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Effects of *Ginkgo biloba* extract on blood pressure and vascular endothelial response by acetylcholine in spontaneously hypertensive rats

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Abstract

We previously demonstrated that *Ginkgo biloba* extract (Ginkgo) produced vasodilation via the nitric oxide pathway in aortic segments isolated from Wistar rats. In this study, we have analysed the effects of daily long-term oral Ginkgo treatment on blood pressure, vascular tone, and calcium mobilization to evaluate the clinical availability. Spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) were fed either a control diet or a diet containing 0.05%–0.5% Ginkgo for 30 days. Administration of Ginkgo did not change systolic blood pressure in WKY, but significantly decreased systolic blood pressure in SHR. In thoracic aortic preparations isolated from SHR, diminished relaxation in response to acetylcholine was improved by a Ginkgo-containing diet. This diet significantly decreased the EC50 value and significantly increased maximum relaxation in response to acetylcholine in SHR. In aortic segments isolated from WKY, acetylcholine-induced relaxation was not affected by a Ginkgo-containing diet. Sodium nitroprusside-induced relaxation was unchanged by a Ginkgo-containing diet in SHR and WKY. We also examined the effects of a Ginkgo-containing diet on the intracellular calcium level of aortic endothelium using a fluorescent confocal microscopic imaging system. Calcium Green 1/AM preloading indicated that acetylcholine significantly increased the endothelial intracellular calcium level. The Ginkgo-containing diet significantly enhanced this increase in the aortic endothelium of SHR, but did not change that of WKY. The results suggested that Ginkgo enhanced endothelium-dependent vasodilation and elevation of the endothelial intracellular Ca^{2+} level in SHR, resulting in hypotension. This accelerative effect of Ginkgo on Ca^{2+} mobilization seemed to be associated with restoration of impaired dilatory function induced by acetylcholine in endothelial cells.

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Introduction

Over the last decade, interest by the general public in the use of herbal dietary supplements has risen exponentially. One of the most popular herbal supplements is *Ginkgo biloba* extract (Ginkgo), which is commonly used in the treatment of early-stage Alzheimer's disease, vascular dementia, peripheral claudication, and tinnitus of vascular origin (Sierpina et al 2003). Ginkgo is marketed as a dietary supplement in the United States and Japan. However, in some European countries, it is prescribed clinically and is recognized as being particularly effective in the amelioration of peripheral vascular diseases such as intermittent claudication and cerebral insufficiency (Kleijnen & Knipschild 1992). Ginkgo exerts various pharmacological actions, including the scavenging of free radicals, improvement of the microcirculation, and antagonism of platelet-activating factor (Spinnewyn et al 1987; McKenna et al 2001; De Smet 2002; Ernst 2002). Ginkgo and its constituents, specifically terpenoids (bilobalide and ginkgolides A, B, and C) and flavonoids (quercetin and rutin), are also reported to possess vasorelaxant properties (Duarte et al 2001a; Ibarra et al 2003). This finding has led to the postulation that Ginkgo might have protective effects in cardiovascular disease. Indeed, Sasaki et al (2002) showed that Ginkgo decreased blood pressure in stroke-prone spontaneously hypertensive rats. In this study, we have analysed the effects of daily long-term oral Ginkgo treatment on blood pressure and endothelial function in spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY), to elucidate the mechanism of hypotension by Ginkgo.

Materials and Methods

Materials

Ginkgo powder containing 24.2% flavone glycosides, 2.7% ginkgolide A, 1.1% ginkgolide B, 1.7% ginkgolide C, 3.9% bilobalide, and less than 1 ppm ginkgolic acid was supplied by Tama Biochemical Co., Ltd (Tokyo, Japan); this mixture was similar to that of EGb 761, a preparation used in European countries. Calcium Green 1/AM was obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents used were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan).

Animals and diets

Male spontaneously hypertensive rats (SHR/Izm) and Wistar Kyoto rats (WKY/Izm) were obtained from SLC (Hamamatsu, Japan). They were cared for in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Mukogawa Women's University, which was compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Rats were housed individually in stainless steel, wire-bottomed cages at a constant temperature (22–24°C) with a 12-h light–dark cycle. After adaptation to these conditions for three to five days, SHR were divided into five groups of six 6-week-old rats each and WKY were divided into two groups of six 6-week-old rats each, as follows. Group 1, control WKY rats; group 2, WKY rats treated with 0.5% Ginkgo (0.5% Ginkgo WKY); group 3, control SHR rats; group 4, SHR rats treated with 0.05% Ginkgo (0.05% Ginkgo SHR); group 5, SHR rats treated with 0.1% Ginkgo (0.1% Ginkgo SHR); group 6, SHR rats treated with 0.5% Ginkgo (0.5% Ginkgo SHR). Ginkgo was added to the commercial rodent diet (CE-2; Clea Japan Inc., Tokyo, Japan) and was given to animals in those groups other than controls. Animals had free access to drinking water. All rats were initially fed a control diet (CE-2 without Ginkgo) for seven days before receiving the experimental diet for 30 days. Blood pressure was measured by the tail-cuff method (Model MK-2000, Muromachi Kikai Co., Ltd, Tokyo, Japan) in unanaesthetized rats between 1300 and 1700 h (light on), at 23–25°C. Rats were placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Rats were trained to become familiar with this procedure for seven days before the experiment. The first day of blood pressure measurement was the day before the experimental diet was first administered. The final day of blood pressure measurement was the thirtieth day after the experimental diet was first administered.

Relaxation studies

After administration of the experimental diet for 30 days, rats were then anaesthetized with pentobarbital sodium (60 mg kg^{-1}, i.p.) and the thoracic aorta was rapidly removed. A part of the thoracic aorta was immediately placed in a Krebs–Henseleit solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and glucose, 10.0. After periaortic fat and connective tissue were removed, the aorta was cut into ring segments of approximately 3 mm in length, and used for a relaxation study and intracellular calcium ion measurement. Each ring preparation was mounted vertically under a resting tension of 1 g in a 5-mL water-jacketed organ bath filled with Krebs–Henseleit solution and attached to a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd, Tokyo, Japan). The bath solution was maintained at 37°C and bubbled with a 95% O_2/5% CO_2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min before the initiation of experimental procedures, and during this period the incubation media were changed every 10 min. After this equilibration period, the ring preparation was contracted with noradrenaline (10^{-7} M) before the putative relaxing agents were cumulatively added. The relaxation response was expressed as a percentage of the maximal relaxation produced by papaverine (10^{-4} M).

Measurements of intracellular Ca^{2+} level

Using microscissors, a window of approximately 1 mm in diameter was opened in the thoracic aorta wall to observe the endothelium. The remaining aortic sections were then immediately immersed in physiological saline solution (PSS) of the following composition (mM): NaCl 140.0; KCl 4.0; $MgCl_2$ 2.0; $CaCl_2$ 2.0; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 10.0; and glucose 10.0, adjusted to pH 7.4 with NaOH. The aorta was loaded with 5 μM Calcium Green 1/AM for 30 min at room temperature and then rinsed three times with PSS. The aorta was then allowed to incubate in PSS for an additional 15 min at 37°C in a 95% O_2/5% CO_2 atmosphere to permit complete hydrolysis of any intact ester linkages in the intracellular Calcium Green 1/AM.

A U-shaped stainless steel wire (diam. 0.3 mm) was inserted into the aortic lumen to flatten the surface (endothelial layer). This aortic preparation was placed in a glass-bottomed chamber so that the window faced the objective lens of a microscope. The chamber was immediately filled with PSS and placed on a microscope stage (ECLIPSE TE 300, Nikon, Tokyo, Japan) coupled to a Nipkow disk confocal scanner (CSU10, Yokogawa Electric Corporation, Tokyo, Japan). An excitation wavelength of 488 nm was provided by an argon-krypton laser (Omnichrom Inc., Chino, CA, USA) and the emitted light was collected with a 510-nm long-pass diachronic reflector and a 515-nm long-pass emission filter through the planfluor objective (× 20). The fluorescence images were acquired with an ICCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and captured on a personal computer using the ARGUS-50 program (Hamamatsu Photonics, Hamamatsu, Japan). To quantify the Ca^{2+} responses in endothelial cells, images constructed from 195 × 130 pixels were collected every 5 s. Calcium Green 1 fluorescence intensity was directly indicated from the resulting values.

Statistics

All values are reported as mean ± s.e.m. Statistical analysis of data for the groups was carried out using analysis of variance followed by post-hoc tests. A probability of less than 0.05 was considered significant. Statistical analyses were carried

out with a computer program (StatView 4.5, Abacus Concepts, Cupertino, CA, USA).

Results

Body weight and food intake during Ginkgo administration

The effects of 30 days of feeding with a Ginkgo-containing diet on the body weight and food intake in WKY and SHR are shown in Table 1. The dose of Ginkgo was also calculated. Addition of Ginkgo (0.05%–0.5%) to the diet of WKY and SHR did not significantly influence body weight or food intake.

Effect of a Ginkgo-containing diet on blood pressure

Figure 1 shows the systolic blood pressure of WKY and SHR after 15 and 30 days on the 0.5% Ginkgo diet. Systolic blood pressure in SHR increased with age and was significantly higher than that in WKY. This increase was significantly suppressed by the 30-day administration of Ginkgo. A Ginkgo-containing diet did not affect systolic blood pressure in WKY over the 30-day administration period.

Effect of a Ginkgo-containing diet on vasorelaxation

Figure 2 shows the influence of 30 days of a Ginkgo-containing diet (0.05–0.5%) on relaxation induced by acetylcholine (ACh) in WKY and SHR aortic rings precontracted with noradrenaline (10^{-7} M). Ginkgo significantly enhanced the maximum relaxation induced by ACh in a dose-related manner. The EC50 values of relaxation for ACh in SHR aortic rings were significantly decreased by administration of a 0.5% Ginkgo diet, as shown in Table 2. In contrast, 30 days of a 0.5% Ginkgo diet did not affect ACh-induced relaxation in WKY (Figure 2, left panel). In the control diet groups of SHR, ACh-induced maximum relaxation (88.8 ± 1.2%) was significantly lower than that observed in WKY (94.6 ± 1.5%). In the aortas of SHR fed 0.1% or 0.5% Ginkgo for 30 days, maximum relaxation induced by ACh was significantly increased relative to that in the control diet groups of SHR (Figure 2, right panel). In other words, Gingko completely restored the relaxation of SHR aortas to that of WKY aortas. Sodium nitroprusside-induced relaxation was not changed after 30-day administration of a Ginkgo-containing diet in either SHR or WKY (Figure 3).

Effects of a Ginkgo-containing diet on the ACh-induced increase in intracellular Ca^{2+} level

Figure 4 shows typical changes in intracellular calcium level induced by ACh in the endothelial layer of aorta isolated from SHR. The left panel is a phase-contrast microscopic image of the endothelial layer in the absence of drugs. Parts of the stainless steel wire inserted to the arterial lumen are observed in the top and bottom of the image. As shown in the middle panel, the distance between the wires was shortened by contraction induced by noradrenaline. This distance was lengthened by the vasorelaxant action of ACh (right panel). Furthermore, the intensity of Calcium Green fluorescence was increased in the presence of ACh. This ACh-induced increase in intracellular calcium ion level was enhanced in the endothelial layer of SHR fed with a 0.5% Ginkgo diet for 30 days (Figure 5).

Discussion

Ginkgo is a well-defined plant extract. Its main constituents are terpenoids (such as bilobalide and ginkgolides A, B, and C) and 30 types of flavonoids (such as quercetin and rutin) (Kleijnen & Knipschild 1992). Our previous study (Kubota et al 2001) demonstrated that Ginkgo produced dose-dependent vasodilation via the nitric oxide pathway in the isolated Wistar rat aorta and suggested that one of the principal ingredients of Ginkgo responsible for this vasodilation was quercetin. This evidence suggested that Ginkgo may produce a hypotensive effect. Sasaki et al (2002) have shown that the age-related increase in blood pressure observed in SHR was suppressed significantly by Ginkgo at 60–120 mg kg^{-1} each day for three weeks. Umegaki et al (2000) have already found that Ginkgo produced antihypertensive effects in deoxycorticosterone acetate-salt hypertensive rats. However, this hypotensive action was observed on systolic blood pressures of 120–140 mmHg, but was not observed on systolic blood pressures more than 140 mmHg. Thus, Ginkgo seemed to improve mild hypertension. Indeed, Jezova et al (2002) suggested that

Table 1 Effects of 30-day treatment with *Ginkgo biloba* extract (Ginkgo) on body weight, food intake, and dose of Ginkgo in Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

	WKY		SHR			
	Control	0.5% Ginkgo	Control	0.05% Ginkgo	0.1% Ginkgo	0.5% Ginkgo
Body weight (g)[a]	260.5 ± 2.6*	254.5 ± 4.8*	278.4 ± 3.3	276.0 ± 3.7	276.6 ± 5.8	281.1 ± 1.6
Intake of diet (g/day)[b]	14.3 ± 0.2*	14.8 ± 0.3*	17.3 ± 0.3	17.0 ± 0.2	17.3 ± 0.2	17.2 ± 0.3
Dose of Ginkgo (mg/day)[c]	0	73.7 ± 1.3	0	8.5 ± 0.1	17.3 ± 0.2	86.3 ± 1.4

[a]The average body weight after 30 days. [b]The average intake of each diet per day for 30 days. [c]The average dose of Ginkgo per day for 30 days. Each value is the mean ± s.e.m. for six rats. *$P < 0.05$ vs each control.

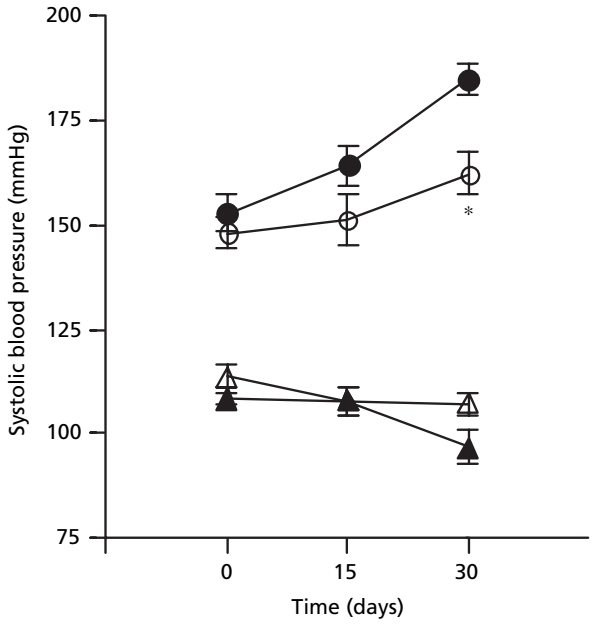

Figure 1 The effect of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on systolic blood pressure in normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate denotes systolic blood pressure, while the abscissa indicates the time course (days) after control diet (▲, WKY; ●, SHR) and 0.5% Ginkgo diet (△, WKY; ○, SHR). Each point represents the mean ± s.e.m. (n = 6). *$P < 0.05$ vs control SHR.

Ginkgo inhibited a rise in blood pressure by cortisol release during stress in healthy volunteers. In this study, 30-day administration of Ginkgo caused a significant inhibition of any increase in blood pressure in SHR, but had no effect on normotensive WKY. These results indicated that Ginkgo inhibited the development of hypertension.

It is well known that vascular resistance is regulated by the endothelium via the synthesis and secretion of a variety of vasoactive substances, such as nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factors, and endothelium-derived contracting factor. The stable balance of these factors released from the endothelium is disturbed in diseases such as hypertension, atherosclerosis, and diabetes. In hypertension, endothelium-dependent relaxation induced by a variety of vasodilator agents, such as ACh, is markedly impaired; this has been documented by various investigators (Lockette et al 1986; Linder et al 1990; Jameson et al 1993; Taddei et al 1993; Vanhoutte & Boulanger 1995). The overproduction of vasoconstrictor prostanoids (Diederich et al 1990; Vanhoutte & Boulanger 1995) and super oxide anions generated in this pathologic process have been proposed as factors that contribute to the impaired relaxation of vessels to endothelium-dependent vasodilators (Fu-Xiang et al 1992; Jameson et al 1993). Akpaffiong & Taylor (1998) suggested that either excess production of oxidants or deficiency of antioxidant systems may have contributed to high blood pressure and vascular endothelial impairment in SHR.

In these in-vitro experiments, diminished relaxation in response to ACh was confirmed in SHR aorta, and relaxation

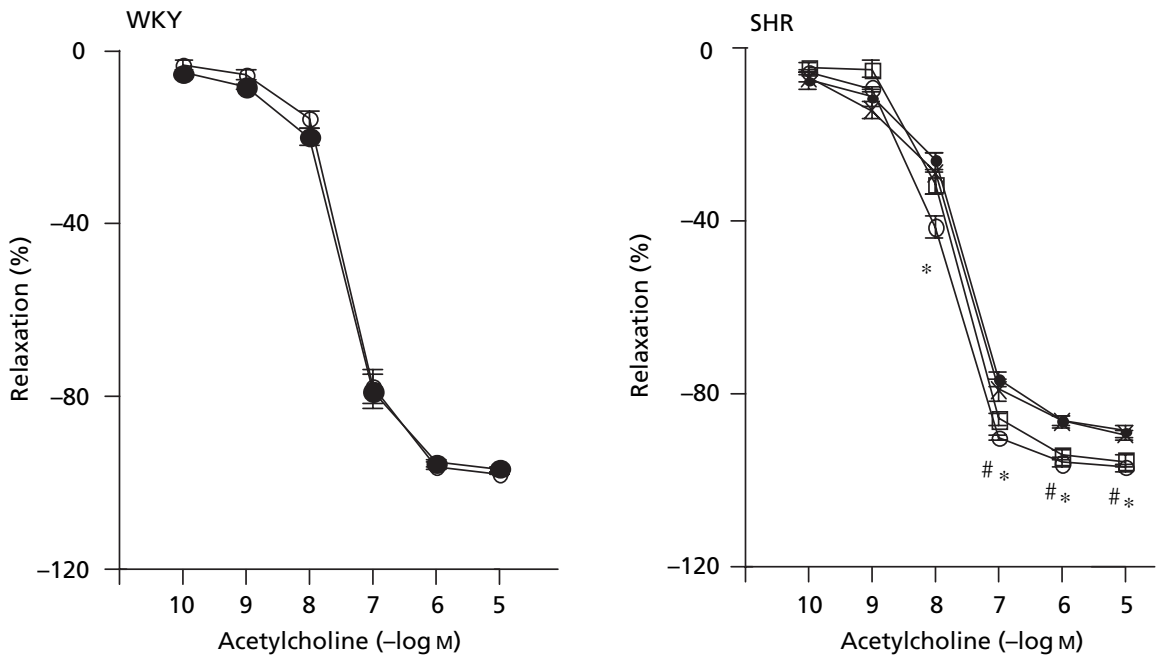

Figure 2 The effect of *Ginkgo biloba* extract (Ginkgo) diet on relaxation induced by acetylcholine in aortic rings preconstricted with noradrenaline (10^{-7} M) isolated from normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate indicates the ratio of relaxation (%) to maximum relaxation in response to papaverine at 10^{-6} M and the abscissa shows the concentration of acetylcholine (M) in rats fed the control diet (▲, WKY; ●, SHR), the 0.05% Ginkgo diet (×, SHR), the 0.1% Ginkgo diet (□, SHR), and the 0.5% Ginkgo diet (△, WKY; ○, SHR). Each point represents the mean ± s.e.m. (n = 6). #$P < 0.05$ 0.1% Ginkgo vs control. *$P < 0.05$ 0.5% Ginkgo vs control.

Table 2 Effects of *Ginkgo biloba* extract (Ginkgo) treatment on EC50 of relaxation produced by acetylcholine in the aortic rings preconstricted with noradrenaline (10^{-7} M) of spontaneously hypertensive rats

Diet	EC50 (nM)
Control	21.5 ± 2.6
Ginkgo 0.05%	16.2 ± 3.4
Ginkgo 0.1%	17.9 ± 2.5
Ginkgo 0.5%	12.6 ± 1.0*

Each value is the mean ± s.e.m. for six rats. *$P < 0.05$ vs control.

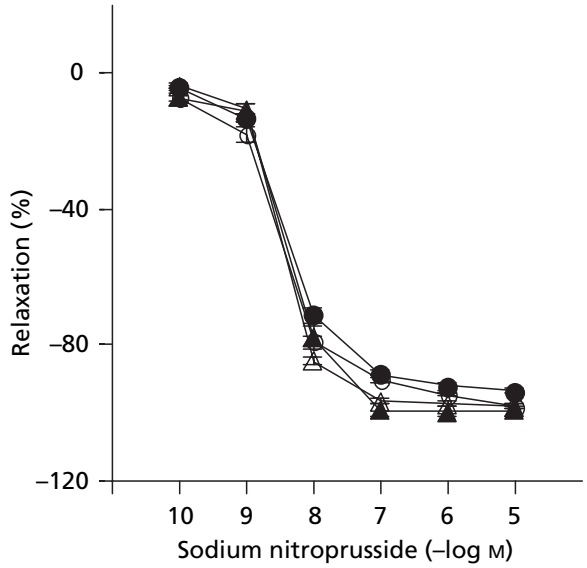

Figure 3 The effect of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on relaxation induced by sodium nitroprusside in aortic rings preconstricted with noradrenaline (10^{-7} M) isolated from normotensive Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). The ordinate indicates the ratio of relaxation (%) to maximum relaxation in response to papaverine (10^{-6} M) and the abscissa denotes the concentration of sodium nitroprusside (M) in rats fed the control diet (▲, WKY; ●, SHR) or the 0.5% Ginkgo diet (△, WKY; ○, SHR). Each point represents the mean ± s.e.m. (n = 6).

was restored by long-term administration of a Ginkgo-containing diet. This effect of Ginkgo was not observed in WKY. However, a significant decrease in EC50 values was observed only with 0.5% Ginkgo diet and a significant increase in maximum relaxation values was observed only with 0.1% and 0.5% Ginkgo diets. These results indicated that ACh-induced relaxation at 10^{-7}–10^{-5} M concentrations was enhanced by 0.1% and 0.5% Ginkgo diets in SHR. Furthermore, relaxation in response to nitric oxide, such as sodium nitroprusside-induced relaxation, was not affected by a Ginkgo-containing diet in either SHR or WKY. These findings suggested that enhanced relaxation resulting from Ginkgo administration was due to increased nitric oxide production/release from the endothelium or to greater nitric oxide bioavailability.

Flavonoids are considered important dietary antioxidants (Robak & Gryglewski 1988). Ginkgo also has antioxidant properties (Haramaki et al 1994; Koc et al 1995; Miyajima et al 1997; Pietri et al 1997). Sasaki et al (2002) reported that Ginkgo produced antioxidant effects, increased nitric oxide metabolites, and increased the expression of endothelial nitric oxide synthase mRNA in stroke-prone SHR. One of the major active ingredients of Ginkgo is considered to be quercetin. Indeed, quercetin was reported to inhibit hypoxanthine-xanthine oxidase activity and scavenge superoxides, hydroxy radicals, and peroxynitrite in-vitro (Rice-Evans & Packer 1998). Moreover, metabolites of orally administered quercetin have been demonstrated to retain the antioxidant properties of the parent compound (Manach et al 1998). Duarte et al (2001a, b) demonstrated that while quercetin reduced elevated blood pressure and restored the endothelium-dependent vasodilation in response to ACh in SHR, no such effects were apparent in WKY. They suggested that the effects of quercetin were associated with a reduced oxidant status due to its antioxidant properties. Superoxide is generally recognized to impair endothelium-dependent vasodilation via inactivation of synthesis and/or release of nitric oxide and consequently to elevate blood pressure (Taniyama & Griendling 2003). Taken together, these findings suggested that the antioxidant properties of the flavonoids within Ginkgo, such as quercetin, may have participated in the effects of Ginkgo on blood pressure and endothelium-dependent relaxation observed in this study.

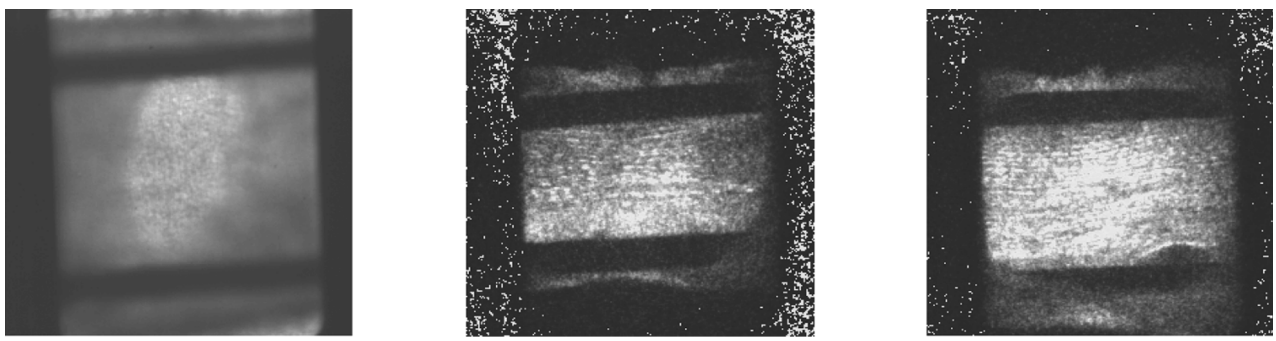

Figure 4 The effect of acetylcholine on intracellular calcium ion level in aortic endothelium isolated from spontaneously hypertensive rats (SHR). Left panel: phase-contrast microscopic image of the endothelial layer. Stainless steel wires are identified in the top and bottom of the image. The degree of vasoconstriction can be estimated from the distance between these two wires. The remaining panels show fluorescent confocal images of intracellular calcium ion level in the endothelial layer of the aorta precontracted with noradrenaline (10^{-6} M) in the absence (middle panel) and presence (right panel) of acetylcholine (10^{-6} M).

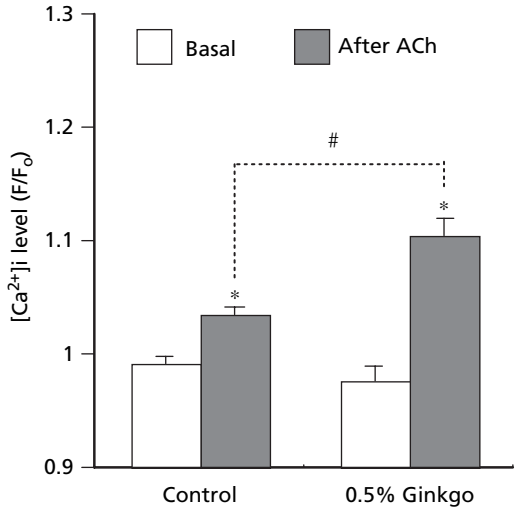

Figure 5 The effects of 0.5% *Ginkgo biloba* extract (Ginkgo) diet on the intracellular calcium ion increase induced by acetylcholine (ACh) in the aortic endothelium isolated from spontaneously hypertensive rats (SHR) fed with control and 0.5% Ginkgo diets. The ordinate shows the intracellular calcium ion level (F/F_0) (fluorescence intensity was the peak fluorescence intensity (F) divided by fluorescence intensity at the beginning of each experiment (F_0)). Each column represents the mean ± s.e.m. (n = 6). $^*P < 0.05$ vs each baseline value. $^{\#}P < 0.05$ vs control acetylcholine.

Indirect and direct experimental evidence demonstrated that the entry of extracellular Ca^{2+} and the liberation of Ca^{2+} from intracellular stores could contribute to an increase in free cytoplasmic Ca^{2+} concentration in endothelial cells, which was an essential step in the synthesis and release of nitric oxide (Rubanyi & Vanhoutte 1988). Endothelial nitric oxide synthase is constitutively expressed in endothelial cells lining the blood vessels and heart. Its activity is tightly controlled by an intramolecular auto-inhibitory element that hinders calmodulin binding and this molecular hindrance is removed by elevated intracellular Ca^{2+} levels (Wu et al 2002; Fleming & Busse 2003). Thus, many studies have indicated that the release of nitric oxide must require an increase in cytoplasmic Ca^{2+} within endothelial cells. Pogan et al (2001) have shown that the Ca^{2+} signalling process in SHR endothelial cells was affected by increased oxidative stress, resulting in a depletion of releasable Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive and -insensitive Ca^{2+} pools. They suggested a possible beneficial action of antioxidants on Ca^{2+} signalling in endothelial cells from models of hypertension. In our study, a greater ACh-induced increase in intracellular Ca^{2+} level was observed in endothelial cells of the aorta isolated from SHR that had been fed a Ginkgo-containing diet than in those isolated from control SHR. This accelerated response may have been caused by recovery of an intracellular Ca^{2+} mobilization mechanism in endothelial cells resulting from the antioxidative action of Ginkgo flavonoids. However, further study is necessary to elucidate the site of action and mechanism of Ginkgo.

In conclusion, the results confirmed that Ginkgo reduced the elevation of blood pressure and improved the dysfunction of the endothelial nitric oxide synthase/nitric oxide pathway in the endothelium in SHR. Ginkgo enhanced the increase in intracellular Ca^{2+} level to ACh in endothelial cells. This acceleratory influence of Ginkgo on intracellular Ca^{2+} mobilization may have participated in the restoration of endothelium-dependent vasodilation in response to ACh. To our knowledge, this was the first study to have investigated the influence of long-term Gingko administration on intracellular calcium mobilization in vascular endothelium. The pharmacological activity was considered to contribute to the possible beneficial properties of Ginkgo in clinical practice, including the regulation of hypertension.

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