Effects of Keishibukuryogan on Vascular Function in Adjuvant-Induced Arthritis Rats

Kazuya Nozaki,a Hiroto Goto,b,c Takako Nakagawa,b Hiroaki Hikiami,a Keiichi Koizumi,d Naotoshi Shibahara,b,c and Yutaka Shimadaa,c

a Department of Japanese Oriental Medicine, Faculty of Medicine, University of Toyama; b Department of Kampo Diagnostics, Institute of Natural Medicine, University of Toyama; c 21st Century COE Program, University of Toyama; and d Department of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan. Received January 4, 2007; accepted March 12, 2007; published online March 15, 2007

It is known that rheumatoid arthritis (RA) accelerates atherosclerosis. Further, the soluble form of vascular adhesion molecule-1 (VCAM-1) is known as a predictive marker of atherosclerosis in RA patients. We reported that keishibukuryogan, one of the Kampo formulas, improved articular symptoms and decreased soluble VCAM-1 in patients with RA. In adjuvant-induced arthritis (AIA) rats, an animal model of RA, it is known that endothelial function is injured by inflammation. So, we investigated the effect of keishibukuryogan on endothelial function in AIA rats. Lewis rats were divided into control, AIA control, and AIA with keishibukuryogan groups. The AIA with keishibukuryogan group was fed 3% keishibukuryogan contained in normal chow. On day 25 after injection of Mycobacterium butyricum, endothelium-dependent relaxation by acetylcholine in the AIA control group was suppressed, but it was improved in the AIA with keishibukuryogan group. The contractions by xanthine/xanthine oxidase in both AIA rats increased, but that in keishibukuryogan decreased compared to the AIA control group. Plasma levels of lipid peroxide increased in the AIA control group, but keishibukuryogan decreased these levels. Plasma levels of nitric oxide (NO) increased in both AIA groups. The expressions of endothelial NO synthase, inducible NO synthase and VCAM-1 of thoracic aorta were investigated by western blotting. These expressions increased in the AIA control group, but were restricted in the AIA with keishibukuryogan group. We considered that keishibukuryogan protected the endothelial function of AIA rats mainly by its anti-oxidative effect.

Key words keishibukuryogan; adjuvant-induced arthritis; endothelial function; vascular cell adhesion molecule-1; nitric oxide synthase; anti-oxidative effect

Rheumatoid arthritis (RA) is the most common inflammatory form of arthritis. It is known that the activity of daily living is constrained by joint pain, and that joint function can become severely affected with long-term RA. Also, the average life span of RA patients is reported to be shorter than that of healthy people, based on the apparent increase in diseases of the cardiovascular system in RA patients.1) Further, it was recently reported that one of the causes of vascular diseases is clearly related to inflammation,2) and that the development of vascular injury is remarkable in a systemic inflammatory disease like RA.3)

Keishibukuryogan is the one of the Kampo formulas often used clinically in Japan. It is used against many diseases, such as gynecologic and psychiatric diseases, and so on. Moreover, as it improves the microcirculation, it is used for cerebral-vascular and cardio-vascular diseases to improve blood circulation. Keishibukuryogan has been shown to have a vasoprotective effect in animal models of diabetes and hypercholesterolemia.4–7) We reported that keishibukuryogan improved the symptoms of RA patients, and it was determined that the soluble form of vascular cell adhesion molecule-1 (sVCAM-1) of RA patients decreased by the administration of keishibukuryogan.8) As sVCAM-1 is one of the predictive factors of atherosclerosis,9,10) keishibukuryogan is suggested to have an inhibitory effect against atherosclerosis in patients with RA. In the present study, we examined the effects of keishibukuryogan on arthritis and vascular function in adjuvant-induced arthritis (AIA) rats.

MATERIALS AND METHODS

Drugs and Chemicals Analytical grades of the following reagents were purchased: Mycobacterium butyricum from Difco Laboratories (Detroit, MI, U.S.A.); pentobarbital sodium from Tokyo Chemical Ind. (Tokyo, Japan); paraffin oil, norepinephrine (NE), acetylcholine (Ach) and sodium nitroprusside (SNP), all from Wako Pure Chemical Ind. Ltd. (Osaka, Japan); Nω-nitro-L-arginine methylester (L-NNAME), xanthine, xanthine oxidase (XOD), phospholipase A2 (PLA2) and anti-mouse β-actin antibody from Sigma (St. Louis, MO, U.S.A.); antibodies against endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and VCAM-1 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); enhanced chemiluminescence reagents (ECL); and pentobarbital sodium from Tokyo Chemical Ind. (Tokyo, Japan); paraffin oil, norepinephrine (NE), acetylcholine (Ach) and sodium nitroprusside (SNP), all from Wako Pure Chemical Ind. Ltd. (Osaka, Japan); Nω-nitro-L-arginine methylester (L-NNAME), xanthine, xanthine oxidase (XOD), phospholipase A2 (PLA2) and anti-mouse β-actin antibody from Sigma (St. Louis, MO, U.S.A.); antibodies against endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and VCAM-1 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.); enhanced chemiluminescence reagents (ECL) and ECL plus from GE Healthcare UK Ltd. (Amersham Place, Little Chalfont, Buckinghamshire, England).

Powdered keishibukuryogan was purchased from Uchida Wakanyaku (Tokyo, Japan). It consisted of equal amounts of the following 5 crude drugs: Cinnamomi Cortex (Cinnamomum cassia Blume), Poria (Poria cocos Wolf), Moutan Cortex (Paeonia suffruticosa Andrews), Persicae Semen (Punum persicae Batsch), and Paeonia Radix (Paeonia lactiflora Pall.).

Figure 1 shows the three-dimensional HPLC chart of keishibukuryogan. The HPLC conditions were as follows: keishibukuryogan extract (2.5 g) was obtained with 20 ml of methanol under ultrasonication for 30 min. The solution was filtered with membrane filter (0.45 μm) and then subjected to HPLC analysis. HPLC equipment was controlled with an

* To whom correspondence should be addressed. e-mail: hiro510@med.u-toyama.ac.jp © 2007 Pharmaceutical Society of Japan
SLC-10A (Shimizu, Tokyo, Japan) using a TSK-GEL ODS-80TS column (ϕ 4.6×250 mm), eluting with solvents (A) 0.05 M AcONH₄ (pH 3.6) and (B) CH₃CN. A linear gradient of 100% A and 0% B changing over 60 min to 0% A and 100% B was used. The flow rate was controlled with an SLC-10AD pump at 1.0 ml/min. The effluent from the column was monitored, and three-dimensional data were processed by SPD-M10A diode array detectors (Shimadzu, Kyoto, Japan). All assigned peaks were identified by co-injection test with authentic samples and compared with UV spectral data.

**Animals**

Twenty-four 10-week-old male Lewis rats obtained from Japan SLC (Shizuoka, Japan) were used. They were kept in an animal room at an ambient temperature of 20±2 °C and humidity of 55±5% under a 12-h dark–light cycle.

Experimental protocols met the “Guidelines for Animal Experimentation” approved by the Japanese Association of Laboratory Animal Science and the Japanese Pharmacological Society.

**Drug Treatment**

Lewis rats were randomly assigned to 3 groups (control group, AIA control group, AIA with keishibukuryogan group). Rats in the control and AIA control groups received standard chow (CE-2, CLEA Japan Inc., Tokyo, Japan), and rats in the AIA with keishibukuryogan group received standard chow containing 3% (wt/wt) keishibukuryogan. They had free access to both food and water. RA patients normally take 6 g of the 5 crude drugs (0.12 g/kg/d). In this study, rats took keishibukuryogan-containing pellets at about 1.0—1.2 g/kg/d, a dose was about 10-fold that of RA patients.

After 1 week conditioning, on day 0, the rats of AIA groups were injected intradermally at the base of the tail with Freund’s complete adjuvant containing 0.5 mg of heat-killed *Mycobacterium butyricum* suspended in 0.1 ml of paraffin oil (5 mg/ml).

**Evaluation of Arthritis**

Between day 0 and 24, rats were assessed every 4 d for body weight and arthritic scores using an arthritic scoring system. The maximal arthritic score per rat was set at 16 (maximum of 4 points×4 paws). All 4 paws were examined and graded for severity and loci of erythema, swelling and induration using a 5-point scale: 0=no signs of disease; 1=signs involving the ankle/wrist; 2=signs involving the ankle plus tarsals (proximal part of the hind paw) and/or wrist plus carpals of the forepaw; 3=signs extending to the metatarsals or metacarpals; and 4=severe signs involving the entire hind or fore paws. The sum of the 4 paws was then calculated. Paw volumes were measured on day 0 and 24 using water displacement plethysmometry. The percent changes in body weight and mean paw volume were evaluated.

**Preparation of Aortic Rings**

On day 25, rats were anesthetized with pentobarbital (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 length) were made. The rings were suspended on steel hooks in a Magnus chamber (Kishimoto UC-5TD, Kyoto, Japan). One end of the hook was attached to a force-displacement transducer (Kishimoto UM-203) and then its isometric contraction was recorded (Niko Bioscience T-634, Tokyo, Japan). The chamber was filled with 5 ml of Krebs solution with the following composition (mM): NaCl 120, KCl 4.7, NaHCO₃ 25.0, KH₂PO₄ 1.2, MgSO₄·7 H₂O 1.2, CaCl₂ 2.5, glucose 10.0. The solution was maintained at 37 °C and bubbled continuously with 5% CO₂ in O₂ at pH 7.4. The rings were equilibrated for 45 min at an initial resting tension of 1 g, and then contracted with 60 mM KCl. After the contraction reached a steady maximal re-

---

**Fig. 1. Three-Dimensional HPLC Analysis of Keishibukuryogan**
response, the Krebs solution was replaced 4 times at 15-min intervals. Endothelial function was studied by evaluating relaxation to Ach.

**Relaxation Experiments** The rings were then precontracted with $5 \times 10^{-6} \text{M}$ NE. For endothelium-dependent relaxation, vessels were relaxed with Ach ($10^{-9}$ to $10^{-4} \text{M}$). To study the direct relaxation of vascular smooth muscle, vessels were relaxed with SNP ($10^{-9}$ to $10^{-4} \text{M}$). Relaxation was expressed as percentage of the decrease in maximal tension obtained by NE-induced contraction.

**Contraction Experiments. Contraction Induced by Xanthine-XOD** To determine the endothelium-dependent contraction of aorta induced by oxygen-derived free radicals, we placed a segment of an aorta in medium containing xanthine ($10^{-4} \text{M}$) and $10^{-5} \text{M}$ L-NAME. Oxygen-derived free radical-induced endothelium-dependent contraction of aorta was determined by the addition of $10 \text{mU/ml}$ XOD to the medium containing xanthine. This contraction was expressed as a percentage of the relative increase to the maximal tension obtained by 60 mM KCl-induced contraction.

**Contraction Induced by PLA$_2$** To examine the effect against thromboxane A$_2$ and prostaglandin H$_2$-induced contraction, PLA$_2$ (1 U/ml) was administered and transient contraction was induced in medium containing $10^{-4} \text{M}$ L-NAME. Contraction was expressed as a percentage of 60 mM KCl maximum contraction.

**Measurement of Serum Lipid Peroxides and NO$_2$/NO$_3$** At the time of sacrifice, 7 ml of blood was withdrawn from the heart. Using this sample, as a marker of oxidative stress, lipid peroxide (LPO) was measured by lipid peroxidation assay kit (Determine LPO; Kyowa Medics, Tokyo, Japan), based on the hemoglobin-methylene blue (Hb-MB) method. Nitric oxide (NO) is an extremely unstable molecule and rapidly undergoes oxidative degradation to stable inorganic nitrogen oxides NO$_2$/NO$_3$ that were used here as indices of in vivo NO generation. Serum NO$_2$/NO$_3$ was measured with an automated system (ENO-10; EICOM Co., Kyoto, Japan), based on the Griess reaction method.

**Western Blot Analysis** Residual thoracic aortas were crushed using SK-Mill (Tokken, Tokyo, Japan) and homogenized in lysis buffer ($20 \text{mm Tris–HCl (pH 7.5)}, 137 \text{mm NaCl, 1% NP-40, 10% glycerol, 1 mm phenylmethyl sulfonyl fluoride (PMSF), 10 \mu g/ml aprotinin, 1 \mu g/ml leupeptin}$). Large tissue debris and nuclear fragments were removed by centrifuge (5500 rpm, 10 min 4 °C). Protein concentration in the supernatant was determined using a Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, U.S.A.). Protein samples (22.5 \mu g) were separated by electrophoresis using a 6% sodium dodecyl sulfate–polyacrylamide gel, and transferred to polyvinylidene difluoride membrane. After blocking with 5% skim milk solution for 1 h, the membranes were incubated with primary antibodies against eNOS, iNOS and VCAM-1, or anti-mouse β-actin antibody for 2 h at room temperature. Horseradish peroxidase-conjugated immunoglobulin antibodies were used as secondary antibodies for 1 h at room temperature. Then the protein bands of β-actin, eNOS and iNOS were detected with ECL, and the band of VCAM-1 with ECL plus. Chemiluminescent signals were detected using X-ray film and analyzed using an NIH image program. The data for individual rats were corrected for β-actin.

**Statistical Analysis** Statistical analysis was performed with Stat View J-4.5 (Abacus Concept, Berkeley, CA, U.S.A.). Data were presented as mean±S.E. Statistical comparisons were evaluated by one- or two-way analysis of variance (ANOVA) followed by Fisher’s PLSD.

**RESULTS**

**Severity of Arthritis** Body weights of the AIA groups decreased as compared to the control group ($p<0.01$), and that of the AIA control group decreased significantly compared to that of the AIA with keishibukuryogan group ($p<0.01$). Mean paw volume and arthritic scores of the AIA groups increased significantly compared to the control group (Table 1).

**Relaxation of Thoracic Aorta** Typical endothelium-dependent relaxations of AIA control (Fig. 2A) and AIA with keishibukuryogan (Fig. 2B) were traced. Ach-induced endothelium-dependent relaxation of aorta of the AIA control group was significantly reduces compared to that of the AIA with keishibukuryogan groups, respectively ($p<0.05$). Relaxation of the AIA with keishibukuryogan group was increased to a greater degree than that of the AIA control group, with statistically significant difference ($p<0.05$). Maximum relaxations were $10.6\pm2.2\%$, $31.3\pm5.0\%$ and $17.1\pm3.5\%$ in the control, AIA control and AIA with keishibukuryogan groups, respectively (mean±S.E., n=8) (Fig. 2C). In endothelium-independent relaxations with SNP, there was no significant difference among the 3 groups (Fig. 2D).

**Constriction of Thoracic Aorta** The XOD-induced contractions of both AIA groups were significantly increased as compared to the control group ($p<0.01$). But the contraction of the AIA with keishibukuryogan group was significantly decreased as compared to the AIA control group ($p<0.05$). Constrictions at 10 mU/ml XOD were $13.1\pm1.5\%$, $48.3\pm3.2\%$ and $37.1\pm3.4\%$ in the control, AIA control, AIA with keishibukuryogan groups, respectively (Fig. 3). PLA$_2$-induced contractions showed no significant differences among the 3 groups.

**Serum Levels of LPO and NO$_2$/NO$_3$** Serum LPO levels of both AIA groups significantly increased as compared to the control group ($p<0.05$). That of the AIA with keishibukuryogan group was significantly decreased as com-

---

Table 1. Severity of Arthritis on Day 24 after Injection of Freund’s Complete Adjunct

<table>
<thead>
<tr>
<th></th>
<th>Body weight (%)</th>
<th>Arthritis score</th>
<th>Mean paw volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>113.1±1.2</td>
<td>0</td>
<td>104.3±2.2</td>
</tr>
<tr>
<td>AIA control</td>
<td>80.5±1.0***</td>
<td>8.3±1.1***</td>
<td>161.5±8.6***</td>
</tr>
<tr>
<td>AIA with keishibukuryogan</td>
<td>83.7±0.7***</td>
<td>7.8±0.8***</td>
<td>162.2±6.7***</td>
</tr>
</tbody>
</table>

Data are from day 24 after FCA injection compared to day 0 (n=8, each group). Values are mean±S.E. ***p<0.0001 vs control group. +p<0.05 vs AIA control group.
pared to that of the AIA control group ($p<0.05$) (Table 2). Serum NO$_2$/NO$_3$ levels were significantly higher in both AIA groups compared to the control group ($p<0.05$) but there was no difference between the AIA groups (Table 2).

**Aortic Expression of Adhesion Molecules and Nitric Oxide Synthase**  
Aortic expression levels of eNOS, iNOS and VCAM-1 were higher in the AIA control group than in the other groups, significantly (Fig. 4). There was no significant difference between the control and AIA with keishibukuryogan groups in the expressions of eNOS, iNOS and VCAM-1.

Table 2. Serum Concentrations of Lipid Peroxides and NO$_2$/NO$_3$  

<table>
<thead>
<tr>
<th>LPO (μmol/ml)</th>
<th>NO$_2$/NO$_3$ ($\times 10^{-5}$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>AIA control group</td>
<td>34.5±5.3**</td>
</tr>
<tr>
<td>AIA with keishibukuryogan group</td>
<td>13.4±2.3**,#</td>
</tr>
</tbody>
</table>

Data are from day 25 after FCA injection (n=8, each group). Values are mean±S.E.  
$**p<0.01$, $***p<0.001$ vs. control group;  
$p<0.05$ vs. AIA control group.

DISCUSSION

RA patients are often complicated with atherosclerotic diseases, and they are regarded as factors predicting the prognosis of RA patients. There is a report that the average expectation of life of RA patients is shorter than that of the general public.1 The frequent use of corticosteroids by RA patients is thought to be related to their higher rates of atherosclerosis.12 However, it has also been reported that serum levels of cholesterol of RA patients, a risk factor of atherosclerosis, are lower than those of healthy people,13 and the body mass index of RA patients is also lower than that of healthy people.14 This means that the cause of advancing atherosclerosis in RA patients remained unclear. Recently, however, atherosclerosis has come to be regarded as an inflammatory disease,2 and mild inflammation as seen in RA patients is also reported to induce injury to the endothelium.15 Accordingly, systemic inflammation is now considered to be one of the central causes of atherosclerosis in RA patients.16

Clinically, there are some reports of anti-inflammatory therapy and anti-tumor necrosis factor-alpha therapy improving endothelial function in RA patients.17—19 It is thought that improving the state of inflammation will have a favorable effect on endothelial function. Basically, AIA rats have a condition resembling the early lesions of atherosclerosis in RA patients, and have been used to study endothelial function. In AIA rats, as inflammation takes place not only in joints but also in the whole body, it was reported that the induction of endothelial dysfunction can be studied by the organ bath method.20—22 Furthermore, it was reported that free radicals play an important role in endothelial dysfunction in AIA rats.22 Drugs such as vitamin E and tetrahydrobiopterin,21,22 which have no anti-inflammatory effect, improve endothelial dysfunction in AIA rats.22

Keishibukuryogan is the Kampo formula often used to improve Oketsu syndrome, a blood stagnation syndrome. One of the causes of Oketsu is insufficiency of microcirculation in modern medicine.23 Keishibukuryogan has been reported to improve blood circulation and vasoendothelial disorders in animal models of diabetes and hypertension.5,6,24,25 The mechanism for this improvement by keishibukuryogan was an anti-oxidative effect on microcirculation.24 We earlier reported that keishibukuryogan was effective against the symptoms of arthritis in RA patients.9 We therefore studied whether keishibukuryogan improved arthritis and abnormality of vascular function in AIA rats. We found that keishibukuryogan did not directly change the arthritis, but rather that it improved the disorder of endothelial function. These results suggested that, in AIA rats, keishibukuryogan affects the vascular function obstructed by inflammatory cytokines rather than suppressing inflammation. As one of the reasons, in this study, serum NO₂⁻/NO₃⁻ levels were not different between the two AIA groups, but aortic expression levels of eNOS, iNOS and VCAM-1 were higher in the AIA control group than in the keishibukuryogan group. Recently, serum NO₂⁻/NO₃⁻ levels have been reported to be increased in the AIA rat.26 The origin for this is thought to be iNOS upregulation by arthritis. Meanwhile, the mechanism of the increase in aortic expression levels of eNOS, iNOS and VCAM-1 is thought as follows. Inflammatory cytokines such as TNF-α activated NAD(P)H oxidase and produced O₂⁻,27 and this leads to increased production of free radicals on vessels. Keishibukuryogan affects this mechanism and suppresses the production of free radicals.

To clarify the mechanism involved, we performed a contraction study with xanthine/XOD, based on the fact that the production of free radicals increased in disordered vessels. The results suggested that keishibukuryogan ameliorated the vascular disorder and decreased the serum levels of LPO. Thus, the anti-oxidative effect of keishibukuryogan is thought to be one of the important factors in these mechanisms. In patients with RA, the plasma levels of sVCAM-1 are increased28 and are reportedly a predictive marker of cardiovascular disease.29 Generally, VCAM-1 is highly expressed on the injured endothelium. VCAM-1 is related to the firm adhesion of leukocytes to the endothelium and plays an important role in the development of atherosclerosis.31 In collagen-induced arthritis, another type of RA model, an increase in VCAM-1 expression has been observed.32 It is reported that oxidative stress plays a major role in inducing the expression of VCAM-1 on human umbilical vein endothelial cells (HUVEC), adding tumor necrosis factor-α.33 There are some reports that antioxidants such as α-tocopherol and probucol restrict the expression of VCAM-1 on HUVEC.34,35 In our study, keishibukuryogan suppressed the expression of VCAM-1 on aorta significantly, improving endothelial dysfunction in AIA rats. As a result, we supposed that keishibukuryogan might protect the endothelium by restricting VCAM-1 expression.

Endothelial NOS is recognized as playing a major role in protecting the endothelial function. Endothelial NOS activity and NO release decrease in the endothelium in spontaneously hypertensive rats, and endothelium-dependent vasorelaxation is weakened. They are then reportedly improved by the administration of vitamin C or E.36 While the expression of eNOS in AIA rats is increased, its endothelial function is disturbed. As overexpression of eNOS accelerates atherosclerosis by generating superoxide,37 it is important to regulate the expression of eNOS in AIA rats. In the present study, keishibukuryogan was observed to reduce the expression of eNOS in aorta of AIA rats. It is supposed that keishibukuryogan scavenged free radicals, thereby suppressing eNOS expression. Moreover, because NO reportedly regulates the expression of VCAM-1,38 we consider that keishibukuryogan might regulate the expression of VCAM-1 by reducing eNOS activity.

Keishibukuryogan consists of 5 crude drugs. Paeonol, a main component of Moutan Cortex, has an anti-thrombotic effect.39 Galloylglucose of Paeoniae Radix, and polyphenol of Cinnamomi Cortex, reportedly have endothelium-dependent relaxative effects,40,41 as well as anti-oxidant effects.42 Thus, these effects were assumed to positively influence vascular function, radical generation, and so on.

Because of the frequent use of keishibukuryogan in daily clinical practice, long-term clinical studies are needed to definitively reveal its potentially far-reaching effects against atherosclerosis in patients with RA.

Acknowledgements This study was supported by Grants-in-Aid for the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technol-
REFERENCES
