Ferulic Acid Restores Endothelium-Dependent Vasodilation in Aortas of Spontaneously Hypertensive Rats

Atsushi Suzuki, Masaki Yamamoto, Hiroko Jokura, Akihiko Fujii, Ichiro Tokimitsu, Tadashi Hase, and Ikuo Saito

Background: Ferulic acid (FA), a phytochemical constituent, has antihypertensive effects, but a detailed understanding of its effects on vascular function remains unclear. The vasoreactivity of FA was assessed using aortic rings isolated from normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR).

Methods: The effects of FA (10⁻³ to 10⁻⁵ mol/L) on vasodilatory responses were evaluated based on contractile responses induced by phenylephrine (10⁻⁶ mol/L) in thoracic aortic rings from male WKY rats and SHR. Basal nitric oxide (NO) bioavailability in the aorta was determined from the contractile response induced by the NO synthase inhibitor N⁵-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴ mol/L). The effects of FA on the production of NADPH-dependent superoxide anion were examined in SHR aortas. The impact of hydroxyhydroquinone, a generator of superoxide anions, on the FA-induced enhancement in acetylcholine-stimulated vasodilation was also investigated.

Results: The FA (10⁻³ mol/L)-induced relaxation was partially blocked by removal of the endothelium or by pretreating SHR aortas with L-NAME. FA increased NO bioavailability, and decreased NADPH-dependent superoxide anion levels in SHR aortas. Ferulic acid improved acetylcholine-induced endothelium-dependent vasodilation in SHR, but not in WKY. Furthermore, the simultaneous addition of hydroxyhydroquinone significantly inhibited the increase in acetylcholine-induced vasodilation by FA.

Conclusions: Ferulic acid restores endothelial function through enhancing the bioavailability of basal and stimulated NO in SHR aortas. The results explain, in part, the mechanisms underlying the effects of FA on blood pressure (BP) in SHR. Am J Hypertens 2007;20:508–513 © 2007 American Journal of Hypertension, Ltd.

Key Words: Endothelial function, nitric oxide, vasodilation.

Epidemiologic studies have shown that the consumption of fruits and vegetables is associated with a reduced risk of cardiovascular disease.¹ Ferulic acid (FA; 4-hydroxy-3-methoxycinnamic acid) is a lignin constituent, produced by plants.² Various studies have indicated that FA has antioxidant effects, photoprotective effects, antitumor activity, and has a protective effect against β-amyloid peptide toxicity.³⁻⁶ We previously demonstrated that FA showed an antihypertensive action in spontaneously hypertensive rats (SHR).⁷ The blood pressure (BP)-lowering effect of intravenously administered FA was largely blocked by pretreatment with a nitric oxide synthase (NOS) inhibitor, suggesting that a nitric oxide (NO)-dependent vascular response was involved in the effect. A detailed understanding of the mechanisms responsible for the action of FA on vascular function, however, remains unclear.

The regulation of reactive oxygen species (ROS) for enhancing the bioavailability of NO is currently attracting attention as a therapeutic target for inhibiting the development and progression of hypertension.⁸ Because FA is reported to have antioxidant action,⁹ we hypothesized that FA improves the bioavailability of NO by reducing ROS levels in the hypertensive vasculature. The goal of this study was to investigate the effects of FA on the involvement of NO and endothelium-dependent and -independent vasoreactivity using thoracic aortas excised from normotensive Wistar Kyoto (WKY) rats and SHR.

Methods
Chemicals
Ferulic acid, N⁵-nitro-L-arginine methyl ester (L-NAME), N⁵-monomethyl-L-arginine (L-NMMA), sodium nitropruss-
side (SNP), 3-morpholino-sydnonimine (SIN-1), phenylephrine, catalase, and superoxide dismutase (SOD) were purchased from the Sigma Chemical Co. (St. Louis, MO). Acetylcholine, dimethyl sulfoxide (DMSO), hydroxyhydroquinone (HHQ), and other inorganic salts were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For contractile experiments, FA was solubilized in DMSO at a concentration of 1 mol/L and diluted to the desired concentration with DMSO immediately before use. When DMSO (vehicle) was added to the bath in control preparations, no effect was observed (data not shown). The other drugs were dissolved in distilled water.

**Animals**

Experiments were conducted with the approval of the Ethics Review Committee for Animal Experimentation of the Kao Corporation. Male WKY rats (WKY/Izm) and SHR (SHR/Izm) (20 to 24 weeks old), purchased from SLC, Inc. (Shizuoka, Japan), were anesthetized with intraperitoneal injections of sodium pentobarbital.

**Ex Vivo Vascular Reactivity**

Experiments were performed according to previously published methods. The descending thoracic aorta was excised, freed of fat and connective tissue, cut into rings approximately 2 to 3 mm in length, and placed in a Magnus tube (5 mL) filled with gassed (95% O2 and 5% CO2) Krebs-Henseleit solution with the following composition (in mmol/L): NaCl 110.8, KCl 5.9, NaHCO3 25.0, MgSO4 1.07, CaCl2 2.49, NaHPO4 2.33, and glucose 11.51. The endothelium was removed by gently rolling the vessel between the forceps and palm. The aortic rings were maintained at 37°C under a 1 g tension and equilibrated for 1 h before initiating the experimental protocols. During this period, the incubation medium was changed at 15-min intervals. The presence of functional endothelium was assessed by the ability of acetylcholine (10⁻⁶ mol/L) to induce more than a 60% relaxation of vessels that had been precontracted by treatment with phenylephrine (10⁻⁶ mol/L). Tissue responses were recorded using isometric transducers (Kishimoto Medical Instruments Co., Ltd., Kyoto, Japan) and recorders (SEKONIC Co., Tokyo, Japan). Vasodilation was calculated as the percent change in phenylephrine-induced contractile tension.

**Vasorelaxant Responses of Ferulic Acid**

The vasorelaxant activity of FA (10⁻⁴ to 10⁻³ mol/L) was determined in WKY or SHR aortic rings (n = 4 to 6) with or without a functional endothelium. To verify the participation of endothelium-derived products in the relaxant effect of FA, experiments were performed in the presence of L-NAME (10⁻⁴ mol/L), a nonselective NOS inhibitor; indomethacin (10⁻⁵ mol/L), a nonselective cyclooxygenase inhibitor; thapsigargin (10⁻⁵ mol/L), a sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor, which were added to the bath 20 min before the addition of phenylephrine (10⁻⁶ mol/L). Ferulic acid was added when the contractile response to phenylephrine reached a steady-state tension. In an alternate set of experiments, the aorta was pretreated for 30 min with FA (10⁻⁵ and 10⁻⁴ mol/L) before the addition of phenylephrine, and acetylcholine-induced (10⁻⁹ to 10⁻⁶ mol/L) relaxation, a measure of the stimulating bioavailability of NO, was examined in WKY or SHR aortas.

**Effect of Ferulic Acid on L-NAME-Induced Contraction**

This experiment was performed following a previously described method. The aortic rings (n = 4 to 6) were preconstricted to 20% effective concentration (EC20) with phenylephrine (5 × 10⁻⁸ mol/L), and the NOS inhibitor L-NAME was added to a final concentration of 10⁻⁴ mol/L. The contraction in rings from rats that had been treated with FA (10⁻⁵ to 10⁻³ mol/L) for 30 min was measured. The resulting contraction, as a percentage of the phenylephrine EC20 contraction, was taken as a measure of the basal bioavailability of NO.

**Effect of Ferulic Acid on Nitric Oxide Donor-Induced Vasodilation**

An SHR aortic ring preparation from which the functional endothelium had been mechanically removed (n = 4 to 5) was pretreated with FA (10⁻⁴ mol/L) for 30 min before the addition of phenylephrine (10⁻⁶ mol/L). Endothelium-independent vasodilation was induced by treatment with SNP and SIN-1 (10⁻⁹ to 10⁻⁶ mol/L). The SNP has been used as a NO donor and SIN-1 is known to release both NO and superoxide anions (O₂⁻). The functional approach for the determination of the antioxidant property of polyphenols is based on their ability to potentiate the vasorelaxant response triggered by SIN-1. Superoxide dismutase (150 units/mL) was added 5 min before the addition of phenylephrine.

**Effect of Hydroxyhydroquinone on Endothelium-Dependent Vasodilation**

In preliminary experiments, HHQ, a generator of superoxide anions, (10⁻⁵ and 10⁻⁶ mol/L) induced contractile responses in phenylephrine precontracted aortas, but 10⁻⁷ mol/L did not in SHR. An SHR aortic ring preparation was treated with FA (10⁻⁵ mol/L) for 30 min before the addition of phenylephrine (10⁻⁶ mol/L). HHQ (10⁻⁷ mol/L) was added, followed by contraction by phenylephrine after 5 min, and the extent of acetylcholine (3 × 10⁻⁸ mol/L)-induced vasodilation was measured. Superoxide dismutase (150 units/mL) or catalase (1000 units/mL) was added 5 min before the addition of HHQ.

**Determination of Vascular Cyclic GMP or Thromboxane B₂ Production**

The concentration of cyclic GMP in SHR aortas was determined using a previously described method with
minor modifications. Aortas with a functional endothelium were incubated in Krebs-Henseleit solution containing isobutylmethylxanthine (10^{-3} \text{ mol/L}, an inhibitor of cyclic nucleotide phosphodiesterases) and were exposed to FA (10^{-3} \text{ mol/L}) for 15 min (n = 5). After homogenization and centrifugation, the supernatant was collected for cGMP determination using an enzyme immunoassay (Amersham Life Science, Bucks, UK). To determine the production of thromboxane B_{2} (TXB_{2}), endothelium-intact aortas were placed in Krebs-Henseleit solution in the presence of FA (10^{-3} \text{ mol/L}) for 15 min (n = 5). At the end of this period, 0.5 mL of medium was collected and TXB_{2} was determined by an enzyme immunoassay (Amersham Life Science).

### Vascular NADPH-Dependent Superoxide Anion Production

The NADPH-dependent production of superoxide anion (O_{2}^{-}) (NADPH oxidase activity) was determined using a chemiluminescence assay,\(^{10}\) with minor modifications (n = 4). Approximately 10-mm long aortic rings from rats were incubated at 37°C for 15 min in Krebs-HEPES buffer containing FA (10^{-5} to 10^{-4} \text{ mol/L}) and L-NMMA (10^{-4} \text{ mol/L}). Rings were transferred tubes containing FA (10^{-4} \text{ mol/L}), NADPH (10^{-4} \text{ mol/L}), L-NMMA (10^{-4} \text{ mol/L}), or lucigenin (5 \times 10^{-5} \text{ mol/L}). Luminescence was calculated as the rate of counts per milligram of tissue after subtracting the counts from a buffer blank using Luminescence-PSN (ATTO, Tokyo, Japan).

### Data Analysis

The results are expressed as the means ± SEM. Data were initially analyzed using analysis of variance for each group. When a significant F-value (P < .05) was obtained, a Dunnett’s test was performed in a post hoc analysis. Dose–response curves were fitted by nonlinear regression with the simplex algorithm. Relaxant responses are given as the percentage of phenylephrine-induced precontraction. Comparisons of dose–response curves were evaluated by two-way ANOVA for repeated measures. Probability values < .05 were considered to be statistically significant.

### Results

#### Vasorelaxant Responses to Ferulic Acid

The tension induced by phenylephrine (10^{-6} \text{ mol/L}) was 0.63 ± 0.08 g in WKY aortas and 0.61 ± 0.05 g in SHR aortas. In SHR vascular ring preparations, FA relaxed phenylephrine-induced contraction in a concentration-dependent manner (Fig. 1). Relaxation was also induced in WKY, but the degree was significantly lower than that for the SHR aortic rings. The relaxation induced by FA was partially inhibited by removing the endothelium or by pretreatment of the aorta with L-NAME. The blocking of cyclooxygenase activity by indomethacin (Fig. 1) or SERCA activity by thapsigargin (data not shown) had no effect on FA-induced vasorelaxation.

#### Vascular Cyclic GMP or Thromboxane B_{2} Production

Ferulic acid (10^{-3} \text{ mol/L}) did not produce a significant change in cGMP content in aortic rings with a functional endothelium (Table 1). Thromboxane B_{2} is the stable metabolite of the vasoconstrictor and platelet proaggregant prostanoid TXA_{2} and, thus, TXB_{2} production was used to estimate TXA_{2} synthesis. The TXB_{2} production was significantly lower in the incubation media of aortic rings from SHR that had been treated with FA (10^{-3} \text{ mol/L}) compared to those from vehicle-treated aortas (Table 1), but treatment with a lower concentration of FA (10^{-4} \text{ mol/L}) did not produce any significant change in TXB_{2} release (539 ± 72 \mu g/mg tissue).

### Table 1. Cyclic GMP and TXB_{2} levels in SHR aortas

<table>
<thead>
<tr>
<th></th>
<th>cGMP</th>
<th>TXB_{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>83 ± 15</td>
<td>613 ± 42</td>
</tr>
<tr>
<td>FA (10^{-3} \text{ mol/L})</td>
<td>59 ± 10</td>
<td>450 ± 57*</td>
</tr>
</tbody>
</table>

\(\text{cGMP} = \text{cyclic GMP}; \ FA = \text{ferulic acid}; \ SHR = \text{spontaneously hypertensive rats}; \ TXB_{2} = \text{thromboxane B}_{2}.

Each value represents the mean ± SE (n = 5).

\(\ast P < 0.05\) vs vehicle.
than the values for vehicle-treated aorta (Fig. 2).

Chemiluminescence of lucigenin induced by NADPH-Superoxide Anion Production

Vascular NADPH-Dependent O2 production was detected using a vascular NADPH oxidase activity measurement system10 (Table 2). The NADPH oxidase activity was significantly lower in SHR (460 ± 31) compared to WKY (264 ± 17). Furthermore, the contraction in rings from SHR that had been treated with FA (10−2 and 10−3 mol/L) was significantly greater than the values for vehicle-treated aorta (Fig. 2).

Acetylcholine-Induced or Nitric Oxide Donor-Induced Vasodilation

Acetylcholine-induced vasodilation was significantly impaired in SHR aortas compared to WKY aortas (Fig. 3A). Treatment with L-NAME (10−4 mol/L) completely inhibited the acetylcholine-induced vasodilatory effect in SHR, suggesting that the acetylcholine-induced vasodilation is largely due to NOS-derived NO. Ferulic acid (10−4 mol/L) had no effect on acetylcholine-induced vasodilation in WKY aortas. In contrast, FA significantly potentiated the acetylcholine-induced vascular response of SHR aortas. In endothelium-denuded rings that had been precontracted by treatment with phenylephrine (10−6 mol/L), SNP or SIN-1 (10−9 to 10−6 mol/L) produced a concentration-dependent dilation. Ferulic acid had no effect on SNP-induced vasodilation, suggesting that it is ineffective on endothelium-independent vasodilation (Fig. 3B). Ferulic acid also had no effect on SIN-1-induced vasodilation (Fig. 3C). In contrast, SOD (150 U/mL) enhanced the vasodilatory effect of SIN-1, demonstrating the protective effect of SOD on NO destruction by O2− under the experimental conditions used here. The lack of a leftward shift in the concentration–response curve of SIN-1 in the presence of FA indicates an inability to protect against NO breakdown at the dose used (Fig. 3C).

Effect of Hydroxyhydroquinone on Acetylcholine-Induced Vasodilation

Ferulic acid augmented acetylcholine-induced vasodilation, and HHQ inhibited the improving effect on endothelial function, suggesting that HHQ inhibited the FA-induced improvement in acetylcholine reactivity (Fig. 4). Furthermore, we investigated whether the inhibition of the FA-induced improvement in acetylcholine-dependent vasodilation by HHQ was mediated by ROS. The effect of FA on acetylcholine-induced vasodilation was significantly improved by the addition of SOD, but not by catalase, suggesting that the inhibitory effect of HHQ on the FA-induced potentiation of the acetylcholine-induced vasodilatory response is mediated by O2−.

Discussion

The effects of FA, a phytochemical constituent that has an antihypertensive action, on rat vascular function were investigated. The findings indicate that FA induces endothelium-dependent vasodilation through enhancing basal and stimulated NO bioavailability in SHR, but not in WKY aortas. The results explain, in part, the mechanisms underlying the effects of FA on blood pressure (BP) in SHR.

Ferulic acid caused a relaxation in phenylephrine-precontracted aortic ring preparations isolated from SHR with an intact endothelium. The FA-induced endothelium-

<table>
<thead>
<tr>
<th>Table 2. NADPH oxidase activity in rat aortas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NADPH oxidase</strong></td>
</tr>
<tr>
<td>WKY</td>
</tr>
<tr>
<td>SHR</td>
</tr>
<tr>
<td>SHR + 10−5 mol/L FA</td>
</tr>
<tr>
<td>SHR + 10−4 mol/L FA</td>
</tr>
</tbody>
</table>

WKY = Wistar Kyoto rats; other abbreviations as in Table 1.

NADPH-dependent production of O2− (NADPH oxidase activity) was determined using a chemiluminescence assay.

Each value represents the mean ± SE (n = 4).

* P < .05 v WKY; † P < .05 v SHR.
dependent relaxation was significantly inhibited by L-NAME, whereas indomethacin had no effects on the relaxation in SHR aorta, suggesting that FA evoked an NO-dependent relaxation. Furthermore, the findings herein demonstrate that FA increases basal NO bioavailability in the L-NAME-induced contractile response in SHR aortas. These findings suggest that the vasorelaxant effect of FA on phenylephrine-induced contraction is partially mediated by endothelial NO in SHR aortas. Unexpectedly, however, no effects of FA (10^{-3} mol/L) on cGMP levels or thapsigargin treatment in SHR aortas were found. It is possible that FA-induced NO was insufficient to stimulate soluble guanylate cyclase or SERCA in this experiment. Otherwise, the FA-induced relaxation persisted, even after the removal of the intact endothelium or treatment with L-NAME. These findings demonstrate that FA has a direct effect on vascular smooth muscle cells in addition to its effect on endothelial cells. We found that the release of TXB_{2} from SHR aortas was significantly reduced by the FA treatment (Table 1), but was unchanged by 10^{-4} mol/L of FA, suggesting that the high concentration of FA might partially relax aortas through the inhibition of TXB_{2} release.

In the experiment using an NO donor, FA (10^{-4} mol/L) had no effect on SNP-induced endothelium-independent vasodilatation, suggesting that FA at this dose does not affect the NO-dependent pathway in vascular smooth muscle in SHR. The report that FA scavenges O_{2}^{·-} derived from xanthine and xanthine oxidase^{9} led us to hypothesize that the ROS scavenging ability of FA might explain the im-

FIG. 3. Effects of ferulic acid (FA) on vasodilatory responses induced by acetylcholine (A), sodium nitroprusside (SNP) (B), and 3-morpholino-sydnonimine (SIN-1) (C) in WKY or SHR thoracic aortas in the presence (A) or absence (B and C) of an intact endothelium. Superoxide dismutase (SOD) (150 units/mL) was added to scavenge superoxide anions derived from SIN-1. The results are the means ± SEM (n = 4 to 6). *P < .01 versus WKY. #P < .05 and $P < .001 versus SHR. Abbreviations are as in Fig. 1.

FIG. 4. Effect of hydroxyhydroquinone (HHQ) on the vasodilatory response in aortic rings excised from SHR. The influence of superoxide anions derived from HHQ (10^{-7} mol/L) on the ferulic acid (FA) (10^{-5} mol/L)-induced enhancement in the acetylcholine (3 × 10^{-8} mol/L)-stimulated vasodilatory response was investigated by adding superoxide dismutase (SOD, 150 units/mL) or catalase (1000 units/mL). The results are the means ± SEM (n = 4). *P < .05 versus vehicle-treated group. Abbreviations are as in Figs. 1 and 3.
bioavailability of NO, thereby impairing endothelial function.16 Contrary to our expectations, FA failed to augment SIN-1-induced endothelium-independent vasodilation, whereas SOD potentiated the SIN-1-induced vasodilation. Based on these findings, it appears unlikely that FA scavenges O$_2^-$ derived from SIN-1 in this ex-vivo system.

The issue of how NO production is stimulated by FA in endothelial cells is unclear based on the findings of this study. In WKY aortas, FA had little influence on either the direct relaxation or the acetylcholine-induced endothelium-dependent vasodilatory effects. In addition, the NO system should not be assumed to reflect the increase in NO production in the endothelium. Studies using SHR suggest that excessive O$_2^-$ reacts with NO, which decreases the bioavailability of NO, thereby impairing endothelial function.17,18 Ortho-methoxy-substituted catechols (apocynin, vanillin, and 4-nitroguaiacol) have been reported to inhibit the activity of NAD(P)H oxidase, a main source of ROS in the vasculature.19 Because FA is also an ortho-methoxy-substituted catechol, it is likely that FA inhibits NAD(P)H oxidase activity and improves the bioavailability of NO in blood vessels. We found in the present study that FA (10$^{-4}$ mol/L) significantly decreased NADPH-dependent O$_2^-$ production from SHR aortas, suggesting that FA inhibited NADPH oxidase activity. Furthermore, HHQ inhibited the FA-induced improvement in endothelium-dependent vasodilation in an O$_2^-$-dependent manner. HHQ-derived O$_2^-$ most likely interferes with the FA-induced increase in available NO in endothelial cells in SHR.

In conclusion, FA restored endothelial function through enhancing basal and stimulated NO bioavailability through the ROS reduction in SHR aorta. The results explain, in part, the mechanisms underlying the effects of FA on BP in SHR.

References