Effects of Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan) on endothelial function in spontaneously hypertensive rats

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Abstract

We examined the protective effect of Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan) (KB) against vascular endothelial disorder in spontaneously hypertensive rats (SHR). We administered KB extract (400 mg/kg/day, p.o.) to SHR for 14 weeks. Blood pressure and plasma viscosity in the KB group were significantly lower than in the SHR control group without KB, and the endothelium-dependent relaxation rate by acetylcholine in the KB group was significantly higher than that in the SHR control group. The rate of endothelium-dependent contraction induced by oxygen-derived free radicals produced by the xanthine-xanthine oxidase system was significantly lower in the KB group than in the SHR control group, and plasma lipid peroxide concentration was also significantly lower in the KB group than in the SHR control group. These results suggest the possibility that KB prevents vascular complications due to hypertension.

Key words Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan), spontaneously hypertensive rat, endothelium-dependent relaxation, endothelium-dependent contraction, lipid peroxide, plasma viscosity.

Abbreviations Ach, acetylcholine; KB, Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan), 桂枝茯苓丸; NE, norepinephrine; NO, Nitric oxide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SHR, spontaneously hypertensive rat; SNPS, sodium nitroprusside; WKY, Wistar-kyoto rat.

Introduction

It is known that the progression of high blood pressure increases the frequency of occurrence of arteriosclerotic angiopathies such as cerebral apoplexy and myocardial infarction in humans.

Arteriosclerosis causes various vascular lesions. When considering the episode mechanism of arteriosclerosis, damage of the vascular endothelium is one of the important aspects.[1] Hypertension is in one of the basic diseases that initiates the disorder of vascular endothelial cells. Mechanical stimulation, a shear stress toward vascular endothelial cells occurring due to hypertension, is thought to be one of the causes of the disorder.[2]

Keishi-bukuryo-gan (KB) is a Kampo medical formulation composed of five kinds of crude drugs. It possesses an improving effect on the microcirculation of the ocular conjunctiva in human,[3] an improving effect on hemorheology,[4] and an inhibitory effect on arteriosclerosis in the rabbit.[5] It is the prescription used for thrombogenesis and arteriosclerotic disease clinically.[6] However, the protective effect of KB against vascular endothelial disorder caused by hypertension has not been confirmed.

In this study, KB extract was administered orally to the spontaneously hypertensive rat (SHR), and vascular endothelial function, nitric oxide (NO) function, hemorheological factors, endothelium-dependent constriction induced by oxygen-derived free radicals produced by the xanthine-xanthine oxidase system, plasma fibrinogen and lipid peroxide were examined to clarify the protective effect of KB against vascular

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endothelial disorder caused by hypertension.

Materials and Methods

Preparation of extracts and chemicals: The extract from KB was composed of 5 kinds of crude drugs: 4.0 g of Keihi (桂皮), Cinnamomi Cortex, *Cinnamomum cassia* BLUME., (China), 4.0 g of Shakuyaku (芍薬), Peoniae Radix *Paeonia lactiflora* PALL., (Japan), 4.0 g of Bukuryo (茯苓), Hoelen *Poria cocos* (FR.) WOLF, (North Korea), 4.0 g of Botampi (牡丹皮), Moutan Radicis Cortex *Paeonia suffruticosa* ANDREWS, (China), 4.0 g of Tohnin (桃仁), Persicae Semen *Prunus persica* (L.) BATSCH., (China). All crude drugs were purchased from Tochimoto Tenkaido (Osaka, Japan). The extract was obtained by boiling them in water for 50 minutes and then freeze-drying into a resultant powder. We obtained 7.5 g of KB extract from 100 g of the above raw materials. The powder was then dissolved in distilled water for the animal experiments. The dose of drug used in this study corresponded to about 10 times the clinical dose. L-Norepinephrine bitartrate (NE), acetylcholine chloride (Ach), sodium nitroprusside dihydrate (SNPS), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), xanthine and xanthine oxidase in suspension were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Test animals: Sixteen 11-week-old male spontaneously hypertensive rats and eight 11-week-old male normotensive Wistar-Kyoto rats (WKY) obtained from Sankyo Labo Service (Toyama, Japan) were used. They were kept in an animal room at an ambient temperature of 23±1°C under a 12 h dark-light cycle. They were allowed an adaptation period of 1 week, during which they were fed a commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan). SHR rats were assigned to two groups: the SHR control group (distilled water), and the KB group (400 mg/kg/day in drinking water). Body weight and blood pressure were monitored indirectly using the tail-cuff method with MK-100 (Neuroscience, Tokyo, Japan) and were measured at biweekly intervals from the baseline period until sacrifice. Groups of 8 animals were sacrificed after 14 weeks on the diet. Experimental protocols met the "Guidelines for Animal Experimentation" approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

Relaxation experiments: The rats were anesthetized with Nembutol (50 mg/kg, i.p.) and killed by drawing blood from the heart. A section of the thoracic aorta was carefully cleaned by removing fat and connective tissues, and ring preparations (3 mm wide) were prepared. The endothelial lining of the rings was removed by pressing the ring slightly and rolling it gently onto filter paper a few times. Removal of the endothelium was then functionally confirmed. The rings were mounted on steel hooks in a Magnus chamber (Kishimoto UC-5TD, Kyoto, Japan). One end of the aorta was attached to a force-displacement transducer (Kishimoto UM-203) and then its isometric contraction was recorded (Niko Bioscience T-634, Tokyo, Japan). Baths were filled with 5 ml of Krebs solution with the following composition (mM): NaCl 120, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5 and glucose 10.0. The solution was maintained at 37°C and bubbled continuously with 5 % CO_2-95 % O_2 at pH 7.4. The rings were equilibrated for 40 min at an initial resting tension of 1 g, and then contracted with 60 mM KCl. When contraction reached a steady maximal response, 10^{-6} M Ach was added. When the endothelium was removed, the relaxation induced by Ach disappeared. Then the Krebs solution was replaced 4 times at 15-min intervals. The rings were then precontracted with 5×10^{-7} M NE. For endothelium-dependent relaxations, vessels were relaxed with Ach (10^{-9} to 10^{-4} mol/L).

To study the direct relaxation of vascular smooth muscle, vessels were relaxed with SNPS (10^{-9} to 10^{-4} mol/L). Relaxation was expressed as a percentage of the decrease in maximal tension obtained by NE-induced contraction.

Contraction experiments: To determine the endothelium-dependent contraction of aortas induced by oxygen-derived free radicals, we placed a segment of an aorta in the medium containing xanthine (10^{-4} M), and precontracted it with 3×10^{-6} M $PGF_{2\alpha}$. Oxygen-derived free radical-induced endothelium-dependent contraction of aortas was determined by the addition of xanthine oxidase (1 to 9 mU/ml) to the medium containing xanthine and $PGF_{2\alpha}$. This contraction was expressed as percentage of the relative increase to the

maximal tension obtained by $PGF_{2\alpha}$-induced contraction.

Blood samples: At the time of sacrifice, 7 ml of blood was withdrawn from the heart. This was anticoagulated in EDTA-2Na (1.5 mg/ml) to measure the hemorheological parameters of whole blood viscosity, plasma viscosity, and other parameters such as NO, fibrinogen and lipid peroxide.

NO measurement: NO is an extremely unstable molecule and rapidly undergoes oxidative degradation when exposed to stable inorganic nitrogen oxides NO_2^-/NO_3^-, which were used as indices of in vivo NO generation. Plasma NO_2^-/NO_3^- were measured with an automated system, ENO-10 (EICOM Co., Kyoto, Japan), based on the Griess technique.

Measurement of blood viscosity: The details of blood viscosity measurement were explained in our previous study.[7] Whole blood and separated plasma were measured by cone-plate rotational viscometer (Bio-rheolizer, Tokyo Keiki Co., Ltd., Tokyo, Japan) at five different shear rates (γ) (19.2, 38.4, 76.4, 192.0, 384.0 sec^{-1}), and the averages of the five values were calculated. This measurement was repeated again by using the remaining blood samples. The final viscosity was estimated at each rate by the average of the two tests. Plasma viscosity was estimated at the high shear rate of 384.0 sec^{-1} by the average of five values. All measurements were performed at a constant temperature of 37°C.

Plasma fibrinogen and lipid peroxide measurements: Plasma fibrinogen was measured by the thrombin time method,[8] and plasma lipid peroxide was measured by Yagi's method.[9]

Statistical analysis: Data were presented as mean±standard error. Statistical comparisons were made using the Mann-Whitney test and repeated measures ANOVA. The level of statistical significance was defined as $p<0.05$.

Results

At 14 weeks of experimental duration, no significant difference was seen between the KB group and SHR control group in terms of body weight. Systolic and average blood pressures in the KB group were significantly lower than those in the SHR control group (Table I).

Ach-induced endothelium-dependent relaxation of aortas reached a maximum at 10^{-4} M in the KB and WKY groups, and at 10^{-6} M in the SHR control group, within the range of tested concentrations from 10^{-9} M to 10^{-4} M. (Figure 1 a). The relaxation of the KB group was significantly greater than that of the SHR control group. The maximum relaxations were 88.0± 2.6, 63.6± 5.9 and 36.0± 5.9 % in the WKY, KB and SHR control groups, respectively (Figure 1a). There was no significant difference in endothelium-independent relaxation of aortas with SNPS between the KB and SHR control groups (Figure 1b). The maximum relaxations were 99.4±0.4, 90.9±1.9 and 88.2±1.4 % in the WKY, KB and SHR control groups, respectively (Figure 1b).

Treatment with xanthine oxidase (1-9 mU/ml) in the presence of xanthine (10^{-4} M) after $PGF_{2\alpha}$ treatment caused a concentration-dependent contraction of the aortal endothelium (Figure 2a). The contrac-

Table I Characteristics of SHR and WKY in different experimental groups.

Group	Start (SHR)	Control (SHR)	KB (SHR)	WKY
Body weight (g)	279.3 ±2.6	390.9 ± 6.6	377.0 ±5.6	406.3 ±2.9
Systolic pressure (mmHg)	192.5 ±2.4	213.3 ± 2.4	204.5 ±2.0*	124.9 ±4.1
Mean pressure (mmHg)	150.2 ±2.6	172.6 ± 2.8	163.5 ±1.4**	98.9 ±4.8
Plasma NO2⁻/NO3⁻ (μM)	8.01±0.70	9.82± 1.29	10.65±1.88	10.91±1.37
Plasma viscosity (cp)	–	1.42± 0.015	1.33±0.016*	1.32±0.011
Plasma fibrinogen (mg/dl)	–	46.1 ±10.6	25.6 ±2.8	21.4 ±1.8
Plasma lipid peroxide (nmol/ml)	–	2.68± 0.12	2.29±0.09*	2.09±0.11

The experimental group (control and KB) consisted of 12-week-old SHR (n=16).
Asterisks indicate significant differences from SHR control.
(*: $p<0.05$, **: $p<0.01$ mean±S.E., n=8 each).

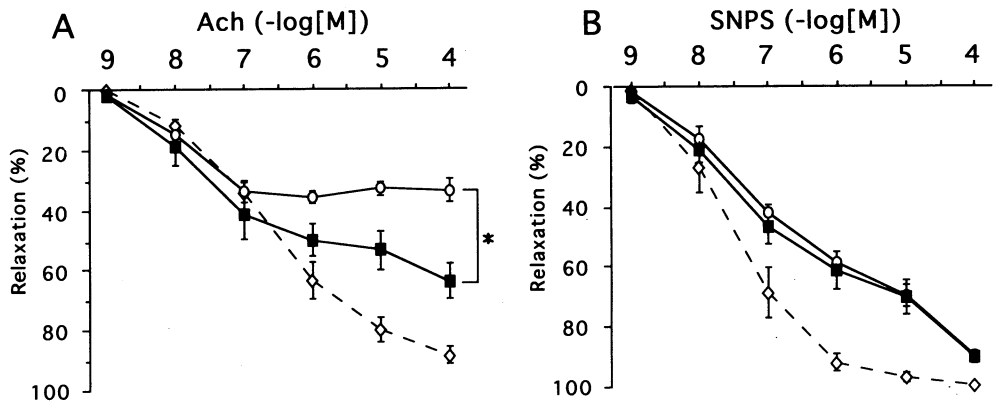

Figure 1 Endothelium-dependent relaxation with Ach (A) and endothelium-independent relaxation with SNPS (B) in arorta of SHR treated with KB for 14 weeks. SHR control group (○), KB group (■), WKY group (◇), Values are expressed as percentage of decrease in the maximal tension contracted with 5×10^{-7}M NE. Shown are mean±S.E. of 8 determinations. Asterisk indicates significant difference from SHR control group (*: $p<0.05$).

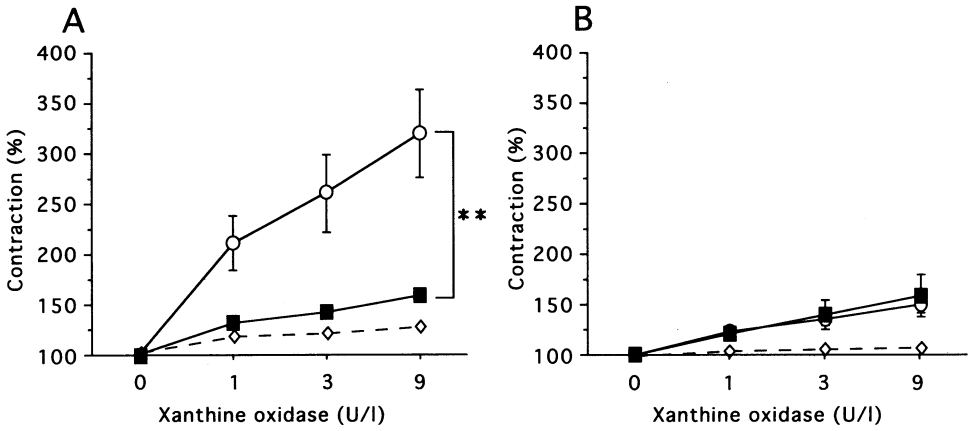

Figure 2 Effect of xanthine oxidase in presence of xanthine (10^{-4}M) on contraction of isolated SHR aortas with (A) and without (B) endothelium after $PGF_{2\alpha}$ treatment as a function of concentration. SHR control group (○), KB group (■), WKY group (◇).
Asterisks indicate significant difference from SHR control group (**$p<0.01$, mean±S.E., n=8).

tion of the aortal endothelium in the KB group was significantly less than that in the SHR control group; the contraction at 9 mU/ml of xanthine oxidase was 126.1±3.9 % in the WKY group, 159±6.2 % in the KB group, and 319±43.6 % in the SHR control group (Figure 2a). Removal of the endothelium decreased these increased contractions, but there was no significant difference in the contraction of aortas without the endothelium between the KB and SHR control groups (Figure 2b). The contraction at 9 mU/ml xanthine oxidase was 108.0±1.9 % in the WKY group, 159±21.0 % in the KB group, and 150±8.5 % in the SHR control group (Figure 2b).

There was no significant difference in plasma NO_2^-/NO_3^- concentration between the KB and SHR control groups (Table I). The KB group (1.33±0.016 cp) had significantly lower plasma viscosity than the SHR control group (1.42±0.015 cp) ($p<0.05$), and plasma fibrinogen concentration was poorer in the SHR control group (46.1±10.6 mg/dl) than in the KB group (25.6±2.8 mg/dl), but the difference was not statistically significant ($p=0.0547$) (Table I).

There was no significant difference in whole blood viscosity between the KB and SHR control groups (data not shown).

The concentration of plasma lipid peroxide in the WKY, KB and SHR control groups were 2.09±0.11, 2.29±0.09 and 2.68±0.12 nmol/ml, respectively, with that of the KB group being significantly lower than that of the SHR control group (Table I).

Discussion

In this study it was clearly evident that there was a significant increase in the rate of endothelium-dependent vascular relaxation in the thoracic aorta of SHR, and this suggested that KB exerted a protective action against vascular endothelial dysfunction in SHR. In other words, KB appears to have an inhibitory effect on the development of arteriosclerosis from hypertension, which can be construed as an early lesion of arteriosclerosis from vascular endothelial hypoactivity.[10]

To clarify the mechanism of the protective action against vascular endothelial dysfunction in SHR, we measured plasma lipid peroxide and found a significant decline in its concentration in the KB group. From the observation that intravascular oxygen-derived free radicals are involved in the production of blood lipid peroxide,[11] it was suggested that KB had an inhibitory effect on the production of oxygen-derived free radicals. Superoxide radical, one of the oxygen-derived free radicals, plays an important role as an attenuation factor of NO function by inactivating NO rapidly.[12] The effect of oxygen-derived free radicals produced by the xanthine - xanthine oxidase system on extirpated blood vessels has been examined by the constrictive response.[13] In the present study, it was shown that the constrictive response of the aortal endothelium to oxygen-derived free radicals was inhibited in the KB group.

In earlier studies, it was reported that NO not only had a vasodilation effect but also an inhibitory effect on the proliferation of vascular smooth muscle cells,[14] as well as a platelet aggregation inhibitory effect.[15]

At the same time, we also performed a hemorheological study, and we found a significant improvement of blood viscosity in the KB group and also that fibrinogen tended to decrease in the KB group compared to the control group. This result was in agreement with a previous report from our laboratory.[16] It has also been reported that an increase in shear stress occurs with a rise in blood viscosity,[17] and that the increase in shear stress is a feature of endothelial cell disorder.[18]

Thus, it may be considered that KB exerts a vascular endothelial protective effect by its pleiotropic action, i.e., scavengeing oxygen-derived free radicals and improving blood viscosity, actions to increase NO function. Future studies should examine the long-term effect of KB on arteriopathy in SHR and, most importantly, the favorable anti-hypertensive effect of the drug in a clinical setting.

Conclusion

When KB was administered to SHR, the drug improved the hemorheology and appeared to decrease the production of free radicals in the aortal endothelium. It became clear that KB exerted inhibitory actions on the rise in blood pressure and the comcomitant vascular endothelial hypoactivity action in SHR. Taken together, our findings suggested that KB has the ability to prevent vascular complications caused by hypertension.

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和文抄録

自然発症高血圧ラット（SHR）における血管内皮障害に対する桂枝茯苓丸（KB）の保護作用について検討した。KBエキス（400 mg/kg/day）は，経口で14週間SHRに投与した。KB群の血圧と血漿粘度は，SHR対照群と比較して有意に低下した。内皮依存性血管弛緩作用はSHR対照群と比較してKB群で有意に増加した。キサンチン-キサンチンオキシダーゼ系による内皮依存性血管収縮作用はSHR対照群と比較してKB群で有意に

減少し，血漿過酸化脂質はKB群で有意に低下した。このことから，桂枝茯苓丸が，高血圧症による血管合併症を予防する可能性が示唆された。

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