Findings from epidemiologic studies indicate that ingestion of fruits and vegetables ameliorates the development of cardiovascular disease (1). Hesperidin (HES) is a well-known flavonoid abundant in citrus fruit peel that exhibits various physiologic activities, such as capillary fragility reduction (2), antioxidative behavior (3), cholesterol-lowering actions (4), and antihypertensive effects (5). Orally ingested HES and glucosyl hesperidin (GHES), a water soluble derivative of HES, are hydrolyzed into hesperetin in the gastrointestinal tract and absorbed as conjugated metabolites (6). Ohtsuki et al. (7) showed that HES and GHES had antihypertensive effects in spontaneously hypertensive rats (SHRs). Little is known, however, about the mechanism of the antihypertensive effects of HES and GHES.

The function of vascular endothelium, which plays an important role in controlling blood pressure, is impaired in hypertensive patients (8, 9), as well as in SHRs (10, 11). We previously reported that continuous ingestion of chlorogenic acids, which are present in coffee beans, apples, western pears, tomatoes, and eggplants, attenuated hypertension in SHRs via improvement of endothelial dysfunction by changing gene expression in the vasculature (12). Therefore, to investigate the mechanisms of the antihypertensive effect of HES and GHES in SHRs, we examined the effects of continuous ingestion of HES and GHES on vascular gene expression and regulatory substances that are responsible for vascular endothelial function.

Materials and Methods

Animals and experimental protocol. Male SHRs (SHR/ Izm) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animal experiments were conducted in the Experimental Animal Facility of Kao Corporation R&D Department. The Animal Care Committee of the Kao Tochigi Institute approved the present study, and all experiments were performed strictly according to the guidelines set by the committee. SHRs (14 wk old upon initiation of the experiment) were randomly assigned to three groups (n = 6/group): a control group treated with the control diet (CE-2, CLEA Japan, Inc.), an HES group treated with the CE-2 diet containing 0.1% HES (MW: 610.56, Lot. 518, Hamari Chemicals, Ltd., Osaka, Japan), and a GHES group treated with the CE-2 diet containing 0.1% GHES (MW: 772.7, Lot. 6C271, Haya-shibara Co., Ltd., Okayama, Japan). The animals were fed with each of the diets for 8 wk. Systolic blood pressure (SBP) and heart rate were measured in awake rats by the tail-cuff method using an automatic blood pressure monitoring system (BP-98A, Softron, Tokyo, Japan). At week 8 of the experiment, urine was collected for 12 h using metabolic cages (Natsume Seisakusho, Tokyo, Japan) and urine volume was measured.

Real-time quantitative polymerase chain reaction (RT-qPCR). At week 8, thoracic aortas were collected and total RNA was extracted with an RNasy mini kit (QIAGEN Inc., Valencia, CA) according to manufacturer’s instructions. Total RNA was reverse-transcribed for cDNA synthesis with Omniscript Reverse Transcriptase (QIAGEN Inc.) for mRNA quantification. Expression of specific mRNAs was determined using an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies Inc., Carlsbad, CA). Commercially available PCR primers and FAM-labeled TaqMan probes (TaqMan® Gene Expression assays, Life Technologies Inc.)
were used for assays. The genes assessed in this experiment were as follows: NADPH oxidase subunits (NOX2, Rn00576710_m1; p22phox, Rn00577357_m1; and p47phox, Rn00586945_m1); and thromboxane A2 synthase (TXA2S), Rn00562160_m1. As a control for RNA integrity and for assay normalization, the glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) gene, Rn01775763_g1, was also amplified.

Effect of hesperetin on thromboxane B2 release from SHR aorta. Male SHRs (15–20 wk) were anesthetized by intraperitoneal injection of sodium pentobarbital. The thoracic aorta was excised, freed of fat and connective tissue, and cut into rings approximately 3 mm in length. The aorta sections were mounted in organ chambers filled with Krebs-Henseleit solution (composition in mmol/L: NaCl, 118; KCl, 4.7; NaHCO3, 25; MgSO4, 1.2; CaCl2, 2.5; KH2PO4, 1.2; and glucose, 10), and gassed with 95% O2 and 5% CO2. The tissues were maintained at 37°C and equilibrated for 10 min before beginning the experiments. After equilibration, hesperetin (10−5, 10−4 m) was added to the chamber and incubated for 15 min. Since TXA2 is very unstable and rapidly converted to the stable metabolite TXB2, the amount of TXB2, which reflects TXA2 production (13, 14), was measured in the cultured buffer using a commercial enzyme immunoassay kit according to the manufacturer’s instructions (Cayman Chemical Company, Ann Arbor, MI).

Statistical analysis. All data are presented as the means±standard error (SE). Statistical significance was determined with one-way analysis of variance followed by Fisher’s protected least significant difference test, using StatView™ (SAS Institute, Cary, NC).

Results and Discussion

SBP, heart rate, body weight, relative heart and kidney weight, and urine volume after continuous ingestion of the experimental diets supplemented with either 0.1% HES or 0.1% GHES for 8 wk are shown in Table 1. The initial SBP values were similar among the groups (190.9±4.0, 192.2±4.4, and 191.8±2.5 mmHg in the control, 0.1% HES, and 0.1% GHES groups, respectively). SBP after 8 wk was significantly decreased in the HES (193.8±1.1 mmHg, p<0.01) and GHES (188.8±3.2 mmHg, p<0.01) groups compared to the control (198.7±1.4 mmHg) group (Table 1). There was no significant difference between the HES and GHES groups. As HES and GHES share metabolic pathways (6), their biologic activities are likely similar. Heart rates tended to be lower in the HES and GHES groups compared to the control group, but the difference was not statistically significant. Ohtsuki et al. reported that ingestion of either dietary HES or GHES for 25 wk led to not only a decrease in blood pressure but also a decrease in heart rate in SHRs (7). Since the mechanisms involved in heart rate lowering effects remain unclear, further research is needed to explain the difference between their findings and ours. Body weight, relative heart weight (heart weight in relation to body weight), relative kidney weight, and urine volume were not affected by HES or GHES treatment (Table 1).

The major finding of the present study was that dietary hesperidin changed the gene expression of vascular regulatory molecules. First, HES and GHES significantly suppressed the gene expression of NOX2 and p22phox, a membrane-bound heterodimer of NADPH oxidase, to a similar extent (Fig. 1). Dietary GHES decreased and HES tended to decrease the expression of p47phox, a cytoplasmic subunit of NADPH oxidase (Fig. 1). SHRs exhibit increased generation of NADPH oxidase driven superoxide anions (O2−) in resistance (mes-

Table 1. SBP, HR, BW, relative HW and KW, and UV in SHRs treated with HES and GHES for 8 wk.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.1% HES</th>
<th>0.1% GHES</th>
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<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>198.7±1.4</td>
<td>193.8±1.1*</td>
<td>188.8±3.2*</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>341.1±15.6</td>
<td>319.6±8.3</td>
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<td>BW (g)</td>
<td>313.3±7.2</td>
<td>313.9±5.6</td>
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<tr>
<td>HW/BW (g/kg)</td>
<td>3.27±0.04</td>
<td>3.36±0.03</td>
<td>3.30±0.04</td>
</tr>
<tr>
<td>KW/BW (g/kg)</td>
<td>3.66±0.08</td>
<td>3.70±0.05</td>
<td>3.72±0.08</td>
</tr>
<tr>
<td>UV (mL)</td>
<td>5.76±0.54</td>
<td>5.73±0.85</td>
<td>5.74±0.74</td>
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</table>

Data are expressed as means±SE (n=6/group). *p<0.05 vs. control.

HES, hesperidin; GHES, glucosyl hesperidin; SHRs, spontaneously hypertensive rats; SBP, systolic blood pressure; HR, heart rate; BW, body weight; HW, heart weight; KW, kidney weight; UV, urine volume.

Fig. 1. Effect of continuous ingestion of HES and GHES on mRNA expression of NAD(P)H oxidase subunits in aortas from SHRs. Data are expressed as means±SE (n=6/group). ***p<0.001, **p<0.01, *p<0.05 versus control group.

Hypotensive Mechanism of Hesperidin and Glucosyl Hesperidin
enteric) and conduit (aortic) vessels (15). O\textsuperscript{2−} reacts with nitric oxide (NO), an endothelium-derived relaxing factor, to form peroxynitrite (ONOO\textsuperscript{−}). Thus, under conditions of excess O\textsuperscript{2−} production, such as hypertension, increased oxidative stress and reduced bioavailability of NO impair endothelial function, leading to the progression of hypertension (16, 17). NADPH oxidase is the main generator of reactive oxygen species in the vasculature (18), while reactive oxygen species promote further activation of NADPH oxidase in a feed-forward mechanism (19). Continuous activation of NADPH oxidase is accompanied by the elevation of mRNA levels of the subunits (20). We previously reported that hesperetin, a common metabolite of HES and GHS, inhibited NADPH oxidase activity in vascular endothelial cells of SHRs (21). Moreover, continuous ingestion of GHS decreased urinary 8-OHdG, a systemic oxidative stress marker, in SHRs (22). These may contribute to the regulation of mRNA levels of NADPH oxidase subunits after continuous ingestion of HES and GHS.

Second, HES and GHS ingestion also suppressed the expression of TXA\textsubscript{2}S (Fig. 2). Further, it was found that hesperetin, a common metabolite of HES and GHS (6), reduced the release of TXB\textsubscript{2} from the SHR aorta (Fig. 3). TXA\textsubscript{2}, an endothelium-derived contracting factor (EDCF), and its synthase (TXA\textsubscript{2}S) are also involved in hypertension. It has been shown that inhibition of TXA\textsubscript{2}S prevents hypertension in SHRs (23) and the levels of plasma TXB\textsubscript{2} are increased in patients with essential hypertension (24). More interestingly, TXA\textsubscript{2}S expression and activity are reportedly mediated by an a priori upregulation of NOX in human smooth muscle cells (25). Together, these findings suggest that decreased NADPH oxidase gene expression by dietary HES and GHS results in the downregulation of TXA\textsubscript{2}S gene expression, followed by a decrease in its product, TXA\textsubscript{2}. These events may partly contribute to the antihypertensive effects of dietary HES and GHS.

In conclusion, this study provides evidence that the continuous ingestion of hesperidin alters the gene expression of vascular regulatory molecules, such as NADPH oxidase and TXA\textsubscript{2}S in the aorta, which leads to prevention of hypertension in SHRs. These findings support the notion that fruit flavonoids have beneficial effects on the vasculature. The degree to which these results generalize to the beneficial effects of fruits and vegetables on the cardiovascular system is unclear but further study is warranted.

REFERENCES

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