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Antiatherogenic Effect of Isoflavones in Ovariectomized Apolipoprotein E-Deficient Mice

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The consumption of isoflavone-containing foods such as soybean and soybean products has been reported to have beneficial effects on the cardiovascular system in postmenopausal women. The present study was carried out to examine the mechanism underlying the beneficial effects of isoflavones in apolipoprotein (apo) E-deficient mice subjected to ovarian resection. Compared with sham-operated mice, ovariectomized mice had a larger arterial lesion area in the aortic root. Feeding the ovariectomized mice an isoflavone-containing diet (0.055 mg/kJ of total isoflavones/cal of diet) reduced the size of these lesions more than did feeding them with an isoflavone-free diet. Neither ovariectomy nor diet had a significant effect on the concentration of cholesterol in serum and urinary levels of isoprostanes, which are biomarkers for oxidative stress in vivo. The ovariectomized mice showed a greater increase in mRNA abundance for monocyte chemoattractant protein (MCP)-I in the aorta and in the level of nitric oxide (NO) secreted by peritoneal macrophages in culture than did the sham-operated mice. The isoflavone-containing diet lowered the MCP-I expression and the NO secretion more than did the isoflavone-free diet. These results suggest that dietary isoflavones confer an antiatherogenic effect by preventing the activation of macrophages due to the removal of ovaries.

KEYWORDS: Apolipoprotein E-deficient mice; isoflavone; ovariectomy; monocyte chemoattractant protein-I; nitric oxide; peritoneal macrophages

INTRODUCTION

Postmenopausal women often suffer from cardiovascular diseases due to a lack of estrogen (*1*). Isoflavone-containing foods such as soybean have beneficial effects on postmenopausal women, since isoflavones display estrogenic activity in certain target tissues. Furthermore, isoflavones potentially reduce cardiovascular risk (*2*) by reducing the level of low-density lipoprotein (LDL) cholesterol, through their strong antioxidative characteristics, and because of their adverse thromboembolic effects. In animal experiments on the role of isoflavones in preventing atherosclerosis, inhibitory effects of dietary soy isoflavones on atherosclerosis have been demonstrated not to be dependent on levels of lipoproteins in plasma (*3*–*5*). Furthermore, although the mechanisms by which dietary soy isoflavones inhibit atherosclerosis are unclear, Adams et al. (*4*, *6*) showed that the inhibitory effect in apo E-deficient mice was related to estrogen receptor α-dependent processes. These results suggest that isoflavones have estrogenic activity that ameliorates atherosclerosis.

As reviewed by Anthony (*7*), soy isoflavones have been shown to inhibit tyrosine kinase activity, the expression of cytokines in macrophages, the migration and proliferation of arterial smooth muscle cells, and the activation and aggregation of platelets. Furthermore, soy isoflavones also have strong antioxidative activity (*5*, *8*, *9*). However, it is not yet known which of these effects helps to prevent atherosclerosis.

The purpose of this study was to identify potential mechanisms for the cardioprotective effects of soy isoflavones. Ovariectomized apolipoprotein (apo) E-deficient mice were chosen as an animal model, because treating these mice with estrogen has been demonstrated to lower the arterial lesion development (*10*). Using this model, we have examined the extent of urinary excretion of isoprostanes as an index for oxidative stress (*11*), the expression of monocyte chemoattractant protein (MCP)-1 mRNA in the aorta as an index for monocytic infiltration of the arterial wall (*12*), and the production of nitric oxide by peritoneal macrophages as an index for inducible nitric oxide production, which may contribute to the formation of lesions by increasing oxidative stress in the vessel wall (*13*–*15*). We show that soy isoflavones are absorbed and consequently suppress arterial mRNA expression for MCP-1 and reduce secretion of nitric oxide metabolites by peritoneal macrophages but have no effect on the serum cholesterol concentration and urinary excretion of isoprostanes.

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MATERIALS AND METHODS

Animals and Diets. Apo E-deficient mice purchased from Jackson Laboratory (Bar Harbor, ME) in 1994 were used (*3*). The animals were individually housed in a temperature-controlled room at 22–25 °C with a 12-h light–dark cycle (lights on 0800–2000 h). Food and water were provided every other day, and body weight and food intake were recorded simultaneously. All diets were based on the AIN-93G formulation, as described previously (*3*), except that, as a source of dietary fat, olive oil (100 g/kg diet) was used instead of soybean oil. The basal diet contained (g/kg) casein 200.0, olive oil 100.0, α-corn starch 132.0, sucrose 100.0, cellulose 50.0, vitamin mixture (AIN-93G) 35.0, L-cysteine 3.0, choline bitartrate 2.5, *tert*-butylhydroquinone 0.014, and α-corn starch to 1000 g. Isoflavones were prepared by treating isoflavone-enriched embryonic axis of soybean (Soya-flavone E, Fuji Oil, Osaka, Japan) with 70% ethanol as described previously (*3*). The isoflavones were composed of the following (mol %): 38.1 daidzin, 9.0 genistin, 21.0 glycitin, 2.7 malonyldaidzin, 8.4 malonylgenistin, 0.2 malonylglycitin, 0.6 acetyldaidzin, 0.3 acetylgenistin, 11.6 acetylglicitin, 3.3 daidzein, 1.1 genistein, and 3.7 glycitein. Experiments were carried out under the Guidelines for Animal Experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and Law No. 105 and Notification (No. 6) of the Government of Japan.

In the first experiment, 10 female apo E-deficient mice, 8 weeks old, were divided into two groups, were anesthetized for surgery with an intraperitoneal injection of Nembutal (50 mg/kg body weight), and underwent a bilateral ovariectomy (OVX) or sham ovariectomy (sham OVX). After being fed the basal diet for 9 weeks, the mice were deprived of food for 4 h prior to killing, anesthetized with Nembutal, and killed by withdrawing blood from the left ventricle. The heart and aorta were removed. The blood was transferred into 1-mL microcentrifuge tubes containing 50 mg of butylated hydroxytoluene (BHT). The serum was then separated, bubbled with Ar, and stored at −80 °C.

In the second experiment, 10 female apo E-dificient mice, 8 weeks or 18 weeks old, underwent OVX and were fed the basal diet or the basal diet supplemented with isoflavones (0.055 mg/kJ of total isoflavones/cal of diet) for 9 weeks. A week before being sacrificed, the older mice were placed in a metabolic cage where they were freely given food and water, and their urine was collected in a container containing BHT for 24 h (*16*). The heart and aorta were removed. The urine was used for measuring concentration of isoflavones.

In the third experiment, 20 apo E-deficient mice, 18 weeks old, underwent an ovariectomy or sham ovariectomy and were fed the basal diet or the basal diet supplemented with isoflavones (0.055 mg/kJ of total isoflavones/cal of diet) for 8 weeks. The urine was collected and used to measure concentrations of isoprostanes and nitric oxides. Four days before sacrifice, the mice were injected intraperitoneally with 3 mL of thioglycolate (40 g/L in saline) to harvest the mouse peritoneal macrophages (MPM) from the peritoneal fluid (*17*). The aorta from the root to the iliac bifurcation was immediately isolated and used for the determination of MCP-I mRNA.

Macrophage-Mediated Nitric Oxide Production. The cells ((10–20) × 10^6/mouse) were washed and centrifuged three times with phosphate-buffered saline at 1000 × *g* for 10 min and then resuspended to 10^9 cells/L in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Beth Haemek, Israel) containing 100 mL/L fetal bovine serum (FBS) (heat-inactivated at 56 °C for 30 min), 100 000 U/L penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine. The cell suspension was dispensed into 35-mm plastic Petri dishes and incubated in a humidified incubator (5% CO_2, 95% air) for 2 h at 37 °C. The dishes were washed once with 5 mL of DMEM to remove nonadherent cells, and the monolayer was then incubated under similar conditions for 36 h. The nitrite plus nitrate (NO_2 plus NO_3) concentration in the supernatant was determined as the final metabolites of NO as described previously (*18*).

Determination of Isoprostanes. The purification and measurement of urinary concentration of 8-iso-prostaglandin F2α (8-iso-PGF2α) or 2,3-dinor-5,6-dihydro-8-iso-PGF2α (15-F2t-IsoP-M) was carried out as described (*19*). The isoprostanes in urine were analyzed by GC negative ion chemical ionization MS; GC negative ion chemical ionization MS was performed using a Shimadzu QP5050A GC/MS (Shimadzu, Kyoto, Japan). The quantification of endogenous 8-iso PGF2α or 15-F2t-IsoP-M was accomplished by SIM analysis of the ratio of *m*/*z* 569 to *m*/*z* 573 or *m*/*z* 543 to *m*/*z* 547. A standard curve was constructed by adding various amounts of unlabeled 8-iso-PGF2α to 1 ng of 8-isoPGF2α-d4 or 15-F2t-IsoP-M to 1 ng of 15-F2t-IsoP-M [$^{18}O_2$]. The 15-F2t-IsoP-M [$^{18}O_2$] was kindly donated by Dr. J. D. Morrow (Department of Medicine and Pharmacology, Vanderbilt University, Nashville, TN 37232). The concentration of isoprostanes in urine was expressed as a function of urinary creatinine (Wako Pure Chemicals, Osaka, Japan).

Morphometric Determination of Atherosclerosis. Apo E-deficient mice were perfused with 50 mL of PBS (pH 7.4) and 10% (v/v) neutral formalin-buffered solution, respectively, *via* a cannula inserted into the left ventricle, which allowed unrestricted efflux from an incision in the vena cava. The heart was removed and fixed in the formalin-buffered solution. To determine the cross-sectional area of lesions, hearts containing aortic roots were processed for a quantitative assay as previously described (*16*).

Determination of Aortic Monocyte Chemoattractant Protein-I mRNA Levels by Reverse Transcription Polymerase Chain Reaction. Total RNA from the mouse aorta was extracted and the reverse transcription polymerase chain reaction (RT-PCR) was carried out as previously described (*19*). The total RNA (5 mg) was transcribed into first-strand cDNA using a You-primed cDNA single step kit. The primers for mouse MCP-I were designed as described (*20*). The sequence was 5′-CACCAGCAAGATGATCCCAATG-3′ for the 5′-primer and 5′-AAGGCATCACAGTCCGAGTCACAC-3′ for the 3′-primer. The aortic β-actin mRNA level was measured to confirm a stable extraction from these aortic samples. PCR amplification of MCP-I as well as β-actin was carried out in 10-μL reaction mixtures as described. The amplification products obtained using the two primer pairs contained 357 bp of MCP-I or 254 bp of β-actin. The PCR products on the nitrocellulose filter were hybridized to a ^{32}P-labeled probe of the cloned mouse MCP-I or β-actin. The intensities of these bands were then quantified with a Bioimaging FLA-5000 analyzer (Fuji Photo Film, Tokyo, Japan).

Determination of Isoflavones in Urine. Urine (200 mL) was subjected to enzymatic hydrolysis with β-glucuronidase for 18 h at 37 °C (*11*). The reaction was terminated by adding 10% trichloroacetic acid. Samples were extracted with diethyl ether, and the extracts were dried at room temperature with a stream of nitrogen before dissolution by 70% ethyl alcohol. Isoflavones were separated on a CAPCELL PAC C18 AG120 column (4.6 mm × 250 mm, Shiseido, Tokyo, Japan) using a linear gradient of 15–35% acetonitrile containing a constant 0.1% acetic acid in 50 min, as described by Ni et al. (*3*). The solovent flow rate was 1 mL/min, and the absorption was measured at 260 nm. The instrument used was a Water HPLC system.

Analyses of Serum Lipids. Serum lipid levels were determined using commercially available kits (cholesterol C test, triglyceride G test, and phospholipid B test from Wako Pure Chemicals, Osaka, Japan, and HDL-C·2 from Daiichi Chemicals, Tokyo, Japan).

Statistics. Reported values represent means ± SEM. A statistical analysis was performed with Student's *t*-test or a one-way analysis of variance (ANOVA) followed by LESD to identify significantly different means. Log transformations of raw data were done in cases of unequal variances.

RESULTS

Effect of Ovariectomy. Eight-week-old ovariectomized and sham-operated female apo E-deficient mice were fed the control diet for 9 weeks. Ovariectomized apo E-deficient mice (OVX) had larger lesions in the aortic root than did sham-operated mice (**Figure 1**). However, there were no significant differences in the concentrations of serum cholesterol (544 ± 68 and 676 ±

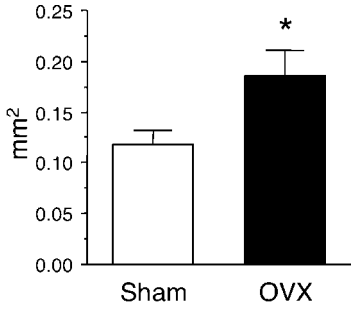

Figure 1. Size of arterial lesions in sham-operated apo E-deficient mice (Sham) or ovariectomized apo E-deficient mice (OVX) fed a control diet. Data are means ± SEM for 5 mice. Asterisk shows a significant difference at $P < 0.05$.

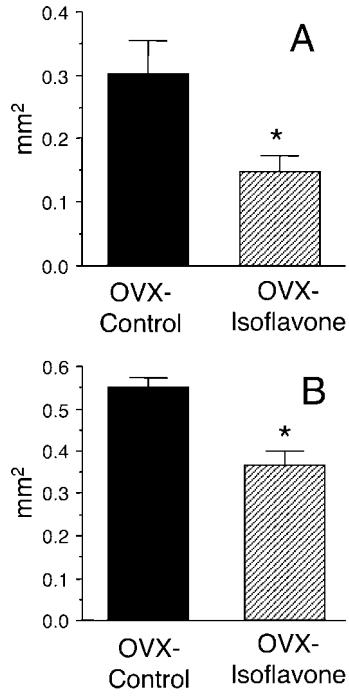

Figure 2. Size of arterial lesions in ovariectomized apo E-deficient mice fed a control diet (OVX-control) or isoflavone-containing diet (OVX-isoflavone): (**A**) eight-week-old mice; (**B**) eighteen-week-old mice. Data are means ± SEM for 5 mice. Asterisk shows a significant difference at $P < 0.05$.

108 mg/dL for sham and OVX group, respectively, $n = 5$), triacylglycerols (137 ± 24 and 253 ± 58 mg/dL, respectively, $n = 5$) and phospholipids (574 ± 67 and 782 ± 146 mg/dL, respectively, $n = 5$).

Effect of Isoflavones in Ovariectomized Mice. Eight-week- and eighteen-week-old ovariectomized female apo E-deficient mice were fed the control diet (OVX-control) and control diet supplemented with isoflavones (OVX-isoflavone) for 9 weeks. Those mice fed the isoflavone containing diet, both the younger and older animal, had smaller lesions than did the corresponding mice fed the control diet (**Figure 2A,B**). There were no significant effect of diets on the concentrations of serum cholesterol, triacylglycerols, and phospholipids in young or old mice (data not shown). The urine from the older mice fed the isoflavone-containing diet contained the following amounts of isoflavones (mmol/g creatine): 236 ± 98 daidzein, 50 ± 18 genistein, and 149 ± 48 glycitein, $n = 5$. The urine from the older mice fed the control diet contained no detectable amounts of isoflavones. These results indicate that the absorption and transport of dietary isoflavones actually occurred under the present experimental conditions.

Table 1. Isoprostane Levels in Urine, NO Production by Cultured Macrophages, and MCP-1 mRNA Abundance in the Aorta in Sham-Operated Apo E-Deficient Mice Fed a Control Diet (Sham-Control), Ovariectomized Apo E-Deficient Mice Fed a Control Diet (OVX-Control), or Ovariectomiced Apo E-Deficient Mice Fed an Isoflavone-Containing Diet (OVX-Isoflavone)[a]

	Sham-Control	OVX-Control	OVX-Isoflavone
	Isoprostane Level in Urine (ng/mg Creatine)		
8-iso $PGF_{2\alpha}$	0.43 ± 0.06	0.76 ± 0.27	0.53 ± 0.08
2,3-dinor	9.93 ± 2.56	8.42 ± 1.66	10.6 ± 0.08
	NO Levels in Culture Medium (μmol/10^6 cells)		
	1.89 ± 0.22 a	3.50 ± 0.45 b	2.00 ± 0.14 a
	MCP-1 mRNA (% to Sham-Control)		
	100 ± 7.5 a	179 ± 10.6 c	151 ± 8.7 b

[a] Data are means ± SEM for 6–7 mice. Means not sharing a common letter are significantly different at $P < 0.05$.

Mechanism for Antiatherogenic Effect of Isoflavones. Eighteen-week old ovariectomized (OVX) or sham-operated (Sham) female apo E-deficient mice were fed the control diet or isoflavone-containing diet for 8 weeks. There was no significant difference in the concentrations of the urinary isoprostanes (8-iso PGF2α and 15-F2t-IsoP-M) between sham-operated mice fed the control diet (Sham-control), ovariectomized mice fed the control diet (OVX-control), and ovariectomized mice fed the isoflavone-containing diet (OVX-isoflavone) (**Table 1**).

The concentration in the culture medium of the NO_2/NO_3 produced by the macrophages prepared from the OVX-control was higher than that produced by the macrophages of the Sham-control. Increased production of NO_2/NO_3 by macrophages from the OVX-control was lowered by the macrophages from the OVX-isoflavone (**Table 1**).

The level in the aorta of the MCP-1 mRNA expression from the OVX-control was higher than that of the Sham-control. The OVX-isoflavone had a lower level of the MCP-I mRNA than did the OVX-control (**Table 1**).

DISCUSSION

Consistent with a previous study (*10*), ovariectomized apo E-deficient mice developed larger lesions in the aortic root than did sham-operated mice. These results indicate a role for estrogen in protection from the development of atherosclerosis (*9*), because exogenous estrogen clearly inhibits lesion progression in numerous animal models of atherosclerosis including apo E-deficient mice (*10*, *21*, *22*). Ovariectomy was expected to result in altered serum lipid levels in comparison with the sham operation. However, this was not the case in the present experiment. Accordingly, it is likely that a lack of estrogen due to the ovariectomy induced the development of atherosclerotic lesions through a mechanism other than changes in the serum lipids levels.

In subsequent experiments, ovariectomized apo E-deficient mice were fed isoflavones derived from the embryonic axis of soybean because isoflavones, in particular genistein (*23*) and equal (bacterial metabolite of daidzein) (*24*, *25*), exert estrogenic actions, thereby ameliorating the development of atherosclerotic lesions in mice (*6*), rabbits (*5*) and female ovariectomized apo E-deficient mice (*4*). In the present study, dietary isoflavones reduced the size of lesions in the aortic root from the ovariectomized young (17 weeks old) and older (27 weeks old) mice.

These beneficial effects appear to be due to the isoflavones absorbed because the dietary consumption was reflected in an elevation in the urinary concentrations of these isoflavones (genistein, daidzein, and glycitein), whereas there was no detectable amount of isoflavones in the urine from ovariectomized mice fed the control diet. According to Holder et al. (*26*), once absorbed soy isoflavones circulate as glucuronidated or sulfated metabolites in rat blood (*26*). Dietary isoflavones, however, had no significant effect on serum lipid concentrations, indicating that the antiatherogenic effect of isoflavones are independent of serum lipid concentrations. Since the isoflavones mixture used in the present study was quite complex, it was difficult to assign which isoflavone species was particularly responsible for the beneficial effect.

According to our previous results (*3*), dietary isoflavones derived from soy protein isolate were not effective to lower atherosclerosis development in apo E-deficient male and female mice without ovariectomy. We have also done a preliminary experiment using testis-removed apo E-deficient mice to examine the effect of dietary isoflavones on the lesion development (unpublished observation), because testosterone is peripherally converted to estrogen. In contrast to the ovariectomy, there was no significant effect of the testis removal on the lesion development and dietary isoflavones resulted in no significant effect on the lesion development. Based on these results, we have used ovariectomized female apo E-deficient mice to examine an underlying mechanism of beneficial effect of isoflavones.

The present study showed that ovariectomy resulted in increased production of NO metabolites by peritoneal macrophages, which were incubated for 24 h. The elevated levels of NO metabolite production were in the apo E-deficient mice fed the isoflavone-containing diet. The inhibitory action appears to be due to the isoflavones absorbed since it is reported that genistein and daidzein inhibit IFN-γ plus lipopolysaccharide (LPS)-induced NO production in RAW 264.7 macrophages (*27*). The NO metabolites are likely derived from the actions of iNOS since activated macrophages generate large amounts of NO from L-arginine by the actions of iNOS (*28, 29*). Overproduction of NO has been associated with oxidative stress (*30*) and chronic inflammation (*31*). However, under these conditions, an increase in the secretion of urinary isoprostanes, oxidative markers in vivo, did not accompany enhanced production of NO in peritoneal macrophages. Alternatively, iNOS-deficient apo E-deficient mice had fewer atherosclerotic lesions than did iNOS-intact apo E-deficient mice (*32*). Therefore, it is likely that suppressing iNOS-induced production of NO by dietary isoflavones may result in less atherosclerotic development in apo E-deficient mice.

The present study showed that ovariectomy resulted in increased levels of MCP-1 mRNA in the aorta, but the increase was lowered in the apo E-deficient mice fed the diet containing isoflavones. It has been reported that the administration of estrogen to ovariectomized rabbits resulted in reduced MCP-1 mRNA expression in thoracic aorta than that seen in untreated ones (*33*). MCP-1 is a cc-chemokine and plays a key role in atherosclerosis by recruiting monocytes and T-cells into the arterial wall (*34*). Furthermore, mice deficient in MCP-1 receptor CCR-2 have smaller atherosclerotic lesions, as well as fewer macrophages and monocytes in their aorta (*35*). Therapeutic drugs and dietary factors targeting MCP-1 or its receptor have proven useful in the prevention of atherosclerosis. Therefore, it is considered that once absorbed, isoflavones regulate MCP-1 expression in aortic endothelial cells through estrogen receptor α, thereby preventing monocytes from infiltrating the arterial wall. In fact, it is known that the atheroprotective effect of dietary soy isoflavones in apo E-deficient mice requires the presence of estrogen receptor a (*4*), and genistine and daidzein dose dependently down-regulate the secretion of MCP-1 induced by TNF-α in human umbilical vein endothelial cells (*27*).

In summary, the present investigation shows that there are at least two mechanisms, a down regulation of iNOS expression induced by activated macrophages and suppressive expression of MCP-1 in the arterial endothelial cells, by which dietary isoflavones prevent atherosclerosis in apo E-deficient mice in vivo.

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