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

Rocio VERA*, Manuel SÁNCHEZ*, Milagros GALISTEO*, Inmaculada Concepcion VILLAR*, Rosario JIMENEZ*, Antonio ZARZUELO*, Francisco PÉREZ-VIZCAÍNO† and Juan DUARTE*

*Department of Pharmacology, School of Pharmacy, University of Granada, 18071 Granada, Spain, and †Department of Pharmacology, School of Medicine, University Complutense of Madrid, 28040 Madrid, Spain

ABSTRACT

The soya-derived phytoestrogen genistein has been suggested to be protective in cardiovascular diseases. In the present study, we have analysed whether chronic oral genistein might influence endothelial function in male SHRs (spontaneously hypertensive rats) via ERs (oestrogen receptors), changes in eNOS (endothelial NO synthase) activity and vascular $\text{O}_2^{-}$ (superoxide) production. Rats (23-weeks old) were divided into the following groups: WKY (Wistar–Kyoto)-vehicle, SHR-vehicle, WKY-genistein (10 mg · kg$^{-1}$ · day$^{-1}$ of body weight · day$^{-1}$); SHR-genistein; SHR-genistein-faslodex (ICI 182780; 2.5 mg · kg$^{-1}$ · day$^{-1}$ of body weight · day$^{-1}$). Vascular expression of eNOS, caveolin-1 and calmodulin-1 were analysed by Western blotting, eNOS activity by conversion of $[^{3}\text{H}]$arginine into L-$[^{3}\text{H}]$citrulline and $\text{O}_2^{-}$ production by chemoluminescence of lucigenin. In SHRs, after 5 weeks of treatment, genistein reduced systolic blood pressure and enhanced endothelium-dependent aortic relaxation to acetylcholine, but had no effect on the vasodilator responses to sodium nitroprusside. Compared with WKY rats, SHRs had up-regulated eNOS and down-regulated caveolin-1 and calmodulin-1 expression, increased NADPH-induced $\text{O}_2^{-}$ production, but reduced eNOS activity. Genistein increased aortic calmodulin-1 protein abundance and eNOS activity, and reduced NADPH-induced $\text{O}_2^{-}$ production in SHRs. The pure ER$\alpha$ and ER$\beta$ antagonist faslodex did not modify any of the changes induced by genistein in SHRs, suggesting that these effects are unrelated to ER stimulation. In conclusion, genistein reduced the elevated blood pressure and endothelial dysfunction in SHRs. This latter effect appears to be related to increased eNOS activity associated with increased calmodulin-1 expression and decreased $\text{O}_2^{-}$ generation.

INTRODUCTION

Isoflavones (e.g. genistein and daidzein) constitute the main class of phytoestrogens, and are found in abundance in soya beans and their derivative foods. There is evidence that the beneficial cardiovascular effects of consumption of soya are mostly due to its isoflavone fraction [1–3]. The cardioprotective ability of these plant products

Key words: calmodulin, caveolin, endothelial nitric oxide synthase (eNOS), hypertension, isoflavone, spontaneously hypertensive rat (SHR), superoxide.

Abbreviations: ACh, acetylcholine; BP, blood pressure; DPI, diphenyleneiodonium; ER, oestrogen receptor; HR, heart rate; $\text{l}$-NAME, $\text{N}^{\text{G}}$-nitro-$\text{l}$-arginine methyl ester; NOS, NO synthase; eNOS, endothelial NOS; $\text{O}_2^{-}$, superoxide; PPAR-$\gamma$, peroxisome proliferator-activated receptor-$\gamma$; SBP, systolic BP; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside; WKY, Wistar–Kyoto.

Correspondence: Dr Juan Duarte (email jmduarte@ugr.es).
has been attributed, at least in part, to their ability to
decrease plasma cholesterol, to lower BP (blood pressure)
and to improve arterial compliance [1–3]. Genistein
(4′,5,7-trihydroxy-isoflavone) in vitro relaxes rat arteries
through an NO-dependent mechanism [4], and enhances
the dilator response to ACh (acetylcholine) in coronary
arteries from atherosclerotic female macaques [5] and
in aortae from both ovariectomized rats [6] and male
SHRs (spontaneously hypertensive rats) [7]. Moreover,
the phytoestrogen daidzein enhanced eNOS (endothelial
NOS (NO synthase)) 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 endo-
toaxenic rats [9] and SHRs [10]. In addition, genistein
or soya supplements have been proven to be effective
in protecting endothelial function in men and healthy
postmenopausal women [11,12]; however, the mecha-
nisms involved in its beneficial effect on endothelial
function are unclear.

Genistein binds to ERs (oestrogen receptors) α and
β, but it shows greater affinity for ERβ [13]. Besides its
activity on ERs, the effects of genistein have also been
attributed to its inhibitory properties on a broad range of
tyrosine kinases [9,10,14,15], its PPAR-γ (peroxisome-
proliferator-activated receptor-γ) agonist activity [16]
or its antioxidant activity [17]. We have demonstrated
recently [7] that, in vitro, genistein and its analogue
daizdein acutely improved endothelial function in arteries
from SHRs, an effect unrelated to its oestrogenic
properties.

Therefore the aim of the present study was to
categorize further the mechanisms of the in vivo effects
of genistein on endothelial function and, particularly, the
role of ERs. Thus we analysed the effects of chronic
oral genistein administration on endothelial function and
the potential mechanisms involved, focusing on the NO
pathway: eNOS activity and the expression of eNOS
and its regulatory proteins (calmodulin-1 and caveolin-1).
The role of oestrogenic mechanisms was studied using a
pure ERα and ERβ antagonist. We selected male hyper-
tensive animals to analyse the female sex-independent
effects of dietary genistein. Genistein was used at doses
which resulted in serum genistein concentrations simi-
lar to those in humans eating a traditional Asian diet high
in soya [18,19].

MATERIALS AND METHODS

Animals and experimental groups

Male WKY (Wistar–Kyoto) rats and SHRs aged 23 weeks
were obtained from Harlan Laboratories. All rats were
maintained, five animals per cage, at a constant temper-
ature (24 ± 1°C), with a 12 h dark/light cycle and on
standard rat chow without soya 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.

Experiment I: analysis of the influence of genistein
on BP and endothelial function in SHRs

Rats were divided into the following groups (n = 10 in
each group): WKY-vehicle [1 % (w/v) methylcellulose],
SHR-vehicle [1 % (w/v) methylcellulose], WKY-
genistein (10 mg·kg⁻¹·day⁻¹, by gavage); and SHR-genistein (10 mg·kg⁻¹·day⁻¹, by gavage). Drugs were given for 5 weeks.
Body weight was measured every week. Genistein treat-
ment was stopped 2 days before the end of the exper-
iment in order to study the long-term effects of
genistein without the involvement of the effects of acute
administration.

Experiment II: analysis of the involvement of oestrogenic
mechanisms in the effects of genistein in SHRs

Rats were divided into the following groups (n = 7 in
each group): SHR-vehicle; SHR-genistein (10 mg·kg⁻¹·day⁻¹,
by gavage) and SHR-genistein-faslodex [genistein, 10 mg·kg⁻¹·day⁻¹,
by gavage; faslodex (ICI 182780; kindly provided by
AstraZeneca), 2.5 mg·kg⁻¹·day⁻¹, subcutaneously]. Drugs were given for 5 weeks. Body
weight was measured every week. Genistein treatment
was stopped 2 days before the end of the experiment.

BP measurements and plasma sexual
hormone levels

SBP (systolic BP) was measured 18–20 h after adminis-
tration of the drugs in conscious 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.
At the end of experiment I, direct BP and HR (heart
rate) were measured in four conscious rats per group.
For this purpose, the rats were anaesthetized intraperi-
nitoneally with 2.5 ml of equitensin/kg of body weight
[500 ml containing 43 % (w/v) chloral hydrate in 81 ml of
ethanol, 4.86 mg of nembutal, 198 ml of propylene glycol
and 10.63 g of MgSO4 in distilled water]. A polyethylene
catheter (PE-50) containing 100 units of heparin in iso-
tonic sterile NaCl solution was inserted into the right
femoral artery for intra-arterial BP and HR measure-
ments in conscious rats. The catheter was tunnelled sub-
cutaneously, 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), BP
and HR were recorded continuously for 60 min. Plasma
testosterone and oestradiol were measured by electrochemiluminescence immunoassay (Roche Diagnostics).

**Vascular reactivity studies**

After the treatment period, rats were injected with a lethal dose of sodium pentobarbitone (120 mg/kg of body weight, intraperitoneal administration). Descending thoracic aortic rings (3 mm) were dissected and mounted in organ chambers filled with Krebs solution (118 mmol/l NaCl, 4.75 mmol/l KCl, 25 mmol/l NaHCO3, 1.2 mmol/l MgSO4, 2 mmol/l CaCl2, 1.2 mmol/l KH2PO4 and 11 mmol/l glucose) at 37 °C and gassed with 95 % O2 and 5 % CO2. 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 described previously [20]. The concentration–relaxation response curves to ACh (10⁻⁸–10⁻⁴ mol/l) were performed in rings pre-contracted by 10⁻⁶ mol/l phenylephrine. The concentration–relaxation response curves to SNP (sodium nitroprusside; 10⁻⁹–10⁻⁶ mol/l) were performed in the dark in rings pre-contracted by 10⁻⁶ mol/l phenylephrine. Endothelium-dependent contractions to ACh were tested in rings which were initially stimulated with 80 mmol/l KCl. After washing in Krebs solution and incubation for 30 min with L-NAME (N^G^-nitro-L-arginine methyl ester; 10⁻⁴ mol/l), ACh was added in a cumulative fashion (10⁻⁶–10⁻⁴ mol/l). In these experiments, the contractile responses to ACh 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-[³H]arginine into L-[^α]-citrulline. Briefly, isolated aortic rings were homogenized, on ice in a buffer containing 10 mmol/l Hepes (pH 7.4), 0.32 mol/l sucrose, 100 µmol/l EDTA, 1 mmol/l dithiothreitol, 1 mg/ml PMSF and 10 µg/ml leupeptin. Homogenates were incubated at 37 °C for 30 min in 50 mmol/l Tris/HCl buffer (pH 7.4) in the presence of 1 mmol/l NADPH, 10 mmol/l L-valine and a mixture of unlabelled and 10 µmol/l L-[³H]arginine (1 µCi/ml). Reactions were terminated by the addition of 1 ml of 20 mmol/l Hepes (pH 5.5) containing 1 mmol/l EGTA and 1 mmol/l EDTA. L-[³H]Citrulline was separated from arginine by adding 1.5 ml of a 1:1 (v/v) 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 Ca^2+^-dependent NOS was determined from the difference between the L-[³H]citrulline produced from samples containing Ca^2+ and samples without Ca^2+ and with 1 mmol/l EGTA.

Results were expressed as pmol of L-citrulline · mg⁻¹ of protein · 30 min⁻¹.

**Western blotting analysis**

Frozen aortic rings were homogenized with a glass Potter homogenizer. eNOS, caveolin-1 and calmodulin-1 protein expression was measured in the supernatant of aortic homogenates. Proteins were quantified using the Bradford method. Western blotting was performed with 40 µg of protein/lane for eNOS, caveolin-1 and calmodulin-1. SDS/PAGE [8 % (w/v) polyacrylamide for eNOS or 15 % (w/v) polyacrylamide for caveolin-1 and calmodulin-1] was performed in a mini-gel system (Bio-Rad Laboratories). The proteins were transferred on to PVDF and incubated overnight with primary mouse monoclonal anti-eNOS (1:2500 dilution; Transduction Laboratories), mouse monoclonal anti-caveolin-1 (1:600 dilution; Transduction Laboratories) or goat polyclonal anti-(caveolin-1) (1:1000 dilution; SantaCruz Biotechnology) antibodies. The membranes were then washed five times for 10 min in TBST (Tris-buffered saline containing 0.1 % Tween 20) and incubated with the corresponding secondary peroxidase-conjugated goat anti-mouse or rabbit anti-goat antibodies (1:2000; Sigma–Aldrich). All incubations were performed at room temperature for 2 h. After washing the membranes, antibody binding was detected by an ECL system (Amersham Biosciences). Films were scanned and densitometric analysis was performed on the scanned image using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). As an additional control, samples were re-probed for expression of skeletal α-actin. Protein abundance/α-actin ratio was calculated, and results are presented as comparisons with 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 O₂⁻ (superoxide) production**

O₂⁻ production in intact aortic segments was quantified by lucigenin-enhanced chemiluminescence, as described previously by Ohara et al. [21]. Aortic rings from all experimental groups were incubated for 30 min at 37 °C in Heps-containing physiological salt solution [119 mmol/l NaCl, 20 mmol/l Hepes, 4.6 mmol/l KCl, 1 mmol/l MgSO4, 0.15 mmol/l Na2HPO4, 0.4 mmol/l KH2PO4, 1 mmol/l NaHCO3, 1.2 mmol/l CaCl2 and 5.5 mmol/l glucose (pH 7.4)]. Aortic production of O₂⁻ was stimulated by addition of NADPH (100 µmol/l). Rings were then placed in tubes containing physiological salt solution with NADPH, and lucigenin was injected automatically at a final concentration of 5 µmol/l. Changes in O₂⁻ release were determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507) in 5-s intervals. In a set of experiments of control SHRs and WKY rats, vessel segments were incubated for 30 min with 10 µmol/l DPI (diphenyleneiodonium), an inhibitor of NADPH oxidase. Vessels were then
dried, and dry weight was determined. O₂⁻ release is expressed as nmol·min⁻¹·mg⁻¹ of dry aortic tissue using combinations of xanthine and xanthine oxidase as an internal standard.

Statistical analysis

Results are means ± S.E.M. of measurements. Two-way ANOVA was used to compare responses of control (vehicle-treated) animals with those of treated ones, and control SHRs with control WKY rats. In experiment II, comparisons were made by one-way ANOVA, with post-hoc multiple comparisons using Dunnet’s test. In all cases, a *P < 0.05 was considered statistically significant.

Concentration–response curves were fitted to the logistic equation:

\[ E = E_{\text{max}} \left( \frac{1}{1 + 10^{-k \times (\log \text{[drug]} - \text{pD2})}} \right) \]

where \( E_{\text{max}} \) is the maximal effect, \( k \) is a factor which represents the slope of the curve, and pD₂ is the drug concentration exhibiting 50% of the \( E_{\text{max}} \) expressed as negative log molar.

RESULTS

BP, HR and plasma sexual hormones levels

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

Plasma testosterone and oestradiol levels were similar in control WKY rats (1.60 ± 0.50 ng/ml and 24.02 ± 3.34 pg/ml respectively) and in control SHRs (1.88 ± 0.54 ng/ml and 22.67 ± 2.76 pg/ml respectively; *P > 0.05). Chronic treatment with genistein did not modify the levels of plasma sexual hormones in either WKY rats (1.21 ± 0.46 ng/ml and 22.67 ± 1.89 pg/ml respectively) or SHRs (2.08 ± 0.74 ng/ml and 29.25 ± 5.31 pg/ml respectively).

Ex vivo vascular reactivity

In aortae from WKY rats and SHRs treated with genistein, no differences were observed in the vasoconstrictor responses induced by noradrenaline or the endothelium-independent vasodilator responses to the NO donor SNP compared with their respective control WKY and SHR groups (Table 1). Aortae from vehicle-treated SHRs had significantly reduced endothelium-dependent vasodilator responses to ACh in arteries stimulated by phenylephrine compared with aortae from control WKY rats (\( E_{\text{max}} = 32.5 ± 5.2 \) compared with 71.9 ± 4.1% respectively, *P < 0.05) (Figure 2A). In addition, in the presence of L-NAME (10⁻⁴ mol/l), aortae from SHRs also had greater endothelium-dependent vasoconstrictor responses to ACh than their nor-motensive WKY rat counterparts (48.7 ± 3.9 compared with 15.6 ± 4.8% of the response to 80 mmol/l KCl respectively). ACh was unable to induce a contraction in denuded vessels from WKY rats or SHRs. Genistein only produced a significant increase in the relaxation induced by ACh in SHRs (Figure 2A), without changes in the endothelium-dependent vasoconstriction to ACh.
Table 1  Parameters of the concentration–response curves to endothelium-independent vasoactive factors

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<td>Values are means ± S.E.M.  $E_{\text{max}}$ (maximal effect) for noradrenaline is expressed as g of contraction, and for SNP as a percentage of relaxation of pre-contraction with phenylephrine. Potency ($-\log EC_{50}$) is the drug concentration exhibiting 50% of the $E_{\text{max}}$ expressed as negative log molar. Potency ($-\log IC_{50}$) is the drug concentration relaxing 50% of the pre-contraction with phenylephrine expressed as negative log molar.</td>
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<th>Noradrenaline ($10^{-9}$–$10^{-6}$ mol/l)</th>
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<td>$-\log EC_{50}$</td>
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<td>Experiment I (n = 10)</td>
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<td>WKY-vehicle</td>
<td>8.27 ± 0.10</td>
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<tr>
<td>WKY-genistein</td>
<td>8.20 ± 0.05</td>
<td>2.64 ± 0.33</td>
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<tr>
<td>SHR-vehicle</td>
<td>7.94 ± 0.17</td>
<td>1.92 ± 0.26</td>
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<tr>
<td>SHR-genistein</td>
<td>7.79 ± 0.10</td>
<td>1.92 ± 0.21</td>
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<td>Experiment II (n = 7)</td>
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<tr>
<td>SHR-vehicle</td>
<td>7.83 ± 0.06</td>
<td>1.30 ± 0.18</td>
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<tr>
<td>SHR-genistein</td>
<td>7.89 ± 0.09</td>
<td>1.45 ± 0.17</td>
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<td>SHR-genistein-faslodex</td>
<td>7.80 ± 0.06</td>
<td>1.31 ± 0.08</td>
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Figure 2  Endothelium-dependent relaxation induced by ACh

(A) Aortae from the SHR-vehicle, SHR-genistein, WKY-vehicle and WKY-genistein groups in experiment I (n = 10). (B) Aortae from the SHR-vehicle, SHR-genistein and SHR-genistein-faslodex groups in experiment II (n = 7). Ach-induced relaxations were induced in arteries contracted by $10^{-6}$ mol/l phenylephrine. Values are expressed as means ± S.E.M. *P < 0.05 and **P < 0.01 compared with the WKY-vehicle group; #P < 0.05 and ##P < 0.01 compared with the SHR-vehicle group.

in both SHRs and WKY rats (45.7 ± 8.1 and 9.6 ± 1.7% of the response to 80 mmol/l KCl respectively). Figure 2(B) shows that faslodex was unable to modify the improvement in endothelial function induced by genistein in SHRs (experiment II).

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

Compared with the WKY rat control group, untreated SHRs exhibited a significant reduction in aortic eNOS activity (Figure 3A). Genistein treatment increased eNOS activity in SHRs but not in WKY rats (experiment I; Figure 3A), and this increase was not modified by faslodex (experiment II; Figure 3B). Paradoxically, protein expression of eNOS in the aorta was much higher in SHRs than in the corresponding WKY rat 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 control SHRs compared with WKY rats (Figures 4B and 4C). After treatment with genistein, caveolin-1 protein expression was unchanged in both WKY rats and SHRs (Figure 4B), whereas calmodulin-1 protein was increased only in SHRs (Figure 4C). In arteries from SHRs without endothelium, the expression of calmodulin-1 was lower than in intact arteries, but there were no differences among groups (results not shown), indicating that the changes in calmodulin-1
occurred in the endothelium and not in the smooth muscle. In experiment II, up-regulated eNOS protein was observed in aortic rings from the faslodex + genistein group compared with the genistein group (Figure 5A). The expression of caveolin-1 and calmodulin-1 in the faslodex + 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$_2^-$**

No significant differences were found in basal vascular production of O$_2^-$ within groups, as measured by the chemiluminescence of lucigenin (ranging between 0.16 and 0.26 nmol · min$^{-1}$ · mg$^{-1}$ of tissue). Aortic production of O$_2^-$ stimulated by NADPH was significantly higher in SHRs than in control WKY rats (Figure 6). Pre-incubation of aortic rings with DPI, an inhibitor of NADPH oxidase, almost suppressed O$_2^-$ production induced by NADPH. Chronic treatment with genistein induced a significant decrease in NADPH-stimulated O$_2^-$ production only in SHRs, but did not have any effects in WKY rats (Figure 6).

**DISCUSSION**

Long-term oral treatment of male SHRs with the soya isoflavone genistein restores the impaired aortic endothelium-dependent vasodilatation and reduces BP, but is without effect in normotensive WKY rats. The major new findings of the present study were: (i) genistein-induced improvement of endothelial function in SHRs was associated with enhanced eNOS activity, increased expression of its modulator calmodulin-1 and reduced vascular production of the NO scavenger O$_2^-$; and (ii) changes in endothelial function, BP and protein expression induced by genistein appear to be unrelated to ER activation, because co-administration of the pure ER$\alpha$ and ER$\beta$ antagonist faslodex did not modify the effects of genistein.

*In vitro*, the soya-bean-derived phytoestrogens genistein and daidzein and the mammalian oestrogen 17$\beta$-oestradiol improved endothelial function in isolated arteries from male SHRs, restoring the impaired relaxant response to ACh [7]. However, there are multiple factors (including pharmacokinetics, homoeostasis and feedback mechanisms, hormonal control and changes in gene expression) which are not retained in *in vitro* studies and...
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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 means ± S.E.M. (n = 4). ###P < 0.01 compared with SHR-vehicle group. S, SHR-vehicle; SG, SHR-genistein; SGF, SHR-genistein-faslodex.

Figure 6  NADPH (100 µmol/l)-stimulated O$_2^-$ production in arteries from SHR-vehicle, SHR-genistein, WKY-vehicle and WKY-genistein groups

Rings from SHR-vehicle and WKY-vehicle groups were incubated with DPI (10 µmol/l). Values are means ± S.E.M., n = 10. *P < 0.05 and **P < 0.01 compared with WKY-vehicle group; #P < 0.05 and ###P < 0.01 compared with SHR-vehicle group.

may influence the long-term in vivo effects. In the present in vivo study, and in agreement with Kitayama et al. [10], we have shown that chronic oral genistein treatment increased endothelium-dependent vasodilatation to ACh in male SHRs 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 SNP was similar in SHRs and WKY rats and was 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 vasodilatation 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], SHRs had increased endothelium-dependent vasoconstriction induced by ACh in arteries treated with the NOS 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 ACh, 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 SHRs 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, posttransductional modification of the enzyme, interactions with its endogenous modulators, or suboptimal concentrations of the substrate l-arginine or the cofactor BH$_4$ (tetrahydrobiopterin) [24]. As reported previously [25, 26], the basal aortic eNOS activity of SHRs was significantly lower than that of WKY rats. Interestingly, genistein enhanced eNOS activity in SHRs, explaining, at least partly, the improvement in endothelium-dependent relaxation. In addition, in SHRs, 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 SHRss 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, compared with vehicle-treated SHRss. 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 elevation of NO production in ACh-induced vasodilation in intact aorta.

In addition, reduced endothelial NO levels may result from an increase in the oxidative inactivation of NO by O2•−. Endothelial dysfunction in SHRss has been associated in many reports with an excess of O2•− generation [28]. In our present study, NADPH-stimulated O2•− production was increased in aortic rings from SHRss compared with WKY rats. We also found that genistein only reduced NADPH-stimulated aortic O2•− production in rings from SHRss but not from WKY rats. These results suggest that genistein specifically reduces NADPH oxidase overactivity in SHRss. Several subunits of the NADPH oxidase complex have been shown to be overexpressed in SHRss [25,28]. We speculate that chronic genistein treatment might also restore this protein expression as observed previously with the flavonoid quercetin [25]. Therefore increased NO bioavailability induced by chronic genistein appears to be due to both increased NO synthesis and decreased NADPH-dependent O2•− production. The increase in NO bioavailability, together with the decrease in O2•− production, may account for the reduction in BP induced by genistein in SHRss.

Genistein is a ligand for ERα and ERβ, having greater affinity for ERβ [13,14]. Squadrito et al. [6] found that genistein exerted similar protective effects on endothelial function to 17β-oestradiol in ovariectomized rats. The role of the interaction of genistein with ER was analysed using the pure ER antagonist faslodex. This drug was unable to alter the changes induced by genistein in BP, endothelial function, eNOS activity and calmodulin aortic protein expression in SHRss, 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 [7], showing that genistein-induced improvement in endothelial function in SHRss in vitro was unaffected by antagonism of ERα and ERβ. In contrast, faslodex has been reported to prevent genistein-induced increases in eNOS mRNA in ovariectomized rats [29], although Wang et al. used a lower dose of faslodex (1.5 mg · kg−1 · day−1) in intact aorta over a shorter period of time (10 days). Faslodex is classically considered as a gold-standard pure ERα and ERβ antagonist [30]; however, it may fail to inhibit certain acute ERβ-mediated effects [31]. Therefore we cannot categorically exclude the involvement of ERβ in the effects of genistein in our present experiments. Surprisingly, in our present study, faslodex + genistein increased eNOS protein expression compared with genistein. The significance 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 ERα and ERβ on the activity of other transcriptional factor involved in eNOS protein expression, such as PPAR-γ. In fact, activation of ERα and ERβ down-regulates PPAR-γ transcriptional activity [16]. Thus it might be speculated that faslodex could facilitate PPAR-γ agonist activity of genistein [16], leading to an increase in eNOS expression.

The major conclusion to be drawn from the present study is that oral treatment with the isoflavone genistein resulted in an enhanced vascular eNOS activity in SHRss associated with increased calmodulin-1 protein expression and decreased vascular O2•− production. Faslodex did not prevent these effects, suggesting that ERs are not involved. These findings provide novel evidence to explain how genistein improves vascular endothelial function in hypertension and support further the beneficial effects of this dietary factor in cardiovascular diseases associated with endothelial dysfunction.

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