Effects of Phytoestrogens on Acetylcholine- and Isoprenaline-Induced Vasodilation in Rat Aorta

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ABSTRACT—The influence of the phytoestrogen, isoflavones, on vasodilating responses of the thoracic aorta precontracted with norepinephrine, together with the stimulatory effect on uterine weight (uterotrophic effect), was investigated in ovariectomized rats. In comparison with intact rats, acetylcholine (ACh)-induced vasodilation showed a tendency to be decreased by ovariectomy. On the other hand, isoprenaline (ISO)-induced vasodilation was significantly increased by ovariectomy. Estrogen replacement (17 β -estradiol dipropionate, 300 μ g/kg per week, for 1 month) completely restored the impaired ACh- and ISO-induced vasodilation caused by ovariectomy. Dietary isoflavone aglycones (containing 52% genistein, 42% daidzein and 6% glycitein) of 157 mg/kg per day (not 67 mg/kg per day) for 1 month, in addition to the effects of estrogen replacement, completely restored the impaired vasodilation caused by ovariectomy. However, the uterotrophic effect of dietary isoflavones of 157 mg/kg per day was incomplete as compared with that by estrogen replacement. These results indicate that phytoestrogen, isoflavones, certainly possess estrogenic actions on the vasodilating responses caused by ACh and ISO, as well as a weaker uterotrophic effect.

Keywords: Isoflavones (genistein and daidzein), Estradiol, Acetylcholine, Isoprenaline, Rat aorta

After menopause, the risk for cardiovascular diseases is promoted by some metabolic and vascular changes, which may be partly due to estrogen deficiency. There are many reports that estrogen replacement therapy reduces cardiovascular disease mortality in post-menopausal women (1, 2). Evidence for the influences of estrogen on cardiovascular diseases is partly derived from the alterations in vascular reactivity. High levels of circulating estrogen have been reported to modulate the vasoactive reagents-induced responses. Acetylcholine (ACh)-induced endothelium-dependent vasodilations are increased during the late stage of pregnancy in guinea pig mesenteric artery (3) and rat aorta (4), and it has also been found in 17 β -estradiol (E₂)-treated rat aorta (5) or rabbit femoral artery (6). Furthermore, we have reported that the relaxing response to isoprenaline (ISO) was suppressed by a short-term treatment with E₂ in rat aorta (7). These evidences indicate that the agonists-induced vasodilation is certainly affected by endogenous or exogenous estrogens.

It is known that phytoestrogens with nonsteroidal structures such as isoflavones are naturally-occurring plant compounds, which bind to estrogen receptor (ER) and

exhibit weak estrogen-like biological activity (8). The isoflavones, genistein and daidzein, are present in high concentrations in soybean products (9). Recently, their components in phytoestrogens are believed to be important for the prevention of cardiovascular diseases, reproductive cancers and osteoporosis (8, 10, 11). In the vasoprotective actions of phytoestrogens, a study of cholesterol-fed rabbits showed that isoflavone aglycone attenuates atherosclerosis development by inhibiting the oxidation of low density lipoprotein (12). Furthermore, a soy diet enhanced the dilating response induced by ACh in atherosclerotic coronary arteries of female rhesus monkeys (13). Genistein supplement improved the endothelial dysfunction induced by ovariectomy in rats through increased nitric oxide synthase (NOS) activity (14). These effects of phytoestrogens mimicking the actions of endogenous estrogen may contribute to their vasoprotective effects.

ER has been reported to exist as two subtypes, the “classical” estrogen receptor ER α and the novel ER β , in rat aorta (15) and other tissues (16). The physiological roles of each ER are not clear at present, but the uterus clearly expressed the messenger RNAs (mRNAs) of both ER subtypes, and the predominant ER subtype in the uterus was ER α (16). Mäkelä et al. demonstrated that the expression of ER β mRNA increased after endothelial denudation

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of carotid artery in female rats (17). Genistein showed 20-fold higher binding affinity to ER β than to ER α (18). Furthermore, isoflavones in soy protein improved cardiovascular risk factors without deleterious effects on the reproductive system of monkeys (19). These results suggest that ER β plays a pivotal role in vasoprotection and that phytoestrogen, isoflavones, may be valuable compounds for estrogen-based drug therapy for cardiovascular diseases. However, it is not clear whether isoflavones exhibit estrogen-like actions on the relaxing responses to ACh and/or ISO and whether they accompany modulated vascular responses with a risk of affecting the reproductive system.

With these experimental backgrounds, we examined whether or not phytoestrogens have estrogen-like actions on the vascular reactivity of ACh and ISO and the uterine weight.

MATERIALS AND METHODS

Animals and treatment with estrogen

All procedures were carried out in accordance with the institutional guidelines for animal research of Tokyo University of Pharmacy and Life Science. Female Wistar-Imamichi strain rats (150–180 g) were purchased from the Institute for Animal Reproduction (Ibaraki). Rats were divided into 5 groups: 1) Estrous intact rats, 2) Ovariectomized (OVX) rats, 3) OVX rats with estrogen replacement, 4) and 5) OVX rats with different doses of isoflavones supplement. After ovariectomy, the rats were immediately injected with 17 β -estradiol dipropionate (EDP) (300 μ g/kg, s.c.). Then the rats were injected with it once a week for 3 weeks (totally 4 times in 4 weeks), and the samples were isolated 1 week after the final injection.

Isoflavones and diet

The isoflavone aglycones in SoyAct™, which was provided by Kikkoman Corporation (Chiba), consist of 52% genistein, 42% daidzein and 6% glycitein. The OVX rats orally received a diet containing SoyAct™. The rats were fed with isoflavone aglycones of 67 mg/kg body weight (BW) per day or 157 mg/kg BW per day for 1 month. The rats fed with 67 mg/kg BW per day and 157 mg/kg BW per day were designated as groups I-67 and I-157, respectively. In our preliminary study, we chose two doses of isoflavone aglycones; a lower dose had no influence on uterine weight and a higher dose increased it.

Preparation of aorta

All animals were anesthetized with ether and euthanized by exsanguination. Blood samples were collected from the femoral artery and the serum was frozen (–80°C) for analyzing the E₂ levels. The thoracic aortae were isolated and cleaned of the adherent fat and connective tissue

in modified Krebs-Henseleit solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 11 mM glucose at 37°C, gassed with 95% O₂ and 5% CO₂. The aortic preparations were cut into rings of about 5-mm-long, and 5 ring preparations were obtained from each animal.

Measurement of isometric tension

The relaxing responses were measured by suspending the rings between two stainless-steel hooks, one of which was attached to the end of a bathing tube and the other to a force transducer (45196A; NEC San-ei Instruments, Inc., Tokyo). The isometric tension changes were recorded on a polygraph (LECT-HORIZ-8K; NEC San-ei Instruments, Inc.). The resting tension was 0.8 g, which was the optimal preload for force development in these blood vessels, as previously described (20). For the relaxing response studies, the submaximal tone was induced with 6×10^{-7} M norepinephrine (NE) and then ACh or ISO was added in a cumulative fashion. Relaxation in response to them was expressed as the percent decrease from the NE-induced tension.

Radioimmunoassay (RIA)

Levels of E₂ in peripheral serum were determined by double-antibody RIA systems using ¹²⁵I labeled radio-ligands. The labeled steroid was oestradiol-6-(*O*-carboxymethyl) oximino-2-[¹²⁵I]iodohistamine (IM135), which was purchased from Amersham Pharmacia Biotech (Tokyo). Antisera against E₂ (GDN 244) were used (21). The RIA techniques used for determination of E₂ were the same as the procedures previously described (22). The intra- and inter-assay coefficients of variation were 6.2 and 7.4% for E₂, respectively.

Drugs

Norepinephrine bitartrate, isoprenaline bitartrate, sodium nitroprusside dihydrate (Sigma Chemical Co., St. Louis, MO, USA) and acetylcholine chloride (Daiichi Pharmaceutical Co., Tokyo) were dissolved in distilled water. 17 β -Estradiol dipropionate (Sigma) was dissolved in sesame oil (Miyazawa, Tokyo). Genistein was obtained from the Laboratory of Medical Plant Science, Tokyo University of Pharmacy and Life Science. The other chemicals were of analytical grade and obtained from Wako Pure Chemical Co. (Osaka).

Data analyses

Values were expressed as the mean $\pm$ S.E.M. When the dose-response curves were compared among the groups, the statistical significance was assessed by an analysis of variance (ANOVA) for repeated measures followed by

the Tukey test. The differences were considered significant for $P < 0.05$.

RESULTS

Comparisons of isoflavones and estrogen supplement for physiological characteristics

In OVX rats, the growth rate was larger than that of intact rats, but EDP or I-157 supplement to OVX rats markedly inhibited the increased growth rate. Changes in the increased body weight depended on food consumption. EDP or I-157 (not I-67) supplement to OVX rats increased uterine wet weight per animal, and EDP completely restored the uterine weight to the levels in intact rats. No significant differences were seen in the levels of serum E_2 between isoflavones-treated OVX rats (I-67 or I-157) and OVX rats (Table 1). The concentrations in EDP-treated OVX rats were similar to those in proestrous rats (54.8

± 3.4 pg/ml).

Effects of isoflavones and estrogen supplement on ACh-induced vasodilation in OVX rats

In this study, the tensions induced by NE (6×10^{-7} M) were 155 ± 8 and 114 ± 8 mg in intact and OVX rat aortic rings, respectively ($P < 0.01$). The tensions induced by NE were 137 ± 11 and 116 ± 8 mg in I-67- and I-157-treated OVX rat aortic rings, respectively. In agreement with Miller and Vanhoutte (23), the NE-induced contraction in EDP-treated OVX rats (151 ± 8 mg) was greater than that in OVX rats ($P < 0.05$).

The endothelium-dependent vasodilation by ACh (10^{-9} – 3×10^{-6} M) showed a tendency to decrease in OVX rats, compared with the response in intact rats, although it was not significant. Supplement with EDP (Fig. 1A, Table 2) and I-157 (Fig. 1B, Table 2) restored the decreased vasodilating response induced by ovariectomy to the levels in

Table 1. Physiological characteristics in isoflavones and estrogen supplement

Groups	GR (g)	FC (g/day)	UW (mg/animal)	Serum E_2 (pg/ml)
Intact	$60 \pm 4^{**}$	14 ± 0.2	$420 \pm 39^{**}$	19.6 ± 2.6
OVX	103 ± 5	16 ± 0.7	112 ± 23	19.3 ± 2.0
OVX + EDP	$37 \pm 4^{**}$	$12 \pm 0.4^{**}$	$417 \pm 37^{**}$	$67.1 \pm 14.6^{**}$
OVX + I-67	88 ± 5	15 ± 0.8	130 ± 10	14.9 ± 3.5
OVX + I-157	$43 \pm 6^{**}$	$12 \pm 0.4^{**}$	$317 \pm 32^{**}$	13.7 ± 1.7

OVX rats were subcutaneously injected with EDP ($300 \mu\text{g/kg}$ per week) for 1 month. OVX rats were fed with 67 mg/kg per day or 157 mg/kg per day of isoflavones for 1 month. They were defined as I-67 and I-157, respectively. GR: growth rate, FC: food consumption, UW: uterine wet weight. Each value represents the mean $\pm$ S.E.M. from 5 to 10 rats. $^{**}P < 0.01$, from OVX.

Fig. 1. Effects of EDP (A) and isoflavones (B) on ACh-induced vasodilation in aortic rings from OVX rats. OVX rats were subcutaneously injected with EDP ($300 \mu\text{g/kg}$ per week) for 1 month. OVX rats were fed with 67 mg/kg per day or 157 mg/kg per day of isoflavones for 1 month. They were defined as I-67 and I-157, respectively. Each value represents the mean $\pm$ S.E.M. from 6 to 10 rats.

Table 2. EC_{50} and E_{max} to ACh and ISO in isoflavones and estrogen supplement

Groups	EC_{50}		E_{max}	
	ACh	ISO	ACh	ISO
Intact	7.64 ± 0.09	7.74 ± 0.06	98.1 ± 1.7	$71.7 \pm 2.1^{**}$
OVX	7.48 ± 0.10	7.75 ± 0.04	96.9 ± 2.5	81.5 ± 2.2
OVX + EDP	$7.74 \pm 0.06^*$	7.70 ± 0.06	99.6 ± 3.5	$74.0 \pm 2.4^*$
OVX + I-67	7.61 ± 0.09	7.65 ± 0.06	95.3 ± 2.2	83.5 ± 2.6
OVX + I-157	$7.76 \pm 0.07^*$	$7.59 \pm 0.04^{**}$	$104.1 \pm 1.7^*$	$71.9 \pm 3.4^*$

EC_{50} are expressed as pD_2 values, maximal relaxation (E_{max}) are expressed as percent precontraction with NE. Each value represents the mean $\pm$ S.E.M. from 6 to 10 rats. $^*P < 0.05$, $^{**}P < 0.01$, from OVX.

intact rats, but I-67 had no influence on it. Both the values of EC_{50} and the maximal relaxation were significantly increased in I-157-treated OVX rats compared with OVX rats (Table 2). In an additional study, we investigated the effects of a short-term treatment with genistein (10 mg/kg per day, s.c., for 1 week) on ACh-induced vasodilation using OVX rats. The effects of genistein treatment on ACh-induced vasodilation were not observed (data not shown).

Effects of isoflavones and estrogen supplement on ISO-induced vasodilation in OVX rats

ISO-induced vasodilation (10^{-9} – 3×10^{-6} M) was significantly increased by ovariectomy, compared with that in intact rats, and EDP supplement to OVX rats completely restored the increased vasodilating response induced by ovariectomy to the levels in intact rats (Fig. 2A, Table 2). I-157 supplement also completely restored it to those in intact rats. However, I-67 supplement did not affect ISO-

induced vasodilation, compared with the response in OVX rats (Fig. 2B, Table 2). The effects of a short-term treatment with genistein on ISO-induced vasodilation were not observed, either (data not shown).

DISCUSSION

In the present study, we used OVX rats to investigate pure actions of phytoestrogens on vasodilating responses and uterotrophic effects without the modulation of endogenous ovarian hormones, because it has been reported that the consumption of soymilk containing >200 mg of isoflavones daily for 1 month effectively reduced the circulating levels of E_2 and increased menstrual cycle length in premenopausal women (24). Dietary I-67 and I-157 did not change the levels of serum E_2 , compared with those in OVX rats. There was no significant difference in the levels of serum E_2 between estrous and OVX rats, suggesting that

Fig. 2. Effects of EDP (A) and isoflavones (B) on ISO-induced vasodilation in aortic rings from OVX rats. OVX rats were subcutaneously injected with EDP (300 μ g/kg per week) for 1 month. OVX rats were fed with 67 mg/kg per day or 157 mg/kg per day of isoflavones for 1 month. They were defined as I-67 and I-157, respectively. Each value represents the mean $\pm$ S.E.M. from 6 to 10 rats.

both of these E_2 levels were derived from the adrenal gland. Supplement with EDP and a high dose of isoflavones, I-157, exhibited a stimulatory effect on uterine weight, and the potency of I-157 was less than that of EDP supplement. On the other hand, I-67 did not show any uterotrophic effect at all. The effects of supplement with EDP and I-157 were the same on the growth rate and food consumption. Based on these physiological characteristics of phytoestrogens, we investigated their influence on the endothelium-dependent vasodilation in isolated aortic preparations. ACh-induced vasodilation was somewhat decreased in 4 weeks after ovariectomy, but ISO-induced vasodilation was significantly increased. Estrogen replacement by EDP supplement completely restored these modulated vasodilating responses. Although these opposite effects of ACh- and ISO-induced vasodilation induced by ovariectomy are not clear, it is suggested that ovarian steroid hormones may be associated with the regulation of vascular tone (2, 25, 26). High-dose isoflavones, I-157, also completely restored these modulated vascular responses, but not I-67, meaning that high-dose isoflavones, which have a sufficient stimulatory effect on uterine weight, exhibit estrogenic actions on ACh- and ISO-induced vasodilation. However, the uterotrophic effect of I-157 was less than that of EDP supplement. These results suggest that phytoestrogens may exert a greater influence on the vascular system than on the reproductive system. This specificity of isoflavones may be explained by the expression levels of ER subtypes in different tissues and by the binding affinity of phytoestrogens to ER subtypes. That is, genistein shows a higher binding affinity to $ER\beta$ than to $ER\alpha$, while E_2 does not differentiate between the two receptors (18). The $ER\alpha$ mRNA is highly expressed in the uterus (16). The present study is the first observation showing that actions of phytoestrogens affect the regulation of vascular tone rather than uterotrophic effects.

The mechanisms for the vasodilating response to ACh increased by estrogen have been suggested to be mainly associated with an increase in NO release stimulated by ACh (25, 27). It has been reported that E_2 rapidly stimulates NOS activity in endothelial cells in a nongenomic manner, and the activation is antagonized by ER antagonists such as tamoxifen and ICI 182,780 (28, 29). Recently, genistein has also been suggested to cause acute NO-dependent vasodilation in the forearm vasculature of human subjects with a similar potency to E_2 (30). However, a long-term treatment with estrogen induces the expression of some target genes (including NOS) (29, 31). In the present study, the modulation induced by treatment with estrogen including phytoestrogens for 1 month in ACh-induced vasodilation may not be contributable to its rapid actions. The endothelium-independent vasodilation by sodium nitroprusside was hardly affected by ovariectomy and/or addi-

tional EDP or isoflavones (EC_{50} : Intact, 8.02 ± 0.05 ; OVX, 7.91 ± 0.07 ; OVX + EDP, 7.95 ± 0.05 ; OVX + I-67, 8.05 ± 0.04 ; OVX + I-157, 7.94 ± 0.08), suggesting that estrogen does not alter the sensitivity of vascular smooth muscle to exogenous NO.

The mechanism for the relationship between β -adrenoceptor-mediated vasodilation and estrogen is not clear. However, it has been reported that isoprenaline-induced vasodilation of rat aorta may involve the cytochrome P450 (CYP) pathway (7, 32). We have also observed that the levels of CYP2C11 mRNA expression in rat aorta were modulated by estrogen (K. Yamaguchi et al., unpublished data). It has been reported that the members of the CYP2C gene family synthesize both epoxyeicosatrienoic acids and 20-hydroxyeicosatetraenoic acid from arachidonic acid (33, 34). Indeed, those CYP2C-derived products cause the vasodilating (35) and contractile response (36), respectively. The mechanisms for the vasodilating response to ISO decreased by estrogen may be partly associated with an alteration in the amounts of CYP2C-derived products. Furthermore, we have observed that the actions of E_2 on ISO-induced vasodilation were almost antagonized by ICI 182,780 (K. Yamaguchi et al., unpublished data). Therefore, ER may mediate the decrease in β -adrenoceptor-mediated vasodilation by estrogen and phytoestrogens.

Furthermore, we studied the effects of a short-term treatment with genistein on the vascular system and the reproductive system. Administration of genistein (10 mg/kg per day, s.c.) for 1 week to OVX rats exhibited a slight uterotrophic effect (OVX: 100 ± 2 mg vs OVX + genistein: 136 ± 12 mg, $n = 4$, $P < 0.05$). However, there was no significant difference in ACh- and ISO-induced vasodilation between OVX rats and genistein-treated OVX rats (data not shown). Therefore, in order to observe the influence of estrogenic effects on vasodilating responses, phytoestrogens may need to be administered over a long period of time.

Our findings indicate that phytoestrogens certainly possess estrogenic actions on the vasodilating responses to ACh and ISO and exhibit a higher sensitivity for the vascular system than the reproductive system. These results suggest that the estrogenic effects of isoflavones on vasodilating responses may contribute to one of their vasoprotective actions.

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