Long-Term Feeding of *Ginkgo biloba* Extract Impairs Peripheral Circulation and Hepatic Function in Aged Spontaneously Hypertensive Rats

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Dietary supplements are often taken for a healthy lifestyle, and their number and type have greatly increased. *Ginkgo biloba* extract (GBE), which is the leaf extract of *Ginkgo biloba*, is one of the most used herbal dietary supplements in the world. GBE products are marketed as a dietary supplement in Japan. In some European countries, GBE, designated as EGb 761, is clinically used for cerebral insufficiency, such as intermittent claudication, dementia and equilibrium disorders, as a dietary supplement.

We previously demonstrated that *Ginkgo biloba* extract (GBE) produces an anti-hypertensive effect via enhanced vasodilation responses in young spontaneously hypertensive rats (SHR) and hepatic hypertrophy occurs with increased cytochrome P450 (CYP) mRNA expression in young rats. In the present study, to clarify whether these actions of GBE are observed in older rats, we investigated cardiovascular functions and hepatic CYP protein expression in aged SHR fed a control diet or a diet containing 0.5% GBE for 4 weeks. In aged SHR, GBE feeding significantly increased liver weight per 100 g body weight without changing the body weight. Furthermore, significant increases in alanine aminotransferase level in serum and marked increase in CYP2B protein expression in the liver were observed in aged SHR fed GBE. On the other hand, GBE feeding did not affect blood pressure, but significantly reduced heart rate and blood flow velocity in tail arteries of aged SHR. Furthermore, GBE feeding did not affect contractile response to phenylephrine, relaxation responses to not only sodium nitroprusside but also acetylcholine, and protein levels of endothelium nitric oxide synthase and soluble guanylate cyclase in aortas of aged SHR. These results suggested that long-term GBE feeding impairs peripheral circulation due to bradycardia and hepatic function in aged SHR. Thus, in the elderly population with hypertension, the use of GBE may need to be assessed for effects on heart rate and liver function.

**Key words** aged; *Ginkgo biloba* extract; P450; cardiovascular system; spontaneously hypertensive rat

**MATERIALS AND METHODS**

**Animals and Diets** Male spontaneously hypertensive rats (SHR/Izm; SHR) were purchased from Japan SLC (Hamamatsu, Japan). Experiments were performed in accordance with the Guiding Principles for Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. A standardized powder form of GBE was kindly supplied by Tama Biochemical Co., Ltd. (Tokyo, Japan). GBE contained 24.2% flavonoids (12.4% quercetin) and 9.4% terpenes, and its composition was similar to that of EGb 761 used in European countries. At 50 weeks of age, the rats were divided into two groups by determination of body weight, blood pressure and heart rate, and then administrated a commercial rodent diet (CE-2; Clea Japan Inc., Tokyo, Japan) (control group, n=5) or a GBE-containing diet (0.5% GBE addition to CE-2) (GBE group, n=6) for 4 weeks, respectively. We have already demonstrated that 4-week treatment with GBE has beneficial effects on cardiovascular function in young SHR; therefore, in the present study, the same conditions of dose and period used in young rats were used to investigate the effects of GBE in aged SHR. In general, the dosage of GBE is not limited by age when used as a supplement.

Once a week during the experiment, in anesthetized rats, blood pressure and heart rate were measured using a blood pressure monitor (tail-cuff method, Model MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan), and subcutaneous blood volume, flow rate and flow velocity in the tail were monitored using a laser Doppler blood flow meter of the contact type (FLO-C1, Neuroscience, Tokyo, Japan), re-
spectively. Pulse pressure was calculated by subtracting diastolic blood pressure from systolic blood pressure. Subcutaneous blood flow velocity, blood volume and flow rate were calculated as the rate of change divided by baseline levels measured before treatment with GBE.

After 4 weeks' treatment, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and blood was taken from the abdominal aorta into a tube containing EGTA. Serum was separated by centrifugation (3000 g for 10 min, 4 °C), and serum levels of total protein, albumin and transaminases, i.e. aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were measured using commercial diagnostic kits (Wako Pure Chemical Industries, Osaka, Japan).

**Measurement of Contractile and Relaxation Responses in Arteries** At the end of the experiment, the thoracic aorta was rapidly removed and placed in a Krebs–Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4 · 7H2O, 2.5 mM CaCl2 · 2H2O, 25.0 mM NaHCO3, 1.2 mM KH2PO4 and 11.1 mM glucose). After removing periaortic fat and connective tissue, the artery was cut into ring segments of approximately 3 mm length. Each ring preparation from aortas was mounted isometrically at an optimal resting tension of 1 g in a 10-ml organ bath filled with Krebs–Henseleit solution. The bath solution was maintained at 37 °C and bubbled with a 95% O2–5% CO2 gas mixture. Each preparation was allowed to equilibrate for at least 60 min before the start of the experiments. Isometric tension change was measured with a force-displacement transducer (Model T-7, NEC San-ei Instruments, Ltd., Tokyo, Japan) coupled to a dual channel chart recorder (Model 8K21, NEC San-ei Instruments). Phenylephrine (1 nM—3 μM) was cumulatively added to the bath to determine contractile response in ring preparation with and without endothelium. The ring preparation was contracted with phenylephrine (0.1 μM), and after this equilibration period, acetylcholine (0.1 nM—1 μM), sodium nitroprusside (0.1 nM—1 μM) or isoproterenol (0.1 nM—1 μM) were cumulatively added to the bath. The relaxation response was expressed as a percentage of the maximal relaxation produced by papaverine (100 μM). The relaxation responses to acetylcholine and isoproterenol were determined using the ring preparation with endothelium, and the responses to sodium nitroprusside and isoproterenol were determined using the ring preparation without endothelium.

**Western Blot Analysis** At the end of the experiment, the liver was removed from each rat and weighed. It was rinsed with 0.9% NaCl solution, weighed and homogenized in a Teflon homogenizer in 150 mM KCl solution (approximately 1 g/4 ml). To determine CYP in the microsome fraction, the homogenate was centrifuged at 9000—10000 g for 1 h at 4 °C, and then the supernatant was further centrifuged at 4 °C), and serum levels of total protein, albumin and transaminases, i.e. aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were measured using commercial diagnostic kits (Wako Pure Chemical Industries, Osaka, Japan).

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In addition, the protein expressions of endothelium nitric oxide synthase (eNOS) and soluble guanylate cyclase (sGC), synthetase in endothelium and effector in smooth muscle cells for nitric oxide, were determined as described previously.12) The data were presented as the eNOS/beta-actin or sGC/alpha-actin ratio.

**Materials** Drugs used in the present experiments were as follows: sodium pentobarbital (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); L-phenylephrine hydrochloride (Sigma Chemical, St. Louis, MO, U.S.A.); acetylcholine chloride (Daichichi Pharmaceutical, Tokyo, Japan); sodium nitroprusside, dl-isoproterenol hydrochloride and papaverine hydrochloride (Nacalai Tesque, Kyoto, Japan). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque.

The antibodies used in the present experiments were as follows: antibodies of goat anti-rat CYP2B1/2B2 (Daichichi Pure Chemical Co., Ltd., Tokyo); horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology, Inc., CA, U.S.A.); mouse monoclonal antibodies to eNOS (BD Transduction Laboratories, Lexington, KY, U.S.A.); beta-actin (Sigma Chemical); alpha-actin (Progen Biotechnik, Heidelberg, Germany); rabbit antibody to beta-1 subunit of sGC (Abcam plc, CB4, U.K.); horseradish peroxidase-conjugated anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, CA, U.S.A.).

**Statistical Analyses** Results are presented as the means ± standard error of the mean (S.E.M.). The findings were evaluated for statistical significance using Student’s t test. When the variances of two groups were different, the Welch test was used. A probability of less than 0.05 was considered significant. Statistical analyses were carried out with a computer program (Stat View 4.5, Abacus Concepts, Inc., CA, U.S.A.).

**RESULTS**

**Effects of GBE Treatment on Body Weight, Liver Weight, and Serum Levels of Total Protein, Albumin, AST and ALT** Neither water nor diet intake changed in aged SHR by treatment with 0.5% GBE diet (data not shown). Table 1 shows the effects of treatment with GBE on the body weight, ratio of liver-to-body weight, and serum levels of total protein, albumin, AST and ALT in aged SHR. Administration of the GBE diet for 4 weeks did not significantly affect body weight, but significantly increased the ratio of liver-to-body weight by approximately 1.4-fold as compared with the control group. After 4-week treatment with GBE, no significant changes in serum levels of total protein and albumin were observed. On the other hand, serum AST level of aged SHR was not changed, but serum ALT level significantly increased to greater than 1.2-fold.

**Effects of GBE Treatment on Hepatic Metabolizing Enzymes** GBE is known to increase CYP2B1/2 mRNA
and protein in the liver of young normotensive rats\textsuperscript{8,11}; therefore, in the present study, protein expression of CYP2B1/2 of aged SHR was determined. As shown in Fig. 1, the protein level in the liver was dramatically increased in the GBE group by approximately 8.5-fold as compared with the control group.

**Effects of GBE Treatment on Blood Pressure, Heart Rate and Pulse Pressure** During the experiment, both the systolic and diastolic blood pressure of aged SHR were not significantly changed by treatment with GBE. After 4-week treatment, systolic blood pressure was 220±8 and 198±6 mmHg in the control and GBE groups, respectively (Fig. 2A). Diastolic blood pressure was 160±10 and 138±8 mmHg in the control and GBE groups, respectively (Fig. 2B). Slightly decreased pulse pressure was observed in aged SHR after 4-week treatment with GBE, but the changes were not significant (Fig. 2C). On the other hand, the heart rate in the GBE group was significant lower than that in the control group (Fig. 2D).

**Effects of GBE Treatment on Blood Flow Velocity, Blood Volume and Flow Rate** Blood flow velocity in GBE-fed aged SHR was lower than that in the control group during the experiment, and the difference was significant after 4-week treatment (Fig. 3A). Also, the blood volume and blood flow rate were slightly decreased in aged SHR by feeding the 0.5% GBE diet for 4 weeks (Figs. 3B, C).

**Effects of GBE Treatment on Contractile and Relaxation Responses in Isolated Aortas** Treatment with GBE did not affect the contractile response to phenylephrine in the aortic rings neither with nor without endothelium isolated from aged SHR (Fig. 4A). There were no significant differences in relaxation responses to either acetylcholine or...
sodium nitroprusside between aortas from aged SHR fed the normal or 0.5% GBE-containing diet for 4 weeks (Figs. 4B, C). The GBE diet did not also affect the relaxation response to isoproterenol (data not shown).

Effects of GBE Treatment on Protein Expressions of eNOS and sGC in Aortas

The GBE diet did not influence the protein levels of eNOS, mainly NO synthesis enzyme in the endothelium, and sGC, an important effector protein of NO in vascular smooth muscle cells, in the aortas from aged SHR (Fig. 5).

DISCUSSION

The present study demonstrated that in aged SHR, feeding a 0.5% GBE diet for 4 weeks impaired the peripheral circulation and promoted bradycardia, while coincidentally inducing hepatic hypertrophy and hypofunction as well as increased hepatic metabolic enzyme expression.

Previously, we found that long-term intake of GBE increased hepatic CYP mRNA expression, especially CYP2B1/2 mRNA expression and its activity, and caused potential interactions with anti-hypertensive and anti-diabetic agents in rats. Similarly, in the present study, GBE feeding induced hepatic hypertrophy and markedly up-regulated the expression of CYP2B1/2 proteins, a metabolic enzyme, in aged SHR. Based on these findings, it is reasonable to postulate that long-term GBE intake would enhance the expression of hepatic metabolic enzyme, and then increase the risk of interaction with drugs in aged SHR, similar to that observed in young rats. In other words, these findings may lead to concern about the interaction of GBE with other drugs in the elderly. There is a report that the effects of Ginkgo extract (EGb 761) on drug metabolizing enzymes are specific to rats and may not be extrapolated to humans; however, according to more recent clinical reports, the interaction of GBE with anti-diabetic agents, anti-ulcer agents and anti-platelet/anti-coagulant agents has been demonstrated. These reports support our postulation that long-term intake of GBE as a supplement in elderly people should be carefully monitored because of the likelihood of interaction with other drugs.

Furthermore, long-term GBE intake caused a slight increase in serum ALT level and significantly increased serum AST level in aged SHR. We have preliminarily observed data in which these levels were unaffected by GBE feeding in young SHR. These findings suggest that long-term GBE intake in the elderly with hypertension may induce hepatic hypofunction. In contrast, unchanged ALT and AST levels have been reported in aged normotensive Wistar rats treated with GBE. Also, GBE displays ameliorating effects on elevated serum ALT and AST levels in Wistar albino rats (200—250 g) with liver fibrosis. The reason for this discrepancy is unclear, but the effects of GBE on hepatic function may be associated with high blood pressure and aging. GBE intake does not seem to cause severe hepatic damage, because serum levels of total protein and albumin were unchanged;
however, we preliminarily obtained data that the serum ALT level increase with age in SHR, suggesting that people with age-related hepatic hypofunction should refrain from taking GBE. Further studies are needed to clarify the mechanism underlying this phenomenon.

Our previous studies demonstrated the safety and utility of GBE for the cardiovascular system in young rats. Namely, in young SHR, GBE shows a hypotensive effect and improves endothelium-dependent relaxation via enhanced NO production. Ginkgo biloba extract EGB761 has been reported to decrease blood pressure and augment eNOS mRNA expression in stroke-prone SHR (10-week-old). Furthermore, recent study demonstrated that EGB761 upregulates eNOS protein expression and reduces blood pressure in Sprague-Dawley rats (approx. 200 g BW). Therefore, we determined the effects of GBE on protein expression of the key enzyme for NO-induced vasorelaxation, eNOS (main synthetase of NO) and sGC (activator of NO in smooth muscle cells), in aged SHR. However, in the present study it was clear that GBE did not show significant inhibitory effects on blood pressure increases via improvement of the vasorelaxation response and protein expression of eNOS and sGC in aged SHR. Further studies are needed to resolve these discrepancies. Since vasorelaxation response in untreated aged SHR was smaller than that in the young SHR used in previous studies, the exacerbated vasorelaxation response due to aging or prolonged exposure to high blood pressure may explain the ineffectiveness of GBE on impaired vasorelaxation in aged SHR.

GBE caused a decrease in the heart rate of aged SHR. Similarly, the long-term intake of high-dose GBE (2%) has been reported to induce a negative chronotropic response in young DOCA-salt hypertensive rats. However, GBE feeding does not reduce heart rate in aged normotensive rats, 72-week-old Wistar rats. These findings suggest that the inhibitory effect on heart rate by GBE may be highly associated with blood pressure rather than aging. We have obtained data that the heart rate of young SHR is faster than that of WKY, and GBE intake induces a decrease in heart rate in 11-week-old SHR (unpublished observation). Thus, the negative chronotropic response to GBE seems to be beneficial for tachycardia in SHR. However, the degree of the decrease in heart rate by GBE in aged SHR is greater than that in young SHR (decrease 12% and 9%, respectively). Furthermore, a decrease in velocity was shown in aged SHR after 4-week intake of GBE. Together with unchanged vasorelaxation response, it may be plausible that GBE impairs the peripheral circulation resulting from inhibition of cardiac function in aged SHR. The mechanism underlying the negative chronotropic response to GBE remains unclear, but it has been reported that GBE causes a negative chronotropic response due to the prolonged action potential duration resulting from inhibition of the Ca2+ current, delayed rectifier K+ current in sinoatrial nodal cells isolated from Wistar rats (200—300 g).

In conclusion, as a result of these studies using aged SHR, long-term GBE intake may not be useful for the cardiovascular system, and may impair cardiac and hepatic function. In general, the elderly are thought to show decreased cardiac and hepatic metabolic function, and have a high probability of taking multiple drugs for several diseases. Thus, we consider that the use of GBE in hypertensive elderly may need to be monitored for effects on heart rate and liver function, as well as interactions with other drugs. We need to further investigate the detailed mechanism underlying these events, e.g., whether the effects are caused by hypertension or aging, or the reduction of peripheral circulation and liver function.

Acknowledgements We thank Ms. R. Higashino and Ms. K. Yasumoto for their technical assistance. This research was partly supported by a grant from the Ministry of Health, Labor and Welfare of Japan, and the Open Research Center Project of Mukogawa Women's University for studying lifestyle-related diseases.

REFERENCES