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Regulative Actions of Dietary Soy Isoflavone on Biological Antioxidative System and Lipid Metabolism in Rats

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Male Sprague−Dawley rats, 4 weeks of age, were fed purified diets either with or without 0.2% soy isoflavones rich powder for 5 weeks to elucidate their direct functions such as antioxidative action and regulation of lipid metabolism. Dietary soy isoflavones decreased serum lipid peroxide level in rats. Levels of liver and serum α-tocopherol were higher in the rats fed isoflavone than in those fed isoflavones-free diet. Thus, dietary soy isoflavones exhibited mild antioxidative function in this animal experiment. Isoflavone metabolites from diet may act as scavengers of reactive oxygen species. Dietary soy isoflavones lowered hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity, although liver cholesterol level was not modulated. However, the levels of serum cholesterol and triglyceride decreased by consumption of soy isoflavones. Therefore, dietary soy isoflavones may exhibit hypocholesterolemic and hypolipidemic functions. Moreover, dietary soy isoflavones lowered hepatic Δ6 desaturase activity. Reflecting this observation, Δ6 desaturation indices ((18:2($n = 6$) + 18:3($n = 6$))/20:4($n = 6$)) of tissue lipids tended to be lower in rats fed isoflavones than in those fed isoflavones-free diet. This action may contribute to the prevention of inflammatory response by imbalance of eicosanoids. These observations suggest that the positive intake of soy isoflavones may reduce the risk of some cardiovasucular diseases through their radical scavenging function and hypocholesterolemic action.

KEYWORDS: Soy isoflavones; diet; antioxidation; lipid metabolism; rats

INTRODUCTION

Isoflavones are phytochemicals found in various beans, particularly in soy-hypocotyl. Many reports have shown that soy isoflavones exert various biological activities, such as the actions that contribute to healthy maintenance and promotion. Moreover, much physiology activity has been clarified until now. Among these activities, it is well-known that isoflavones have anticarcinogenic action (*1*), antiosteoporosis (*2*), and hypocholesterolemic effect (*3*).

The primary isoflavones in soybeans are genistein and daidzein and their respective β-glycosides, genistin, and daidzin. Much lower amounts of glycitein and its β-glycoside, glycitin, are also present in soybeans (*4*). The portion having the highest isoflavone content in soybean is soy-hypocotyl, and it has approximately equal amounts of daidzin and glycitin. Soybeans and soy products have about 1−3 mg isoflavones/g protein, and the many isoflavones are malonyl- and β-glycoside derivatives (*4*).

The soy isoflavones are structurally similar to estrogen (*5*). Some investigations have focused on the estrogenic activities of isoflavones as active components of soy protein and responsible for many of its beneficial effects. Soy isoflavones are effective in preventing bone loss (*2*). Moreover, consumption of intact soy protein resulted in a significant decrease in total cholesterol and low-density lipoprotein cholesterol plus very low-density lipoprotein cholesterol compared with a diet containing soy protein without isoflavones in female rhesus macaques (*3*). They speculated that the estrogenic activities of isoflavones might be responsible for the lipid-lowering effects of soy protein.

Some reports have shown that soy isoflavones have been shown to exert preventive actions of the development of atherosclerosis and subsequent coronary heart disease in vitro studies (*6*). Moreover, the isoflavones have scavenging activity of reactive oxygen species and antioxidant properties (*7*,*8*). In fact, isoflavones can inhibit lipoprotein oxidation in serum and LDL oxidation in vitro studies (*9*). Therefore, dietary isoflavones also may reduce development of atherosclerosis through their lipid-lowering and antioxidant activity.

However, these numerous activities of soy isoflavones have been mainly reported from in vivo trial using crude soy powder containing low content of isoflavones and in vitro studies, and the direct activity by isoflavone itself is not completely elucidated. The purity of used isoflavones in experiment also may contribute much confusion. Moreover, the direct effects of

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dietary soy isoflavones on lipid metabolism and antioxidative effects in vivo are still not clear. Therefore, in this study, we focused directly on the regulation by dietary soy isoflavones on lipid metabolism in rats using powder containing a high content of soy isoflavones.

MATERIALS AND METHODS

Materials. 3-Hydroxy-3-methyl[1-^{14}C]glutaryl-CoA (51 mCi/mmol) was purchased from Amersham International (Buckinghamshire, UK). [1-^{14}C]Linoleic acid (55 mCi/mmol) and [4-^{14}C]cholesterol (52 mCi/mmol) were the products of New England Nuclear (Boston, MA). Radioactive linoleic acid and cholesterol were purified by thin-layer chromatography before use. Isoflavone powder, Fujiflavone P40, was purchased from Fujicco Co., Ltd (Hyogo, Japan). Other solvents and chemicals were of reagent grade or better quality and purchased from nacalai tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Animals and Diets. Male Sprague−Dawley rats (4 weeks old, CLEA Japan, Tokyo, Japan) were housed individually in a room with controlled temperature (20−23 °C) and light (08.00−20.00 h). After rats were acclimatized for 1 week, they were divided into two groups of six or eight each; one group was fed isoflavone-free diet (Control group, six rats) and a second group was fed a 0.2% isoflavone-added diet (Isoflavone group, eight rats). The composition of isoflavones was 15.6 mg of daidzin, 10.6 mg of glycitin, and 5.9 mg of genistin per 100 mg of isoflavone powder. Experimental diets were prepared according to the recommendation of the American Institute of Nutrition and contained (weight %): casein, 20; α-cornstarch, 13.2; sucrose, 10; safflower oil, 10; cellulose, 5; mineral mixture (AIN-93G), 3.5; vitamin mixture (AIN-93), 1.0; L-cystine, 0.3; choline bitartrate, 0.25; *tert*-butylhydroquinon, 0.0014; cholesterol, 0.3%; and cornstarch to 100. Diets were prepared weekly and were packed in pouches and stored at 4 °C to prevent autoxidation of lipids. After 5 weeks, rats were decapitated at night (01.00) for analysis of hepatic cholesterol metabolism. The liver was excised immediately, and microsome was prepared at 4 °C. Plasma was prepared by centrifugation after allowing blood to clot at room temperature. These samples were kept at −30 °C until analysis to avoid oxidation. Feces were collected for 3 days, beginning 10 days before killing to analyze fecal neutral and acidic steroids, and then lyophilized. The Hirosaki University Animal Policy approved this animal study, and rats were maintained according to the guidelines for the care and use of laboratory animals of Hirosaki University.

Analyses. The activities of 3-hydroxy-3-methlglutaryl (HMG)-CoA reductase, cholesterol 7α-hydroxylase, and Δ6 desaturase in liver microsomes were measured by the methods of Shapiro et al. (*10*), Van Contfort et al. (*11*), and Svensson (*12*), respectively. The concentration of microsomal protein was determined by the method of Bradford (*13*). Liver and serum lipids were extracted by the method of Folch et al. (*14*), and the concentrations of liver triglyceride, total cholesterol, and serum total cholesterol were measured as described previously (*15*). The levels of serum triglyceride and HDL-cholesterol were measured using commercially available kits (triglyceride-test Wako and HDL-cholesterol-test Wako (Wako Pure Chemical Industries, Ltd)). Each lipid class of liver and serum were separated by TLC, and their fatty acid composition was analyzed by gas−liquid chromatography (GLC, Shimadzu GC-8A, Shimadzu, Kyoto, Japan) with a flame ionization detector using a Silar 10C column (2-mm × 3-m) after transmethylation (*16*). The levels of fecal neutral and acidic steroids were analyzed by GLC using 5α-cholestane and 23-nordeoxycholic acid (Steraloids, Wilton, NH) as internal standards (*17*). Liver and serum lipoperoxides were measured by the method of Yagi and Ohokawa et al. (*18*, *19*) as thiobarbituric acid reactive substances (TBARS). The levels of serum and liver α-tocopherol were measured by the method of Ueda et al. (*20*).

Determination of Aorta 6-Keto-PGF$_{1\alpha}$. The aorta was cut into 1.5-cm portions and placed in tubes containing Krebs−Henscleit bicarbonate buffer (1.54 M KCl, 0.77 M KH2PO4, 0.77 M MgSO4, 0.77 M NaHCO3, pH 7.4) and incubated for 30 min at room temperature (25 °C). The aorta was then blotted dry on filter paper and weighed.

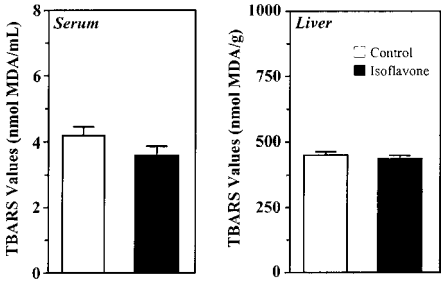

Figure 1. Effects of dietary soy isoflavones on levels of lipid peroxide of serum and liver in rats. Data are presented as mean ± SE for eight rats in each group. Control, rats fed isoflavone-free diet; Isoflavone, rats fed 0.2% isoflavone-added diet.

Reaction mixture was acidification to pH 4.5 with 3% formic acid for prostacyclin extraction. The samples were then extracted with ethyl acetate and dried at low temperature under a nitrogen gas atmosphere. The residues were resolved in acetonitrile (200 μL), and then fluorescence derivatization was carried out. To aliquots of 125 μL was added 2.5−5 mg $KHCO_3$ in 1.5-mL Eppendorf tubes. The samples were added to 50 μL of 10 mM Br-DMEQ (in acetonitrile) and 25 μL of 11.4 mM 18-Crown6 (in acetonitrile), vortex mixed, and then incubated for 15 min at 50 °C. The samples were analyzed by Shimadzu LC-10AT Liquid Chromatograph with RF-550 Spectrofluorometric Detector (Ex370 nm, Em450 nm) using Econosil C18 (4.6-mm × 0.25-mm, 5-μm, Alltech). The solvent system consisted of 0.1% TFA in water/acetonitrile (67:33, v/v) (A) and 0.1% TFA in water/acetonitrile (50:50, v/v) (B). Elution was carried out using a mobile phase consisting of 0% B for 20 min, 100% B for 15 min, 100% B for 20 min, 0% B for 1 min, and 0% B for 10 min. The flow rate was 1.5 mL/min. Concentrations were quantified using 6-keto-PGF1α as external standard. All procedures were carried out at low temperature (*21*).

Determination of PGE$_2$ and PGF$_{2\alpha}$. The kidney was cut about 0.5 g and exactly weighed. Kidney was homogenized in cooled physiological buffered saline containing 1 mM disodium dihydrogen ethylenediamine tetraacetate dihydrate and 0.1 mM indomethacin and then rehomogenaized in cool ethanol in a homogenizer. The mixture was centrifuged at 10 000 rpm for 20 min at 4 °C. The supernatant fraction was collected and dried at low temperature under nitrogen gas atmosphere. The residues were added to cool distilled water and acidified to pH 3.0 with 1 N HCl for prostaglandin E2 and F2α extraction. The samples were then extracted with ethyl acetate and dried at low temperature under a nitrogen gas atmosphere. The residues were resolved in actonitrile (200 μL). Fluorescence derivatization of extracted samples and high performance liquid chromatography analyses of derivatives were done as described above.

Statistical Analysis. Data were analyzed by Student's *t*-test to determine the exact nature of the difference ($P < 0.05$) among the groups.

RESULTS AND DISCUSSION

Growth and Liver Weight. On average, a rat weighing 136 g was fed 11.5 g per day and gained 133 g of body weight over 35 days. There were no significant differences in these indices between the two groups. The average liver weight was the same in two groups, 3.8 g /100 g body weight.

Lipid Peroxides Levels of Serum and Liver. The serum TBARS values tended to be lower in the rats fed isoflavone than they were in those fed the isoflavone-free diet (**Figure 1**). Contrary to this observation, there was no difference in liver TBARS values.

α-Tocopherol Level of Serum and Liver. The level of serum α-tocopherol was significantly higher in the rats fed isoflavone than it was in those fed the isoflavone-free diet (**Figure 2**). The same observation was made in the level of liver α-tocopherol.

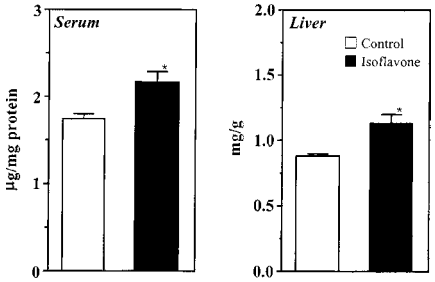

Figure 2. Effects of dietary soy isoflavones on levels of α-tocopherol of serum and liver in rats. Data are presented as mean ± SE for eight rats in each group. *, Significantly different from the corresponding value in rats fed isoflavone-free diet at $P < 0.05$. Abbreviations are the same as those in **Figure 1**.

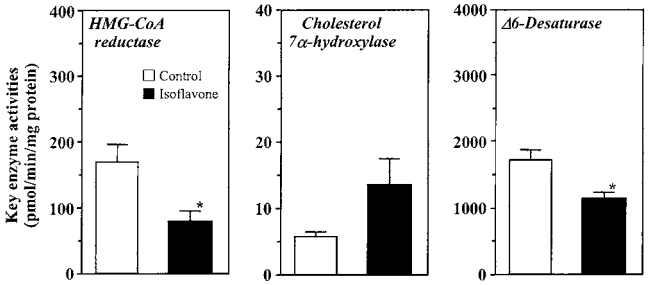

Figure 3. Effects of dietary soy isoflavones on hepatic microsomal key enzyme activities of cholesterol biosynthesis and linoleic acid desaturation in rats. Data are presented as mean ± SE for eight rats in each group. *, Significantly different from the corresponding value in rats fed isoflavone-free diet at $P < 0.05$. Abbreviations are the same as those in **Figure 1**.

Table 1. Effect of Dietary Soy Isoflavones on Levels of Liver and Serum Lipids in Rats[a]

	liver		serum		
groups[b]	total cholest mg/g	triglycds mg/g	total cholest mg/dL	HDL cholest mg/dL	triglycds mg/dL
control	45.0 ± 2.4	69.7 ± 1.3	151 ± 4	17.1 ± 1.2	207 ± 16
isoflavone	45.1 ± 0.8	65.1 ± 12.0	128 ± 6[c]	19.9 ± 1.0	60.2 ± 2.8[c]

[a] Data are presented as means ± SE for 6 or 8 rats in each group. [b] Control, rats fed isoflavone-free diet. Isoflavone, rats fed 0.2% isoflavone added diet. [c] Significantly different from the corresponding value in rats fed isoflavone-free diet at $P < 0.05$ (Student's *t*-test).

Key Enzyme Activities of Liver Microsomes. The activity of hepatic HMG-CoA reductase was significantly lower in the rats fed the isoflavone-added diet than it was in those fed the isoflavone-free diet (**Figure 3**). The activity of hepatic cholesterol 7α-hydroxylase tended to be higher in the Isoflavone group than in the Control group. Dietary isoflavones, compared with the isoflavone-free diet, also significantly lowered the hepatic Δ6 desaturase activity.

Levels of Liver and Serum Lipids. The levels of liver total cholesterol and triglycerides were not shown to be significantly different in the rats fed the isoflavone-added diet compared to those fed the isoflavone-free diet. (**Table 1**). The levels of serum total cholesterol and triglycerides were significantly lower in the rats fed the isoflavone-added diet than in those fed the isoflavone-free diet. On the other hand, dietary isoflavone, compared with the rats fed the isoflavone-free diet, tended to increase the serum HDL-cholesterol level.

Fatty Acid Composition of Liver and Serum Lipids. The proportion of linoleic acid (18:2($n = 6$)) in liver phosphatidylcholine (PC) and liver phosphatidylethanolamine (PE) tended to be slightly higher in the Isoflavone group than in the Control group, although that of serum PC in the Isoflavone group was lower than that in the Control group. On the other hand, dietary isoflavone tended to slightly decrease the proportion of arachidonic acid (20:4($n = 6$)) in liver PC, serum PC, and serum cholesterylester (CE). The ratio of Δ6 desaturation indices [(20:3 + 20:4)/18:2] of liver PC, liver PE, and serum CE tended to be lower in the Isoflavone group than in the Control group (**Table 2**).

Concentrations of Tissues Eicosanoids. Productive level of 6-keto-$PGF_{1\alpha}$ of thoracic aorta was significantly lower in the rats fed isoflavone than in those fed the control diet (**Table 3**). Moreover, dietary isoflavone, compared with the isoflavone-free diet, significantly lowered the levels of PGE_2 and $PGF_{2\alpha}$ in kidney.

Fecal Steroid Excretion. The excreted level of total neutral steroids into feces tended to be higher in the rats fed isoflavone than that of those fed the isoflavone-free diet (**Table 4**). Dietary isoflavone, compared with the isoflavone-free diet, significantly increased the excreted level of total acidic steroids into feces. The composition of acidic steroids was modified by dietary isoflavone, and the ratio of secondary to primary bile acid (deoxycholic acid/cholic acid and lithocholic acid/chenodeoxycholic acid) was decreased in the rats fed isoflavone.

We found that soy isoflavone exhibits mild antioxidative function in this animal experiment. Dietary isoflavone decreased serum TBARS value in rats. Anderson et al. (*7*) also suggested that dietary genistein lowered lipoprotein TBARS. Therefore, some isoflavone metabolites derived from dietary isoflavone may prevent lipid peroxidation in tissues through their radical scavenging action.

On the other hand, levels of liver and serum α-tocopherol were higher in the rats fed isoflavone than in those fed isoflavone-free diet. Dietary soy isoflavone significantly enhances the activities of antioxidative enzymes in mice experiment (*8*). Isoflavone metabolites from diet may act as scavengers of reactive oxygen species in tissue, as described above. Therefore, the consumption of vitamin E seems to be inhibited by scavenging action of reactive oxygen species by isoflavone metabolites. Thus, the positive intake of soy isoflavone may strengthen the biological antioxidative system.

Dietary isoflavone regulated lipid metabolism in rats. Hepatic HMG-CoA reductase activity was decreased by dietary isoflavone. However, liver total cholesterol level was not modulated by dietary isoflavone, although serum cholesterol level was lowered by intake of isoflavone. We do not have any explanation to elucidate this conflict; however, liver cholesterol level may be lowered by a more long-term feeding trial of isoflavone.

Anthony et al. (*3*) observed that soy protein containing isoflavone, compared with soy protein without isoflavone, resulted in significantly decreased LDL cholesterol levels and increased HDL cholesterol levels in a cynomolgus monkey experiment. Thus, dietary isoflavone seems to improve lipoprotein metabolism.

Hepatic cholesterol 7α-hydroxylase activities tended to be higher in the rats fed isoflavone than it was in those fed the control diet. Reflecting modulation of enzyme activities, the excreted level of acidic steroids into feces tended to increase in the isoflavone-fed group compared with the Control group. This observation may be attributed to the decrease of serum cholesterol level by intake of isoflavone. Ni et al. (*23*) observed that dietary soy protein containing isoflavone, but not soy protein without isoflavone, increased mRNA levels of cholesterol 7

Table 2. Effect of Dietary Soy Isoflavones on Fatty Acid Composition of Liver and Serum Lipids in Rats[a]

	fatty acids (weight%)							
groups	16:0	18:0	18:1	18:2	20:3	20:4	22:6	20:3 + 20:4/18:2
				liver				
phosphatidylcholine								
control	21.3 ± 1.5	15.5 ± 1.1	17.9 ± 1.1	14.0 ± 2.7	1.8 ± 0.8	23.0 ± 3.6	2.3 ± 0.2	1.9 ± 0.5
isoflavone	20.9 ± 1.0	14.8 ± 1.1	18.2 ± 1.3	16.6 ± 1.7	1.9 ± 0.8	21.2 ± 3.7	2.0 ± 0.4	1.4 ± 0.3
phosphatidylethanolamine								
control	26.5 ± 10.2	18.5 ± 7.2	13.3 ± 5.2	6.6 ± 3.0	0.7 ± 0.3	27.2 ± 10.3	4.9 ± 1.9	4.2 ± 3.5
isoflavone	18.8 ± 1.7	19.3 ± 2.8	13.2 ± 1.6	8.1 ± 1.5	0.5 ± 0.3	29.6 ± 0.9	8.0 ± 0.9[c]	3.7 ± 0.7
				serum				
phosphatidylcholine								
control	24.5 ± 1.7	14.7 ± 1.3	21.0 ± 0.6	22.8 ± 1.2	2.0 ± 0.4	10.8 ± 1.7	2.1 ± 1.3	0.6 ± 0.1
isoflavone	32.0 ± 6.4[c]	17.8 ± 1.4[c]	18.9 ± 1.9[c]	15.6 ± 3.3[c]	1.7 ± 1.0	9.4 ± 3.5	1.8 ± 0.6	0.7 ± 0.2
cholesterolester								
control	13.9 ± 2.3	1.5 ± 0.2	35.7 ± 6.3	10.0 ± 2.4	0.3 ± 0.1	21.2 ± 5.1	0.8 ± 0.8	2.2 ± 0.6
isoflavone	19.9 ± 4.9[c]	3.9 ± 2.2[c]	36.6 ± 3.9	10.4 ± 2.8	0.3 ± 0.2	14.2 ± 6.5	2.1 ± 1.5	1.5 ± 0.8

[a] Data are presented as means ± SE for 6 or 8 rats in each group. [b] Abbreviations are the same as those in **Table 1**. [c] Significantly different from the corresponding value in rats fed isoflavone-free diet at $P < 0.05$ (Student's *t*-test).

Table 3. Effect of Dietary Soy Isoflavones on Levels of Tissues Eicosanoids in Rats[a]

	aorta	kidney	
groups	6-keto-$PGF_{1\alpha}$ pmol/g tissue	PGE_2 pmol/g tissue	$PGF_{2\alpha}$ pmol/g tissue
control	121 ± 32	50.6 ± 11.1	11.1 ± 1.1
isoflavone	34.4 ± 12.4[c]	23.8 ± 1.7[c]	8.09 ± 0.50*

[a] Data are presented as means ± SE for 6 or 8 rats in each group. [b] Abbreviations are the same as those in **Table 1**. [c] Significantly different from the corresponding value in rats fed isoflavone-free diet at $P < 0.05$ (Student's *t*-test).

Table 4. Effect of Dietary Soy Isoflavones on Fecal Steroids Excretions in Rats[a]

	control	isoflavone
dried fecal weight (mg/d)	796.1 ± 46.4	774.2 ± 48.3
neutral steroid (mg/d)		
coprostanol	6.09 ± 0.46	6.74 ± 0.95
cholesterol	2.84 ± 0.21	3.58 ± 0.26
total neutral steroid	7.91 ± 1.05	10.32 ± 1.06
acidic steroid (mg/d)		
lithocholic	0.97 ± 0.15	1.03 ± 0.10
deoxycholic	0.59 ± 0.08	0.86 ± 0.11
chenodeoxycholic	0.53 ± 0.15	0.52 ± 0.10
hyodeoxycholic	1.86 ± 0.69	2.00 ± 0.44
ursodeoxycholic	0.45 ± 0.06	0.55 ± 0.07
cholic	0.13 ± 0.01	0.33 ± 0.05[c]
α-murichollic	1.24 ± 0.59	1.59 ± 0.35
β-murichollic	0.17 ± 0.06	0.50 ± 0.17
total acidic steroid	5.93 ± 0.78	7.32 ± 0.67
deoxycholic/cholic	4.67 ± 0.55	2.81 ± 0.34
lithocholic/chenodeoxycholic	3.00 ± 1.05	2.27 ± 0.33
total steroids (mg/d)	13.9 ± 0.98	17.6 ± 1.58

[a] Data are presented as means ± SE for 6 or 8 rats in each group. [b] Abbreviations are the same as those in **Table 1**. [c] Significantly different from the corresponding value in rats fed isoflavone-free diet at $P < 0.05$ (Student's *t*-test).

α-hydroxylase in liver. This observation may be associated with the promotion of cholesterol 7 α-hydroxylase activities

Dietary isoflavone significantly decreased serum triglyceride level, although liver triglyceride levels were not changed.

Peluso et al. (*24*) also observed that extracted isoflavone lowered liver triglyceride levels by 33% relative to the isoflavone-free diet in male Sprague−Dawley rats. Thus, dietary soy isoflavones seem to decrease tissue triglyceride levels; however, more studies will be needed to clarify the mechanism of this observation because triglyceride-lowering effect by isoflavones has not been shown in other animal experiments.

Dietary isoflavone lowered the hepatic Δ6 desaturase activity. Reflecting this activity, Δ6 desaturation indices of tissue lipids in the rats fed isoflavone tended to be lower than the Control group. Moreover, the production of eicosanoids from arachidonic acid also decreased by dietary isoflavone. Raso et al. (*25*) showed that various naturally occurring flavonoids inhibit PGE2 production and cyclooxygenase-2 enzyme expression. Similarly, the mRNA level of hepatic Δ6 desaturase also may be modulated by dietary isoflavone. These actions of soy isoflavone may lead to the possibility of inhibition on inflammatory response.

We found that soy isoflavones have antioxidative activity and regulative function of lipid metabolism in this animal experiment using rat. We also found approximately 7.5 ug/mL of absorbed isoflavones in serum when rats were fed the 0.3% isoflavone-added diet (unpublished data). Therefore, these activities of isoflavone may be direct functions of absorbed and metabolized isoflavones, but not indirect functions such as estrogenlike action or some activities accompanying its action, although we did not show exact explanation about this opinion. Thus, the positive intake of isoflavone may reduce the risk of some cardiovasucular disease such as atherosclerosis. More studies using more purified isoflavones and a large number of animals will need to elucidate more exact biological activities of isoflavone to apply to functional foods.

ABBREVIATIONS USED

CE, cholesterylester; GLC, gas liquid chromatography; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low-density lipoprotein; TBARS, thiobarubituric acid reactive substances; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin

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