FEBS Letters 580 (2006) 2317–2322

# Improvement of hypertension and vascular dysfunction by hydroxyhydroquinone-free coffee in a genetic model of hypertension

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Received 14 February 2006; revised 7 March 2006; accepted 12 March 2006

Available online 24 March 2006

Edited by Barry Halliwell

**Abstract** **Chlorogenic acid, a polyphenol found in coffee, has antihypertensive actions, but epidemiologic data on the effects of coffee on blood pressure are controversial. Specific coffee components that inhibit the hypotensive effect of chlorogenic acid and the physiologic mechanisms underlying the effects of coffee without these components were investigated. One component, hydroxyhydroquinone (HHQ), inhibited the hypotensive effects of chlorogenic acid in spontaneously hypertensive rats (SHR). The attenuation of hypertension by HHQ-free coffee was associated with nitric oxide, the suppression of mRNA expression of NAD(P)H oxidase, and the improvement in endothelium-dependent vasodilation in the aorta. Thus, HHQ-free coffee might regulate vascular tone by improving the bioavailability of nitric oxide in SHR.**


*Keywords:* Blood pressure; Chlorogenic acid; Nitric oxide; Spontaneously hypertensive rat; Vasodilation

## 1. Introduction

Endothelial function is impaired in diseases such as hypertension, atherosclerosis, and diabetes; and reactive oxygen species (ROS) are generally thought to be involved [1]. NAD(P)H oxidase is considered to be the main source of ROS, an underlying factor in vascular disease [2]. A number of studies have suggested that excess superoxide anions ($O_2^-$) react with nitric oxide (NO), an endothelium-derived relaxing factor, resulting in the impairment of endothelial function [3,4]. $O_2^-$ production is increased in blood vessels of hypertensive animals, including spontaneously hypertensive rats (SHR) [2]. Thus, a strategy aimed at regulating ROS production is a therapeutic target for inhibiting the development and progression of hypertension [2].

Epidemiologic studies indicate that the ingestion of vegetables and fruits inhibits the development of cardiovascular disease [5]. Flavonoids and other phenols in vegetables and fruits appear to play an important role in preventing the development of circulatory disorders [6]. Chlorogenic acid, a well-known antioxidative polyphenol, is present in foods such as coffee beans, apples, Western pears, Japanese apricots, tomatoes, potatoes, and eggplants [7]. Several chlorogenic acid isomers can be found in coffee: the best known of which is 5-caffeoylquinic acid (Fig. 1) [8]. Green coffee bean extract (GCE), a source of chlorogenic acid, reduces blood pressure in essential hypertensive subjects and in SHR, and improves vasoreactivity in humans, suggesting that chlorogenic acid could play a preventative role in vascular disease [9–12]. NO-mediated vasodilation is partly involved in the hypotensive mechanism of chlorogenic acid [11].

Epidemiologic studies and interventional trials have demonstrated that, in spite of an adequate intake of chlorogenic acid (15–325 mg per cup), the ingestion of roasted coffee has little effect on hypertension or aortic elasticity [13–15]. Coffee contains a number of pharmacologically active components including caffeine (1,3,7-trimethylxanthine). Since caffeine is an inducer of hypertension, it has been extensively studied, but a recent study demonstrated that caffeine intake has little effect on blood pressure in regular coffee drinkers [13]. In sharp contrast, GCE reduces blood pressure in human hypertensives in spite of a caffeine content equal to that in roasted coffee [9,10]. Otherwise, coffee intake increases the urinary excretion of hydrogen peroxide in humans; and a candidate source of ROS in coffee is hydroxyhydroquinone (HHQ; 1,2,4-trihydroxynbenzene) [16] (Fig. 1). Thus, coffee contains a prooxidative component that might lead to the breakdown of NO.

These findings led us to hypothesize that a coffee component, other than caffeine, inhibits the hypotensive effect of chlorogenic acid, and that the elimination of such an inhibitory component might permit the preparation of coffee that improves hypertension. To test this hypothesis, we fractionated a coffee solution and investigated the components of the fractions that interfered with chlorogenic acid-induced antihypertensive effects in SHR. HHQ was identified as a candidate component that prevents chlorogenic acid-induced antihypertension. We further investigated the antihypertensive effect of HHQ-free coffee, and the mechanisms of action of HHQ-free coffee on the involvement of NO, endothelium-dependent vasodilation, and the NAD(P)H oxidase expression, which is a major ROS-producing enzyme in the vasculature.

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*Abbreviations:* DCC; droplet countercurrent chromatography; GCE, green coffee bean extract; HHQ, hydroxyhydroquinone; HPLC, high performance liquid chromatography; L-NAME, $N^G$-nitro-L-arginine methyl ester; NO, nitric oxide; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat

0014-5793/$32.00 
doi:10.1016/j.febslet.2006.03.047

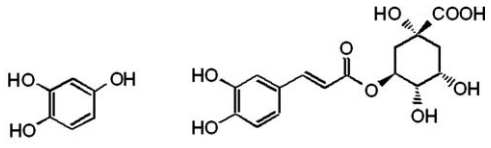


Fig. 1. Chemical structures of hydroxyhydroquinone (1,2,4-trihydroxybenzene) and 5-caffeoylquinic acid, a representative chlorogenic acid.

## 2. Materials and methods

### 2.1. Chemicals and materials

5-Caffeoylquinic acid (5-CQA), $N^G$-nitro-L-arginine methyl ester (L-NAME), phenylephrine hydrochloride, and acetylcholine chloride were purchased from Sigma Chemical Co. (St. Louis, MO). HHQ was purchased from Wako Pure Chemical Industries (Osaka, Japan). GCE, a hot-water extract of green coffee beans, was purchased from the T. Hasegawa Corporation (Tokyo, Japan). The main component of GCE is chlorogenic acid (28%), and other components included caffeine (6%), ethanol (5%), water (50%), and others (11%).

### 2.2. Isolation of coffee components that inhibit chlorogenic acid-induced antihypertension

The procedure used for fractionating the preparation is shown in Fig. 3A. Commercially available decaffeinated instant coffee was dissolved in ion-exchanged water, filtered through a glass filter under reduced pressure, and divided into soluble and insoluble fractions. The soluble fraction was fractionated as follows. The mobile phase was 0.5% acetic acid. The soluble fraction was dissolved with the mobile phase, and adsorbed on a Chromatorex ODS column (Fuji Silysia Chemical, Aichi, Japan), followed by elution with the mobile phase containing methanol. The following eluates were collected; fractions 1 and 2 as F1, fractions 3 to 12 as F2, and fractions 13 to 18 as F3. F2 was further divided into fractions 3 to 7 (F2-1) and fractions 8 to 12 (F2-2). F2-1 was fractionated by preparative high performance liquid chromatography (HPLC) under the conditions described below. Instrument: HPLC preparative system PLC-561 (GL Science, Tokyo, Japan), column: Inertsil ODS-3 (∅30 × 250 mm, GL Sciences, Tokyo, Japan), eluent: A, 50 mM AcOH aq.; B, 50 mM AcOH–$CH_3CN$ sol., gradient time table: A, 100% (0–30 min), 0% (30–35 min); B, 100% (35–45 min), flow rate: 40 ml/min, temperature: 35°C, and detection: UV 258 nm. The resulting eluates were divided into three fractions based on the HPLC chromatogram: F2-1-A, retention time (RT) 0 to 8.3 min; F2-1-B, RT 8.3 to 11 min; and F2-1-C, RT 11 to 46 min. F2-1-B was fractionated by droplet countercurrent chromatography (DCC) using the following conditions. Instrument: DCC system DCC-3000 (Tokyo Rikakiki, Tokyo, Japan), column: glass (∅2 mm), solution supply pump: L-6000 (Hitachi, Tokyo, Japan), fraction collector: DC-1000 (Tokyo Rikakiki), and solvent: chloroform: methanol: 50 mM AcOH aq. = 7:13:8. The resulting eluates were collected into the three fractions described below, concentrated, and freeze–dried: fractions 1–19 (F2-1-B-a), fractions 20–26 (F2-1-B-b), and fractions 27–30 (F2-1-B-c).

### 2.3. Removal of HHQ from coffee extract

Roasted Columbia beans (*Coffea robusta*) were extracted with hot water at 95 °C or higher, and then cooled to 25 °C. The coffee extract was treated with activated charcoal (Japan EnviroChemicals, Osaka, Japan) to remove low molecular weight compounds including HHQ by stirring the suspension with for 1 h at room temperature. The activated charcoal-treated coffee extract was then filtered through a membrane filter and dried under reduced pressure. The chlorogenic acid content, determined by HPLC, was 25 mg/g powder in the coffee that was not treated with activated charcoal and 27 mg/g powder in activated charcoal-treated coffee. The HHQ content, determined by HPLC, was 62 mg/kg of powder in the coffee that was not treated with activated charcoal and 2 mg/kg of powder in the activated charcoal-treated coffee, in which 97% of HHQ had been removed. The activated charcoal-treated dried powder was used as HHQ-free coffee in the following experiments.

### 2.4. Blood pressure measurement

Male SHR/Izm rats, a genetic model of hypertension, were purchased from Japan SLC Co., Ltd (Shizuoka, Japan). The Ethics Review Committee for Animal Experimentation of the Kao Corporation approved the animal studies. In the single oral administration studies, SHR (males, 13–14-week-old) were used. GCE, caffeinated instant coffee (chlorogenic acid content, 6%; caffeine content, 5%), 5-CQA, coffee fractions, and HHQ were dissolved in physiological saline and were orally administered to conscious SHR at 5 ml/kg of body weight using a stomach probe. The dose of chlorogenic acid was 200 or 300 mg/kg of body weight, doses at which a stable reduction in blood pressure was previously reported [11]. To determine whether HHQ-free coffee reduces blood pressure through interactions with the NO pathway in SHR, the blood pressure response to HHQ-free coffee was determined in SHR pretreated with the NO synthase inhibitor L-NAME (3 mg/kg, subcutaneous injection into the neck) before the administration according to a previously described method [17]. Physiological saline was administered to the control group. The tail artery systolic blood pressure (SBP) was measured using a non-invasive blood pressure measurement system, BP-98A (Softron, Tokyo, Japan), as described previously [11]. The change in systolic blood pressure (SBP) (%) was calculated by: [(SBP after administration) − (SBP before administration)] × 100/(SBP before administration). To study the effects of continuous HHQ-free coffee administration, two groups of SHR ($n = 6$ per group, 14-week-old at the initiation of the experiment) were given the control diet or a dried HHQ-free coffee diet (9.3%) for 8 weeks ad libitum. A powdery CE-2 diet (CLEA Japan, Inc., Tokyo, Japan) was used as the control diet. Tail arterial SBP was measured at 2 week intervals for 8 weeks. The chlorogenic acid dose was approximately 300 mg/kg per day.

### 2.5. Ex vivo vascular reactivity

This experiment was performed according to a previously described method [18]. At the end of the 8-week experimental period, the descending thoracic aorta was excised, freed of fat and connective tissue, cut into rings approximately 2–3 mm in length, and placed in a gassed (95% $O_2$ and 5% $CO_2$) Krebs–Henseleit solution with the following composition (in mM): NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, $NaHPO_4$ 2.33, and glucose 11.51. The tissues were maintained at 37 °C under a 1-g tension and equilibrated for 1 h before initiating the experimental protocols. Vascular reactivity was measured in aortic rings with the functional endothelium pre-contracted with submaximal concentrations of phenylephrine ($10^{-6}$ M). Endothelium-dependent vasodilation was evaluated by comparing the concentration response curves to acetylcholine ($10^{-9}$ M–$10^{-6}$ M), and calculated as the percent of precontractile vascular tone. The responses of the tissues were recorded using isometric transducers (Kishimoto Medical Instruments, Kyoto, Japan) and recorders (SEKONIC, Tokyo, Japan).

### 2.6. Real-time polymerase chain reaction (PCR)

At the end of the 8-week experimental period, total RNA was isolated from the thoracic aorta with ISOGEN™ (NIPPON GENE, Tokyo, Japan) according to a previously described method [19]. Total RNA (50 ng) was reverse-transcribed for cDNA synthesis with Omniscript™ Reverse Transcriptase (Qiagen Inc., Valencia, CA) to quantify the mRNA expression of NAD(P)H oxidase components; Nox-2, $p22^{phox}$, and $p47^{phox}$, and 36B4 as a control. Real-time PCR was performed using ABI PRISM-7000 and -7700 Sequence Detector (PE Applied Biosystems, Tokyo, Japan). TaqMan® 1000 RXN Gold with Buffer A Pack (PE Applied Biosystems) was used to detect the PCR. Each set of primers and probes was designed using Primer Express™ Software v2.0 (PE Applied Biosystems). The NCBI Blast program (http://www.ncbi.nlm.nih.gov/blast/) was used to check the sequence specificity of the primers and probes.

### 2.7. Statistical analysis

All values are expressed as means ± S.E. Statistical analyses of the data were performed using StatView (SAS Institute, Cary, NC). Data for each group were initially analyzed using the analysis of variance. When a significant $F$-value ($P < 0.05$) was obtained, a Dunnett's test was performed in a post hoc analysis. Comparisons of the dose–response curves were evaluated by the two-way analysis of variance for repeated measures.

## 3. Results

### 3.1. Antihypertensive effects of roasted coffee in SHR

Fig. 2 shows the changes in SBP in SHR after a single oral administration of GCE and roasted instant coffee in which the dose of chlorogenic acid was 200 mg/kg. The initial SBP values were 216 ± 4, 226 ± 6, and 202 ± 3 mmHg in the saline group, the GCE group, and the coffee-treated group, respectively. There were no significant changes in SBP in the coffee group throughout the experimental period. In contrast, the GCE-induced SBP change reached a maximum at 12 h. When caffeine was added to GCE to adjust the caffeine content (200 mg/kg) of the coffee, SBP was reduced to a level similar to that shown in Fig. 2 (data not shown).

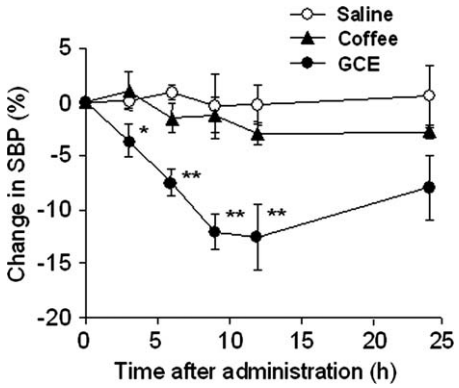


Fig. 2. Effect of a single oral administration of coffee and green coffee bean extract (GCE) in awake SHR (200 mg/kg chlorogenic acid). The results are the means ± S.E.M. ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus saline or coffee group.

### 3.2. Isolation of coffee components that inhibit chlorogenic acid-induced antihypertension

The fractionation procedure and recovery rates in each fraction are shown in Fig. 3A. Chlorogenic acid was eluted in fraction F3, which reduced SBP 12 h after administration to SHR (data not shown). Following repeated isolation steps using reversed phase HPLC on an ODS column and DCC chromatography, the F2, F2-1, F2-1-B, and F2-1-B-b fractions were found to contain components that inhibited 5-CQA-induced antihypertension. Changes in SBP at 12 h after the administration of 5-CQA (200 mg/kg) alone and each of the above fractions were as follows: −12.8 ± 1.3% in the 5-CQA group; −3.4 ± 3.0% in the 5-CQA plus the F2 group; −2.3 ± 0.5 % in the 5-CQA plus the F2-1 group; −3.5 ± 1.2% in the 5-CQA plus the F2-1-B group; and −3.7 ± 1.2% in the 5-CQA plus the F2-1-B-b group. These fractions significantly inhibited the hypotensive effect of 5-CQA ($P < 0.05$). On the HPLC chromatogram (Fig. 3B) and three-dimensional spectrum (data not shown), the main peak of the F2-1-B-b fraction was identical to that of standard HHQ. The F2-1-B-b fraction contained approximately 40% HHQ. The high-polarity and lack

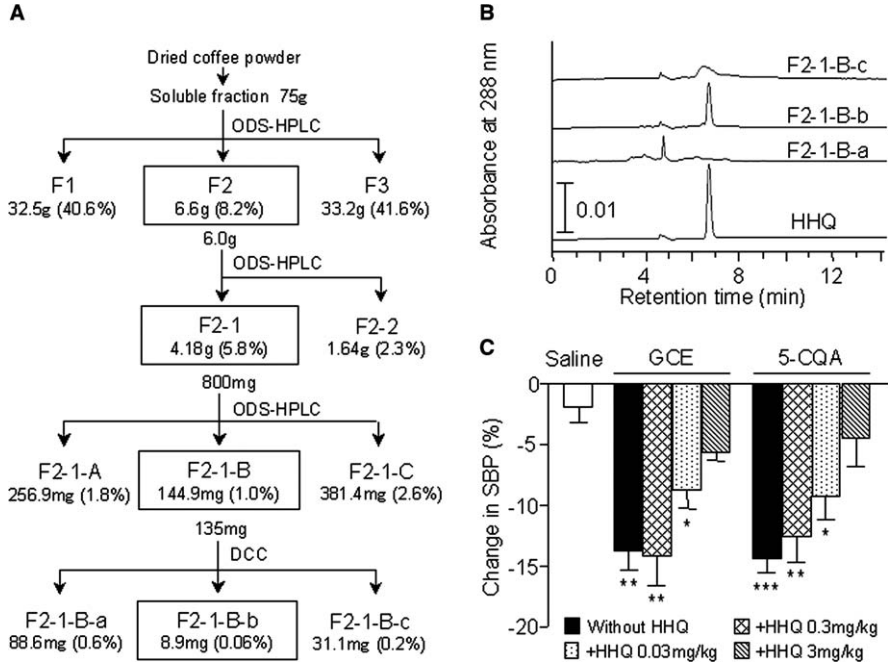


Fig. 3. Isolation of inhibitory components in coffee related to chlorogenic acid-induced antihypertension. (A) Purification of coffee by reversed phase ODS-HPLC and DCC. (B) The DCC pattern indicated that the main peak, F2-1-B-b, corresponded to an authentic sample of hydroxyhydroquinone (HHQ). (C) Change in SBP 12 h after a single oral administration of green coffee bean extract (GCE, 300 mg/kg chlorogenic acid) or 5-caffeoylquinic acid (5-CQA, 300 mg/kg) co-administered with HHQ (0.03, 0.3, and 3 mg/kg) in SHR. The results are the means ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus saline group.

of UV absorbing compounds of the fraction in HPLC suggested that mono- and disaccharides were also present in the F2-1-B-b fraction. The SBP reducing effects of a single oral administration of GCE (300 mg/kg of chlorogenic acid) or 5-CQA (300 mg/kg) were significantly inhibited by the concomitant ingestion of HHQ in a dose-dependent manner (Fig. 3C).

### 3.3. Effects of oral administration of HHQ-free coffee in SHR

After confirming the removal of HHQ by HPLC, we orally administered HHQ-free coffee to SHR. HHQ-free coffee significantly decreased SBP at 12 h after its administration (Fig. 4A). It is noteworthy that the co-administration of HHQ (3 mg/kg) or the administration of the NO synthase inhibitor L-NAME with the HHQ-free coffee significantly inhibited the HHQ-free coffee-induced antihypertensive effect in SHR. When HHQ-free coffee was orally administered for a period of 8 weeks to SHR, the increase in SBP was significantly inhibited in the HHQ-free coffee group compared to the control diet group (Fig. 4B). The levels of expression of the mRNA of NAD(P)H oxidase components Nox-2, $p22^{phox}$, and $p47^{phox}$ in the aorta were significantly suppressed as the result of the HHQ-free coffee treatment compared to the control diet group (Fig. 4C). In SHR aortas, acetylcholine-induced endothelium-dependent vasodilation was significantly improved in the HHQ-free coffee diet group compared to the control diet group (Fig. 4D). At the end of the 8-week study period, no significant differences were found in daily food intake ($23.2 \pm 1.8$ g and $22.5 \pm 0.1$ g) and body weight ($375 \pm 6$ g and $377 \pm 8$ g) between the HHQ-free coffee diet group and the control diet group, respectively.

## 4. Discussion

The literature contains conflicting reports on the effect of coffee intake on blood pressure [13,14]. Blood pressure rises after coffee consumption in non-habitual coffee drinkers, whereas the effect of coffee on blood pressure is smaller or not detectable in habitual coffee drinkers [14]. In SHR, we demonstrated that a single administration of a coffee solution had little effect on SBP, while GCE significantly decreased SBP in spite of the presence of caffeine. This suggests that the antihypertensive components of coffee are inhibited by some other, as yet unidentified component in coffee. The fraction containing HHQ significantly inhibited the hypotensive effect of chlorogenic acid. Of note is the finding that HHQ clearly inhibited the chlorogenic acid-induced antihypertension in a dose-dependent manner. The amount of chlorogenic acid in coffee

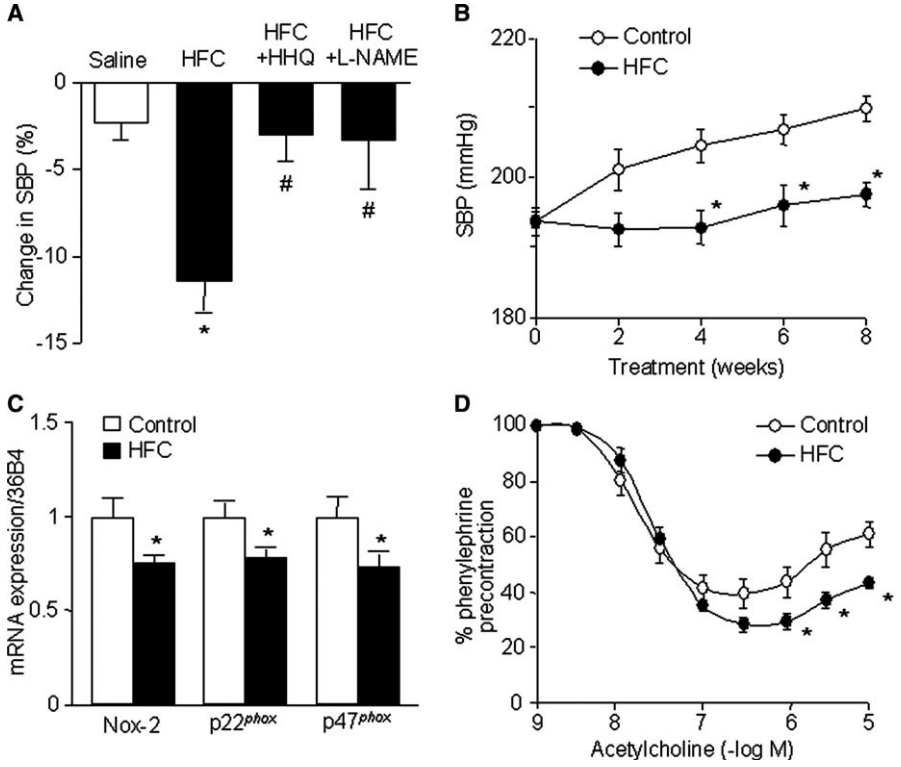


Fig. 4. Effects of an oral administration of HHQ-free coffee (HFC) in SHR. (A) Change in SBP 12 h after a single oral administration of HHQ-free coffee (300 mg/kg chlorogenic acid) co-administered with HHQ (3 mg/kg) or L-NAME (3 mg/kg) in SHR. The results are the means ± S.E.M. ($n = 6$). $^{*}P < 0.05$ versus saline group. $^{\#}P < 0.05$ versus HHQ-free coffee group. (B) Effect of continuous intake of HHQ-free coffee on SBP in SHR during the 8-week experimental period. HHQ-free coffee diet (approximately 300 mg/kg/d chlorogenic acid) significantly inhibited the increase in SBP in SHR. (C) Effect of the continuous intake of HHQ-free coffee on the mRNA expression of NAD(P)H oxidase components Nox-2, $p22^{phox}$, and $p47^{phox}$. Eight weeks after treatment with HHQ-free coffee, mRNA expression was measured in SHR aortas using real-time PCR. (D) Effect of the continuous intake of HHQ-free coffee on the endothelium-dependent vasodilatory response to acetylcholine in thoracic aortic rings from SHR. (B–D) Each value represents the mean ± S.E.M., $n = 6$. $^{*}P < 0.05$ versus control diet group.

varies from 0.2 to 3.8 g/kg and that of HHQ varies from 6 to 20 mg/kg [20]; thus, the ratio of HHQ/chlorogenic acid is approximately 1/100, which is the same ratio of HHQ (3 mg/kg) and chlorogenic acid (300 mg/kg) used in the present study (Fig. 3C). Collectively, these findings suggest that HHQ is the main component in coffee that inhibits chlorogenic acid-induced antihypertension.

A single oral ingestion of HHQ-free coffee led to a significant decrease in blood pressure, which was blocked by pretreatment with L-NAME, suggesting the involvement of NO in the mechanism of action. Since the antihypertensive effect of chlorogenic acid is reported to be associated with NO [11], a possible candidate for the active substance in HHQ-free coffee is chlorogenic acid. Chlorogenic acid in coffee might mainly be degraded by microflora elastases in the large intestine, and then absorbed as caffeic and ferulic acids [21]. Caffeic acid reduces cell proliferation through the Janus kinase/signal transducer and activators of transcription and the Ras/Raf/ERK1/2 pathways, followed by the down-regulation of NADPH oxidase activity in angiotensin II-induced vascular smooth muscle cells in stroke-prone SHR [22,23]. In addition, we previously reported that the hypotensive effect of ferulic acid is inhibited by pretreatment with an NO synthase inhibitor, but not a cyclooxygenase inhibitor, a β- and an α-adrenoceptor antagonist, or a calcium channel blocker [24]. Thus, coffee metabolites, such as caffeic and ferulic acids, appear to improve vascular function through reducing ROS production and enhancing the bioavailability of NO.

The production of $O_2^-$ and hydrogen peroxide ($H_2O_2$) by HHQ has been confirmed in vitro and in vivo [16,20]. The effects of $H_2O_2$ on vascular reactivity are controversial. $H_2O_2$ has been reported to act as an endothelium-derived hyperpolarizing factor in physiological levels in resistant arteries [25]. On the other hand, $H_2O_2$ (0.1–10 mM) has been reported to induce the contractile response in intrapulmonary arteries [26]. In the present study, a single HHQ treatment (3 mg/kg) failed to change the blood pressure in SHR; the change in SBP was $-4 \pm 1\%$ 12 h after the administration that was not significantly different compared with the saline group. Thus, HHQ-derived $H_2O_2$ is less likely to contribute to the regulation of blood pressure, based on the results of this experiment. Collectively, the above findings suggest that HHQ-derived $O_2^-$ might mainly inhibit the chlorogenic acid-induced increase in NO activity. Because HHQ-free coffee improved endothelium-dependent vasodilation, an effect that was accompanied by a reduction in the levels of NAD(P)H oxidase mRNA, the possibility that HHQ-derived $O_2^-$ inhibited the coffee chlorogenic acid-induced increase in NO bioavailability cannot be excluded. More detailed experimentation will be needed, however, to determine the mechanism of action of HHQ-free coffee and physiological interactions of coffee components, HHQ, chlorogenic acid, and others.

In summary, HHQ, a prooxidant component of roasted coffee, clearly inhibited the antihypertensive effect of chlorogenic acid. The removal of low molecular weight components including HHQ by treatment with activated charcoal might allow the chlorogenic acid present in coffee to exert a hypotensive effect. The attenuation of hypertension by HHQ-free coffee was associated with the suppression of NAD(P)H oxidase mRNA expression and the improvement in endothelium-dependent vasodilation in SHR aortas. Accordingly, HHQ-free coffee might help to minimize vascular injury through its ability to improve hypertension and endothelial function.

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