87; daidzein, 2.19; and glycitein, 0.12. Casein and soybean oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). The crude protein contents of the soy protein and casein were 90.5 and 88.7 g/100 g, respectively. Genistein and daidzein were obtained from LC Laboratories, Woburn, MA.

Animals and Experimental Diets. Male Sprague–Dawley rats were obtained from Charles River Japan (Kanagawa, Japan) at 4 weeks of age. The animals were housed individually in a room with controlled temperature and humidity and a 12 h light–dark cycle. After adapting to the conditions for 5 days, rats were divided into six groups, each with similar average body weights (143–147 g, $n = 7-8$), and assigned experimental diets for 14 days as follows: casein diet (C); casein diet fortified with isoflavone (CI); aqueous methanol-treated soy protein diet (S); aqueous methanol-treated soy protein diet fortified with isoflavone (SI); a diet containing two proteins derived equally from casein and freeze-dried tofu (T10); and a diet containing protein derived from freeze-dried tofu (T20) (Table 1). Each diet contained 200 g/kg of protein. CI and SI were supplemented with a mixture of isoflavones to match the isoflavone level of T20 (final isoflavone content = 0.12 g/kg). Soybean oil and cellulose were added to adjust the fat and dietary fiber content of each diet. Animals were allowed free access to food and water. After being deprived of food for 3 h, the animals were sacrificed by bleeding from the abdominal aorta under diethyl ether anesthesia. The study was approved by the review board of animal ethics of the National Food Research Institute (H19-052) and followed institutional guidelines for the care and use of laboratory animals.

Analyses of Lipids and Isoflavones in Serum. Serum lipid concentrations were analyzed using commercial kits (Wako Pure Chemical Industries). Serum isoflavone concentrations were analyzed by HPLC as detailed previously.[13]

Selecting Differentially Expressed Genes from the DNA Microarray Data. In each of the groups, five rats with average triacylglycerol levels, that is, those with the highest and lowest serum levels having been eliminated, were used for microarray analysis. Hepatic total RNA was individually prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA). The total RNA was processed to prepare biotinylated cRNA using One-Cycle Target Labeling and Control Reagents (Afflymetrix, Santa Clara, CA) and then hybridized to an Affymetrix Rat Genome 230_2.0 GeneChip microarray. Each sample was applied to one microarray chip. After the washing and staining procedures, the microarrays were scanned with a GCS 3000 scanner (Affymetrix). The microarray data were normalized using the SuperNORM data processing service (Skylight Biotech Inc., Akita, Japan) in accordance with three-parameter log-normal distribution of perfect match (PM) data, and the expression of each gene was quantified using the trimmed mean of the corresponding normalized z scores.[18] The value for a group in terms of gene expression was estimated by using arithmetic means, and fluctuations within a group were estimated by using SD. The significance of a dietary effect was statistically tested by two-way ANOVA (PM cell sensitivity and dietary group effect).[19] The level of significance used for the selection was 0.01. The magnitude of a difference was estimated by taking trimmed means among differences calculated for the PM data; in comparisons between C and other diets, genes with Δz scores of more or less than 0.2 were selected. This magnitude corresponds to a 1.41-fold difference. Finally, 1295 genes of a total of 31042 genes on the microarray satisfied the criteria for the significance and difference (see the Supporting Information, Table S1). The microarray data have been deposited into the Gene Expression Omnibus (GEO) public repository, accession code GSE23748.

Detection of Biological Functions Frequently Appearing in Annotations of the Genes Selected. To determine whether characteristic biological functions differed among the dietary groups, frequencies with which biological terms appeared in annotations of the genes selected were examined. The list of annotations was obtained from Affymetrix;[20] the categories from Gene Ontology Biological Process[21] were used to speculate on the function of the genes. The significance of the frequency of biological functions was estimated by testing bias according to the binominal distribution model. To solve problems caused by some functions being registered multiple times for a gene, repeated occurrences of biological functions were counted in a gene-wise manner.[19] In all, 52 biological functions that showed p values of <0.01 and had more than six genes were found.

Summarizing Microarray Data. A principal component analysis (PCA) was used to reduce the dimensionality of the data and graphically display characteristics of each experimental group and biological functions.[22] In this study, a matrix of representative values of samples and selected genes was investigated. The matrix was further centered and scaled gene-wise, then decomposed, and the scores and loadings of each principal component (PC) were obtained using R.[23] In total, the methodology rotates the scaled matrix to find a new set of perpendicular axes toward which most of the variations in the matrix data have appeared. The PC score represents the relationships among samples

in the direction of the axes, whereas the loadings represent the correlations of each gene to the axes. The *R* scripts are available upon request. To arrange expressional data selected by biological function, self-organizing maps (SOMs) were made for finding lipid metabolism-related genes that showed a similar pattern of expression in the heat map.[24]

Estimating the Effect of Isoflavone Supplementation on Gene Expression. The effects of isoflavone supplementation in the present study were compared to those in our previous study using a higher concentration of genistein and daidzein (2 g/kg in diet) in the diet than in the present study (0.12 g/kg in diet).[25] The effects were estimated as the differences between representative values of supplied group's data and each of the isoflavone-free diet groups' representative values, that is, differences between CI and C, SI, and S in the present study and between the genistein- or daidzein-fed groups and the isoflavone-free group in the previous study.[25] Then Pearson's correlation coefficient was measured for the differences in a pairwise manner. A total of 4724 genes exhibited significant differences simultaneously in both studies, and those were used to estimate the correlations.

Quantitative Real-Time RT-PCR Analysis. Total RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). mRNA levels were analyzed by real-time RT-PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as detailed.[13] mRNA abundance was calculated as a ratio of the β-actin value in each cDNA sample and expressed as a percentage of the value of rats fed C. The nucleotide sequences of the forward and reverse primers and probe used to detect the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase mRNA were 5′-CGTCTTCAGCACTGTCGTCATT-3′, 5′-GAAAAAAGGG-CAAAGCTTCATTT-3′, and 5′-TTCCTCGACAAAGAATTGACAG-GCT-3′, respectively. The nucleotide sequences of the forward and reverse primers and probe used to detect the SREBP-2 mRNA were 5′-GATCAAGTCAGCAGCCAAGGA-3′, 5′-TGCAGAAAGCCTGGT-GTACCT-3′, and 5′-TACTGCGCCCAGAGGAACCCAGC-3′, respectively. The nucleotide sequences of the forward and reverse primers and probe used to detect the LXRα mRNA were 5′-GCACGCTA-CATTTGCCATAGC-3′, 5′-GGCGACACTCCTGGCATTT-3′, and 5′-TGGCCACTGCCCCATGGACAC-3′, respectively. Other nucleotide sequences are described in previous papers.[13,26]

Analysis of Enzymatic Activities. The enzymatic activities involved in fatty acid synthesis (fatty acid synthase, ATP-citrate lyase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme, and glycerol 3-phosphate dehydrogenase) were measured spectrophotometrically using the supernatant fraction of liver homogenates as described previously.[13] Logarithms of the activities per milligram of protein were compared with the *z* scores of normalized microarray data. The correlation line was robustly estimated using Tukey's line function of the *R*.[23]

Statistical Analysis. Data are presented as the mean ± SD. Except for the microarray analysis, statistical analyses were performed using SPSS 13.0J (SPSS Japan Inc., Tokyo, Japan). A one-way ANOVA followed by Tukey's post hoc analysis was used to determine the significance of dietary effects on the parameters. Statistically significant differences were defined at a level of $p < 0.05$.

RESULTS

Animal Growth, Liver Weight, and Serum Lipid and Isoflavone Levels. Both the soy protein-fed groups (S and SI) and the tofu-fed groups (T10 and T20) showed lower body weight gains than the casein-fed groups (C and CI) (Figure 1A). The difference was significant for S, SI, and T20 (*p* values of Tukey's post hoc analysis test are provided in the Supporting Information, Table S2). A similar tendency was observed in liver weight (Figure 1B) and serum triacylglycerol and cholesterol levels (Figure 1C,D). In contrast to dietary protein sources, the supplemental isoflavone did not have a significant effect on these parameters, although considerably higher serum isoflavone concentrations were detected (Figure 1E,F). Despite similar levels of food consumption during the experimental period (Figure 1G), total energy intake was slightly but significantly lower in the rats fed T20 than in those fed C and CI (Figure 1H).

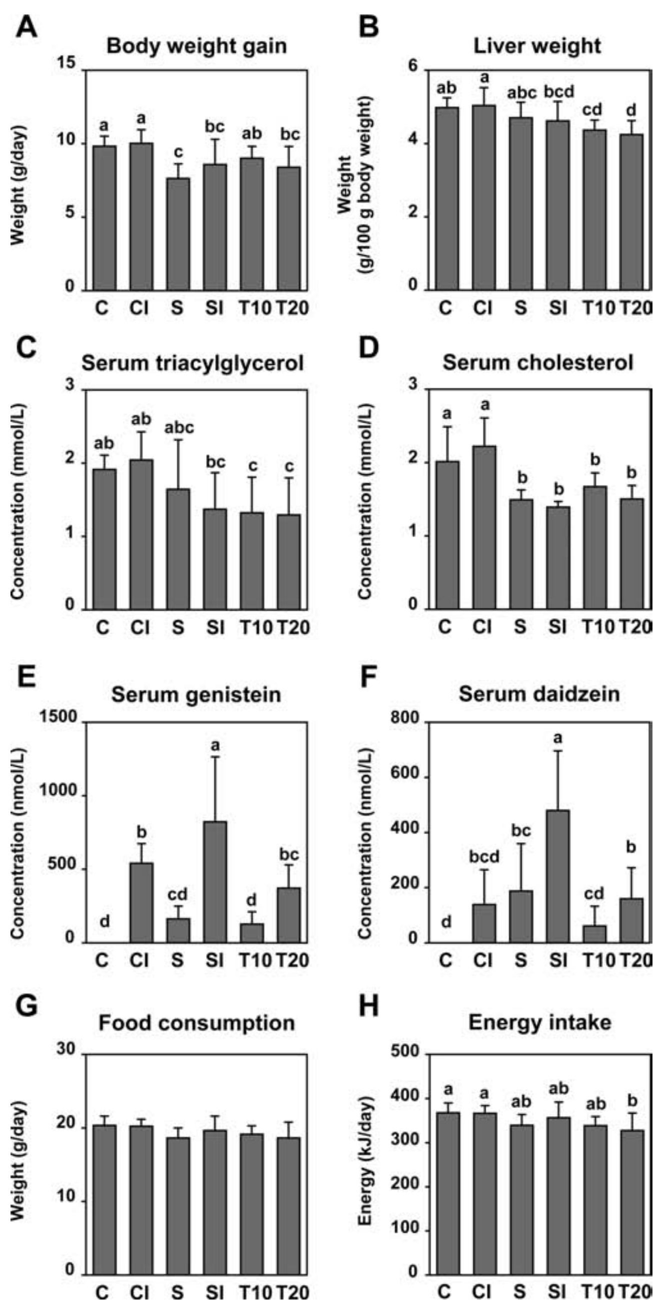

Figure 1. Growth and biochemical parameters of rats. At the end of the 14 day experimental period, rats were weighed and serum lipid and isoflavone levels were measured: (A) body weight gain during the experimental period; (B) liver weight at the end of the experimental period; (C) triacylglycerol, (D) cholesterol, (E) genistein, and (F) daidzein concentrations in serum at the end of the experimental period; (G) food consumption and (H) energy intake during the experimental period. Values are the mean ± SD, $n = 7-8$. Means without a common letter differ, $p < 0.05$.

Table 2. Over-represented Gene Ontology Involving Hepatic Genes Expressed Differently between the C-Fed and Other Groups[a]

rank	gene ontology ID	category of gene ontology biological process	frequency selected	total content	*p* values
1	8152	metabolic process	90	819	0
2	55114	oxidation reduction	65	427	0
3	6629	lipid metabolic process	35	205	7.86×10^{-12}
4	8610	lipid biosynthetic process	23	90	1.53×10^{-11}
5	42493	response to drug	35	289	4.78×10^{-8}
6	1676	long-chain fatty acid metabolic process	8	13	1.13×10^{-7}
7	6631	fatty acid metabolic process	17	87	2.87×10^{-7}
8	6694	steroid biosynthetic process	14	63	7.17×10^{-7}
9	6633	fatty acid biosynthetic process	12	48	1.33×10^{-6}
10	7067	mitosis	16	89	1.83×10^{-6}
11	38	very long-chain fatty acid metabolic process	6	9	2.79×10^{-6}
12	6879	cellular iron ion homeostasis	10	35	3.16×10^{-6}
13	6695	cholesterol biosynthetic process	9	28	3.84×10^{-6}
14	6637	acyl-CoA metabolic process	7	18	1.37×10^{-5}
15	8202	steroid metabolic process	13	72	1.58×10^{-5}
16	51301	cell division	17	119	1.72×10^{-5}
17	10033	response to organic substance	17	123	2.60×10^{-5}
18	14070	response to organic cyclic substance	18	138	3.21×10^{-5}
19	7584	response to nutrient	18	142	4.60×10^{-5}
20	7049	cell cycle	26	267	9.17×10^{-5}

[a] Categories with more than six genes were selected. $n = 5$.

Biological Functions Observed in the Frequencies of Keywords in Gene Annotations. To investigate how the levels of serum lipids were reduced in the soy protein- or tofu-fed groups, we further measured comprehensive gene expression levels in the liver. To focus on genes that showed significant and large expressional changes, 1295 genes were selected and the frequency of each annotation keyword was counted as shown in Materials and Methods. Among the genes selected, 52 keywords that describe biological processes showed significance in their frequencies of appearance as defined by the significance and number of genes involved in the process. It should be noted that half of the top 20 keywords with the lowest *p* values were related to the metabolism of fatty acids and steroids (Table 2). Additionally, three categories related to the cell cycle (mitosis, cell division, and cell cycle) were also found with lower frequencies in the table. In contrast, other specific categories were not selected except several ambiguous categories such as metabolic process, oxidation reduction, and response to nutrient. Despite the lower energy intake in the T20-fed group than in C- and CI-fed groups (Figure 1H), none of the 13 keywords that were found to relate with the low energy intake, such as response to nutrient levels and response to starvation, significantly appeared in annotations of the genes selected (Table 2 and Supporting Information, Table S1).

Genetic Profile of Each Experimental Group and Biological Function Based on PCA. To investigate characteristics of the experimental groups, a PCA was performed on the 1295 selected genes. The analysis extracts tendencies among multivariate matrix data and shows the magnitude of each tendency as a PC score and a loading. A score is given to each sample and shows the characteristics of that sample. A loading is given to each gene and shows the correlation of the gene's expressional changes to the tendencies. In the present case, the first two PCs succeeded in extracting >80% of total variance in the data (PC1, 57.9%; PC2, 25.4%), showing that the characteristics of the diets are largely explained by the two PCs. The sample groups form some clusters according to the protein sources of the diets (Figure 2A); clusters (S and SI) and (C and CI) were distinctly separated in PC1, and clusters T10 and T20 were located near the C and S groups, respectively. PC2 separated tofu-fed groups from the others. The effect of supplementing C and S with isoflavone seemed to be minimal in both PC1 and PC2. Actually, effects were detected in PC5, which contributed to 4.2% of total variance, showing a smaller number of genes had been altered. Additionally, genes related to lipid metabolism did not show strong correlations with the axis of PC5 (data not shown). Therefore, isoflavones have only limited effects on global gene expression, especially among lipogenic genes.

Genes that had contributed to tendencies appeared in the loadings of PCs (Figure 2B). The groups of related genes found for the frequently annotated keywords, lipogenesis and cell cycle, formed clusters alongside the axes: genes related to lipid and/or steroid synthesis (orange) along PC1 and those related to the cell cycle (green) along PC2. According to the commonality of the axes of the PCA, the lipogenesis-related genes contributed to the tendency that had been provided by protein source (PC1), whereas the cell cycle-related genes contributing to the tendency had been provided by the tofu diet (PC2). Indeed, among fatty acid metabolism-related genes, a clear tendency was observed in the expression patterns (Figure 3A). The list of genes covered the entire pathway of lipid synthesis, including key enzymes such as acetyl-CoA carboxylases, fatty acid synthases, and ATP-citrate lyase as well as positive transcription factors such as SREBP-1,[27] and the lipogenic genes showed greater expression in the C- and CI-fed groups than the S-, SI-, T10-, and T20-fed groups. The same tendency was obvious in the genes related to cholesterol synthesis, including the key enzyme HMG-CoA reductase (Supporting Information, Figure S1). In contrast, genes related to lipid degradation or oxidation showed different tendencies among experimental groups (Figure 3).

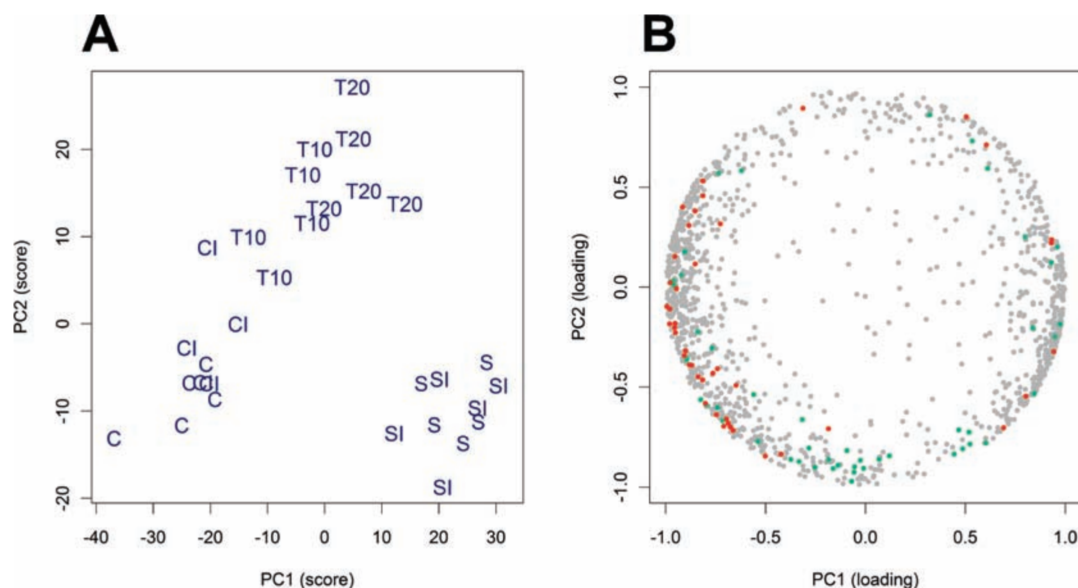

Figure 2. Principal component analysis applied to individual samples and selected genes. Score plots of PC1 compared with PC2 were derived from hepatic gene expression in rats fed C, CI, S, SI, T10, or T20: (A) each dot represents a rat sample, $n = 5$; (B) each dot represents a single gene. Orange and green dots show the genes related to lipid/steroid synthesis and the cell cycle, respectively.

Effect of Isoflavone Supplementation on Hepatic Gene Expression. To elucidate whether the observations regarding the isoflavone supplement (0.12 g/kg in diet) were due to the concentration, the expressional changes were compared with those observed with a much higher concentration of genistein and daidzein (2 g/kg in diet) studied previously.[25] If the addition of isoflavones to different diets or at different concentrations has a common effect, the levels of expressional change would be similar, presenting positive correlations; however, if a threshold or minimum concentration exists, such correlations would not appear. A correlation was observed only between the high concentration of genistein (G−cont) and daidzein (D−cont)-fed groups, showing a common effect between the supplements (Table 3). However, the effects of the lower concentration of isoflavones showed an inverse correlation between different protein sources (casein (CI−C) and soy protein (SI−S)-fed groups), and no correlations were found among any other combinations of the groups. Correlations were not found even after concentration on the genes that contributed to PC5 (data not shown).

Confirmation of the Microarray Data by Real-Time RT-PCR. Real-time RT-PCR was performed to confirm the mRNA expression patterns of six genes detected by microarray analysis. The targets were chosen from among major genes having products involved in fatty acid synthesis (acetyl-CoA carboxylase, fatty acid synthase, and ATP-citrate lyase) and sterol metabolism (HMG-CoA reductase). Furthermore, the amounts of mRNA of two genes, regulated by SREBP-1 and encoding glucose 6-phosphate dehydrogenase and malic enzyme that catalyze pathways closely related to lipid biosynthesis,[27] were measured, thereby confirming the translational regulation of lipogenesis. The results of the real-time RT-PCR analysis were generally consistent with the microarray data for all targets evaluated (Supporting Information, Table S3). To better understand whether changes in these transcription factors were involved in the observed effects, the mRNA expression of transcription factors involved in lipogenesis, SREBP-1a, SREBP-1c, SREBP-2, and LXRα, was analyzed. The expression of SREBP-1c was lower in the soy protein- and tofu-fed groups than in casein-fed groups, confirming the results of the microarray analysis (Srebf1). In contrast, the expression of other transcription factors did not differ among the groups.

Relationship between the mRNA Expression and Activity of Lipogenic Enzymes. The enzymatic activities related to lipogenesis were measured to verify whether any changes in mRNA expression were accompanied by changes in activity, reflecting cell physiology. As in the mRNA expression, significantly lower activity levels of lipogenic enzymes were observed in the S, SI, T10, and T20 groups compared with the C and CI groups (data not shown). The log ratios of the measured activities were compared with those of microarray data obtained from the same liver samples. For all of the enzymes studied, certain correlations were observed with Pearson's r and p values (Figure 4). Some of the genes were measured with two probe sets of different sensitivities, which may alter the intercepts of correlation lines.

DISCUSSION

Our observations showed that freeze-dried tofu diets lowered body weight gain (Figure 1A) and serum lipid levels (Figure 1C, D) compared with casein diets in rats. Notably, the reduction of serum lipid levels in rats fed T20 was reproduced using the diet in which half of the protein source was substituted by casein (i.e., T10). This observation supports the notion that even a moderate intake of soy food that simulates the eating behavior in Asian countries could exert a certain hypolipidemic effect. The data also demonstrated that the lower serum lipid levels in the tofu-fed groups were similar to those levels in the soy protein-fed groups, suggesting that soy protein is the hypolipidemic compound of the soy food. In contrast to the proteinaceous compound, the isoflavones contained in tofu or added to soy protein little affected serum lipid levels. This was inconsistent with our

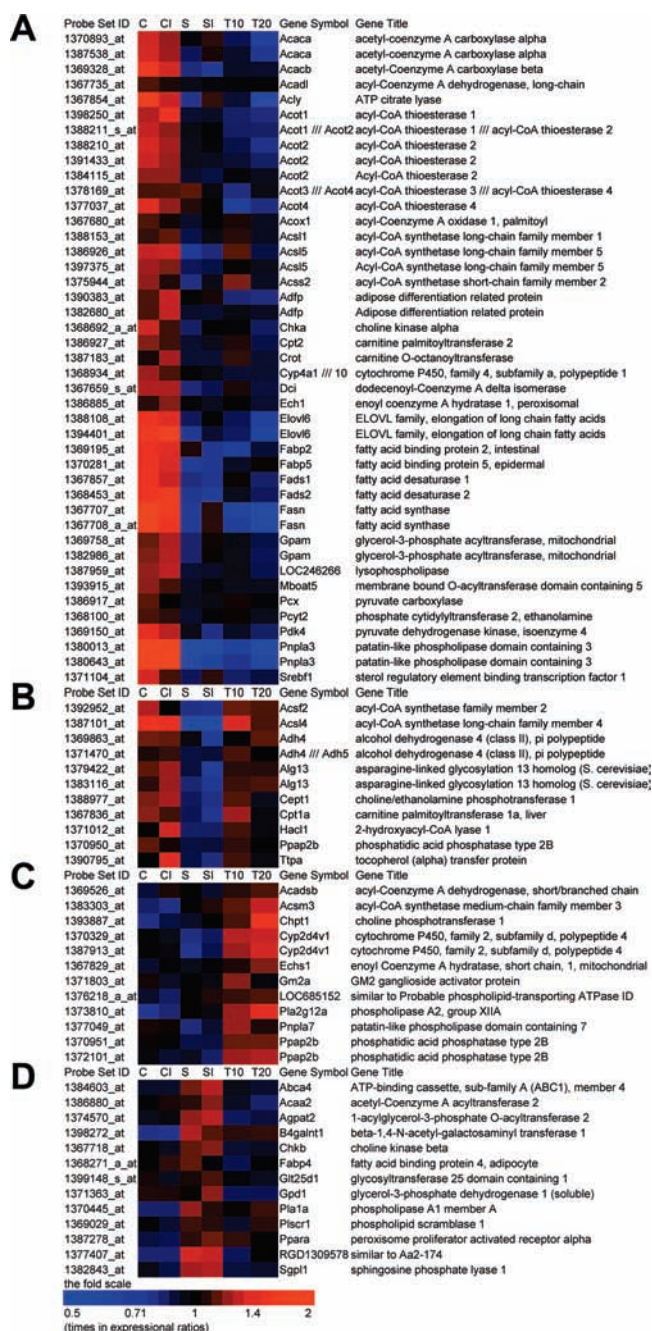

Figure 3. Changes in the expression of hepatic genes involved in lipid metabolism. Heat maps show 79 lipid metabolism-related genes expressed differently between C-fed and other diet-fed groups. These genes were clustered into four groups: (A) C- and CI-fed groups had higher values; (B) S- and SI-fed groups had lower values; (C) T10- and T20-fed groups had higher values; (D) S- and SI-fed groups had higher values than the other groups, $n = 5$.

previous study;[25] however, the concentration of isoflavone in this study was quite low. This issue will be discussed later.

To characterize how freeze-dried tofu was involved in lowering serum lipid concentrations, we investigated the global changes in transcriptomic expression in the liver, where most storage lipids are produced before being exported to the rest of the body,[16] using microarray technology. The list of frequently annotated keywords (Table 2) showed that lipid metabolism-related genes were widely affected in the present study. Actually, genes related to lipid and/or steroid metabolism showed extreme values on the PC1 axis (Figure 2B), and the expression levels were much higher in the groups fed C and CI than in those fed S, SI, T10, and T20 (Figure 3A and Supporting Information, Figure S1). The similarity of such expression patterns suggested that the alterations caused by T10 and T20 are derived from their soy protein component, and the reduced expression of lipogenic genes in the liver may cause changes in serum lipid levels. Indeed, the activity of lipogenic enzymes nearly paralleled the levels of mRNA expression (Figure 4), showing that the transcriptomic changes altered enzymatic activities. By contrast, for other genes related to lipid metabolism, such as fatty acid β-oxidation, and the genes for the transcription factor PPARα,[28] results were inconsistent between tofu-fed groups (T10 and T20) and soy protein-fed groups (S and SI) (Figure 3). Thus, unlike lipogenesis, other pathways of hepatic lipid metabolism may be affected differently by soy protein and freeze-dried tofu. These findings show that the protein component of freeze-dried tofu, but not isoflavone, suppresses lipogenic gene expressions in liver. This would be responsible for the decrease in serum triacylglycerol and cholesterol levels in the corresponding samples.

Table 3. Correlations for the Changes in Gene Expression Observed on the Supplementation of Diets with Isoflavone[a]

	G−cont[b]	D−cont[c]	CI−C[d]	SI−S[e]
G−cont	1	0.366	−0.00157	0.00476
D−cont	0.366	1	0.00185	0.0187
CI−C	−0.00157	0.00185	1	−0.257
SI−S	0.00476	0.0187	−0.257	1

[a] The correlations were determined with the genes whose expression exhibited significant differences between the diets with and without isoflavone, $n = 5$. [b] Differences between a genistein-supplemented diet and a control diet.[25] [c] Differences between a daidzein-supplemented diet and a control diet.[25] [d] Differences between CI and C in the present study. [e] Differences between SI and S in the present study.

Contrary to the protein component of soy food, isoflavones had little effect on serum lipid levels (Figure 1C,D), although isoflavones were detected in the serum of rats fed isoflavone-supplemented and freeze-dried tofu diets (Figure 1E,F). This ineffectiveness was also evident in microarray analyses (Figures 2A and 3 and Supporting Information, Figure S1) in which isoflavones did not exert any significant effect on the mRNA expression related to lipid metabolism in the liver. These observations are inconsistent with our previous studies,[13,25] which employed a 17-fold higher concentration of isoflavone in diet (2 versus 0.12 g/kg). As the Pearson's correlation coefficient shows (Table 3), the effect of isoflavones may be influenced by experimental conditions such as dietary components like proteins and fats;[29,30] the inverse correlation between SI−S and CI−C (Table 3) could be an example of such conditional effects. Alternatively, a pragmatic threshold for the activation of isoflavones may exist for regulating lipid metabolism.[12,13] Anyway, it is clear that the isoflavone supplement little affected hepatic gene expression (Figure 3) or the growth and serum lipids of animals (Figure 1A−D,G,H) regardless of the dietary protein source; hence, any synergistic effects of isoflavones and proteins are negated.

The methanol treatment of soy protein to eliminate isoflavones may affect the higher structure of proteins.[31] However, the hypolipidemic properties of our soy protein were basically consistent with those of the freeze-dried tofu, although

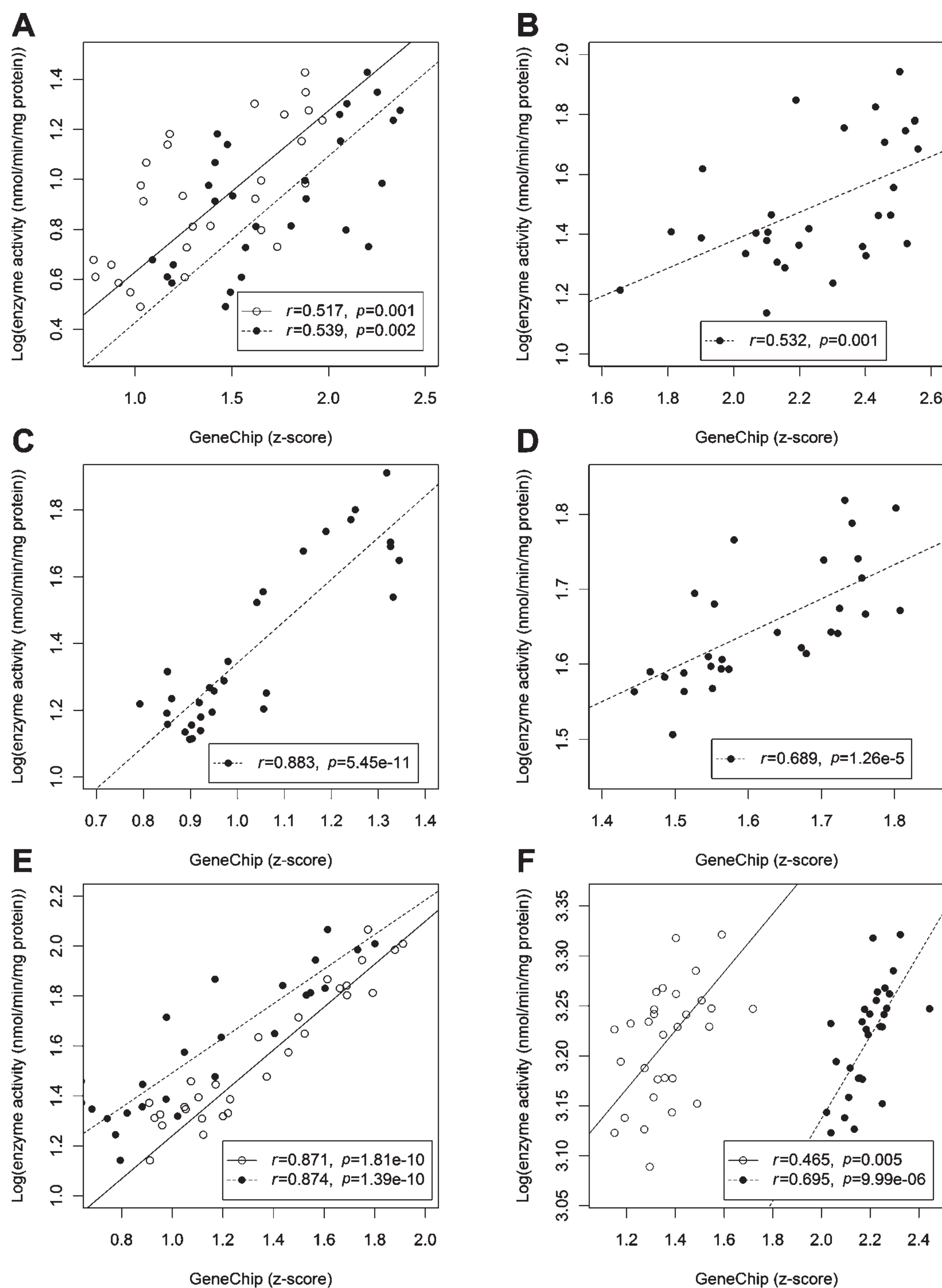

Figure 4. Correlation between the microarray data and the activity of lipogenesis-related enzymes: (A) fatty acid synthase; (B) ATP-citrate lyase; (C) glucose 6-phosphate dehydrogenase; (D) 6-phosphogluconate dehydrogenase; (E) malic enzyme; (F) glycerol 3-phosphate dehydrogenase. Black and white dots in panels A, E, and F represent different probes for the enzyme in the microarray analysis. Values superimposed are Pearson's correlation coefficients (r). $n = 5$.

expression levels of several genes involved in hepatic lipid metabolism were inconsistent, as will be discussed later. Thus, the basic proteinaceous function may be maintained by the treatment, or differences in the higher structure of the protein may be insignificant, even if the protein structure was denatured.

Although freeze-dried tofu and soy protein similarly suppressed hepatic lipogenesis, they differed in some metabolic activities. For example, expression levels of genes related to fatty acid β-oxidation, such as the PPARα gene,[28] differed between the tofu-fed groups (T10 and T20) and soy protein-fed groups (S and SI) (Figure 3). Therefore, freeze-dried tofu and soy protein are not the same dietary protein source in terms of modulators of hepatic lipid metabolism. For changes other than in lipid metabolism, Figure 2A shows that the freeze-dried tofu groups

were distinguishable from the casein- and soy protein-fed groups in the direction of PC2. The physiological meaning of this separation is unclear; however, cell cycle-related genes, which contributed to the separation (Figure 2B, green spots), could have some causal relationship with the smaller liver sizes of the tofu-fed groups (Figure 1B), because progression of the cell cycle in hepatocytes is usually initiated to restore liver mass after a hepatectomy.[32] We presently do not have evidence to estimate the cause of the difference between tofu and soy protein; it could be the methanol treatment of protein or some unidentified components included in tofu other than isoflavone.

The present study demonstrated a protein fraction to be the active component of soy foods that substantially decreased serum lipid concentrations in rats. The dietary factors significantly altered hepatic mRNA levels of genes involved in lipid and cholesterol metabolism. Although expression levels of some of the genes involved in fatty acid β-oxidation and/or the cell cycle were inconsistent, those of lipogenic genes were similar between soy protein- and freeze-dried tofu-fed groups. By contrast, supplementation with isoflavone had limited effects on serum lipid levels and hepatic gene expression and did not enhance the effects of dietary protein sources. Therefore, frequent consumption of soy protein or soy foods may help to improve circulating lipid levels mainly by suppressing hepatic lipogenesis.

■ ASSOCIATED CONTENT

Supporting Information. Genes differentially expressed between the C-fed and other diet groups (Table S1); *p* values of Tukey's post hoc analysis test for growth and biochemical parameters of rats (Table S2); RT-PCR analysis of the expression of selected hepatic genes involved in lipid metabolism (Table S3); changes in expression patterns of hepatic genes involved in cholesterol metabolism (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*Phone: +81 29 838 8083. Fax: +81 29 838 7996. E-mail: youkot@affrc.go.jp.

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■ ABBREVIATIONS USED

C, casein diet; CI, casein diet containing isoflavone; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LXR, liver X receptor; PC, principal component; PCA, principal component analysis; PM, perfect match; PPAR, perxisome proliferator-activated receptor; S, soy protein diet; SI, soy protein diet containing isoflavone; SOM, self-organizing map; SREBP, sterol regulatory element-binding protein; T10, diet containing two proteins derived equally from casein and freeze-dried tofu; T20, diet containing protein derived from freeze-dried tofu.

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