Beneficial effects of *Acer okamotoanum* sap on L-NAME-induced hypertension-like symptoms in a rat model

HYUN YANG1, INHO HWANG1, TAE-HYOUNG KOO1, HYO-JIN AHN1, SUN KIM1, MI-JIN PARK2, WON-SIL CHOI2, HA-YOUNG KANG2, IN-GYU CHOI3, KYUNG-CHUL CHOI1 and EUI-BAE JEUNG1

1Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Chungbuk 361-763; 2Department of Forest Products, Division of Wood Chemistry and Microbiology, Korea Forest Research Institute, Seoul 130-712; 3Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea

Received August 11, 2011; Accepted October 24, 2011

DOI: 10.3892/mmr.2011.650

Abstract. The sap of *Acer okamotoanum* has been termed ‘bone-benefit-water’ in Korea owing to its mineral and sugar content. In particular, the calcium (Ca) and potassium (K) concentrations of the sap of *Acer okamotoanum* are 40- and 20-times higher, respectively, than commercial spring water. In the present study, we examined whether *Acer okamotoanum* sap improves or prevents hypertension-like symptoms in a rat model. Male Sprague-Dawley rats (8-weeks-old) were provided commercial spring water supplemented with 25, 50 and 100% *Acer okamotoanum* sap, 3% potassium ions (K+) or captopril, and treated daily for 2 weeks with NG-nitro-L-arginine methyl ester (L-NAME; 100 mg/kg/day) by subcutaneous injection, in order to induce hypertensive symptoms. Rats were euthanized 6 h following the final injection. To assess the effect of the sap on hypertension-like symptoms, we examined the mean blood pressure (BP), protein levels and localization of endothelial nitric oxide synthase (eNOS) in the descending aorta of the rats. BP levels were significantly lower in hyperensive rats received 25, 50 and 100% sap compared with rats who were administered only commercial spring water. Protein levels of eNOS were repressed in L-NAME-only-treated rats, but were elevated in the descending aorta of rats administered captopril, K+ water and *Acer okamotoanum* sap (25, 50 and 100%) up to the level of the sham group provided commercial spring water, and then injected with dimethyl sulfoxide for the same period of time. Localized eNOS protein was abundantly expressed in the perivascular descending aorta adipose tissue of the rats. Taken together, these results demonstrated that the sap of *Acer okamotoanum* ameliorated high BP induced by L-NAME treatment in a rat model.

Introduction

Hypertension in humans is a prevalent and major predisposing factor to a host of illnesses, including cardiovascular and renal disease. Hypertensive diseases have been associated with dietary and lifestyle choices throughout the duration of a lifetime (1); however, genetics has also been shown to play a role (2) as hypertension appears to be a complex, polygenic trait (3). Most cardiovascular complications are related to alterations in vascular structure and function (4-7). The most important cells of the blood vessel walls are the endothelium and vascular smooth muscle cells (5,7). As endothelial dysfunction occurs during the early stages of hypertension, it is reasonable to consider that adiposity and substances secreted by white adipose tissue may directly or indirectly influence the function of endothelial cells. Adipose tissue consists of a variety of cell types, including adipocytes, preadipocytes, stromal and vascular cells. Each of these cells presents its own secretion profile and specific regulation (8). Perivascular adipose tissue is suggested to play a role in the regulation of arterial tone, as well as in the pathogenesis of hypertension (9).

Awareness of the role of nitric oxide (NO) in cell-to-cell communication has changed the concept of traditional neurotransmission. N-methyl-D-aspartate receptors mediate the dipsogenic response and c-Fos expression induced by intracerebroventricular infusion of angiotensin II (ANG II) (10). NO, a unique biological messenger molecule, has been identified in association with endothelium-derived relaxing factor (EDRF) (11). Many biological activities of EDRF, such as smooth muscle relaxation (12), have been attributed to NO. Chronic administration of NG-nitro-L-arginine methyl ester (L-NAME) blocks NO synthesis and produces arterial hypertension. Administration of L-NAME is associated with myocardial fibrosis and hypertrophy. The rennin-angiotensin system may be involved in these L-NAME-induced effects. Angiotensin-converting enzyme (ACE) is activated with inhibition of NO synthase (NOS). ACE inhibition and ANG II-type 1 (AT1) receptor blockade prevent L-NAME-induced vascular and myocardial re-modeling (13-15).

Sap is the fluid transported in the xylem cells or phloem sieve tube elements of a plant. Sap consists primarily of water.
but also contains mineral elements, sugar, hormones and other nutrients. In Korea, the sap of Acer mono (painted maple) has been termed ‘bone-benefit-water’ owing to its abundant calcium (Ca\(^{2+}\)), potassium (K\(^{+}\)) and magnesium ion (Mg\(^{2+}\)) content. The sap of Acer okamotoanum contains a considerable amount of sugar as well as various minerals, and can be used as a drink or concentrated into a syrup by boiling to prevent extreme dehydration and tiredness (16,17). The sap of Acer mono has been shown to have a medicinal effect on rats with osteoporosis-like symptoms (18). The addition of dietary Acer okamotoanum is known to decrease the ethanol and acetaldehyde concentration of the blood, and as such may play a role in alcohol detoxification (19). However, the pharmacological effects of the sap on cardiovascular health have not yet been examined.

In the present study, we investigated the effect of the sap of Acer okamotoanum on hypertension-like symptoms in a rat model induced by the injection of L-NAME during their growth stage (from 8 to 10 weeks of age). Commercial spring water with increasing percentages of sap, K\(^{+}\) or captopril were supplied to the rats, and mean blood pressure (BP) and body weights (BWs) were analyzed after 2 weeks. We also examined the expression and localization of endothelial NOS (eNOS) protein in the descending aorta in order to determine the potential anti-hypertensive effects of Acer okamotoanum sap.

Materials and methods

Experimental animals. Male Sprague Dawley (SD) rats (8-weeks-old) were obtained from Koatech (Pyeongtaek, Gyeonggi, Korea). All animals were housed in polycarbonate cages and acclimatized in an environmentally controlled room (temperature 23±2°C; relative humidity 50±10%; frequent ventilation and 12:12-h light-dark cycle) prior to use. Rats (n=42) were divided into seven groups (n=6). Hypertension-like symptoms were induced in six groups by daily treatment with L-NAME (Sigma, St. Louis, MO, USA) for 2 weeks.

To assess the preventative effect of Acer okamotoanum sap on hypertension-like symptoms, commercial spring water (50 ml daily) as a negative control (NC); captopril solution 100 mg/kg/day as a positive control (PC); 3% K\(^{+}\) water (T1); or (w/v) solutions of 25% (T2), 50% (T3) or 100% (T4) sap, were restrictively supplied to the rats from 8 to 10 weeks of age. A sham group was provided commercial spring water and injected with dimethyl sulfoxide (DMSO) for the same period of time. BWs were measured pre- and post-experiment. The Ethics Committee of Chungbuk National University approved all experimental procedures.

BP measurements. On days 0 and 14, each animal was housed in an individual metabolic cage and BP was measured using the non-invasive tail-cuff method (LE5002; Panlab, S.L., Barcelona, Spain). Each animal was pre-trained for 3 consecutive days prior to the day 0 BP measurement in order to minimize any stress reactions.

Western blot analysis. To obtain protein samples from the rat descending aortas, PRO-PREP\(^{\text{TM}}\) (Intron Bio, Korea) was used according to the manufacturer's instructions. Samples containing 30 μg of cytosolic protein were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (PerkinElmer Co., Wellesley, MA, USA) using a TransBlot Cell (TE-22; Hoefer Co., CA, USA). The resulting blot was blocked in Tris-buffered saline with Tween-20 (TBS-T) containing 5% skim milk for 1 h and then incubated with primary antibody, mouse anti-eNOS (diluted 1:500; BD, Franklin Lakes, NJ, USA) or mouse anti-rabbit β-actin (diluted 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 4 and 2 h, respectively. After washing four times for 1 h with TBS-T, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody against mouse (diluted 1:5,000; Santa Cruz Biotechnology) for eNOS and against rabbit (diluted 1:5,000; Santa Cruz Biotechnology) for β-actin for 1 h at room temperature. After washing four times for 1 h with TBS-T, each blot was developed by incubation in enhanced chemiluminescent (ECL) reagent (Amersham Biosciences, Little Chalfont, UK) and exposed to BioMax\(^{\text{TM}}\) Light film (Kodak) for 1-5 min. To ensure the specificity of the bands, each blot was stripped and re-stained without primary antibody as a negative control. Finally, to compare expression among samples, band density measurement was performed using NIH ImageJ software.

Immunohistochemistry. Localized expression of eNOS protein was detected by immunohistochemistry. To prepare samples, descending aortas were sliced with a rotary microtome and embedded in paraffin. Sections (4-μm) were deparaffinized with xylene and hydrated by successive incubation in 100, 95, 90, 80 and 70% ethanol, and then in commercial spring water. Hydrogen peroxide 3% in TBS-T was applied for 30 min to block endogenous peroxidase activity after sections were incubated in citrate buffer (pH 6.0), heated in a microwave for 10 min and allowed to cool at room temperature. Non-specific reactions were blocked by incubating sections in 10% normal goat serum for 2 h at room temperature. Sections were incubated with the eNOS primary antibody used for Western blotting (diluted 1:300; BD) at room temperature for 4 h. After washing with TBS-T, sections were incubated with biotinylated secondary antibody, mouse immunoglobulin G (IgG) for eNOS (Vector Laboratories, Burlingame, CA, USA) at 37°C for 40 min, and then incubated with ABC-Elite for 30 min at 37°C. Diaminobenzidine (DAB; Sigma) was used as a chromogen, and the sections were counterstained with Harris hematoxylin, followed by mounting in Canada balsam.

Data analysis. Data are presented as the means ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Statistical analysis was performed using Prism Graph Pad (ver. 4.0; GraphPad Software Inc., San Diego, CA, USA). P<0.05 was considered to denote statistical significance.

Results

BP, BW and water intake. Dietary supplementation of Acer okamotoanum sap affected the BP of rats with L-NAME-induced hypertension (Table 1). L-NAME treatment resulted in a marked increase in BP from 77±9 to 152±8 mmHg, following 2 weeks of treatment. The addition of captopril, K\(^{+}\) water and Acer okamotoanum sap (25, 50
and 100%) with L-NAME treatment attenuated the rise in BP, but daily water intake levels remained similar among the seven groups during the experiment. Rats from three groups had similar BWs (sham, 310±9; NC, 302±9; PC, 300±15). In treatment groups (T1, T2, T3 and T4), rat BWs were reduced compared to the sham, NC and PC groups.

Effect of Acer okamotoanum sap on eNOS expression in the descending aorta of rats. To investigate the expression of descending aortic eNOS, rats were separated into seven groups (sham, NC, PC and T1 to T4). As noted in Fig. 1, the protein level of eNOS was reduced by L-NAME in the NC group compared to the sham group, and effectively induced by captopril supplementation in the PC group. In parallel with the PC group, descending aortic eNOS protein expression was increased by supplementation with Acer okamotoanum sap or K⁺ water. However, the induced-eNOS expression did not show a dose-dependent pattern in groups T2-T4.

Localization of eNOS in the descending aorta of rats. To investigate the spatial expression of eNOS in the descending aorta, we analyzed eNOS expression in the tissue sections by immunohistochemistry. Using a primary antibody against eNOS, we demonstrated abundant expression in the cytoplasm of perivascular adipocytes in the descending aorta, although expression was weaker in vascular endothelial cells than in the cytoplasm of perivascular adipocytes (Fig. 2). In the NC group, eNOS protein l was weakly expressed in the cytoplasm of perivascular adipocytes in the descending aorta (Fig. 2).

Discussion

The sap of Acer okamotoanum contains a considerable amount of sugar and minerals (Ca, K and Mg) (16,17). This

Table I. Mean blood pressure (BP), body weight (BW) and water intake in the rats with L-NAME-induced hypertension according to the various treatments.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Baseline (Day 0)</th>
<th>2 weeks (Day 14)</th>
<th>Daily water intake (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean BP (mmHg)</td>
<td>BW (g)</td>
<td>Mean BP (mmHg)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Spring water + DMSO (sham)</td>
<td>82±10</td>
<td>223±3</td>
<td>82±10</td>
</tr>
<tr>
<td>Spring water + L-NAME (NC)</td>
<td>77±9</td>
<td>213±2</td>
<td>152±8(^a)</td>
</tr>
<tr>
<td>Captopril + L-NAME (PC)</td>
<td>81±9</td>
<td>214±5</td>
<td>78±14</td>
</tr>
<tr>
<td>K⁺ water + L-NAME (T1)</td>
<td>85±9</td>
<td>216±2</td>
<td>80±26</td>
</tr>
<tr>
<td>25% Sap + L-NAME (T2)</td>
<td>89±7</td>
<td>212±3</td>
<td>101±8</td>
</tr>
<tr>
<td>50% Sap + L-NAME (T3)</td>
<td>82±10</td>
<td>215±4</td>
<td>89±13</td>
</tr>
<tr>
<td>100% Sap + L-NAME (T4)</td>
<td>84±9</td>
<td>215±2</td>
<td>76±11(^b)</td>
</tr>
</tbody>
</table>

\(^a\)P<0.05 vs. baseline; \(^b\)P<0.05 vs. 25% group; \(^c\)P<0.05 vs. sham group. DMSO, dimethyl sulfoxide; L-NAME, NG-nitro-L-arginine methyl ester; NC, negative control; PC, positive control.
Acer okamotoanum sap is believed to be beneficial to treat extreme dehydration and tiredness or fatigue owing to its high mineral content. In our previous study, we examined the mineral content of this sap and compared it to commercial spring water, demonstrating that K, Ca, and Mg are present at high levels in Acer okamotoanum sap (18). We also demonstrated that the mineral content of the sap prevents or improves alcohol hangover symptoms (19). Electrolytes and minerals are involved in most cellular metabolism and activities (20). They act on fluids and the acid-base balance in various compartments of the body. In addition, minerals play an important role in nerve conduction, muscle contraction, and transport of substance across membranes (21).

In the present study, after the rats were fed specialized water for 2 weeks, commencing at 8 weeks of age, we observed that dietary Acer okamotoanum sap supplementation reduced BP and BW in hypertensive rats, and that this finding was also observed in the PC and T1 groups. Captopril prevented hypertension in rats treated with L-NAME, and this protective effect was associated with the recovery of NOS activity in rat aortic tissue (22). Another study demonstrated that the preventive addition of K+ to a high-salt diet considerably attenuated the development of salt hypertension in immature Dahl rats (23). These results indicate that high K+ intake targets sympathetic hyperactivity and/or sympathetic vasoconstriction as the principal vasoactive mechanism underlying the development of severe salt hypertension in immature Dahl rats (24). A mechanism similar to that of the anti-hypertensive action of dietary K+ supplementation was also reported in salt-loaded stroke-prone spontaneous hypertensive rats (25), as well as in deoxycorticosterone acetate-salt hypertensive rats (26).

Dietary K supplementation reduced BP and increased NO bioactivity in normotensive sodium-sensitive Asians following salt loading (27). We also observed that the addition of dietary Acer okamotoanum sap decreased the induced-BP and recovered eNOS levels in the adipocytes of descending aortas. Acer okamotoanum contains various minerals, including Ca, K and Mg. K+ concentration was 16-fold higher in the Acer okamotoanum sap than in commercial spring water. These results indicate that the high concentration of Acer okamotoanum had anti-hypertensive activity as a dietary effect of K+ ions. In the PC, T1, T3 and T4 groups, we observed that weight loss effects were induced by specialized supplementation (i.e., captopril, K+ water and Acer okamotoanum sap). We also observed that eNOS protein was abundantly expressed in the cytoplasm of perivascular adipocytes. Notably, NO activates the expression of mammalian sirtuin (SIRT1) in adipose tissue (28). Preliminary results suggest that SIRT1 mediates mitochondrial biogenesis in adipocytes by increasing PGC-1α expression (28). We hypothesized that the level of eNOS is elevated by Acer okamotoanum sap in perivascular adipose tissue, and is involved in mitochondrial fat metabolism through SIRT1 expression in perivascular adipocytes of hypertension-induced rats. eNOS and inducible NOS (iNOS) proteins are expressed in subcutaneous omental adipose tissue in humans (29). ACE is activated under inhibition of NOS. ACE inhibition and ANG II-type AT1 receptor blockade prevent L-NAME-induced vascular and myocardial re-modeling (13-15). In another study clearly demonstrating the role of adipocytes, targeted expression of angiotensin in adipose tissue led to fat mass enlargement and the modulation of BP (30).

In conclusion, the beneficial effect of sap on hypertension-like symptoms was evaluated by BP, BW and histological examination of eNOS. BP levels were significantly lower in rats injected with L-NAME, and then supplemented with Acer okamotoanum sap and K+ water compared to the NC group. BW was significantly reduced in the K+ water and 50 and 100% sap solution groups, but the 25% sap group was not affected. These results indicate that dietary supplementation with 25% sap did not affect adipocytic fat metabolism. In addition, we

---

**Figure 2. Localization of eNOS protein in descending aortic of rats with L-NAME-induced hypertension-like symptoms.** Immunohistochemistry was performed to detect eNOS protein in descending aortic tissue. Descending aortic tissues were divided into seven groups as shown: (a) dimethyl sulfoxide (DMSO) + commercial spring water (sham), (b) L-NAME + commercial spring water (NC), (c) L-NAME + captopril solution (PC), (d) L-NAME + 25% sap solution (T2), (e) L-NAME + 50% sap solution (T3), (f) L-NAME + 100% sap solution (T4), (g) L-NAME + 3% potassium ion (K+) water (T1). Arrowheads indicate eNOS-positive cells. Magnification, x200. eNOS, endothelial nitric oxide synthase; L-NAME, NG-nitro-L-arginine methyl ester; NC, negative control; PC, positive control.
examined the expression of eNOS in the descending aorta and showed that aortic eNOS was enhanced up to the level of the sham group by sap and K+ water supplementation. Based upon these results, we propose that the sap of Acer okamotoanum may improve L-NAME-induced hypertension-like symptoms by increasing K+ levels in a rat model.

Acknowledgements

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2010-0011433).

References