



Original Article

Endothelium-dependent vasodilation by ferulic acid in aorta from chronic renal hypertensive rats

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Background: Ferulic acid (FA) is a naturally occurring nutritional compound. Although it has been shown to have antihypertensive effects, its effects on vascular function have not been intensively established. The aim of this study was to assess the vasoreactivity of FA in chronic two-kidney, one-clip (2K1C) renal hypertensive rats.

Methods: Hypertension was induced in 2K1C rats by clipping the left renal artery and age-matched rats that received a sham treatment served as a control. Thoracic aortas were mounted in tissue baths to measure isometric tension. The effects of FA on vasodilatory responses were evaluated based on contractile responses induced by phenylephrine in the aortic rings obtained from both 2K1C and sham rats. Basal nitric oxide (NO) bioavailability in the aorta was determined by the contractile response induced by NO synthase inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME).

Results: FA induced concentration-dependent relaxation responses which were greater in 2K1C hypertensive rats than in sham-clipped control rats. This relaxation induced by FA was partially blocked by the removal of endothelium or by pretreating with L-NAME. L-NAME-induced contractile responses were augmented by FA in 2K1C rats, while no significant differences were noted in sham rats. FA improved acetylcholine-induced endothelium-dependent vasodilation in 2K1C rats, but not in sham rats. The simultaneous addition of hydroxyhydroquinone significantly inhibited the increase in acetylcholine-induced vasodilation by FA.

Conclusion: These results suggest that FA restores endothelial function by altering the bioavailability of NO in 2K1C hypertensive rats. The results explain, in part, the mechanism underlying the vascular effects of FA in chronic renal hypertension.

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Introduction

Ferulic acid (FA; 4-hydroxy-3-methoxycinnamic acid) is an ubiquitous phenolic compound found in plant tissues and thus is a bioactive ingredient in many foods. Some of the rich sources of FA are rice bran, whole grain food, citrus fruits, banana, beetroot, cabbage, spinach, and broccoli [1]. Several studies have

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indicated that FA has antioxidant effects, antitumor activity, as well as antihyperlipidemic and radioprotective properties [2–4]. Observational epidemiologic studies have shown that dietary consumption of fruits and vegetables is associated with a lower incidence of cardiovascular diseases and mortality resulting from such diseases [5]. It has been previously demonstrated that FA has an antihypertensive action when intravenously administered in spontaneously hypertensive rats and that the blood pressure lowering effect of FA is blocked by pretreatment with a nitric oxide synthase (NOS) inhibitor [6], which suggests that an NO-dependent vascular response is involved in this mechanism. Increasing evidence suggests that FA restores endothelial function by enhancing the bioavailability of basal and stimulated NO in the aortas of hypertensive rats [7].

The endothelium plays a pivotal role in the maintenance of vascular tone and blood pressure by regulating the release of several vasoactive substances, including NO [8]. Functional changes to the endothelium in various pathological conditions are characterized by impaired endothelium-dependent relaxation. A deficient production of endothelium-derived NO results in diminished vasodilator tone, allowing vascular resistance to increase, thereby contributing to the elevated blood pressure levels [9–11]. Therefore, an altered role of NO may be critical in the pathogenesis of hypertension. Indeed, the endothelium-dependent vasodilation is impaired in a number of experimental models, including two-kidney, one-clip (2K1C) renovascular, aortic coarctation, Dahl salt-sensitive, deoxycorticosterone acetate-salt, spontaneously hypertensive rats [10–13]. We have also observed that the endothelium plays an inhibitory role in the aortic contractions of rat by releasing NO, while its role is altered in 2K1C hypertensive rats [14]. Although an improved endothelium-dependent vasodilation by FA has been demonstrated in the aortas of genetically hypertensive rats [7], the effects of FA on vascular function in 2K1C hypertension remain unclear.

This study was designed to examine the effects of FA on the involvement of NO and endothelium-dependent and endothelium-independent vasoreactivity in chronic 2K1C renal hypertensive rats. The thoracic aorta of such rats was isolated and the changes in isometric tension were recorded.

Methods

Development of 2K1C hypertension

Male Sprague-Dawley rats, weighing 160–180 g, were anesthetized with intraperitoneal injections of thiopental sodium (40 mg/kg). An incision was made on the left flank to provide access to the left renal artery, which was separated from the renal vein and was cleared of the connective tissues. A U-shaped solid silver clip with an open gape of 0.2 mm was inserted in the exposed renal artery, resulting in partial occlusion of renal perfusion. The contralateral kidney was left untouched and the wound was closed. A group of age-matched rats received a sham treatment and they were operated like 2K1C rats, except that no clip was inserted. All animals were fed normal chow and given tap water. The rats were tested 10 weeks after the clipping, because the endothelial dysfunction is associated with the duration of hypertension [15]. Hypertensive rats were selected by measuring systolic blood pressure in a conscious state using the tail-cuff method.

Tissue preparation

The thoracic aorta between the aortic arch and the diaphragm was rapidly excised, cleared of adherent connective tissues, and cut into rings (2–3 mm in length) under a dissection microscope. In some preparations, the endothelium was removed by gently rubbing the intimal surface with a moistened cotton swab. Successful removal of endothelial cells from aortic rings was confirmed by the inability of acetylcholine to induce relaxation.

The rings were mounted using two triangle-shaped stainless steel holders in the vessel lumen in organ baths containing 15 mL of physiological salt solution (PSS) of the following composition (in mM): NaCl, 118.3; KCl, 4.7; NaHCO₃, 25; MgCl₂, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5; and glucose, 11.1 at 37 °C. The solution was bubbled with a mixture of 95% O₂ and 5% CO₂. One of the holders was fixed at the bottom of the chambers and the other was connected to a force displacement transducer (Grass FTO3) in order to measure the isometric tension. Before initiating specific experimental protocols, the aortic rings were equilibrated under a resting tension of 2 g for at least 90 minutes. During this period, the incubation medium was changed every 15 minutes.

Protocols

In the first set of experiments, to confirm the vasorelaxant activity of FA, the aortic rings from 2K1C and sham rats were precontracted to 50% effective concentration with phenylephrine (3×10^{-7} M in sham and 4×10^{-8} M in 2K1C rats), the concentrations of which were obtained in preliminary experiments. When the contractile response achieved a steady state, we determined the concentration–response curves with respect to the cumulative addition of FA (10^{-5} – 10^{-3} M) in aortic rings with or without a functional endothelium. To verify the participation of endothelium-derived products in the relaxant effects of FA, experiments were performed in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), a nonselective NOS inhibitor; indomethacin (10^{-5} M), a nonselective cyclooxygenase inhibitor; thapsigargin (10^{-5} M), a sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor, all of which were added to the bath 20 minutes before the addition of phenylephrine.

In the second set of experiments, after an incubation period of 45 min in 25 mM KCl (obtained by equimolar replacement of NaCl by KCl in PSS), aortic rings with intact endothelium from 2K1C and sham rats were treated with L-NAME (10^{-4} M). The contraction in the rings from rats that had been pretreated with FA (10^{-5} – 10^{-3} M) for 30 minutes was measured. The resulting contraction as a percent change of the 25 mM KCl was taken as a measure of the basal bioavailability of NO [7,16].

In the third set of experiments, relaxation responses to acetylcholine (10^{-9} – 10^{-5} M) were performed in the presence or absence of L-NAME (10^{-4} M) in the aortic rings with endothelium that were precontracted with phenylephrine. In addition, the aorta was pretreated for 30 minutes with FA (10^{-4} M) before the addition of phenylephrine. Acetylcholine was then added and the resulting relaxation was taken as a measure of the stimulating bioavailability of NO [7]. In an alternate set of experiments, preparation of an aortic ring involved mechanical removal of the functional endothelium and pretreatment with FA (10^{-4} M) for 30 minutes before the addition of phenylephrine. Endothelium-independent vasodilation was induced by treating the rings from 2K1C rats with sodium nitroprusside (SNP; 10^{-10} – $10^{-6.5}$ M) and 3-morpholinolinosydnonimine (SIN-1; 10^{-10} – $10^{-6.5}$ M). SNP has been used

as an NO donor and SIN-1 is known to release both NO and superoxide anions (O_2^-) [17]. Superoxide dismutase (SOD, 150 U/mL) was added 5 minutes before the addition of phenylephrine in the case of treatment with SIN-1.

In the fourth set of experiments, aortic rings with endothelium from 2K1C rats were treated with FA (10^{-4} M) for 30 minutes before the addition of phenylephrine. Hydroxyhydroquinone (HHQ, 10^{-7} M), a generator of O_2^- [18], was added, followed by contraction with phenylephrine after 5 minutes, and the extent of vasodilation induced by acetylcholine (4×10^{-8} M) was measured. SOD (150 U/mL) or catalase (1000 U/mL) was added 5 minutes before the addition of HHQ.

Drugs

The drugs used in the experiments were FA, L-NAME, phenylephrine, acetylcholine, indomethacin, thapsigargin, SNP, SIN-1, SOD, HHQ, and catalase. HHQ was purchased from Wako Pure Chemical Industries, Ltd (Osaka) and the other chemicals were from Sigma Chemical Co. (St. Louis, MO). FA and indomethacin were dissolved in dimethylsulfoxide (DMSO) and the others were prepared by dissolving in distilled water. The final bath concentrations of DMSO were less than 0.05%, which did not alter the contraction or relaxation responses.

Statistical analysis

Values presented in the figures are expressed as means and standard error of the means. Relaxant responses are given as the percent change in phenylephrine-induced precontraction. Statistical comparisons were performed using the Student *t* test or analysis of variance followed by Duncan's test for multiple comparisons. A *P* value < 0.05 was considered to be statistically significant.

Results

Ten weeks after the operative intervention, systolic blood pressures were 193 ± 4 mmHg ($n=43$, $P < 0.05$) and 138 ± 5 mmHg ($n=36$) in 2K1C hypertensive and sham-clipped control rats, respectively.

Vasorelaxant responses to FA

The tension induced by phenylephrine was enhanced in the aortic rings from 2K1C rats (1.38 ± 0.09 g, $P < 0.05$) than in those from sham rats (1.05 ± 0.08 g). In the aortic rings from 2K1C rats, the addition of FA relaxed phenylephrine-induced contraction in a concentration-dependent manner. Relaxation was also induced in sham rats, but the degree was significantly lower than that for the 2K1C aortic rings (Fig. 1). The relaxation induced by FA was markedly inhibited by removing the endothelium or by pretreating the aorta with L-NAME in 2K1C rats (Fig. 2). Treatment with indomethacin or thapsigargin did not affect FA-induced vasorelaxation (data not shown).

Effect of FA on L-NAME-induced contraction

The magnitude of 25 mM KCl-induced contraction was comparable between the aortic rings from 2K1C (342 ± 47 mg,

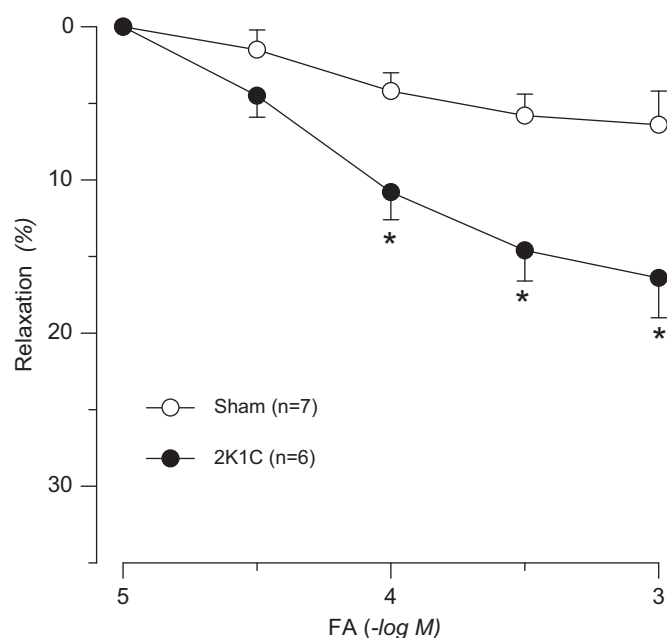


Figure 1. Ferulic acid (FA)-induced vasorelaxation in phenylephrine-precontracted aortic rings from sham-clipped control and 2K1C hypertensive rats. Points represent the means \pm SE with the number (*n*) of experiments carried out in parentheses. * $P < 0.05$, compared with sham values.

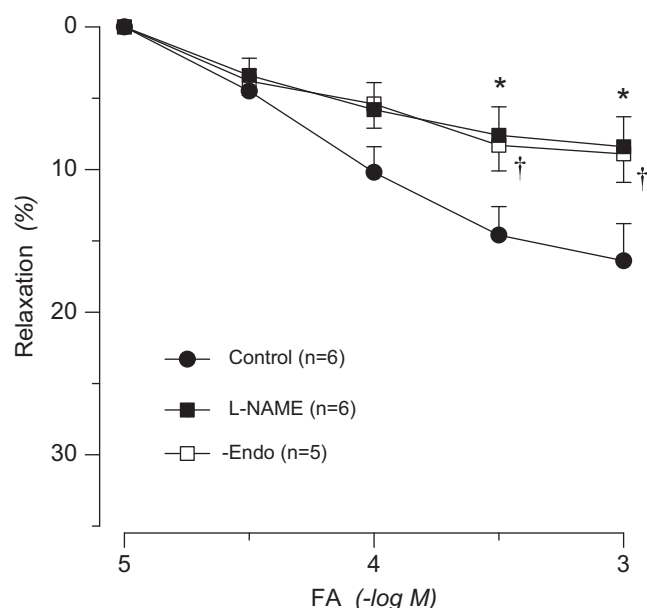


Figure 2. Ferulic acid (FA)-induced vasorelaxation in phenylephrine-precontracted aortic rings without endothelium (-Endo) from 2K1C hypertensive rats. The results obtained by treatment with N^G -nitro-L-arginine methyl ester (L-NAME) are also shown. *, † $P < 0.05$, compared with control values, respectively.

$n=19$) and sham rats (327 ± 33 mg, $n=22$). When L-NAME was added to the rings with 25 mM KCl, the resulting contraction was greater in sham rats ($375 \pm 22\%$, $P < 0.05$, $n=6$) compared with that in 2K1C rats ($185 \pm 19\%$, $n=5$). In addition, the contraction in rings from 2K1C rats that had been treated with FA (10^{-4} or 10^{-3} M) was significantly greater than the values for those with vehicle-treated aorta, while no significant differences were shown in sham rats (Fig. 3).

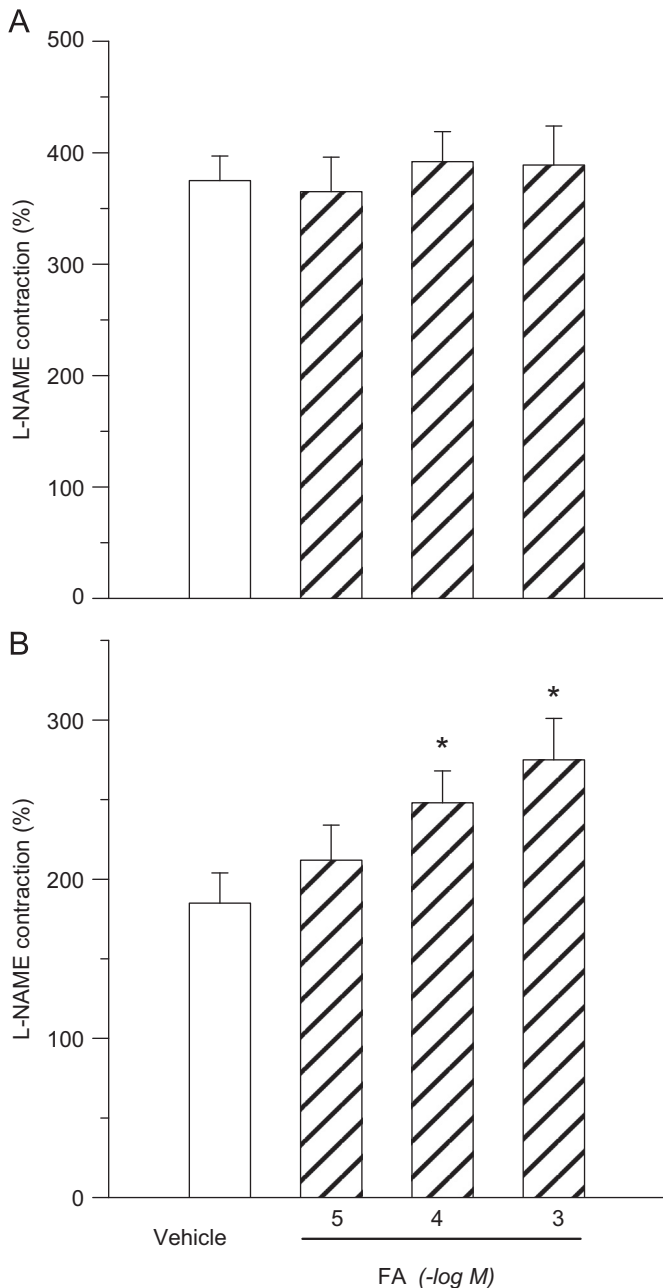


Figure 3. Effects of ferulic acid (FA) on *N*^c-nitro-L-arginine methyl ester (L-NAME)-induced contraction in aortic rings from (A) sham-clipped control and (B) 2K1C hypertensive rats. Contractile responses to L-NAME were obtained in rings submaximally precontracted with KCl (25 mM). The data presented were obtained from five to eight experiments. * $P < 0.05$, compared with the vehicle value.

Acetylcholine-induced or NO donor-induced vasodilation

Acetylcholine-induced vasodilation was significantly attenuated in aortic rings from 2K1C rats compared with those from sham rats. Treatment with L-NAME (10^{-4} M) completely inhibited the acetylcholine-induced vasodilatory effect in both 2K1C and sham groups (data not shown). FA (10^{-4} M) had no effect on the acetylcholine-induced vasodilation in sham rats, while it significantly potentiated the acetylcholine-induced vascular response in aortic rings from 2K1C rats (Fig. 4). In the aortic rings without endothelium from 2K1C rats that had been precontracted with phenylephrine, SNP or

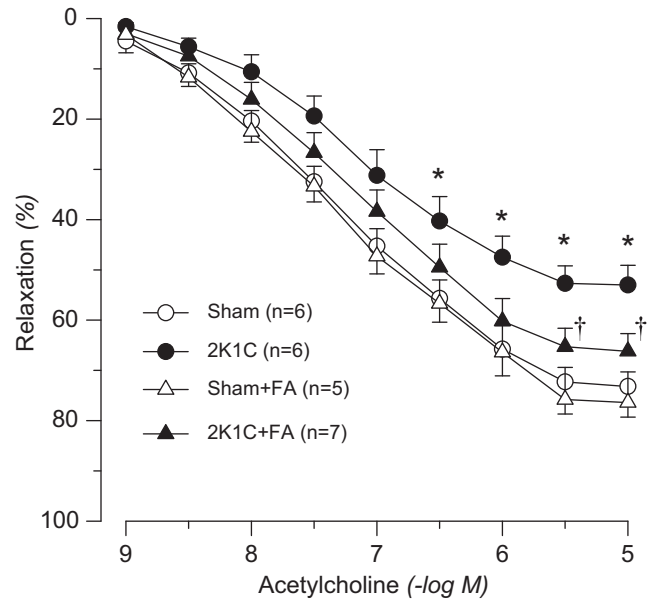


Figure 4. Effects of ferulic acid (FA) on vasodilatory responses induced by acetylcholine in aortic rings with endothelium from sham-clipped control and 2K1C hypertensive rats. * $P < 0.05$, compared with corresponding sham values. † $P < 0.05$, compared with corresponding 2K1C values.

SIN-1 produced a concentration-dependent vasodilation. FA had no effect on SNP-induced vasodilation. FA also did not affect SIN-1-induced vasodilation, while SOD enhanced the vasodilatory effect of SIN-1 (Fig. 5).

Effect of HHQ on acetylcholine-induced vasodilation

HHQ (10^{-7} M) alone had no effect on the phenylephrine-induced contraction in aortic rings with endothelium from 2K1C rats. FA augmented acetylcholine-induced vasodilation, and HHQ inhibited the FA-induced improvement in acetylcholine reactivity. The effect of FA on acetylcholine-induced vasodilation in the presence of HHQ was recovered by the addition of SOD, while the addition of catalase did not affect acetylcholine-induced vasorelaxation (Fig. 6).

Discussion

It has been shown previously that vascular reactivity to contractile agonist is enhanced in disease states such as hypertension [19]. In accordance with our previous findings [14], the contractile response to phenylephrine was augmented in 2K1C hypertensive rats as compared with sham-clipped control rats. In this study, FA caused a relaxation in phenylephrine-precontracted aortic ring preparations with an intact endothelium isolated from 2K1C rats, whereas the relaxant effect of FA was negligible in sham rats. Similar results have also been obtained in spontaneously hypertensive rats [7]. The FA-induced endothelium-dependent relaxation was significantly inhibited by L-NAME, whereas the blocking of cyclooxygenase activity by indomethacin or SERCA activity by thapsigargin had no effects on the relaxation in aortic rings from 2K1C rats. These results suggest that FA-evoked relaxation is NO dependent.

In order to examine whether the basal bioavailability of NO is altered by FA in hypertension, L-NAME was added in aortic rings

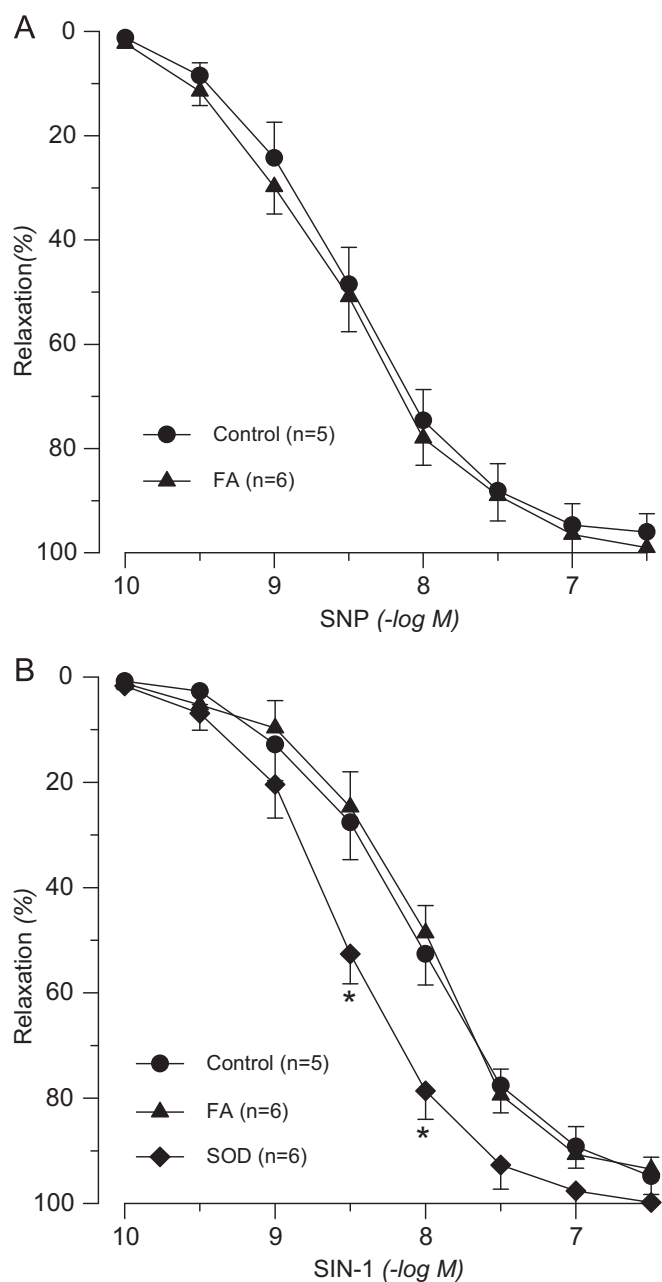


Figure 5. Effects of ferulic acid (FA) on vasodilatory responses induced by (A) sodium nitroprusside (SNP) or (B) 3-morpholinopyridone (SIN-1) in aortic rings without endothelium from 2K1C hypertensive rats. The results obtained by pretreatment with superoxide dismutase (SOD) are also shown. * $P < 0.05$, compared with control values.

with intact endothelium, which were precontracted with 25 mM KCl [16,20]. L-NAME-induced contractions were attenuated in 2K1C rats as compared with sham rats. In agreement with these observations, it has been demonstrated that the basal release of NO is less pronounced in the resistance and conduit arteries of hypertensive rats when compared with normotensive rats [16,21]. We have also shown previously that the contractile response to N^{ω} -nitro-L-arginine, another NOS inhibitor, is impaired in KCl-precontracted aortic rings from 2K1C rats as compared with those from sham rats [20]. Furthermore, the magnitude of L-NAME-induced contractions in rings from 2K1C rats that had been treated with FA was significantly enhanced than that for the vehicle-treated aorta. The results

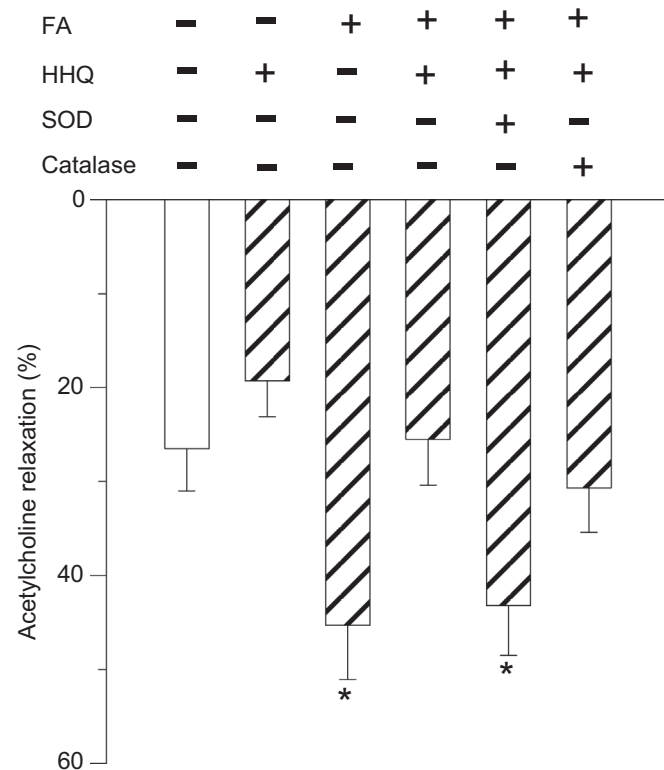


Figure 6. Effects of hydroxyhydroquinone (HHQ) on vasodilatory responses induced by acetylcholine in aortic rings with endothelium from 2K1C hypertensive rats. The effects of HHQ on the ferulic acid (FA)-induced enhancement in the acetylcholine-stimulated vasodilatory response after pretreatment with superoxide dismutase (SOD) or catalase are also shown. Data are attained from five to eight experiments. * $P < 0.05$, compared with the vehicle-treated value.

demonstrate that FA increases basal NO bioavailability in the L-NAME-induced contractile response in 2K1C hypertensive rats, as has been shown previously in genetically hypertensive rats [7]. These findings suggest that the vasorelaxant effect of FA on phenylephrine-induced contractions is partially mediated by endothelial NO in aortic rings from 2K1C rats. By contrast, the FA-induced relaxation persisted even after the removal of the intact endothelium or treatment with L-NAME. Therefore, the possibility of FA having a direct effect on vascular smooth muscle cells in addition to its effects on endothelial cells cannot be ruled out.

The present study confirmed earlier observations [13,14,22] that endothelium-dependent relaxations to acetylcholine are markedly depressed in 2K1C hypertensive rats as compared with sham-clipped control rats. Treatment with L-NAME completely inhibited the acetylcholine-induced vasodilatory effect in both groups, suggesting that the acetylcholine-induced vasodilation is largely due to NOS-derived NO. FA potentiated the acetylcholine-induced vasorelaxation in aortic rings from 2K1C rats, while it did not have any effect in sham rats. Acetylcholine causes NO release by the activation of specific endothelial receptors, resulting in the activation of endothelial NOS [23]. Therefore, the results imply that FA may stimulate the release of NO in this experimental condition in 2K1C hypertensive rats. In association with these observations, it has been suggested that FA enhances the stimulating bioavailability of NO in aortic rings from

spontaneous hypertensive rats [7]. In addition, in the experiment using an NO donor in aortic rings without endothelium from 2K1C rats, FA had no effect on SNP-induced endothelium-independent vasodilation. In this case, the results suggest that FA does not affect the NO-dependent pathway in vascular smooth muscle in 2K1C hypertensive rats.

Impaired endothelium-mediated vasodilation in hypertension has been linked to reduced NO bioavailability. This may be secondary to decreased NO synthesis or to increased NO degradation because of its interaction with O_2^- [24]. It has been demonstrated that NO can be scavenged by O_2^- to form peroxynitrite [24,25], effectively reducing endothelium-derived NO. Indeed, it has been observed that O_2^- generation is increased in hypertension [26,27]. The report that FA scavenges O_2^- derived from xanthine and xanthine oxidase [28] led us to hypothesize that the scavenging ability of FA might explain the improvement in NO bioavailability in aortic rings from 2K1C rats. To investigate this issue, the effects of FA on the SIN-1-induced vasodilation were examined, because SIN-1 is known to release both NO and O_2^- [17]. Contrary to our expectations, FA failed to augment SIN-1-induced endothelium-independent vasodilation, whereas SOD potentiated the SIN-1-induced vasorelaxation. Based on these findings, it appears unlikely that FA scavenges O_2^- derived from SIN-1 in this experimental condition.

The major finding from this study is that the L-NAME-induced vasoconstrictions and the acetylcholine-induced endothelium-dependent vasorelaxations are augmented by FA in aortic rings from 2K1C hypertensive rats, while FA has no effect in sham-clipped control rats. These results suggest that FA may alter the bioavailability of NO in the vascular endothelium in 2K1C rats, as had been shown previously in spontaneously hypertensive rats. The issue of how NO bioavailability is stimulated by FA in endothelial cells is unclear based on the findings of this study. The vascular effects induced by FA in 2K1C hypertensive rats should not be assumed to reflect the increase in NO production in the endothelium, because the NO system is overactive in hypertension [10]. Several studies have reported that excessive O_2^- reacts with NO, which decreases the bioavailability of NO, thereby impairing endothelial function in spontaneous and experimental hypertension [26,27,29]. Therefore, one possible explanation may be attributed to the regulation of O_2^- by FA in endothelial cells in aortic rings from 2K1C rats, although FA did not affect the SIN-1-induced endothelium-independent vasodilation. We found in the present study that HHQ, a generator of O_2^- [18], inhibited the FA-induced improvement in endothelium-dependent vasodilation by acetylcholine in an O_2^- -dependent manner in aortic rings with endothelium from 2K1C rats, as had been shown previously in genetically hypertensive rats [7]. The results imply that HHQ-derived O_2^- most likely interferes with the FA-induced increase in available NO in endothelial cells in 2K1C hypertensive rats.

In summary, FA was found to restore endothelial function by altering the basal and stimulated NO bioavailability in 2K1C hypertensive rats. This finding explains, in part, the mechanism underlying the vascular effects of FA on blood pressure in 2K1C chronic renal hypertension.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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