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Genistein treatment reduces arterial contractions by inhibiting tyrosine kinases in ovariectomized hypertensive rats

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Abstract

The aim of the present study was to evaluate the vascular effects of genistein in a short-term study. The ovariectomized spontaneously hypertensive rats (SHR) were divided into four groups ($n = 8$ in each), which received the following subcutaneous treatments either for 2 days or for 2 weeks: (1) solvent control (96% dimethylsulphoxide (DMSO) 1 ml/kg), (2) estradiol-17β (25 μg/kg), (3) genistein (2.5 mg/kg; low-dose), and (4) genistein (25 mg/kg; high-dose). The renal arterial rings were studied using organ bath system. The renal artery contractions were attenuated by the 2-day low-dose genistein treatment as follows: angiotensin II (46%), noradrenaline (42%) KCl (36%), and endothelin-1 (34%). Only the angiotensin II-induced contractions were reduced by the 2-week treatment with estradiol-17β (38%) and with the low-dose of genistein (31%). The 2-day genistein treatment reduced tyrosine phosphorylation, while the other treatments or treatment times had no effect. The 2-day low-dose genistein treatment had no estrogenic effect on the uterine morphology. The mechanism for attenuated contractility in the renal arteries after the 2-day low-dose genistein treatment is independent of the estrogenic effect of genistein, but is due to the tyrosine kinase inhibitory property of genistein.

Keywords: Genistein; Estradiol-17β; SHR (spontaneously hypertensive rat); Renal artery; Tyrosine phosphorylation

1. Introduction

Genistein is a plant-derived estrogen-like compound, whose main dietary source is soybean (Eldridge and Kwolek, 1983). Genistein can competitively bind to both estrogen α- and β-adrenoceptors (Kuiper et al., 1998), which are expressed in the smooth muscle cells of the aorta, for instance and in the coronary arteries (Register and Adams, 1998). Both estradiol-17β (Jiang et al., 1991) and genistein (Nevala et al., 1998) have been shown to relax arteries endothelium-independently in vitro.

Apart from being an estrogen receptor agonist, genistein is also known to inhibit tyrosine kinases via interaction with the ATP-binding site (Akiyama et al., 1987). Higher tyrosine kinase activity in smooth muscle cells than in skeletal or cardiac muscle tissue (Di Salvo et al., 1997) suggests that tyrosine kinases regulate specialized functions of smooth muscle including contraction, secretory activities, cellular growth, proliferation, and death. Tyrosine kinase inhibitors have been shown to antagonize vascular contraction in response to a wide range of contractile agents, and to reduce resistance in different arteries such as in the rat mesenteric artery (Toma et al., 1995), aorta (Duarte et al., 1997), and renal artery (Giménez et al., 1998) in vitro. The mechanisms of this include the reversible inhibition of the increase of intracellular $[Ca^{2+}]$ in the vascular smooth muscle cell (Di Salvo et al., 1997), and the regulation of the effect of Ca^{2+} on the contractile apparatus of vascular smooth muscle cell (Toma et al., 1995).

Both genistein and estradiol-17β have effects on arterial function after long-term use. Genistein treatment enhances endothelium-dependent relaxations in ovariectomized rats (Squadrito et al., 2000). Prolonged estrogen treatment reduces phenylephrine-induced contractions in the rat arteries (Zhang and Davidge, 1999) and restores impaired

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endothelium-dependent vasodilatation in ovariectomized rabbits (Gisclard et al., 1987) and in spontaneously hypertensive rats (SHR) (Williams et al., 1988). The improved endothelial function is associated with the maintenance of nitric oxide synthesis by estrogen in the arterioles of ovariectomized hypertensive rats (Huang et al., 1997).

In previous studies, we investigated the effect of genistein on arterial tone in vitro (Nevala et al., 1998, 2001) and the effect of long-term exposure of genistein-rich soy extract on hypertension and arterial function (Nevala et al., 2000). The aim of the present study was to evaluate the effect of a short-time genistein treatment on renal arterial reactivity in ovariectomized spontaneously hypertensive rats ex vivo, especially focusing on the mechanism of action of genistein.

2. Methods

2.1. Experimental animals and treatments

Sixty-four ovariectomized SHR 8 to 10 weeks old (Harlan Sprague Dawley, Indiana, IN, USA) were housed five to six animals to a cage in the animal laboratory (illuminated from 6.30 AM to 6.30 PM, room temperature 22–24 °C) for 2 weeks. The rats had free access to tap water and chow (R36, Lactamin, Stockholm, Sweden). At the beginning of the study, the weight- and blood-pressure-matched rats were divided into eight subgroups of eight rats each, for the following treatments given subcutaneously once a day: (1) 96% dimethylsulphoxide (DMSO) 1 ml/kg (solvent), (2) estradiol-17β (25 μg/kg), (3) genistein (2.5 mg/kg; low-dose), and (4) genistein (25 mg/kg; high-dose) either for 2 days or for 2 weeks. The study plan was approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki.

2.2. Measurement of blood pressure and sample preparation

The systolic blood pressure and the heart rate of the rats (which were pretrained for the procedure) were measured at the beginning of the study and at the end of the 2-week treatment. A tail-cuff computerized analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA) was used as described by us previously (Nevala et al., 2000).

After the 2-day or the 2-week treatments, the rats were made unconscious with CO_2/O_2 70/30% (AGA, Riihimäki, Finland) and decapitated. The heart was excised, the large blood vessels, the atria and the free wall of the right ventricle were dissected and the left ventricular was weighed. The kidneys and uteri were excised, washed with ice-cold saline and weighed. Organ weight-to-body weight ratios were calculated to describe the hypertrophy or atrophy of these organs.

2.3. Arterial responses in vitro

The renal arteries were carefully excised and cleaned of adherent connective tissue for functional in vitro studies (Pörsti et al., 1991). Two 3-mm-long sections renal arteries were prepared and placed between stainless steel hooks and mounted in an organ bath chamber in Krebs–Ringer buffer (pH 7.4) of the following composition (mM): NaCl (119.0), $NaHCO_3$ (25.0), glucose (11.1), $CaCl_2 \times 2H_2O$ (1.6), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4 \times 7H_2O$ (1.2), then aerated with 96% O_2 and 4% CO_2. The renal arterial rings were equilibrated for 1 h at +37 °C with a resting tension of 0.2 g. The force of contraction was measured with an isometric force-displacement transducer and registered with a polygraph (FT03 transducer, Model 7P122E Polygraph; Grass Instrument, Quincy, MA, USA). Acetylcholine (1 μM)-induced relaxation was used to test the presence of endothelium.

The contraction response for a single administration of angiotensin I and also of angiotensin II was determined. The cumulative contraction response curves to noradrenaline, KCl, and endothelin-1, and the cumulative relaxation responses curves to nitroprusside, and acetylcholine were determined as described by Kähönen et al. (1993). The application of agonists was always performed in the same order.

2.4. Tissue samples for morphology

Tissue samples from the right renal artery and uterine horns were collected and fixed for 24 to 48 h with 10% formalin. The samples were dehydrated and embedded in paraffin by using the standard protocol. Cross-sections (5 μm) of the arteries and uteri were deparaffinized, hydrated and stained with Masson's trichrome or hematoxylin and eosin. The slides were examined in a blinded fashion by the authors (P.F. and R.N.).

2.5. Tyrosine phosphorylation

To study tyrosine phosphorylation, the aortic rings were homogenized, using an Ultra-Turrax homogenizer, in five volumes of boiling lysis buffer (10 mM Tris, pH 7.4, 1% Na^+ docecyl sulfate (SDS), 1.0 mM Na_3VO_4, 50 mM NaF). The protein content of the supernatants was measured according to the method of Lowry et al. (1951). The supernatants were boiled in reducing Laemmli sample buffer (Laemmli, 1970), and 10-μg samples were separated in 4–15% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Immunodetection was performed using anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, New York, NY, USA), biotinylated anti-mouse immunoglobulins (Dako, Glostrup, Denmark) and streptavidin–biotin horseradish peroxidase-conjugated secondary antibodies (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), followed by detection

by enhanced chemiluminescence (ECL, Amersham Pharmacia).

2.6. Endothelial nitric oxide synthesis (eNOS)

Aorta tissues were homogenized, using an Ultra-Turrax homogenizer, in five volumes of boiling lysis buffer (1% SDS, 1.0 mM Na_3VO_4, 10 mM Tris pH 7.4) (Sigma, St. Louis, MO, USA). The protein content of the supernatants was measured according to the method of Lowry et al. (1951). Equal amounts of protein (30 μg) were resolved by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to the nitrocellulose membranes (Hybond-C Extra, Amersham) [22]. The membranes were blocked with 5% instant nonfat dry milk (Valio, Helsinki, Finland) in Tris-buffered saline containing Tween 20 (TBS-T: 20 mM Tris, 137 mM NaCl, Riedel-de Haën, Seelze, Germany), 0.1% Tween 20 (Fluka, Buchs, Switzerland), pH 7.6) and incubated overnight with the mouse monoclonal anti-eNOS immunoglobulin G_1 (IgG_1) (1:2500,Transduction Laboratories, Lexington, KY, USA) at +4 °C, and for 1 h with the horseradish peroxidase-coupled anti-mouse IgG_1 (1:1000, Zymed Laboratories, San Francisco, CA, USA). The bound antibodies were detected using an enhanced chemiluminescence (ECL) reagent (Amersham). Homogenized rat aortas and prestained molecular marker proteins (Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA) were used as a positive control. Each band was quantified with computer programs (GeneSnap and GeneTools, Synoptics, Cambridge, UK). The intensities of sample bands were compared with the intensity of the positive control band, which was given an arbitrary value of 100.

2.7. Metabolic caging

On the 10th day, the rats from the 2-week experiment were kept for 24 h in the metabolic cages. They had free access to chow and tap water. The urine was collected, and food and water consumptions were determined.

2.8. Measurement of urine creatinine and electrolytes

Urine creatinine was analyzed by the Jaffe method (Bartels et al., 1972) (BM/Hitachi 917 analyzer, Boehringer Mannheim, Germany/Hitachi, Tokyo, Japan) without deproteinization. Urine Na^+ and K^+ were determined by flame photometer using an ion-selective electrode compensator (human serum pool, IL model 943, Instrumentarium Laboratory, Milan, Italy). Urine Ca^{2+} was determined with the method described by Cali et al. (1973).

2.9. Drugs

The following drugs were used: acetylcholine chloride, angiotensin I acetate, angiotensin II acetate, endothelin-1, estradiol-17β, genistein, noradrenaline bitartrate (Sigma) and Na^+ nitroprusside (F. Hoffmann La Roche, Basel, Switzerland). The estradiol-17β and genistein were dissolved in DMSO (96%); the other compounds were dissolved in water. The solutions were prepared just before use and protected from light.

2.10. Statistical analysis

The results are given as mean ± S.E.M. The arterial relaxations are expressed as the percentage relaxation of the precontraction level induced by noradrenaline (1 μM). Cumulative contractions are presented as grams. The sensitivity of the artery to the cumulative relaxation response is presented as pD_2 values, which are calculated as the negative log of the dose required to produce the half-maximal response. Student's *t*-test was used for the comparison of the means. Data for multiple observations over time were analyzed with the two-way analysis of

Table 1
Body weights, organ weights, organ weight indices, and blood pressures after a 2-day or 2-week treatment in control, estradiol-17β, low-dose genistein, and high-dose genistein groups

	Control	Treatment period (2 days)			Control	Treatment period (2 weeks)		
		Estradiol (25 μg/kg)	Genistein (2.5 mg/kg)	Genistein (25 mg/kg)		Estradiol (25 μg/kg)	Genistein (2.5 mg/kg)	Genistein (25 mg/kg)
Body weight (g)	208 ± 1	204 ± 3	206 ± 3	207 ± 2	214 ± 3	196 ± 2[c,d]	214 ± 2	199 ± 3[b]
Left ventricle/heart weight	80 ± 0.7	80 ± 0.6	79 ± 0.4	78 ± 1.2	80 ± 0.8	81 ± 0.8	81 ± 0.5[d]	80 ± 0.5[d]
Kidney weight (mg)	1133 ± 22	1174 ± 20	1133 ± 19	1133 ± 25	1160 ± 23	1192 ± 26	1191 ± 14[d]	1162 ± 16
Uterus weight (mg)	58 ± 4	134 ± 14[c]	78 ± 12	93 ± 6[c]	52 ± 3	200 ± 11[c,e]	84 ± 11[b]	136 ± 4[c,f]
LVH-index (mg/g)	3.9 ± 0.04	3.9 ± 0.07	3.9 ± 0.07	3.8 ± 0.04	3.8 ± 0.09	4.1 ± 0.07[b]	3.8 ± 0.05	4.0 ± 0.08[a,e]
Kidney-index (mg/g)	5.4 ± 0.1	5.7 ± 0.1	5.5 ± 0.1	5.5 ± 0.09	5.4 ± 0.07	6.1 ± 0.08[c,e]	5.6 ± 0.07	5.8 ± 0.06[c,e]
Uterus weight index (mg/g)	0.3 ± 0.02	0.7 ± 0.07[c]	0.4 ± 0.05	0.5 ± 0.03[c]	0.2 ± 0.02	1.0 ± 0.05[b,f]	0.4 ± 0.05[a]	0.7 ± 0.02[b,f]
Blood pressure (mm Hg)	147 ± 3	148 ± 4	148 ± 2	149 ± 3	157 ± 2	152 ± 3	155 ± 3	159 ± 3

Data are mean ± S.E.M. (n = 7–8 in each group).
[a]P < 0.05, [b]P < 0.01, [c]P < 0.01 vs. control group.
[d]P < 0.05, [e]P < 0.01, [f]P < 0.001 vs. treatment period of 2 days.

variance (ANOVA) with repeated measures. For the multiple comparisons of the means, Newman–Keuls's test was used.

3. Results

3.1. Body weights, uterine weights, uterine hypertrophy indexes, and blood pressures

After the 2-week treatment, the body weights were significantly lower both in the estradiol-17β ($P<0.001$) and in the high-dose genistein ($P<0.01$) groups compared to the controls. After the 2-day treatment, the uterine weight index (organ to body weight index), rose in the estradiol-17β ($P<0.001$) and in the high-dose genistein groups ($P<0.001$), but not in the low-dose, genistein group. The 2-week treatment with both low- and high-dose genistein, and with estradiol-17β increased the uterine weight index compared to the controls ($P<0.05$, $P<0.01$, and $P<0.01$, respectively). The 2-week genistein or estradiol-17β treatments did not significantly alter the blood pressure compared to the controls (Table 1).

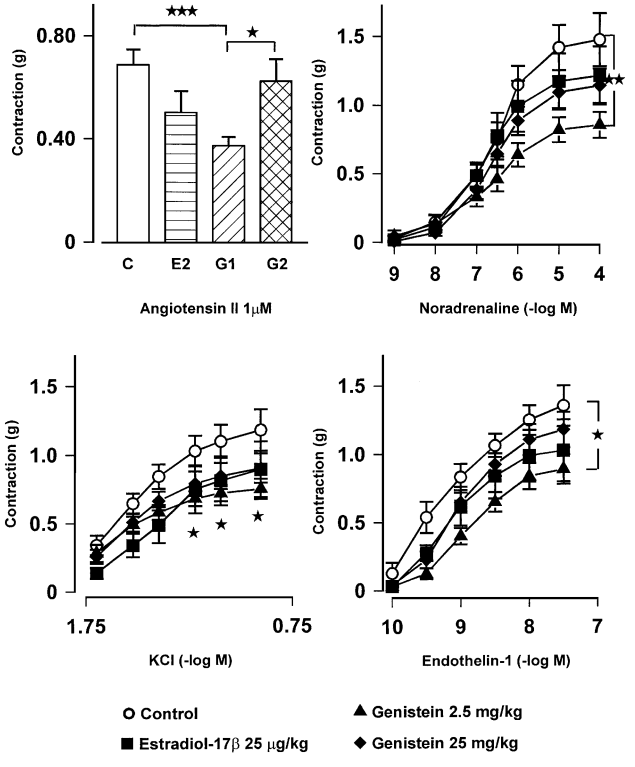

Fig. 1. Effect of 2-day control (○), estradiol, 25 μg/kg (■), genistein, 2.5 mg/kg (▲), and genistein, 25 mg/kg (♦) treatments on renal arterial contractions induced by angiotensin II, noradrenaline, KCl, and endothelin-1. Data are mean ± S.E.M. ($n=7-8$ in each group). * $P<0.05$, * * $P<0.01$, * * * $P<0.001$ vs. control group.

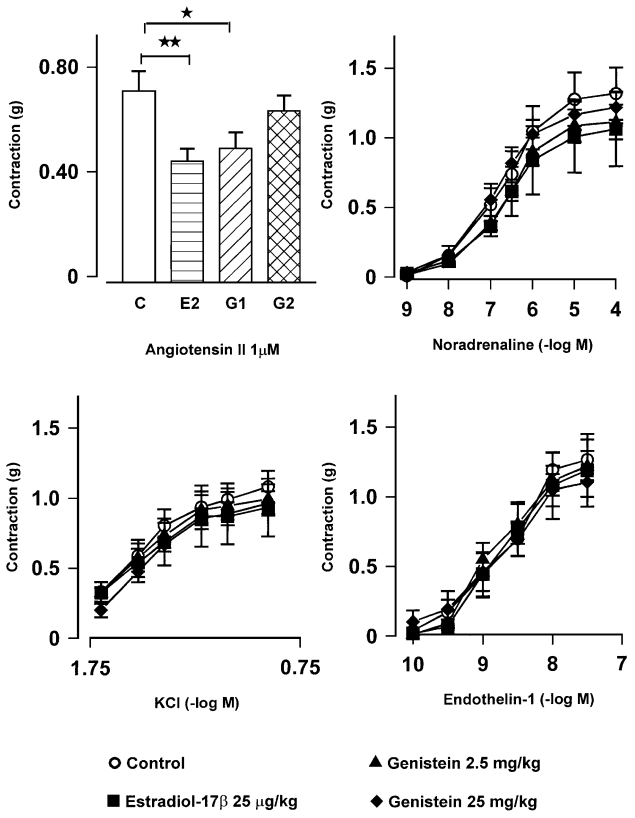

Fig. 2. Effect of 2-week control (○), estradiol, 25 μg/kg (■), genistein, 2.5 mg/kg (▲), and genistein, 25 mg/kg (♦) treatments on renal arterial contractions induced by angiotensin II, noradrenaline, KCl, and endothelin-1. Data are mean ± S.E.M. ($n=7-8$ in each group). * $P<0.05$, * * $P<0.01$ vs. control group.

3.2. Renal arterial responses in vitro

The contractions induced by angiotensin II ($P<0.001$), noradrenaline ($P<0.05$), KCl ($P<0.05$), and endothelin-1 ($P<0.01$) contractions were attenuated in the rats receiving low-dose genistein for 2 days when compared to the control group (Fig. 1). Angiotensin II-induced contraction was also smaller in the low-dose genistein group than in the high-dose group. After the 2-week treatment, angiotensin II-induced contraction was less in the estradiol-17β ($P<0.01$) and the low-dose genistein ($P<0.05$) groups than in the control group, but in the other contractions no differences appeared between the treatment groups (Fig. 2).

The angiotensin I-induced contraction responses after 2-day treatment with control solvent, estradiol-17β, low- and high-dose genistein were 0.7 ± 0.1, 0.5 ± 0.1, 0.5 ± 0.0, and 0.7 ± 0.1 g, respectively. The angiotensin I contractions after the 2-week treatment with the vehicle, estradiol-17β, low- and high-dose genistein were 0.6 ± 0.1, 0.5 ± 0.1, 0.6 ± 0.1, and 0.4 ± 0.1 g, respectively.

Depending on the treatment group, the precontraction induced by noradrenaline (1 μM) was 0.6 ± 0.1–1.2 ± 0.2 g

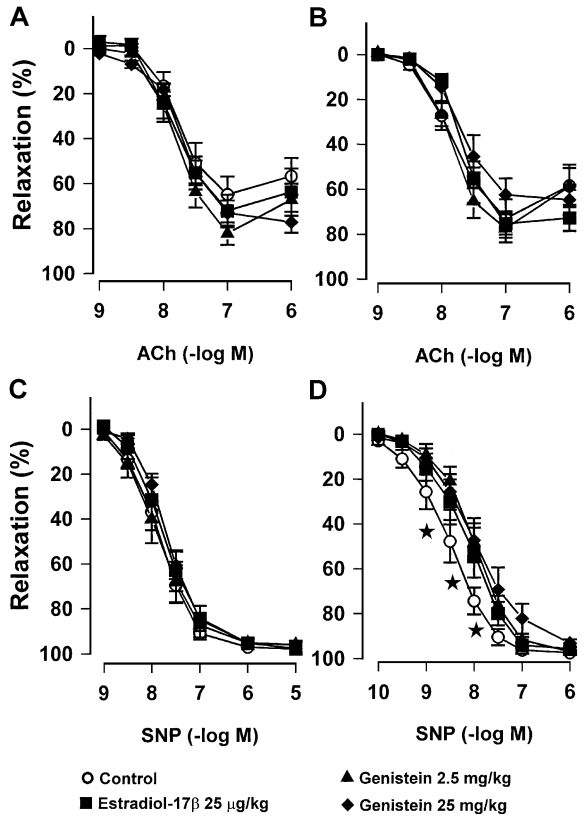

Fig. 3. Effect of 2-day (A, C) and 2-week (B, D) control (○), estradiol, 25 μg/kg (■), genistein, 2.5 mg/kg (▲), and genistein, 25 mg/kg (◆) treatments on renal arterial relaxations induced by acetylcholine and Na^+ nitroprusside. Data are mean ± S.E.M. ($n = 7–8$ in each group). * $P < 0.05$ control vs. other groups.

in renal arteries. The acetylcholine-induced relaxation responses were not altered by the treatments (Fig. 3A,B). The maximum relaxations to nitroprusside did not differ between the treatment groups. The sensitivity (pD_2-value) to nitroprusside increased in all the groups after the 2-week treatments compared to 2-day treatments ($P < 0.05$). At the end of 2 weeks, the renal arteries from the control animals were the most sensitive to nitroprusside relaxation ($P < 0.05$) (Fig. 3C,D).

3.3. Tyrosine phosphorylation

The 2-day low-dose genistein treatment decreased tyrosine phosphorylation in aortic rings. The tyrosine phosphorylation was unaffected by the 2-day estradiol-17β and high-dose genistein treatments as well as by all the treatments of 2-week (Fig. 4).

3.4. Tissue morphology

The 2-day treatments produced moderate differences in the uterine morphology (Fig. 5). The estradiol-17β treatment caused an increase in the height of the luminal and glandular epithelium. The size of the glands as well as the thickness of the endometrium and myometrium exceeded those in the control and genistein groups.

The 2-week treatments produced more distinct alterations between the different groups. The samples from the control and low-dose genistein group had luminal and glandular epithelium of cuboidal shape with small densely stained nuclei. The high-dose genistein group had luminal epithelium of cuboidal or columnar shape. The size of the glands and the thickness of the endo- and myometrium exceeded those in the control or the low-dose genistein groups. The 2-week estradiol-17β group had stratified luminal epithelium, large glands with high epithelium and thicker endo- and myometrium than in the other groups (data not shown).

The morphology of the renal arteries did not differ between the groups. Every sample had normal intimal, medial, and adventitial morphology (data not shown).

3.5. eNOS

The amount of eNOS protein in the aortic tissue was increased by both the 2-day and the 2-week treatments with estradiol-17β (in arbitrary units, mean ± S.E.M., 228 ± 57, 228 ± 38, respectively), and slightly but not significantly, by the 2-week treatment with the low-dose genistein (219 ± 35), when compared to the respective controls (166 ± 50 and 172 ± 25).

3.6. Food intake and electrolyte excretion

The daily food intake was lower in the high-dose genistein group compared to the control group ($P < 0.05$). The daily water consumption and urine excretion were similar in all the groups. The daily urine Na^+ excretion decreased in the high-dose genistein animals vs. the control ($P < 0.05$), while no differences appeared between the groups in the daily urine K^+ excretion. Creatinine excretion was also similar in all groups. Urine Ca^{2+}

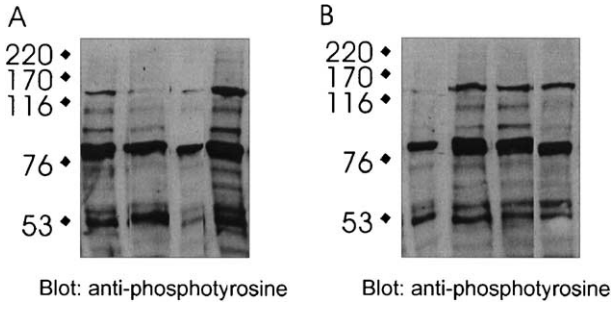

Fig. 4. Effect of control (1), estradiol-17β, 25 μg/kg (2), genistein, 2.5 mg/kg (3), and genistein, 25 mg/kg (4) treatments on tyrosine phosphorylation in aortic smooth muscle after 2-day (A) and 2-week (B) treatment period.

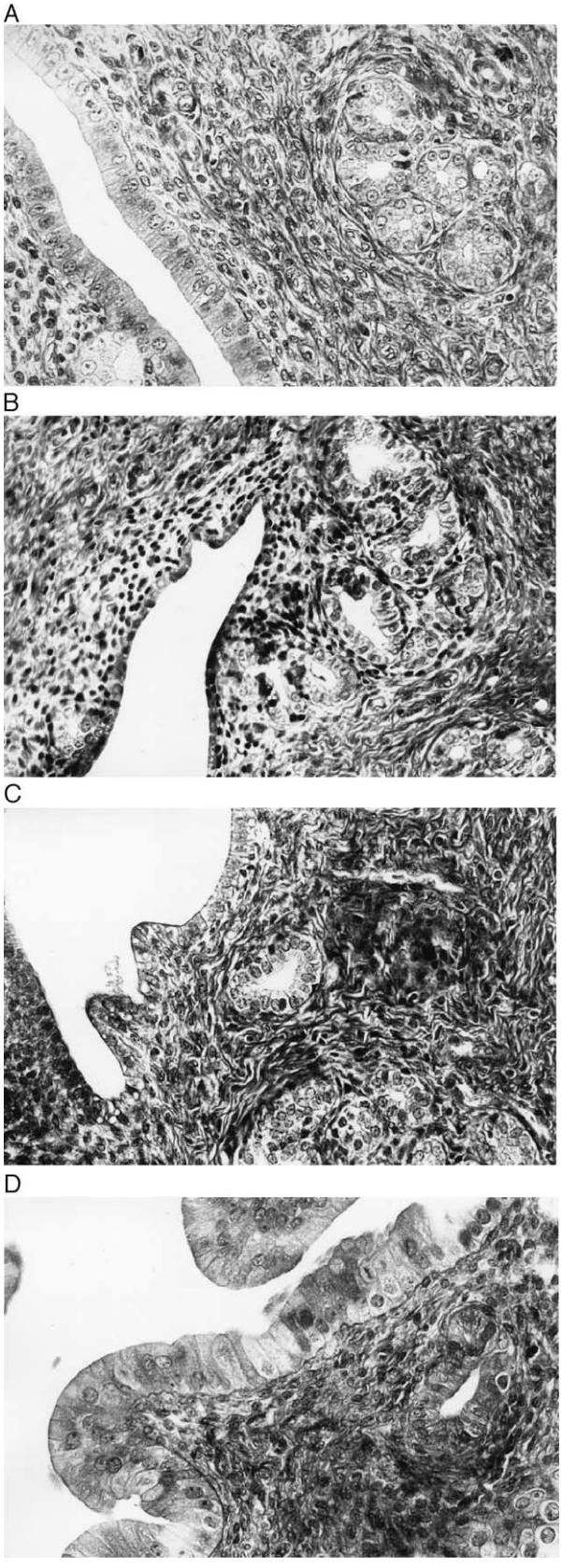

Fig. 5. Uterine morphology of ovariectomized spontaneously hypertensive rat. The rats were treated for 2 days with control (A), genistein, 2.5 mg/kg (B), genistein, 25 mg/kg (C), or estradiol-17β, 25 μg/kg (D). (Masson's trichrome staining. Original magnification × 300).

Table 2
Results of metabolic caging and urine electrolyte excretion after 2-week treatments

	Control	Estradiol (25 μg/kg)	Genistein (2.5 mg/kg)	Genistein (25 mg/kg)
Food intake (g/day)	17.4 ± 0.4	17.3 ± 0.7	17.6 ± 0.8	15.3 ± 0.7[a]
Water intake (ml/day)	26.5 ± 2.0	29.7 ± 2.2	29.3 ± 2.1	23.6 ± 1.2
Urine excretion (ml/day)	13.4 ± 1.3	15.5 ± 1.8	14.1 ± 1.8	10.3 ± 1.1
Potassium excretion (mM/day)	1.9 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	1.7 ± 0.1
Sodium excretion (mM/day)	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.0 ± 0.1[a]
Calcium excretion (mM/day)	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.00[b]
Creatinine excretion (mM/day)	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.06 ± 0.00[a]

Data are mean ± S.E.M. ($n = 7–8$ in each group).
[a]$P < 0.05$, [b]$P < 0.01$ vs. control group.

excretion was smaller in the high-dose genistein group than in the controls ($P < 0.01$) (Table 2).

4. Discussion

The aim of the present study was to evaluate the effect of a short-term genistein treatment on renal arterial reactivity in ovariectomized spontaneously hypertensive rats ex vivo using two different doses. A low-dose genistein treatment for 2 days reduced renal arterial contractility and aortic tyrosine phosphorylation, but the same treatment over for 2 weeks was ineffective in these respects. The high-dose genistein or estradiol-17β treatments for 2 days or 2 weeks had no marked effect on the contractility or tyrosine phosphorylation. In the renal arteries, the treatments did not cause any clear changes in the endothelium-dependent, or -independent relaxation responses, or arterial morphology.

The treatment doses of genistein were selected on the basis of knowledge that isoflavonoids at concentration 100–1000 times higher than that of estradiol-17β have been considered to compete with endogenous mammalian estrogens, bind estrogen receptors, and prevent estrogenic effects in mammals (Adlercreutz et al., 1995). Therefore, the lower dose of genistein was 100 and the higher dose 1000 times higher than that of estradiol-17β. Ovariectomized SHR were used for two reasons. Firstly, ovariectomized rats do not have endogenic estrogen production, which sensitizes the rats to the estrogenic effect of genistein. Secondly, the

tyrosine kinase inhibitors have stronger influence on the arterial responses in hypertensive than in normotensive rats (Zerrouk et al., 1999). The relatively short genistein treatment times were tested, because in our previous experiment (Nevala et al., 2000), we treated the SHR with genistein-rich soy extract for 5 weeks. In the present study, we wanted to investigate the effect of a shorter genistein treatment time on arterial function.

The tyrosine phosphorylation was decreased in aortic smooth muscle by the 2-day low-dose genistein treatment. It is interesting that the aortic smooth muscle tyrosine phosphorylation were unaffected by the 2-day high-dose (25 mg/kg) genistein treatment. The tyrosine kinase inhibitor activity of genistein has been shown to be evident at doses up to 1 mg/kg (Akiyama et al., 1987). Our results suggest that at very high doses, genistein lose its specificity to inhibit tyrosine kinase or that it has some other uncharacterized effects on the function of vascular smooth muscle cell. The decreasing effect of the low-dose genistein treatment on tyrosine phosphorylation was no more evident after 2-week treatment even at the lower dose. It is possible that the tyrosine phosphorylation is compensatorily increased after the decrease of basal level during the first days of genistein treatment.

Both the 2-day and the 2-week treatments with the low-dose genistein reduced angiotensin II-induced contraction responses in the renal arteries. The 2-week, but not the 2-day treatment with estradiol-17β had a similar effect. The treatments in the present study did not markedly alter angiotensin I responses, which do not support the existence of marked differences in angiotensin-converting enzyme activities, even though ovariectomy is known to increase, and hormone replacement therapy to decrease angiotensin-converting enzyme activity in rats (Tanaka et al., 1997). Estrogen replacement therapy attenuated the pressor response to angiotensin II in vascular smooth muscle in ovariectomized normotensive rats (Cheng and Gruetter, 1992), and in transgenic hypertensive rats (Brosnihan et al., 1997). Lack of estrogen increases angiotensin 1 (AT1) receptor expression in vascular smooth muscle cells (Nickenig et al., 1998) and in adrenal gland (Roesch et al., 2000), and the estrogen substitution therapy reverses the overexpression (Nickenig et al., 1998; Roesch et al., 2000). It is unknown whether genistein has effects similar to those of estrogen on the density of angiotensin receptors. On the other hand, AT1 receptors belong to the G-protein-coupled receptor family, which lacks intrinsic tyrosine kinase activity (Berk and Corson, 1997). The activation of tyrosine kinases by several different mechanisms contributes to the effects of angiotensin II on target tissues. Tyrosine kinases activated by angiotensin II include, for example focal adhesion kinase, Janus kinases and the receptor tyrosine kinases, epidermal growth factor, and platelet-derived growth factor (Berk, 1999). Unknown tyrosine kinases may also mediate tyrosine phosphorylation after angiotensin II stimulation (Berk, 1999). These angiotensin II-regulated tyrosine kinases seem to be required for angiotensin II effects such as vasoconstriction (Berk, 1999). Thus, it is also possible that the reduced angiotensin-II-induced contractions were due to tyrosine kinase inhibition by genistein, and that the mechanism is different from that of estradiol-17β.

The noradrenaline-induced contractions were attenuated after the 2-day low-dose genistein treatment. This agrees with the finding that 6 h after single dose intraperitoneal administration of genistein (10 mg/kg), noradrenaline-induced aortic contractions are reduced in rats ex vivo (Duarte et al., 1997) Noradrenaline is one of the regulators of renal perfusion, because it has been shown to contract the renal arteries (Gross and Gharaibeh, 1986), the renal afferent arterioles (Wilson, 1986), and the interlobar arteries of the kidney (Chen et al., 1997). The noradrenaline-induced contractions are associated to the activation of tyrosine kinases in rat aortic (Abebe and Agrawal, 1995) and in pulmonary smooth muscle (Savineau et al., 1996) in vitro. Noradrenaline stimulates both α_1- and α_2-adrenoceptors. This leads to the activation of tyrosine kinase regulated phospholipase D (Jinsi et al., 1996), which is followed by smooth muscle contraction. To sum up, the activation of tyrosine kinases is inhibited by the low-dose genistein treatment for 2 days, which causes the suppression of noradrenaline-induced contractions.

The 2-day low-dose genistein treatment also reduced endothelin-1-induced contractions in renal arteries. Endothelin-1 is the most active pressor substance discovered with a potency of 100 times of that of angiotensin II (Masaki, 1995), and it regulates renal blood flow (Okumura et al., 1990). Endothelin-1 increases renal vascular resistance (Hoffman et al., 1990) and contracts renal arteries by activating endothelin ET_A receptors, which leads to the influx of extracellular Ca^{2+} (Betts and Kozlowski, 2000). However, the activation of tyrosine kinases may be involved in the vasoconstricting effect of endothelin-1, because endothelin-1 enhances tyrosine phosphorylation in vascular smooth muscle cells (Koide et al., 1992), and the increased phosphorylation is associated with smooth muscle contraction (Shimada et al., 1986).

In the present study, the 2-week estradiol-17β and low-dose genistein treatments attenuated only the angiotensin II-induced contractions but not the endothelin- and noradrenaline-induced ones. In some studies, estradiol-17β has been shown to inhibit noradrenaline- and endothelin-1-induced vascular contractions (Ravi et al., 1994; David et al., 2001). However, opposite findings also exist. It has been found that although the 2-week estrogen treatment attenuates the angiotensin II-induced contractions, it has no effect on contractions induced by endothelin-1 in rats (Seo et al., 2000). On the other hand, days to weeks treatment with estradiol-17β can even enhance the contractions caused by noradrenaline in rats (Lydrup and Nilsson, 1996; Seo et al., 2000). α_1-Adrenoceptor-induced contractions are also shown to be more resistant to the relaxing effect of genistein

than the angiotensin II-induced ones (Laniyonu et al., 1994; Garcha et al., 2001). Thus, the arterial contraction caused by angiotensin II seems to be more sensitive to the inhibitory effect of estradiol-17β and genistein than endothelin-1 and noradrenaline-induced ones.

Genistein has been shown to bind to both estrogen α- and β-receptors and to act as an agonist (Kuiper et al., 1998). The relative binding affinity of genistein is greater for estrogen β receptor than for estrogen α receptor (Kuiper et al., 1997). Both of these estrogen receptors are expressed in sites such as in the coronary artery and the aortic smooth muscle cells, for instance (Register and Adams, 1998), but their significance in arterial function is still poorly understood. However, some studies exist. Estrogen inhibits the vascular smooth muscle cell proliferation and the increase in vascular media after injury in both estrogen α receptor (Iafrati et al., 1997) and estrogen β receptor (Karas et al., 1999) deficient mice. This indicates that the protective effect of estradiol-17β requires only one type of functional estrogen receptor at time, or that some other still uncharacterized estrogen receptor is involved. In the present study, the estrogenic effect of 2-day and 2-week estradiol-17β treatment was evident in the uterus with the stratified luminal epithelium, but estradiol-17β did not affect arterial contractility. The low-dose genistein had no estrogenic effect on the uteri, which suggests, in agreement with the ineffectiveness of estradiol-17β in altering the contractility that the reduced renal arterial contractions were not due to estrogen receptor activation.

In the present study, neither low-dose, high-dose genistein nor the estradiol-17β treatments altered the endothelium-dependent relaxations in the renal arteries. On the other hand, long-term estrogen administration has been shown to partially restore impaired endothelium-dependent vasodilatation in ovariectomized rabbits (Gisclard et al., 1987) and SHR (Williams et al., 1988). An oral genistein supplementation of 4 to 5 weeks improves endothelium-dependent relaxations in ovariectomized rats (Squadrito et al., 2000), but fails to do so in normal male and female SHR (Nevala et al., 2000). In the present study, the rats were still young at the end of the experiment and the longer treatment time was only 2 weeks, which may have been too short to influence on the function of endothelium. The different treatments or the treatment times either had no effect on the structure of endothelium or on the lumen media ratio, when studied with a light microscope. This suggests that genistein or estradiol-17β treatments shorter than 2 weeks cannot affect the function of endothelium.

Endothelium-independent relaxation to Na^+ nitroprusside was unaltered by low- or high-dose genistein, or by the estradiol-17β treatment. Sensitivity to Na^+ nitroprusside was even most potent in the renal arteries in the group treated for 2 weeks with a solvent vehicle. Thus, neither the genistein nor the estradiol-17β treatments increased the sensitivity of vascular smooth muscle to nitric oxide. This agrees with other findings, that oral genistein supplementation had no effect on endothelium-independent relaxations in ovariectomized female SHR (Squadrito et al., 2000) or in normal male or female SHR (Nevala et al., 2000).

In the present study, the estradiol-17β and the low-dose genistein treatment slightly upregulated the expression of eNOS in the aorta. In the arterioles of ovariectomized female SHR, both the basal and the shear stress-stimulated release of NO is smaller than in the arterioles of normal female SHR (Huang et al., 1998). In addition, in male SHR, the physiological concentration of estradiol-17β normalizes impaired endothelium-dependent dilator responses by an estrogen-receptor-mediated, transcriptional upregulation of eNOS (Huang et al., 2000). Genistein supplementation has also been shown to increase Ca^{2+}-dependent NOS activity in the lungs in normotensive ovariectomized rats (Squadrito et al., 2000). In short, both genistein and estradiol-17β replacements seem to influence in the regulation of nitric oxide synthesis.

In conclusion, the 2-day, but not the 2-week low-dose genistein treatment reduced contractility, but had no effect on the relaxation responses in the renal arteries of SHR. The mechanism for attenuated contractility is independent of the estrogenic effect of genistein, but can be related to the tyrosine kinase inhibitory property of genistein.

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