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## Chronic administration of genistein improves endothelial dysfunction in spontaneously hypertensive rats: involvement of eNOS, caveolin and calmodulin expression and NADPH-oxidase activity

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Short title: genistein and endothelial dysfunction

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#### Abstract

The soy derived phytoestrogen genistein has been suggested to be protective in cardiovascular diseases. We have analyzed if chronic oral genistein might influence the endothelial function in male spontaneously hypertensive rats (SHR) via estrogen receptors (ERs), changes in endothelial NO synthase (eNOS) activity and vascular superoxide  $(O_2)$  production. Rats (23 week old) were divided into the following groups, Wistar Kyoto (WKY)-vehicle; SHR-vehicle; WKY-genistein (10 mg/kg/day); SHRgenistein; SHR-genistein-faslodex (ICI 182780, 2.5 mg/kg/day). Vascular expression of endothelial NO synthase (eNOS), caveolin-1 and calmodulin-1 were analyzed by western blot, eNOS activity by conversion of  $[^{3}H]$  arginine to L- $[^{3}H]$  citrulline and O<sub>2</sub><sup>-</sup> production by chemoluminescence of lucigenin. In SHR, after 5 weeks of treatment, genistein reduced systolic blood pressure, enhanced the endothelium-dependent aortic relaxation to acetylcholine, but had no effect on the vasodilator responses to sodium nitroprusside. Compared to WKY, SHR showed upregulated eNOS and downregulated caveolin-1 and calmodulin-1 expression, increased NADPH-induced O<sub>2</sub><sup>-</sup> production but reduced eNOS activity. Genistein increased aortic calmodulin-1 protein abundance and eNOS activity, and reduced NADPH-induced  $O_2^-$  production in SHR. The pure  $\alpha$  and  $\beta$ estrogenic receptor antagonist faslodex did not modify any changes induced by genistein in SHR, suggesting that these effects are unrelated to ERs stimulation. In conclusion, genistein reduced the elevated blood pressure and the endothelial dysfunction in SHR. This latter effect seems to be related to the increased eNOS activity, associated with increased calmodulin-1 expression, and the decreased O2<sup>-</sup> generation.

**Keywords**: Genistein, SHR, phytoestrogen, hypertension, eNOS, caveolin, calmodulin, superoxide.

#### Introduction

Isoflavones (e.g. genistein and daidzein), constitute the main class of phytoestrogens. They are found in abundance in soybeans and their derivative foods. There is evidence that the beneficial cardiovascular effects of consumption of soy are mostly due to its isoflavone fraction [1-3]. The cardioprotective ability of these plant products has been attributed, at least in part, to their ability to decrease plasma cholesterol, to lower blood pressure and to improve arterial compliance [1-3]. Genistein (4',5,7-trihydroxy-isoflavone) in vitro relaxes rat arteries through a NO-dependent mechanism [4] and enhances the dilator response to acetylcholine in coronary arteries from atherosclerotic female macaques [5] and in aortae from both ovariectomized rats [6] and male spontaneously hypertensive rats (SHR) [7]. Moreover, the phytoestrogen daidzein enhanced eNOS activity increasing calmodulin, its endogenous activator, and decreasing caveolin-1 in normotensive male rats [8]. In vivo, genistein has also been reported to improve endothelial function in endotoxemic rats [9] and SHR [10]. In addition, genistein or soy supplements have been proven to be effective in protecting endothelial function in men and healthy postmenopausal women [11-12]. However, the mechanisms involved in its beneficial effect on endothelial function are unclear.

Genistein binds to estrogen receptors (ERs)  $\alpha$  and  $\beta$ , but it shows greater affinity for the ER $\beta$  [13]. Besides its activity on ERs, the effects of genistein have been also attributed to its inhibitory properties on a broad range of tyrosine kinases [9,10,14,15], to its peroxisome proliferator-activated receptor  $\gamma$  agonist activity [16] or its antioxidant activity [17]. We have recently demonstrated that, *in vitro*, genistein and its analog daizdein acutely improved endothelial function in arteries from SHR, an effect unrelated to its estrogenic properties [7].

4

Therefore, the aim of the present study was to further characterize the mechanisms of the *in vivo* effects of genistein on endothelial function, and particularly, the role of ER. Thus, we analyzed the effects of chronic oral genistein administration on endothelial function and the potential mechanisms involved, focusing on the NO pathway: eNOS activity, the expression of eNOS and its regulatory proteins (calmodulin-1 and caveolin-1). The role of estrogenic mechanisms was studied using a pure  $\alpha$  and  $\beta$  ER antagonist. We selected male hypertensive animals to analyze the female sex-independent effects of dietary genistein. Genistein was used at doses which resulted in serum genistein concentrations similar to those in humans eating a traditional Asian diet high in soy [18,19].

#### Methods

#### Animals and experimental groups

Male Wistar Kyoto (WKY) and SHR aged 23 weeks were obtained from Harlan Laboratories (Barcelona, Spain). All rats were maintained five per cage at a constant temperature ( $24 \pm 1^{\circ}$ C), with a 12-hour dark/light cycle and on standard rat chow without soy derivatives (AIN 76). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No, 85-23, revised 1996).

Experiment I. Analysis of the influence of genistein on blood pressure and endothelial function in SHR. Rats were divided into the following groups (n = 10 in each group), WKY-vehicle (methylcellulose 1% w/v); SHR-vehicle; WKY-Genistein (10 mg/kg, per day, by gavage); SHR-Genistein. Drugs were given for 5 weeks. Body weight was measured every week. The genistein treatment was stopped 2 days before the end of the experiment in order to study the long-term effects of genistein without the involvement of the effects of acute administration.

*Experiment II. Analysis of involvement of estrogenic mechanisms in the effects of genistein in SHR.* Rats were divided into the following groups (n = 7 in each group), SHR-vehicle; SHR-Genistein (10 mg/kg, per day, by gavage) and SHR-Genistein-faslodex (ICI 182,780, kindly provided from AstraZeneca, Cheshire, England) (2.5 mg/kg, per day, s.c.). Drugs were given for 5 weeks. Body weight was measured every week. The genistein treatment was stopped 2 days before the end of the experiment.

#### Blood pressure measurements and plasma sexual hormone levels

Systolic blood pressure (SBP) was measured 18-20 hours after administration of the drugs in conscious, prewarmed, restrained rats by tail-cuff plethysmography [20]. At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP level. At the end of experiment I, direct blood pressure and heart rate were measured in four conscious rats per group. For this purpose, the rats were anaesthetized with 2.5 ml/kg equitensin (500 mL contain 43% (w/v) chloral hydrate in 81 mL ethanol; 4.86 mg nembutal; 198 mL propylene glycol; 10.63 g MgSO4; distilled water) (i.p.). A polyethylene catheter (PE-50) containing 100 U heparin in isotonic, sterile, NaCl solution was inserted into the right femoral artery for intra-arterial blood pressure and heart rate measurement in conscious rats. The catheter was tunnelled subcutaneously, exteriorized through the skin on the dorsal side of the neck and protected with a silver spring. Rats were allowed to recover for 24 h and, after connecting the catheter to a transducer and a two-channel recorder (TRA-021 and Letigraph 2000, respectively; Letica SA, Barcelona, Spain), blood pressure and heart rate were continuously recorded for 60 min. Plasma testosterone and estradiol were

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measured by electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany).

#### Vascular reactivity studies

Descending thoracic aortic rings (3 mm) were dissected and mounted in organ chambers filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, NaHCO<sub>3</sub> 25, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 11) at 37 °C and gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Rings were stretched to 2 g of resting tension by means of two L-shaped stainless-steel wires inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer (Letigraph 2000), respectively, as previously described [20]. The concentration-relaxation response curves to acetylcholine (10<sup>-8</sup>-10<sup>-4</sup> M) were performed in rings pre-contracted by 10<sup>-6</sup> M phenylephrine. The concentration-relaxation response curves to nitroprusside (10<sup>-9</sup>-10<sup>-6</sup> M) were performed in the dark in rings pre-contracted by 10<sup>-6</sup> M phenylephrine. Endothelium-dependent contractions to acetylcholine were tested in rings which were initially stimulated with 80 mM KCl. After washing in Krebs solution and incubation for 30 min with L-NAME (10<sup>-4</sup> M), acetylcholine was added in a cumulative fashion (10<sup>-8</sup>-10<sup>-4</sup> M). In these experiments, the contractile responses to acetylcholine were expressed as a percentage of the response to KCl.

#### Measurement of vascular NOS activity

NOS activity in aortic homogenates was determined by monitoring the conversion of L-[ $^{3}$ H]arginine to L-[ $^{3}$ H]citrulline. Briefly, isolated aortic rings were homogenized, on ice, in a buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) (pH 7.4), sucrose (0.32 M), EDTA (100  $\mu$ M), dithiothreitol (1 mM),

phenylmethylsulphonyl fluoride (1 mg/ml) and leupeptin (10 µg/ml). Samples homogenates were incubated at 37°C for 30 min in TRIS buffer (50 mM, pH 7.4) in the presence of the NADPH (1 mM), L-valine (10 mM) and a mixture of unlabelled and L-[<sup>3</sup>H]arginine (10 µM, 1 µCi/ml). Reactions were terminated by the addition of 20 mM HEPES (1 ml, pH 5.5) containing 1mM EGTA and 1 mM EDTA. L-[<sup>3</sup>H]citrulline was separated from arginine by adding 1.5 ml of a 1:1 suspension of cation exchange resin (Dowex AG50 W-X8, Sigma) in water. The radioactivity was measured in the supernatants by liquid scintillation counting. The activity of the Ca<sup>2+</sup>-dependent NOS was determined from the difference between the L-[<sup>3</sup>H]citrulline produced from samples containing Ca<sup>2+</sup> and samples without Ca<sup>2+</sup> and with 1 mM EGTA. Results were expressed as picomoles of L-citruline per milligram of protein per 30 minutes.

#### Western blotting analysis

Frozen aortic rings were homogenized with a glass potter. eNOS, caveolin-1 and calmodulin-1 protein expression was measured in the supernatant of aortic homogenates. Proteins were quantified using the Bradford method. Western blottings were performed with 40 μg protein per lane for eNOS, caveolin-1 and calmodulin-1. Sodium dodecyl sulphate (SDS)-polyacrilamide (8% for eNOS or 15 % for the rest of proteins) electrophoresis was performed in a mini-gel system (Bio-Rad Laboratories, Hercules, California, USA). The proteins were transferred to polyvinylidene difluoride membranes (PVDF) and incubated overnight with primary monoclonal mouse anti-eNOS (1:2500 dilution, Transduction Laboratories, San Diego, California, USA), monoclonal mouse anti-calmodulin-1 (1:600 dilution, Transduction Laboratories), or polyclonal goat anti-calmodulin-1 (1:1000 dilution, SantaCruz Biotechnology, Santa Cruz, California, USA). The membranes were then washed five times for 10 min in Tris-buffered saline-0.1 %

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Tween 20 (TBST) and incubated with the corresponding secondary peroxidase conjugated goat anti-mouse or rabbit anti-goat antibodies (1:2000, Sigma-Aldrich, Madrid, Spain). All incubations were performed at room temperature for 2 hours. After washing the membranes, antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Films were scanned and densitometric analysis was performed on the scanned images using Scion Image-Release Beta 4.02 software (<u>http://www.scioncorp.com</u>). As an additional control, samples were re-probed for expression of  $\alpha$ -skeletal actin. Protein abundance/ $\alpha$ -actin ratio was performed and results were presented as comparisons to this ratio in aorta from WKY-vehicle in experiment I or from SHR-vehicle in experiment II on the same gels, expressed as 100%.

#### Detection of vascular superoxide (O<sub>2</sub><sup>-</sup>) production

 $O_2^-$  production in intact aortic segments was quantified by lucigenin-enhanced chemiluminescence, as previously described by Ohara et al. [21]. Aortic rings from all experimental groups were incubated for 30 minutes at 37 °C in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, MgSO<sub>4</sub> 1, Na<sub>2</sub>HPO<sub>4</sub> 0.15, KH<sub>2</sub>PO<sub>4</sub> 0.4, NaHCO<sub>3</sub> 1, CaCl<sub>2</sub> 1.2 and glucose 5.5. Aortic production of O<sub>2</sub><sup>-</sup> was stimulated by addition of NADPH (100 µM). Rings were then placed in tubes containing physiological salt solution with NADPH, and lucigenin was injected automatically at a final concentration of 5 µM. Changes in O<sub>2</sub><sup>-</sup> release were determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals. In a set of experiments of control SHR and WKY, vessel segments were incubated for 30 min with the inhibitor of NADPH oxidase diphenyleneiodonium (DPI, 10 µM). Vessels were then dried, and dry weight was determined.  $O_2^-$  release is expressed as nmol/min/mg dry aortic tissue, using combinations of xanthine and xanthine oxidase as an internal standard.

#### Statistical analysis

Results are expressed as means  $\pm$  s.e. means of measurements. Two way analysis of variance (ANOVA) was used to compare responses of control (vehicle-treated) animals with those of treated ones, and control SHR with control WKY rats. In experiment II, comparisons were made by one way ANOVA, with post-hoc multiple comparisons using Dunnet test. In all cases a P < 0.05 was considered statistically significant. Concentration-response curves were fitted to the logistic equation:  $E = E_{max} / (1 + 10^{-k * ([drug]-pD2)})$ , where  $E_{max}$  is the maximal effect, k is a factor which represents the slope of the curve, and pD<sub>2</sub> is the drug concentration exhibiting 50% of the  $E_{max}$  expressed as negative log molar.

#### Results

#### Blood pressure, heart rate and plasma sexual hormones levels

Long-term genistein administration reduced tail SBP in SHR and this effect reached statistical significance after 17-18 days of treatment while no changes were observed in WKY rats (Figure 1A, Experiment I). Tail SBP values were confirmed by direct mean arterial pressure measurements in conscious animals at the end of the experimental period (Figure 1A). Heart rate was increased in control SHR as compared to control WKY, but genistein did not significantly modify this parameter in both SHR and WKY rats (Figure 1A). The pure ER antagonist faslodex was unable to modify the antihypertensive effect induced by chronic oral genistein treatment (Figure 1B, experiment II).

Plasma testosterone and estradiol levels were similar in control WKY (1.60  $\pm$  0.50 ng/ml and 24.02  $\pm$  3.34 pg/ml, respectively) and in control SHR (1.88  $\pm$  0.54 ng/ml and 22.67  $\pm$  2.76 pg/ml, respectively, P>0.05). Chronic treatment with genistein did not modify the levels of plasma sexual hormones neither in WKY (1.21  $\pm$  0.46 ng/ml and 22.67  $\pm$  1.89 pg/ml, respectively) nor in SHR rats (2.08  $\pm$  0.74 ng/ml and 29.25  $\pm$  5.31 pg/ml, respectively).

#### Ex vivo vascular reactivity

In aortae from WKY and SHR treated with genistein, no differences were observed in the vasoconstrictor responses induced by noradrenaline or in the endothelium-independent vasodilator responses to the NO donor sodium nitroprusside as compared with their respective control WKY and SHR groups (Table 1). Aortae from vehicle-treated SHR showed significant reduced endothelium-dependent vasodilator responses to acetylcholine in arteries stimulated by phenylephrine as compared to aortae from control WKY ( $E_{max} = 32.5 \pm 5.2$  % vs 71.9 ± 4.1 %, respectively, P<0.05) (Figure 2A). In addition, in the presence of L-NAME (10<sup>-4</sup> M), aortae from SHR also showed greater endothelium-dependent vasoconstrictor responses to acetylcholine than their normotensive WKY counterparts (48.7 ± 3.9 % vs 15.6 ± 4.8 % of the response to 80 mM KCl, respectively). Acetylcholine was unable to induce a contraction in denuded vessels from WKY or SHR. Genistein produced a significant increase in the relaxation induced by acetylcholine only in SHR (Figure 2A), without changes in the endothelium-dependent vasoconstriction to acetylcholine in both SHR and WKY (45.7 ± 8.1 % and 9.6 ± 1.7 % of the response to 80 mM KCl, respectively). Figure 2B shows that faslodex

11

was unable to modify the improvement in endothelial function induced by genistein in SHR (Experiment II).

# Effects of genistein on vascular eNOS activity and vascular protein expression of eNOS and its protein regulators

Compared with the WKY control group, untreated SHR exhibited a significant reduction of aortic eNOS activity (Figure 3A). Genistein treatment increased eNOS activity in SHR but not in WKY (Figure 3A, experiment I) and this increase was not modified by faslodex (Figure 3B, experiment II). Paradoxically, protein expression of eNOS in the aorta was much higher in SHR than in the corresponding WKY group and genistein treatment had no effect on either group (Figure 4A). Thus, we examined the changes in expression of caveolin-1, the allosteric negative regulator of eNOS, and calmodulin-1, which positively regulates eNOS activity. The expression of caveolin-1 and calmodulin-1 was markedly lower in aortae from SHR control compared to WKY (Figure 4B, and 4C). After treatment with genistein, caveolin-1 protein expression was unchanged in both WKY and SHR (Figure 4B), while calmodulin-1 protein was increased only in SHR (Figure 4C). In SHR arteries without endothelium the expression of calmodulin-1 was lower than in intact arteries, but there were no differences among groups (not shown), indicating that the changes in calmodulin-1 occurred in the endothelium and not in the smooth muscle. In experiment II, aortic rings from faslodex plus genistein showed up-regulated eNOS protein as compared to genistein group (Figure 5A). The expression of caveolin-1 and calmodulin-1 in the faslodex plus genistein group was similar to the genistein group (Figure 5B and 5C), i.e. faslodex did not prevent the increase in calmodulin-1 expression induced by genistein.

#### Effects of genistein on vascular production of O<sub>2</sub><sup>-</sup>

No significant differences were found in basal vascular production of  $O_2^-$  within groups as measured by the chemiluminescence of lucigenin (ranking between 0.16-0.26 nmol/min/mg tissue). Aortic production of  $O_2^-$  stimulated by NADPH was significantly higher in SHR than WKY control rats (Figure 6). Preincubation of some aortic rings with the inhibitor of NADPH oxidase DPI almost suppressed the  $O_2^-$  production induced by NADPH. Chronic treatment with genistein induced a significant decrease of NADPHstimulated  $O_2^-$  production only in SHR, being without effects in WKY rats (Figure 6).

#### Discussion

Long-term oral treatment of male hypertensive rats with the soy isoflavone genistein restores the impaired aortic endothelium-dependent vasodilatation and reduces blood pressure, without effect on normotensive WKY rats. The major new findings of the present study were: (1) The genistein-induced improvement on endothelial function in SHR was associated with enhanced eNOS activity, increased expression of its modulator calmodulin-1 and reduced vascular production of the NO scavenger  $O_2^-$ . (2) Changes in endothelial function, blood pressure and protein expression induced by genistein seems to be unrelated to ER activation because co-administration of the pure  $\alpha$  and  $\beta$  ER antagonist faslodex did not modify the effects of genistein.

In vitro, soybean-derived phytoestrogens genistein and daidzein and the mammalian estrogen  $17\beta$ -estradiol, improved the endothelial function in isolated arteries from male hypertensive animals, restoring the impaired relaxant response to acetylcholine [7]. However, there are multiple factors (including pharmacokinetics, homeostasis and feedback mechanisms, hormonal control and changes in gene

expression) which are not retained in *in vitro* studies and may influence the long term *in* vivo effects. In the present in vivo study and in agreement with Kitayama et al. [10], we showed that chronic oral genistein treatment increased the endothelium-dependent vasodilatation to acetylcholine in male SHR but not in WKY rats. In the rat aorta, endothelium-dependent vasodilatation relies almost entirely on the endothelial release of NO and the subsequent activation of soluble guanylate cyclase [22]. The endothelium-independent vasodilation induced by the soluble guanylate cyclase activator nitroprusside was similar in SHR and WKY and unaffected by genistein, indicating that endothelial dysfunction and its improvement by genistein are due to changes in endothelium-derived NO availability rather than downstream effects on vascular smooth muscle. Additionally, apparent changes in endothelium-dependent vasodilation may also occur as a consequence of the opposing effects of the release of endothelium-derived vasoconstrictors [23]. In fact, consistent with previous reports [23], SHR showed increased endothelium-dependent vasoconstriction induced by acetylcholine in arteries treated with the NO synthase inhibitor L-NAME. This response was abolished by both endothelial denudation (present results) and incubation with the thromboxane-endoperoxide receptor antagonist SQ-29,548 [23], indicating the involvement of endothelial release of vasoconstrictor prostanoids. However, chronic genistein treatment did not modify the endothelium-dependent vasoconstriction induced by acetylcholine suggesting that the protective effect of genistein on endothelial function is unrelated to changes in endothelium-dependent vasoconstriction. Taken together, these data strongly suggest that genistein restores endothelial function in SHR by increasing NO availability.

The pathological reduction of NO synthesis and its restoration by drugs may be caused by several factors such as changes in the expression of eNOS, postransductional modification of the enzyme, interactions with its endogenous modulators or suboptimal concentrations of the substrate L-arginine or the cofactor tetrahydrobiopterin (BH4) [24]. As previously reported [25,26], the basal aortic eNOS activity of SHR was significantly lower than that of WKY. Interestingly, genistein enhanced eNOS activity in SHR, explaining, at least partly, the improvement of endothelium-dependent relaxation. In addition, in SHR we also found an increase in eNOS expression and a decrease in caveolin-1 expression, a negative eNOS modulator, which may even be viewed as a compensatory mechanism to maintain the production of bioactive NO in the face of increased oxidant stress [27]. The enhancement of aortic eNOS activity in SHR by genistein may be related, at least in part, to the higher endothelial calmodulin (positive regulator of eNOS) abundance despite the unchanged eNOS and caveolin-1 protein expression, as compared to vehicle treated SHR. Cell stimulation with Ca2+mobilizing agonists promotes calmodulin binding to eNOS which disrupts the inactive heteromeric complex formed between eNOS and caveolin, rendering the active form of the enzyme. Thus, calmodulin overexpression induced by genistein may lead to hyperactivation of eNOS and elevations of NO production in acetylcholine-vasodilation in intact aorta.

In addition, reduced endothelial NO levels may result from an increase in the oxidative inactivation of NO by  $O_2^{-}$ . Endothelial dysfunction in SHR has been associated in many reports with an excess of  $O_2^{-}$  generation [28]. In our study, NADPH-stimulated  $O_2^{-}$  production was increased in SHR aortic rings compared to WKY. We also found that genistein only reduced NADPH-stimulated aortic  $O_2^{-}$  production in rings from SHR but not from WKY. These results suggest that genistein specifically reduce the NADPH oxidase overactivity in SHR. Several subunits of the NADPH oxidase complex have been shown to be overexpressed in SHR [25,28]. We speculate that

15

chronic genistein might also restore this protein expression as previously observed with the flavonoid quercetin [25]. Therefore, an increased NO bioavailability induced by chronic genistein, seems to be due to both an increased NO synthesis and a reduced NADPH-dependent  $O_2^-$  production. The increase in NO bioavailability together with the reduced  $O_2^-$  production may account for the reduction of blood pressure induced by genistein in SHR.

Genistein is a ligand for estrogen receptor ER $\alpha$  and ER $\beta$ , showing greater affinity for the ER $\beta$  [13,14]. Squadrito et al. [6] found that genistein exerted similar protective effects in endothelial function to 17ß-estradiol in ovariectomized rats. The role of the interaction of genistein with ER was analyzed using the pure ER antagonist, faslodex. This drug was unable to alter the changes induced by genistein in blood pressure, endothelial function, eNOS activity and calmodulin aortic protein expression in SHR, suggesting that these effects are unrelated to ER stimulation. Potential alternative mechanisms include inhibition of tyrosine kinases, a large family of protein kinases involved in many signalling transduction pathways regulating gene expression. These results agree with our previous in vitro experiments showing that genisteininduced improvement in endothelial function in SHR in vitro [7] were unaffected by antagonism of ER $\alpha$  and ER $\beta$ . In contrast, faslodex has been reported to prevent genistein-induced increases in eNOS mRNA in ovariectomized rats [29] despite that they used a lower dose of faslodex (1.5 mg/kg/day s.c) during a shorter period of time (10 days). Faslodex is classically considered the gold-standard pure ER $\alpha$  and ER $\beta$ antagonist [30]. However, faslodex may fail to inhibit certain acute ER<sub>β</sub>-mediated effects [31]. Therefore, it cannot be categorically excluded the involvement of ER $\beta$  in the effects of genistein in our experiments. Surprisingly, in our study, faslodex plus genistein increased eNOS protein expression as compared to genistein. The significance

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16

of this effect is unclear because it resulted in no changes in eNOS activity. It might be interpreted in terms of the inhibitory effects of ER $\alpha$  and ER $\beta$  on the activity of other transcriptional factor involved in eNOS protein expression, such as peroxisome proliferator-activated receptor (PPAR)- $\gamma$ . In fact, activation of ER $\alpha$  and ER $\beta$  downregulates PPAR- $\gamma$  transcriptional activity [16]. So, it might be speculated that faslodex could facilitate the PPAR- $\gamma$  agonist activity of genistein [16], leading to an increase in eNOS expression.

The major conclusion to be drawn from this study is that oral treatment with the isoflavone genistein resulted in an enhanced vascular eNOS activity in SHR associated with an increased calmodulin-1 protein expression and a reduced vascular  $O_2^-$  production. Faslodex did not prevent these effects suggesting that ERs are not involved. These findings provide novel evidence to understand how genistein improves vascular endothelial function in hypertension and further support the beneficial effects of this dietary factor in cardiovascular diseases associated with endothelial dysfunction.

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20

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#### Figure legends.

**Figure 1**. Effects of genistein on arterial blood pressure and heart rate. Panel A (experiment I) represents the time course of the systolic arterial pressure (SBP) as measured by tail-cuff plethysmography in the SHR vehicle ( $\bigcirc$ , n = 10), SHR genistein ( $\blacklozenge$ , n = 10), WKY vehicle ( $\square$ , n = 10) and WKY genistein ( $\blacksquare$ , n = 10) groups and the direct measurements of mean arterial pressure (MAP) and heart rate in the vehicle (WKY and SHR, solid bars, n = 4) and genistein (WKY and SHR, open bars, n = 4) groups, respectively. Panel B (experiment II) represents the time course of the SBP as measured by tail-cuff plethysmography in the SHR vehicle ( $\bigcirc$ , n = 7), SHR genistein ( $\blacklozenge$ , n = 7) and SHR-genistein-faslodex ( $\blacktriangledown$ , n = 7) groups. Values are expressed as means ± s.e. mean. \*P < 0.05, \*\*P < 0.01 *vs* WKY vehicle group, #P < 0.05, ##P < 0.01 *vs* SHR vehicle group.

**Figure 2.** Endothelium-dependent relaxations induced by acetylcholine (Ach). Panel A (experiment I): aortae from the SHR vehicle ( $\bigcirc$ , n = 10), SHR genistein ( $\blacklozenge$ , n = 10), WKY vehicle ( $\square$ , n = 10) and WKY genistein ( $\blacksquare$ , n = 10) groups, and Panel B (experiment II) aortae from the SHR vehicle ( $\bigcirc$ , n = 7), SHR genistein ( $\blacklozenge$ , n = 7) and SHR-genistein-faslodex ( $\blacktriangledown$ , n = 7) groups. Ach-induced relaxations were induced in arteries contracted by  $10^{-6}$  M phenylephrine. Values are expressed as means  $\pm$  s.e. mean. \*P < 0.05, \*\*P < 0.01 *vs* WKY vehicle group, <sup>#</sup>P < 0.05, <sup>##</sup>P < 0.01 *vs* SHR vehicle group.

**Figure 3.** eNOS activity measured by the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline in aortic homogenates. Panel A (experiment I) SHR vehicle (n = 10), SHR genistein (n = 10), WKY vehicle (n = 10) and WKY genistein (n = 10) groups. Panel B (experiment II) SHR

vehicle (n = 7), SHR genistein (n = 7) and SHR-genistein-faslodex (n = 7) groups. Values are expressed as means  $\pm$  s.e. mean. \*\*P < 0.01 *vs* WKY vehicle group, <sup>#</sup>P < 0.05, <sup>##</sup>P < 0.01 *vs* SHR vehicle group.

**Figure 4**. Western blots of eNOS (A), caveolin-1 (B), and calmodulin-1 (C) protein expression in intact aortae from groups in experiment I. Values are expressed as means  $\pm$  s.e. mean (n = 4). \*\*P < 0.01 *vs* WKY vehicle group, <sup>#</sup>P < 0.05, <sup>##</sup>P < 0.01 *vs* SHR vehicle group.

**Figure 5**. Western blots of eNOS (A), caveolin-1 (B), and calmodulin-1 (C) protein expression in intact aortae from groups of experiment II. Values are expressed as means  $\pm$  s.e. mean (n =4). <sup>##</sup>P < 0.01 *vs* SHR vehicle group.

**Figure 6**. NADPH (100  $\mu$ M)-stimulated O<sub>2</sub><sup>-</sup> production in arteries from SHR vehicle (n = 10), SHR genistein (n = 10), WKY vehicle (n = 10) and WKY genistein (n = 10) groups. Rings from SHR vehicle and WKY vehicle groups were incubated with DPI (10 $\mu$ M). Values are means ± s.e.m. \*P < 0.05 and \*\*P < 0.01 *vs* WKY vehicle group, <sup>#</sup>P < 0.05 and <sup>##</sup>P < 0.01 *vs* SHR vehicle group.

	Noradrenaline $(10^{-9} - 10^{-6} \text{ M})$		SNP (10 <sup>-9</sup> -10 <sup>-6</sup> M)	
	- log EC <sub>50</sub>	$E_{max}\left(g ight)$	- log IC <sub>50</sub>	E <sub>max</sub> (%)
Experiment I				
WKY-vehicle ( $n = 10$ )	$8.27\pm0.10$	$2.27\pm0.27$	$7.36\pm0.10$	98.4 ± 1.0
WKY-genistein $(n = 10)$	$8.20\pm0.05$	$2.66\pm0.33$	$7.58\pm0.17$	97.8 ± 2.4
SHR-vehicle $(n = 10)$	$7.94\pm0.17$	$1.92\pm0.26$	$7.36\pm0.15$	97.0 ± 2.1
SHR-genistein (n = 10)	$7.79\pm0.10$	$1.92\pm0.21$	$7.46\pm0.22$	$96.4\pm4.8$
Experiment II				
SHR-vehicle $(n = 7)$	$7.83\pm0.06$	$1.30\pm0.18$	$7.94\pm0.10$	99.1 ± 1.0
SHR-genistein $(n = 7)$	$7.89\pm0.09$	$1.45 \pm 0.17$	$7.94\pm0.08$	96.8 ± 2.4
SHR-genistein-faslodex $(n = 7)$	$7.80\pm0.06$	$1.31\pm0.08$	$7.86\pm0.15$	97.5 ± 2.7

#### Table 1. Parameters of the concentration-response curves to endothelium-independent vasoactive factors

Values are expressed as mean  $\pm$  SEM.  $E_{max}$  (maximal effect) for noradrenaline is expressed as g of contraction and for sodium nitroprusside (SNP) as a percentage of relaxation of the pre-contraction with phenylephrine. Potency (-log EC<sub>50</sub>) is the drug concentration exhibiting 50 % of the  $E_{max}$  expressed as negative log molar. Potency (log IC<sub>50</sub>) is the drug concentration relaxing 50 % of the pre-contraction with phenylephrine expressed as negative log molar.

## Figure 1







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Figure 3



Figure 4





## Figure 6

