Resveratrol Attenuates Ovariectomy-Induced Hypertension and Bone Loss in Stroke-Prone Spontaneously Hypertensive Rats

Kenichi Mizutani, 1 Katsumi Ikeda, 2 Yasuhiro Kawai, 3 and Yukio Yamori 1

1Life Science, Environmental Conservation and Development, 2Otsuka Department of International Preventive Nutritional Medicine, Graduate School of Human and Environmental Studies, Kyoto University, Kyoto 606-8501, and 3Health Care R & D Department, Research Division, Sunstar Incorporated Company, Osaka 569-1044, Japan

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Summary

We examined the effect of resveratrol (3, 4', 5-trihydroxy stilbene), a phenolic compound found in the skins of most grapes, on blood pressure and bone loss in ovariectomized (OVX), stroke-prone spontaneously hypertensive rats (SHRSP). Nineteen-week-old female SHRSP were divided into a sham-ovariectomized (sham) group fed a control diet and two OVX groups fed either a control diet (OVX-Cont) or a diet supplemented with resveratrol (5 mg/kg per d; OVX-Resv). Ovariectomy induced significant increases in systolic blood pressure (SBP). Resveratrol lowered the SBP by 15% by the third week of administration, and this effect was maintained throughout the study. Resveratrol treatment also significantly enhanced endothelium-dependent vascular relaxation in response to acetylcholine (ACh) in OVX rats. Finally, femur breaking energies measured for the resveratrol-treated (OVX-Resv) group were significantly higher than those of the resveratrol-untreated (OVX-Cont) group. While no significant differences in calcium, magnesium and phosphorus content were found between the femurs of OVX-Cont and OVX-Resv rats, the femur hydroxyproline content in the OVX-Resv group was significantly higher than of the OVX-Cont group. We conclude that, in OVX-SHRSP, resveratrol acts by a similar mechanism to mammalian estrogens, lowering blood pressure by increasing dilatory responses to ACh. The present study also demonstrated that resveratrol was able to prevent ovariectomy-induced decreases in femoral bone strength.

Key Words: stroke-prone spontaneously hypertensive rats, phytoestrogens, hypertension, osteoporosis, ovariectomy

Women of reproductive age are known to have a lower risk of coronary heart disease (CHD) than men (1–3). Following loss of ovarian function, postmenopausal women display an increased incidence of hypertension and CHD (4), and this effect appears to be reversed by estrogen replacement therapy (5, 6). Hypertension develops more rapidly and is more severe in males than in females in genetic and other hypertensive animal models (7, 8). Estrogen deficiency after menopause is also associated with osteoporosis, being the most common cause of age-related bone loss. A sharp decrease in ovarian estrogen production is the predominant cause of rapid bone loss during the first decade after menopause (9). Taken together, these findings suggest that estrogen supplementation may delay both hypertension and bone loss.

Some women are reluctant to take estrogen replacements because of potentially increased cancer risks (10). It would be useful to identify a natural dietary substance that minimizes both vascular diseases and bone loss attributable to loss of ovarian function in postmenopausal women without significantly increasing the risk of estrogen-dependent cancers. Diets containing phytoestrogens have been shown to reduce the risk of cardiovascular disease and osteoporosis in both humans and nonhuman primates (11). Resveratrol (3,4',5-trihydroxystilbene), a phenolic compound found in the skins of most grapes (12, 13), is structurally similar to 17β-E2 and diethylstilbestrol (DES), and has estrogenic activity (14). Several biological actions of resveratrol have been reported. These include antioxidant effects on low-density lipoprotein cholesterol (15) which are expected to prevent atherosclerotic changes, attenuation of platelet aggregation via inhibition of the metabolism of arachidonic acid (16), and vasorelaxing activity in the isolated rat aorta (17).

In the present study, we examined the effect of resveratrol on blood pressure and bone metabolism using ovariectomized (OVX), stroke-prone spontaneously hypertensive rats (SHRSP), which have previously been shown to serve as an animal model of postmenopausal hypertension and osteoporosis (18).

MATERIALS AND METHODS

Materials. Eighteen 18-wk-old female SHRSP/Izm rats were obtained from the Disease Model Co-operative Research Association (Kyoto, Japan). At 19 wk of age, bilateral ovariectomy was performed by the dorsal ap-
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The rats were divided into a sham-ovariectomized (sham) group fed a control diet (Funahashi SP diet, Funahashi Farm, Chiba, Japan) and two OVX groups fed either a control diet (OVX-Cont) or a diet supplemented with resveratrol (5 mg/kg per d; OVX-Resv). All animals were housed in cages in groups of six. Body weight (BW) and blood pressure were checked before assignment to groups to ensure homogeneity of weight and blood pressure in each group. The temperature was maintained at 23 ± 1 °C and animals were subjected to a 12-h light-dark cycle. Systolic blood pressure (SBP) was measured without anesthesia by a photoelectric oscillometric tail-cuff method (UR-1000, Ueda, Tokyo, Japan). BW was checked once a week. At the end of the eighth week of the feeding period, all rats were sacrificed under diethyl ether anesthesia. Hearts, brains, uteri, thoracic aortae and femurs were carefully removed. Weights were recorded for each organ, and aortic rings were prepared as described below.

Preparation of aortic rings and tension recordings. We further examined to determine whether resveratrol treatment augments vasodilation in OVX-SHRSP by NO-mediated mechanisms. Thoracic aortae were carefully removed protecting the endothelial lining, cleared of adhering fat and connective tissues, and cut into 3-mm-wide transverse rings. Aortic rings were placed in a physiological saline solution of the following composition (mM): NaCl, 120; KCl, 4.7; MgSO4, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25; and glucose, 10. Saline solution was aerated with a 95% O2-5% CO2 mixture and maintained at 37 °C and pH 7.4. To investigate mechanical responses, each ring was suspended in an organ bath and subjected to an initial load of 2.5 g. Contractions were recorded isometrically via a force-displacement transducer (Nihondenki Sanei, Tokyo, Japan) and recorded on a polygraph (Nihondenki Sanei). Following 2 h equilibration, submaximal contraction of aortic rings was induced with prostaglandin (PG) F2α (10−5 M). Increasing doses of acetylcholine (ACh) were added to the bath to assess the integrity of the endothelium. Only preparations that showed relaxation in response to ACh were used for further experiments. To assess the basal release of nitric oxide (NO) from PG F2α precontracted aortic rings, responses to 10−4 M Nω-nitro-L-arginine methyl ester (l-NAME) acetate were evaluated. Data were expressed as the percentage of relaxation or contraction relative to the control.

Mechanical bone strength analysis. To determine femur mechanical strength, the three-point bending test was performed at a constant test speed of 10 mm/min using a material testing machine (EZ-test, Shimadzu, Kyoto, Japan) (20). Load-deformation curves were read into a computer using an analytical software program (WinAGS Lite, Shimadzu), and load values for each strain increment of one percent were used to derive stress-strain curves. The breaking load was read from the stress-strain curves as the values at the breaking point for each specimen. The breaking energy (J) was calculated as the area under the stress-strain curves up to the breaking point.

Calcium (Ca), phosphorus (P), magnesium (Mg) and hydroxyproline (Hyp) analysis in femur. After mechanical bone strength measurements, all femurs were lyophilized for 72 h. Right femurs were then dissolved in 6 N HCl and hydrolyzed at 120 °C for 24 h. Hydrolysates were analyzed in an amino acid analyzer. Left femoral bones were dry ashed at 650 °C for 48 h, dissolved in 1 N HNO3 and analyzed by ICP-atomic emission spectrometry.

Statistics. Results are presented as the mean ± SD. Differences between the means were calculated using one-way analysis of variance (ANOVA) and Fisher’s PLSD. Probability values less than 0.05 were considered significant.

RESULTS

Food intake and body weight

Food intake in the OVX-Cont and OVX-Resv groups was higher than that in the sham group. No significant differences in food intake were observed between the OVX-Cont and OVX-Resv groups. Ovariectomy increased BW by 7–17%. However, treatment with resveratrol did not alter BW in OVX-SHRSP.

Blood pressure and heart and brain weights

Figure 2 shows SBP values for each group over the 8-wk feeding period. Ovariectomy induced a significant increase in SBP. However, resveratrol lowered the SBP by 15% by the third week, and this effect was maintained throughout the study. Heart and brain weights, expressed as a percentage of BW (Table 1), were signifi-
cantly lower in the OVX-Resv group than in the OVX-Cont group (p<0.05).

**Relaxation in response to ACh in aortic rings**

ACh induced concentration-dependent relaxation of endothelium-intact aortic rings precontracted with PG Flα. As shown in Fig. 3, aortic rings from the OVX-SHRSP group responded poorly to ACh (maximum relaxation=40.8±2.49%) as compared to the sham-SHRSP group (maximum relaxation=50.4±9.76%). Resveratrol treatment significantly improved endothelium-dependent vascular relaxation responses to ACh as compared to the OVX-Cont SHRSP group.

**Basal NO release from aortic rings**

In studies designed to assess the tone-related release of NO from PG Flα-precontracted aortic rings, responses to L-NAME were evaluated in aortic rings (22, 23) obtained from the three groups. The addition of increasing concentrations of the NO synthesis inhibitor L-NAME (10⁻³-10⁻⁵M) elicited vasoconstriction in a concentration-dependent manner (data not shown). Significantly higher contractile responses (10⁻⁴M L-NAME) were observed in tissues from the OVX-Resv group as compared to the OVX-Cont group (Fig. 4).

**Femoral mechanical strength and its components**

Measured femoral mechanical properties (breaking load and breaking energy) are shown in Table 2. The femoral breaking load of the OVX-Cont group was lower than that of the sham group. The OVX-Resv group had a higher femoral breaking load value than the OVX-Cont group, but the difference was not statistically significant. In contrast, femoral breaking energies for the OVX-Resv group were significantly higher than those for the OVX-Cont group. Femoral Ca, Mg, P and Hyp contents are shown in Table 3. No significant differences in Ca, Mg, or P were found between the OVX-Cont and OVX-Resv groups. However, total femoral Hyp in the OVX-Resv group was significantly higher than in the OVX-Cont group.

**DISCUSSIONS**

Recent publications have highlighted geographical differences in the prevalence of many diseases, including CHD (24), breast cancer (25), osteoporosis (26) and menopausal symptoms (27). The potential biological impact of environmental and dietary estrogens on human health has generated considerable interest (28-30). These agents include phytoestrogens, soy bean isoflavones (31-33) and a variety of synthetic compounds, such as the isofiavone derivative 7-isopropoxy-3-phenyl-4H-1-benzopyran-4-one (ipriflavone) (34). Chemically, many of the known phytoestrogens are flavonoids; others are coumestans (35) or resorcylic acid lactones, and an estrogenic hydroxystilbene was recently reported to occur naturally in wood (14). The phytoestrogen resveratrol has recently been revealed to exhibit variable degrees of estrogen receptor agonism (16). Resveratrol occurs naturally in grapes and a variety of medicinal plants. In plants, resveratrol functions as a phytoalexin, serving as a protective agent against fungal infections. Because of its high concentration in grape skin, significant amounts of resveratrol are present in wine (14, 36).

The risk of hypertension and stroke is lower in postmenopausal women relative to men of the same age (37), but the incidence of cerebrovascular events rapidly increases in women after menopause (38). Alkayed et al. (39) have demonstrated that female rats sustain smaller cortical and striatal infarcts after middle cerebral artery occlusion compared with age-matched

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Table 1. Effects of ovariectomy and resveratrol on relative organ weights in SHRSP.

<table>
<thead>
<tr>
<th>Measure</th>
<th>OVX-Cont</th>
<th>OVX-Resv</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight as percent BW</td>
<td>0.546±0.038</td>
<td>0.415±0.020¹*</td>
<td>0.505±0.024²</td>
</tr>
<tr>
<td>Brain weight as percent BW</td>
<td>1.152±0.173</td>
<td>0.801±0.015¹*</td>
<td>1.042±0.138</td>
</tr>
<tr>
<td>Uterus weight as percent BW</td>
<td>0.034±0.007</td>
<td>0.031±0.002*</td>
<td>0.167±0.073³</td>
</tr>
</tbody>
</table>

Mean values with SD are given (6 rats per group).

¹: Significantly different from OVX-Cont group, p<0.05.

²: Significantly different from sham group, p<0.05.
Fig. 3. Concentration-response curves to ACh cumulatively added to endothelium-intact aortic rings obtained from ovariectomized and sham-operated SHRSP. Mean values with SD are given (6 rats per group).

Aortic rings were submaximally contracted with PGF$_{2\alpha}$ before obtaining cumulatively responses to ACh. †: sham group is significantly different from OVX-Cont group, p<0.05.

*: OVX-Resv group is significantly different from OVX-Cont group, p<0.05.

Aortic rings from OVX-SHRSP showed a poor relaxant response to ACh as compared to sham-operated SHRSP. Resveratrol treatment significantly improved endothelium-dependent vascular relaxation in response to ACh as compared to OVX-Control SHRSP.

Fig. 4. Contraction of endothelium-intact aortic rings from ovariectomized and sham-operated SHRSP in response to L-NAME. Mean values with SD are given (6 rats per group).

Aortic rings were moderately contracted by PGF$_{2\alpha}$ before responses to L-NAME. †: Significantly different from OVX-Cont group, p<0.05.

A significantly greater contractile response was observed in tissues from the OVX-Resv group as compared to the Cont-OVX group.

Table 2. Effects of ovariectomy and resveratrol on the length and strength of the femur in SHRSP.

<table>
<thead>
<tr>
<th>Measure</th>
<th>OVX-Cont</th>
<th>OVX-Resv</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral length (mm)</td>
<td>33.24±2.02</td>
<td>34.14±1.20</td>
<td>33.34±0.67</td>
</tr>
<tr>
<td>Breaking load (N)</td>
<td>83.30±17.16</td>
<td>99.46±9.57</td>
<td>95.53±23.64</td>
</tr>
<tr>
<td>Breaking energy (J)</td>
<td>0.0351±0.0032</td>
<td>0.0523±0.0073†</td>
<td>0.0487±0.0141†</td>
</tr>
</tbody>
</table>

Mean values with SD are given (6 rats per group).

†: Significantly different from OVX-Cont group, p<0.05.

Table 3. Effects of ovariectomy and resveratrol on the mineral and hydroxyproline concentrations of the femur in SHRSP.

<table>
<thead>
<tr>
<th>Measure</th>
<th>OVX-Cont</th>
<th>OVX-Resv</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mg/g bone)</td>
<td>94.35±1.22</td>
<td>95.05±1.69</td>
<td>97.05±1.60†</td>
</tr>
<tr>
<td>P (mg/g bone)</td>
<td>45.70±0.33</td>
<td>45.10±0.38</td>
<td>45.75±0.25</td>
</tr>
<tr>
<td>Mg (mg/g bone)</td>
<td>1.86±0.02</td>
<td>1.86±0.02</td>
<td>1.89±0.04</td>
</tr>
<tr>
<td>Hyp (mg/g bone)</td>
<td>7.18±0.09</td>
<td>7.64±0.10†**</td>
<td>7.33±0.08†</td>
</tr>
</tbody>
</table>

Mean values with SD are given (6 rats per group).

†: Significantly different from OVX-Cont group, p<0.05.

**: Significantly different from sham group, p<0.05.
Cont group after 3-wk of feeding with resveratrol, and this effect was maintained throughout the experimental period. A direct effect of estrogen on the blood vessel wall is suggested by its ability to relax isolated blood vessels (40, 41), and by evidence for the presence of estrogen receptor in isolated arteries and vascular cells (42). We showed that long-term resveratrol consumption in ovariecctomized SHRSP enhanced dilatary response to ACh in aortic rings relative to the OVX-Cont group. The basal release of NO from aortic rings was assessed indirectly, initially by inducing moderately active tone and then observing the effects of L-NAME on changes in basal NO tone (22, 23). Basal NO release from endothelium-intact aortic rings from the OVX-Cont group decreased significantly as compared to the sham-operated group. Resveratrol replacement therapy in the OVX group induced a more marked contractile response to L-NAME than observed in the untreated OVX group. Differences in basal NO formation are thus reflected in the responses of endothelium-intact aortic rings in the presence of L-arginine analogs. The mechanism of action of resveratrol in lowering blood pressure appears to be similar to that of mammalian estrogens, involving enhancement of the dilator response to ACh, possibly by basal NO release. This observation is in agreement with the findings of a previous study suggesting that the vasodilatory action of resveratrol is mediated via an NO-related mechanism (19).

The present study confirms that the oral administration of resveratrol has the potency to attenuate ovariectomy-induced decreases in femoral bone strength, measured as breaking load and breaking energy. Breaking load is the maximum power that is required to break bone by the three-point bending method, while breaking energy represents the integrated power required to make a break. It is believed that breaking load represents a momentary and maximum power, while breaking energy reflects the overall power required to make a break. Correspondingly, breaking load is considered to reflect both bone mineral and bone protein such as collagen, whereas breaking energy primarily reflects bone proteins (43). It is thought that the stimulation of bone strength caused by resveratrol intake arises from an alteration of bone metabolism, and a resulting increase in bone protein. These results are supported by recent observations of the positive effect of soybean products, both soybean milk-based diet and soybean protein, on bone strength in OVX-SHRSP appeared to be due to the stimulation of bone formation.

To our knowledge, this is the first report showing that resveratrol modifies blood pressure and improves bone metabolism in vivo. The results predict that dietary resveratrol could contribute to the prevention of postmenopausal hypertension and osteoporosis.

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REFERENCES
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