

*Original Article*

# Isohumulones Derived from Hops Ameliorate Renal Injury *via* an Anti-Oxidative Effect in Dahl Salt-Sensitive Rats

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Previous studies have reported that isohumulones, the bitter compounds in beer, improve insulin resistance and hyperlipidemia in several animal models. In this study, we examined whether isohumulones ameliorate renal injury. Dahl salt-sensitive hypertensive rats were fed a low-salt diet (LS), a high-salt diet (HS) or a high-salt diet containing 0.3% isohumulones (HS+IH) for 4 weeks. Urinary nitrite/nitrate (NOx) excretion was measured at 4 weeks along with blood pressure and urinary protein excretion. Renal injury was evaluated histologically and reactive oxygen species (ROS) and nitric oxide (NO) production in the renal cortex was visualized. Oxidative stress and NO synthase (NOS) expression were evaluated by immunohistochemical staining and Western blot analysis. Mean blood pressure was significantly decreased in the HS+IH group compared with the HS group at 4 weeks (158.1±8.7 vs. 177.5±3.7 mmHg;  $p<0.05$ ). Isohumulones prevented the development of proteinuria in the HS+IH group compared with the HS group at 2 weeks (61.7±26.8 vs. 117.2±9.8 mg/day;  $p<0.05$ ). Glomerulosclerosis and interstitial fibrosis scores were significantly decreased in the HS+IH group compared with the HS group (0.61±0.11 vs. 1.55±0.23, 23.7±6.8 vs. 36.1±3.5%;  $p<0.05$  for both). In the HS group, increased ROS and decreased NO were observed in glomeruli *in vivo*. Isohumulones reduced the ROS production, leading to the restoration of bioavailable NO. Urinary NOx excretion was significantly increased in the HS+IH group compared with the HS group. Furthermore, renal nitrotyrosine was increased in the HS group compared with the LS group, and this effect was prevented by isohumulones. Renal NOS expression did not differ among the three groups. These results suggest that isohumulones may prevent the progression of renal injury caused by hypertension *via* an anti-oxidative effect. (*Hypertens Res* 2007; 30: 175–184)

**Key Words:** Dahl salt-sensitive rat, nitric oxide, oxidative stress, reactive oxygen species, renal injury

## Introduction

Regular drinking of red wine in France results in low mortality from coronary heart disease, an effect known as the “French Paradox” (1). The polyphenols contained in red wine

have been shown to inhibit oxidation of low-density lipoprotein (2), platelet aggregation (3) and development of atherosclerosis (4). Previous studies have also demonstrated that polyphenols have antihypertensive and anti-arteriosclerotic effects in experimental rat models (5, 6). In addition, a specific kind of polyphenol has been reported to prevent renal

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injury in a renal ischemia/reperfusion rat model (7). In addition to polyphenols, components derived from plants have recently been shown to have beneficial effects. For example, green coffee bean extract was shown to improve vasoreactivity in humans (8).

Isohumulones, the bitter compounds derived from hops, are responsible for the bitter taste in beer. Their three major types are isohumulone, isocohumulone, and isoadhumulone, all of which are structurally related. A recent study indicated that they have beneficial effects, improving glucose tolerance by reducing insulin resistance and hyperlipidemia by increasing liver fatty acid oxidation in both diabetic and high-fat-diet mice (9–12). Isohumulones were also shown to activate peroxisome proliferator-activated receptors (PPAR)  $\alpha$  and  $\gamma$  *in vitro*. In a preliminary study of type 2 diabetic patients, they significantly reduced not only hyperglycemia but also systolic blood pressure, suggesting that they have an antihypertensive effect (9).

Several studies have recently shown an association between the progression of renal injury and oxidative stress in hypertensive rat models. In lead-induced hypertensive rats, reactive oxygen species (ROS) caused inactivation of nitric oxide (NO), which was followed by hypertension and renal injury (13). In another study, increased production of ROS was associated with up-regulation of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase in spontaneously hypertensive rats (SHR) (14). In Dahl salt-sensitive hypertensive (DS) rats, a high salt intake resulted in greater renal oxidative stress and renal damage with a marked decrease in renal medullary superoxide dismutase (SOD) compared with Dahl salt-resistant (DR) rats (15). The SOD mimetic, Tempol, reduced renal superoxide anion release, ameliorated glomerular injury and exerted an antihypertensive effect in this model (16). In addition to decreased SOD activity, up-regulated NAD(P)H oxidase activity caused increased superoxide production in the renal cortex of DS rats on a high-salt diet (17).

In the present study, we examined whether isohumulones can prevent hypertension and the progression of renal injury induced by a high salt intake in DS rats. In addition, we explored whether isohumulones exhibit an anti-oxidative effect with increased NO bioavailability in renal tissue in this model.

## Methods

### Animal Preparation

The experimental protocol was approved in advance by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School, Kurashiki, Japan. Five-week-old male DS rats were purchased from Seac Yoshitomi Inc. (Fukuoka, Japan). The animals were housed in a temperature- and humidity-controlled room with a 12/12 h light/dark cycle, and they had free access to tap water. They were fed standard

rat chow containing low salt (0.3% NaCl) (Oriental Yeast, Osaka, Japan) as preconditioning for 1 week. Then, 6-week-old DS rats weighing 185 to 220 g were randomly divided into three groups ( $n=9$  each): DS rats fed rat chow containing low salt, high salt (8% NaCl), or both high salt and 0.3% isohumulones. As a source of isohumulones, we used isomerized hop extract (IHE) from English Hop Products Co., Ltd. (Kent, England), which is an aqueous solution primarily containing isohumulone, isocohumulone, and isoadhumulone at a ratio of 37:48:15. The chemical structures of these components were shown in a previous report (9). The IHE was known to contain 30% isohumulones. To prepare a high-salt diet containing 0.3% isohumulones, we added 1 ml extract to 100 g powdered high-salt chow (1% IHE) and mixed them thoroughly to spread the extract equally. This diet was replaced every 2 days to prevent oxidation of isohumulones. Four weeks after beginning the special diets, rats were killed by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The kidneys removed from five rats in each group were used for histological and biochemical analysis, and four rats in each group were used for *in situ* detection of ROS and NO, by a method that will be described in a later section. The removed left kidneys were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until protein extraction. The right kidneys were fixed in 4% paraformaldehyde and embedded in paraffin for further histological analysis.

### Physiological and Biochemical Measurements

Body weight, food intake and blood pressure were measured every 2 weeks. Blood pressure was measured in pre-warmed rats by the tail-cuff method (BP-98A; Softron Co., Tokyo, Japan). After these measurements, rats were placed in metabolic cages for 24 h to collect urine samples. Rats undergoing urinary collection were fed no food, but were given tap water. Urinary sodium, protein and *N*-acetyl- $\beta$ -D-glucosaminidase (NAG) levels were measured using iron-sensitive electrodes, pyrogallol sulfonphthalein, and the colorimetric method, respectively. After the final 24-h fasting in metabolic cages, blood samples were obtained from rats *via* an 18-gauge needle inserted into the left ventricle after the rats were killed. The blood urea nitrogen (BUN) level was measured using urease UV. Serum and urinary creatinine and serum glucose levels were also measured using enzymatic methods, and the serum insulin level was measured by enzyme immunoassay.

### Histological Assessment

Renal tissue samples embedded in paraffin were sectioned into 4- $\mu\text{m}$  slices and stained with periodic acid-Schiff (PAS) and Masson-trichrome (MT). Kidney sections were photographed and digitalized into color images using a Nikon Coolscope (Nikon Co., Tokyo, Japan). Two pathologists performed the semiquantitative analysis of PAS-stained sections in a blind fashion. The severity of glomerular injury was eval-

uated by the glomerulosclerosis score according to the following grade: 0, no sclerosis; 1, sclerotic changes in less than 25% of the total area of the glomerulus; 2, from 25% to 50%; 3, from 50% to 75%; 4, greater than 75%. Fifty glomeruli were randomly selected in each rat and the average score was calculated. The severity of tubulointerstitial injury was evaluated by the interstitial fibrosis score. Ten fields (magnification:  $\times 100$ ) were randomly selected in the renal cortex of each rat. The interstitial fibrosis score per field was calculated by the point-counting method ( $11 \times 11$  grids per field) using Adobe Photoshop 7.0 software (Adobe Systems Incorporation, San Jose, USA). This score was expressed as the percentage of interstitial area to tubulointerstitial area, excluding glomeruli and interlobular arteries and veins. The average of ten scores was calculated in each rat.

### ***In Situ* Detection of ROS and NO**

ROS and NO in the kidney were visualized using a slight modification of the perfusion technique described in a previous report (18). Briefly, under general anesthesia, rats were heparinized by injection of 100 units heparin sodium into the tail vein to prevent thrombosis during treatment. After opening the abdomen and collecting a blood sample, an 18-gauge needle connected to an infusion pump was inserted into the left ventricle. After incising the right atrium, the whole body was perfused with 37°C phosphate-buffered saline (PBS) at a flow rate of 5 ml/min. Once blood had been removed, the whole body was perfused with PBS containing 0.01 mmol/l diaminorhodamine-4M AM (DAR-4M AM; Daiichi Pure Chemicals Co., Tokyo, Japan), 0.05 mmol/l dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, USA), 0.1 mmol/l L-arginine, and 2 mmol/l CaCl<sub>2</sub> for 10 min at a flow rate of 3 ml/min. For the purpose of removing all the unreacted DAR-4M AM and DCFH-DA and fixing the tissues, perfusion with 4% paraformaldehyde containing 0.2 mmol/l N<sup>o</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma Aldrich Japan, Tokyo, Japan) was performed for 30 min at a flow rate of 5 ml/min. After fixation, tissues were cut into 1-mm-thick sections and placed on a slide glass. Fluorescent images of NO and ROS were obtained with a confocal laser-scanning microscope TCS-NT (Leica Microsystems Co., Tokyo, Japan). The wavelengths for observation were as follows: DAR-4M AM, excitation at 560 nm and emission at 575 nm; DCFH-DA, excitation at 490 nm and emission at 530 nm.

### **Immunohistochemical Study**

Immunohistochemical staining for nitrotyrosine was performed using a Histofine Simple Stain MAX-PO kit (Nichirei Co., Tokyo, Japan). Microwave heat-induced antigen retrieval in citrate buffer, pH 6.0, was required for optimal staining. After deparaffinization, 4- $\mu$ m-thick tissue sections were incubated in methanol with 3% hydrogen peroxide to

block endogenous peroxidase activity. Sections were incubated for 2 h at 37°C with rabbit anti-nitrotyrosine polyclonal antibody (5  $\mu$ g/ml) (Upstate Biotechnology, Inc., Lake Placid, USA). For negative controls, normal rabbit serum was used instead of primary antibody. The primary antibody was detected using a Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei Co.) and 3,3'-diaminobenzidine (Sigma-Aldrich Japan). Stained sections were photographed and digitalized using a Nikon Coolscope (Nikon Co.).

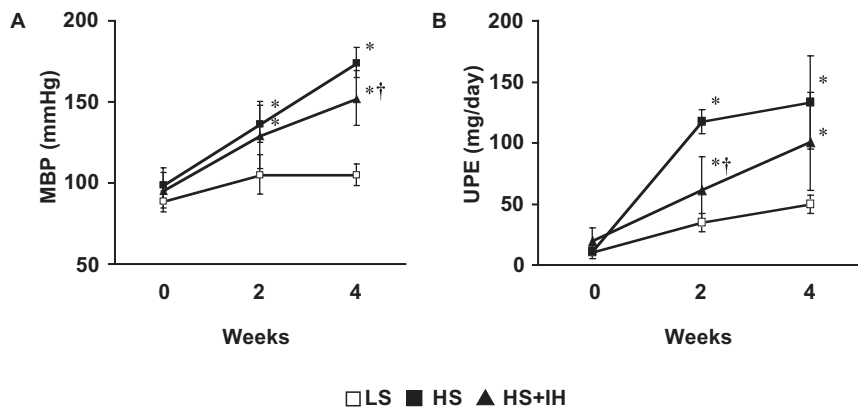
### **Western Blot Analysis**

We performed Western blot analysis for nitrotyrosine, endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). For protein extraction, renal tissue samples were homogenized in lysis buffer containing 0.25 mol/l sucrose, 50 mmol/l dithiothreitol, 3 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.9), 0.5 mmol/l ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mmol/l 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8  $\mu$ mol/l aprotinin, 21  $\mu$ mol/l leupeptin, 36  $\mu$ mol/l bestatin, 15  $\mu$ mol/l pepstatin A, 14  $\mu$ mol/l (4-guanidino) butane, and 4% sodium dodecylsulfate (SDS). After centrifugation (8,000  $\times g$ , 10 min, 4°C), the supernatants were subjected to Western blot analysis. Protein concentrations were determined using a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, USA). Extracted proteins (200  $\mu$ g/lane) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 20 mA per gel for 80 min, using 7.5% and 10% SDS-polyacrylamide gels (Atto Co., Tokyo, Japan) for NOS and nitrotyrosine, respectively. For analysis of nitrotyrosine or NOS, samples were heated at 95°C for 3 min before electrophoresis. Proteins were transferred by semidry electroblotting to polyvinylidene difluoride membranes (Millipore Co., Bedford, USA) at 24 V for 80 min. Membranes were blocked in 5% skimmed milk in PBS containing 0.5% Tween-20 (PBS-T), and incubated with rabbit anti-nitrotyrosine polyclonal antibody (2  $\mu$ g/ml; Upstate Biotechnology), rabbit anti-eNOS, anti-nNOS or anti-iNOS polyclonal antibody ( $\times 1,000$ ; Santa Cruz Biotechnology, Inc., Santa Cruz, USA), respectively, for 3 h at room temperature. Mouse anti-actin monoclonal antibody ( $\times 500$ ; Santa Cruz Biotechnology, Inc.) was used as an internal control. After washing five times with PBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated donkey anti-mouse IgG for 1 h at room temperature ( $\times 5,000$ ; Santa Cruz Biotechnology, Inc.). After washing five times with PBS-T, antibodies were visualized using an enhanced chemiluminescence method (ECL plus) (Amersham Biosciences Co., Piscataway, USA). The relative optical densities of bands were quantified using NIH Image analysis software V1.61 (National Institutes of Health, Bethesda, USA). The value in each group was represented as the fold increase relative to the LS group.

**Table 1. Physiological and Biochemical Characteristics 4 Weeks after Beginning Special Diets**

	LS	HS	HS+IH
Body weight (g)	348±10	350±11	337±12
Food intake (g/kg/day)	53.1±2.7	60.2±5.7	59.0±9.3
Urinary volume (ml/day)	20.1±6.2	22.3±3.8	20.0±8.6
Urinary sodium (mEq/day)	0.44±0.07	2.26±0.53*	2.10±1.10*
Urinary NAG (U/day)	0.22±0.04	0.70±0.35*	0.45±0.08
Blood urea nitrogen (mg/dl)	19.7±3.7	39.5±3.1*	35.7±7.4*
Ccr (mg/min/100 g body weight)	0.76±0.11	0.42±0.10*	0.57±0.16*
Serum glucose (mg/dl)	115.4±12.8	150.8±8.8*	132.0±33.4
Serum insulin (ng/ml)	0.12±0.04	0.32±0.15	0.22±0.22

LS, Dahl salt-sensitive hypertensive (DS) rat on a low-salt diet; HS, DS rat on a high-salt diet; HS+IH, DS rat on a high-salt diet containing 0.3% isohumulones; NAG, *N*-acetyl- $\beta$ -D-glucosaminidase; Ccr, creatinine clearance. Data are expressed as mean±SD. *N*=9 per group. \**p*<0.05 vs. LS rats.



**Fig. 1.** Temporal profile of mean blood pressure (MBP; A) and urinary protein excretion (UPE; B) for 4 weeks in DS rats on a low-salt diet (LS), high-salt diet (HS) or high-salt diet with isohumulones (HS+IH). *N*=9 per group. \**p*<0.05 vs. LS rats; †*p*<0.05 vs. HS rats.

### Urinary Nitrite/Nitrate and 8-Hydroxy-Deoxyguanosine Measurement

Urinary nitrite/nitrate (NO<sub>x</sub>) excretion was determined by a colorimetric method with the Griess reaction, using a BIOMOL Nitric Oxide Assay Kit (BIOMOL Research Laboratories, Plymouth Meeting, USA). As a marker of oxidative stress, urinary excretions of 8-hydroxy-deoxyguanosine (8-OHdG) were measured using a competitive enzyme-linked immunosorbent assay kit (Japan Institute for Control of Aging, Shizuoka, Japan) according to the manufacturer's instructions.

### Statistical Analysis

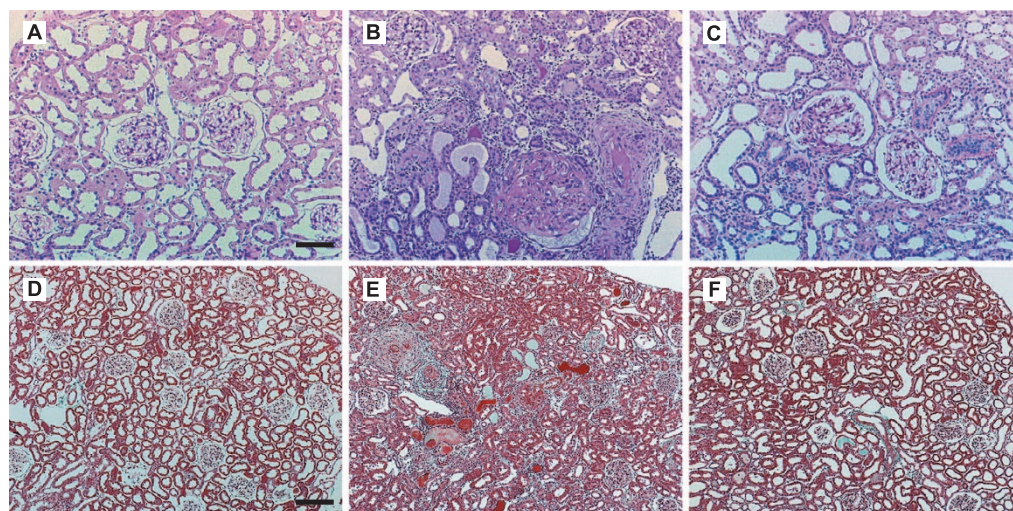
Values are expressed as the mean±SD. All parameters were evaluated by two-tailed unpaired Student's *t*-test or compared by one-way analysis of variance (ANOVA) when multiple mean comparison was required. Mann-Whitney's *U* test was

also used to compare differences between the non-treated group and treated group in Table 1. A value of *p*<0.05 was considered statistically significant.

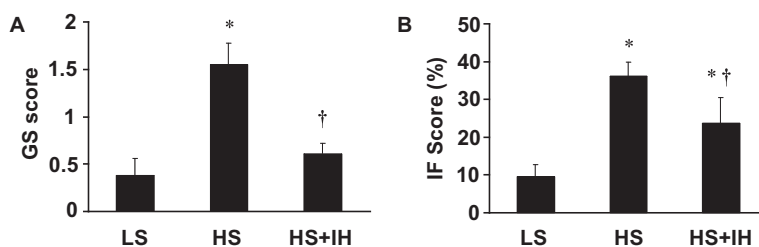
## Results

### Animal Features

Mean blood pressure (MBP) was significantly increased in DS rats on a high-salt diet (HS) compared with DS rats on a low-salt diet (LS) at 4 weeks (177.5±3.7\*, 104.5±7.4 mmHg, respectively, \**p*<0.05 vs. LS), and the administration of isohumulones significantly prevented the elevation of MBP (158.1±8.7 mmHg, *p*<0.05 vs. HS) (Fig. 1A). Urinary protein excretion was significantly increased in HS rats compared with LS rats at 4 weeks (132.9±38.2\*, 49.3±7.5 mg/day, respectively; \**p*<0.05 vs. LS), and was significantly decreased in DS rats on a high-salt diet with isohumulones (HS+IH) compared with HS rats at 2 weeks (61.7±26.8† vs.



**Fig. 2.** Histological findings of the renal cortex in tissue sections stained by PAS (A–C) and Masson-trichrome (D–F). DS rats on a low-salt diet (LS) showed no abnormality in the glomeruli or tubulointerstitial area, with little interstitial fibrosis (A and D). DS rats on a high-salt diet (HS) exhibited focal and segmental sclerosis in glomeruli and moderate tubulointerstitial damage (B). The area of interstitial fibrosis in HS rats clearly increased compared with that in LS rats (E). DS rats on a high-salt diet with isohumulones (HS+IH) revealed little abnormality or segmental sclerosis in glomeruli and only weak tubulointerstitial damage (C). The area of interstitial fibrosis in HS+IH rats was slightly increased compared with that in LS rats (F). Bar in A: 100  $\mu$ m; bar in D: 200  $\mu$ m.



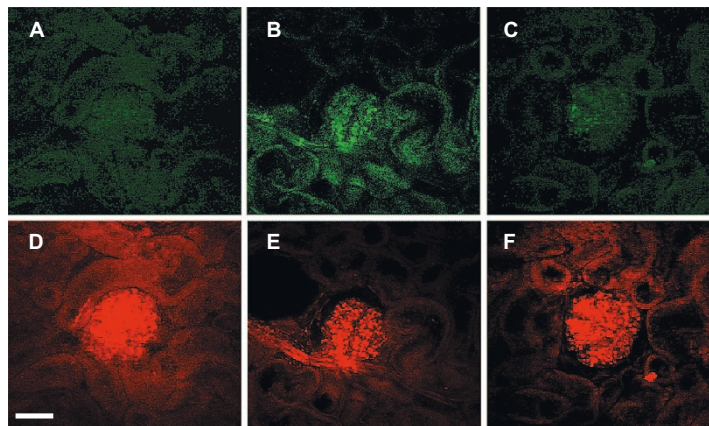
**Fig. 3.** Semiquantitative evaluation of the severity of renal tissue damage in DS rats. The glomerulosclerosis (GS) score was significantly higher in DS rats on a high-salt diet (HS) compared with DS rats on a low-salt diet (LS), and the score in DS rats on a high-salt diet with isohumulones (HS+IH) was significantly lower than that in HS rats (A). The interstitial fibrosis (IF) score was significantly higher in HS rats compared with LS rats, and the score in HS+IH rats was significantly lower than that in HS rats (B). N = 5 per group. \* $p < 0.05$  vs. LS rats; † $p < 0.05$  vs. HS rats.

117.2±9.8 mg/day, respectively; † $p < 0.05$  vs. HS) (Fig. 1B). Physiological and biochemical data 4 weeks after beginning the special diets are summarized in Table 1 ( $n = 9$  in each group). Body weight and food intake were not very different among three groups. Urinary NAG excretion was significantly increased in HS rats but not in HS+IH rats compared with LS rats. Creatinine clearance (Ccr) was significantly lower in HS rats than in LS rats. The administration of isohumulones increased Ccr in HS rats, but not significantly. The serum glucose level was significantly increased in HS rats but not in HS+IH rats compared with LS rats, although the serum insulin level was not very different among the three groups. All parameters were not significantly different between HS

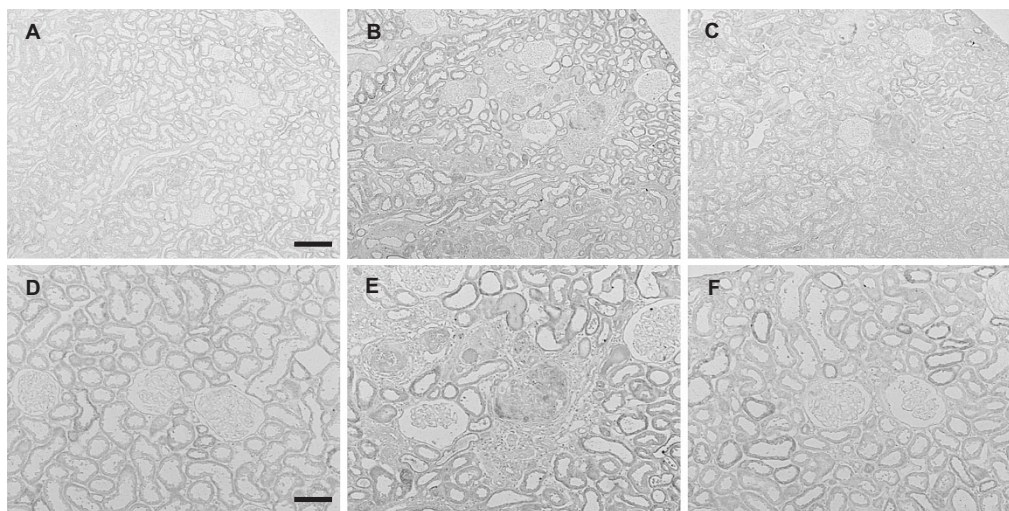
and HS+IH rats by a non-parametric test. This indicated that the intakes of salt and isohumulones were almost equivalent between the two groups.

### Histological Findings

Histological findings in the renal cortex are presented in Fig. 2 (PAS: A–C; MT: D–F). In PAS-stained sections, LS rats exhibited no abnormalities in the glomeruli or tubulointerstitial areas, but HS rats showed focal and segmental sclerosis in the glomeruli and moderate tubulointerstitial damage with tubular atrophy, interstitial fibrosis and monocyte infiltration (Fig. 2A, B). HS+IH rats exhibited little abnormality or seg-



**Fig. 4.** In situ detection of ROS and NO in the renal cortex of DS rats (ROS: A–C; NO: D–F). In the ROS assay, DS rats on a low-salt diet (LS) exhibited little intensity in glomeruli (A). DS rats on a high-salt diet (HS) showed increased intensity in the glomeruli and afferent arterioles (B). DS rats on a high-salt diet with isohumulones (HS+IH) showed an intensity equivalent to that in LS rats (C). With respect to NO, LS rats exhibited strong intensity in glomeruli (D). HS rats showed reduced intensity in glomeruli (E), while HS+IH rats revealed partially restored intensity in glomeruli (F). Bar: 50  $\mu$ m.

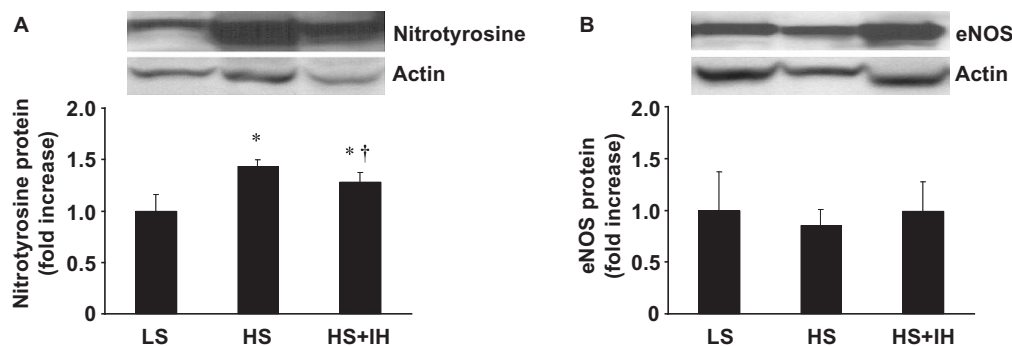


**Fig. 5.** Immunohistochemical findings for nitrotyrosine in the renal cortex of DS rats. DS rats on a low-salt diet (LS) exhibited weak positive staining in the tubular epithelium (A and D). DS rats on a high-salt diet (HS) exhibited strongly positive staining in the sclerotic segments in glomeruli and most of the tubular epithelium (B and E). DS rats on a high-salt diet with isohumulones (HS+IH) revealed positive staining localized in a part of the tubular epithelium (C and F). Bar in A: 200  $\mu$ m; bar in D: 100  $\mu$ m.

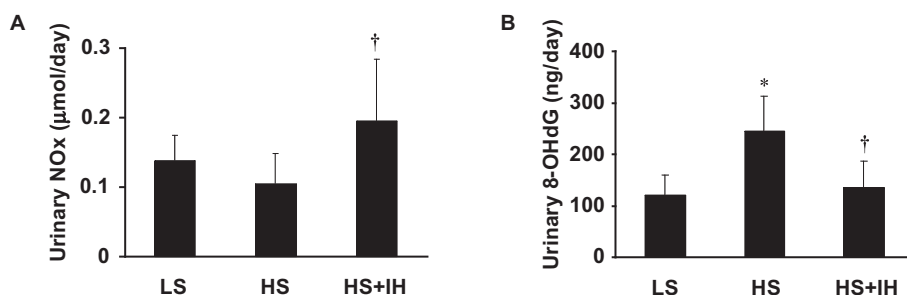
mental sclerosis in glomeruli and mild tubulointerstitial damage (Fig. 2C). This indicates that the administration of isohumulones attenuated renal tissue damage in DS rats on a high-salt diet. MT-stained sections also exhibited moderately severe interstitial fibrosis in HS rats and weak interstitial fibrosis in HS+IH rats (Fig. 2E, F).

The glomerulosclerosis and interstitial fibrosis scores are shown in Fig. 3A and B, respectively ( $n=5$  in each group). The glomerulosclerosis score was significantly higher in HS

rats compared with LS rats ( $1.55 \pm 0.23^*$ ,  $0.38 \pm 0.17$ , respectively;  $*p < 0.05$  vs. LS), and the score in HS+IH rats was significantly lower than that in HS rats ( $0.61 \pm 0.11$  in HS+IH rats;  $^\dagger p < 0.05$  vs. HS) (Fig. 3A). The interstitial fibrosis score was also significantly higher in HS rats compared with LS rats ( $36.1 \pm 3.5^*$ ,  $9.5 \pm 3.1\%$ , respectively,  $*p < 0.05$  vs. LS). Importantly, the administration of isohumulones resulted in a significantly lower score in HS+IH rats compared with HS rats ( $23.7 \pm 6.8\%$  in HS+IH rats;  $^\dagger p < 0.05$  vs. HS) (Fig. 3B).



**Fig. 6.** Western blot analysis for nitrotyrosine and endothelial nitric oxide synthase (eNOS) proteins in the renal tissues of DS rats. Renal nitrotyrosine protein (A) was significantly higher in DS rats on a high-salt diet (HS) compared with DS rats on a low-salt diet (LS). In addition, the level in DS rats on a high-salt diet with isohumulones (HS+IH) was significantly lower than that in HS rats. Renal eNOS protein (B) was not significantly different among the three groups. N = 5 per group. \*p < 0.05 vs. LS rats; †p < 0.05 vs. HS rats.



**Fig. 7.** Urinary nitrite/nitrate (NOx) and 8-hydroxy-deoxyguanosine (8-OHdG) excretion in DS rats. Urinary NOx excretion (4 weeks) in DS rats on a high-salt diet (HS) decreased compared with that in DS rats on a low-salt diet (LS), but not significantly (A). The excretion in DS rats on a high-salt diet with isohumulones (HS+IH) increased significantly compared with that in HS rats. Urinary 8-OHdG excretion (2 weeks) increased significantly in HS rats compared with LS rats (B). In addition, the excretion in HS+IH rats was significantly lower than that in HS rats. N = 9 per group. \*p < 0.05 vs. LS rats; †p < 0.05 vs. HS rats.

**In Situ Detection of ROS and NO**

We successfully visualized the concomitant production of ROS and bioavailable NO in renal tissues according to the previously reported method (18). The representative fluorescent findings of ROS and NO in the renal cortex are presented in Fig. 4 (ROS: A–C; NO: D–F). With respect to ROS, LS rats exhibited little intensity in glomeruli (Fig. 4A). In HS rats, increased intensity in the glomeruli and afferent arterioles was observed compared with LS and HS+IH rats (Fig. 4B, C). This indicates that administration of isohumulones reduced the production of ROS. In the NO assay, LS rats exhibited strong intensity in glomeruli (Fig. 4D). In HS rats, reduced intensity in glomeruli was observed compared with LS rats (Fig. 4E). HS+IH rats showed partially restored intensity in glomeruli compared with HS rats (Fig. 4F).

**Findings for Nitrotyrosine**

Immunohistochemical findings in the renal cortex using anti-nitrotyrosine antibody are shown in Fig. 5. LS rats exhibited weak positive staining in the tubular epithelium (Fig. 5A, D), whereas no staining was confirmed in the negative controls (data not shown). In HS rats, strongly positive staining was observed in sclerotic segments in glomeruli and most of the tubular epithelium (Fig. 5B, E). In HS+IH rats, however, the positive staining was localized to a part of the tubular epithelium (Fig. 5C, F).

**Western Blot Analysis for Nitrotyrosine and NOS Proteins**

Results of the Western blot analysis for renal nitrotyrosine protein (66 kD) are presented in Fig. 6A. Renal nitrotyrosine protein was significantly increased in HS rats compared with LS rats (1.43 ± 0.06\*, 1.00 ± 0.16, respectively; \*p < 0.05 vs.

LS). In addition, the level in HS+IH rats was significantly less than that in HS rats ( $1.28 \pm 0.09^\dagger$  in HS+IH rats;  $^\dagger p < 0.05$  vs. HS). This indicates that the administration of isohumulones reduced the level of nitrotyrosine.

The results of the Western blot analysis for renal eNOS protein (135 kD) are presented in Fig. 6B. Renal eNOS protein was slightly decreased in HS rats compared with LS and HS+IH rats ( $0.85 \pm 0.15$ ,  $1.00 \pm 0.37$ ,  $0.99 \pm 0.28$ , respectively). The level of renal nNOS protein was not significantly different among the three groups (data not shown). The level of renal iNOS protein was too low for evaluation. These results revealed that renal NOS protein levels were not significantly different among the three groups.

### Urinary NOx Excretion

Results of urinary NOx excretion at 4 weeks are shown in Fig. 7A. The excretion decreased in HS rats compared with LS rats, but not significantly ( $0.104 \pm 0.043$ ,  $0.138 \pm 0.036$   $\mu\text{mol/day}$ , respectively). The administration of isohumulones significantly increased urinary NOx excretion compared with that in HS rats ( $0.196 \pm 0.088^\dagger$   $\mu\text{mol/day}$  in HS+IH rats;  $^\dagger p < 0.05$  vs. HS).

### Urinary 8-OHdG Excretion

Importantly, at 2 weeks, HS+IH rats exhibited significantly reduced proteinuria compared with HS rats ( $61.7 \pm 26.8^\dagger$  vs.  $117.2 \pm 9.8$  mg/day, respectively;  $^\dagger p < 0.05$  vs. HS), although MBP was not significantly different between the two groups ( $129.1 \pm 20.7$ ,  $136.3 \pm 11.6$  mmHg, respectively). Therefore, we examined urinary 8-OHdG excretion at 2 weeks to determine whether isohumulones prevent the progression of renal injury directly through an anti-oxidative effect. The results of urinary 8-OHdG excretion at 2 weeks are shown in Fig. 7B. The excretion was significantly increased in HS rats compared with LS rats ( $246 \pm 66^*$ ,  $120 \pm 38$  ng/day, respectively;  $*p < 0.05$  vs. LS). Isohumulones significantly suppressed the increased excretion in HS rats ( $135 \pm 52^\dagger$  ng/day in HS+IH rats;  $^\dagger p < 0.05$  vs. HS). The results thus revealed a concomitant decrease in proteinuria and oxidative stress by the administration of isohumulones.

## Discussion

In the present study, isohumulones, the bitter compounds derived from hops, were shown to ameliorate renal injury and also to exert an antihypertensive effect in a DS rat model. Isohumulones also suppressed the development of proteinuria at the early stage of hypertensive renal injury. In DS rats on a high-salt diet, the accumulation of oxidative and nitrosative injury was observed in renal tissues. Treatment with isohumulones also ameliorated these changes. We successfully demonstrated that ROS production was increased in the renal tissues of HS rats using the *in situ* detection method. The

intensities of ROS production differed among glomeruli. This difference may have been associated with the difference in the severity of the lesions in each glomerulus. Treatment with isohumulones almost completely suppressed the generation of ROS. In contrast to the increased generation of ROS, the level of bioavailable NO was decreased. It is noteworthy that the decreased bioavailable NO in the HS group was simultaneously recovered to nearly the basal level by isohumulone treatment. The oxidative stress demonstrated in the renal tissues of the HS group was accompanied by the accumulation of nitrotyrosine. Increased ROS likely inactivated NO and simultaneously generated peroxynitrite, which modified the tyrosine residue and produced nitrotyrosine. These findings indicated that the ameliorative effect of isohumulones on the imbalance of ROS and NO was due at least in part to an anti-oxidative effect of this compound, because the urinary NOx excretion was increased despite the limited change in renal NOS expression.

The enzyme activity of NOS was deteriorated under the condition of oxidative stress. Oxidative stress decreases the level of tetrahydrobiopterin (BH<sub>4</sub>), which is an essential cofactor of NOS. Under the condition of BH<sub>4</sub> deficiency, NOS conversely produces O<sub>2</sub><sup>-</sup> rather than NO, a phenomenon referred to as uncoupling of NOS. We previously reported that the administration of BH<sub>4</sub> recovered the uncoupling of NOS and ameliorated the imbalance of ROS and NO in a diabetic rat model (18). Thus, isohumulones may ameliorate NOS uncoupling through anti-oxidative effects in the DS rat model.

The ameliorative effects of isohumulones on hypertensive renal injury are presumably associated with the anti-oxidative effect of this compound. Isohumulones exerted an anti-proteinuric effect at 2 weeks, and the blood pressure levels did not differ between the HS and HS+IH groups at this time point. Thus, the effects of isohumulones were independent of a blood pressure-lowering effect, at least at the early stage of hypertensive renal injury. The relevance of ROS in the development of proteinuria and renal injury was well documented. We have previously demonstrated ROS-induced structural changes in molecules involved in regulating permselectivity of the glomerular basement membrane (19). Oxidative stress has also been shown to participate in apoptotic cell death in the process of glomerulosclerosis (20–22). Several studies have shown that high-salt loading exacerbated renal injury, with increased production of ROS in the renal tissues of DS rats (15–17). A variety of anti-oxidative agents have also been shown to ameliorate renal injury in hypertensive models. For example, the thiol antioxidant *N*-acetyl-L-cysteine (NAC) reduced ROS production in the kidney and ameliorated renal injury in hypertensive rats (23, 24). Pravastatin, a 3-hydroxy-3-methyl coenzyme A (HMG-CoA) inhibitor, was also reported to prevent the progression of renal injury *via* an anti-oxidative effect in DS rats (25). Isohumulones may have reduced oxidative stress in the renal tissues of DS rats by restoring SOD activity or inhibiting NAD(P)H oxidase activ-



ity, because decreased SOD activity and increased NAD(P)H oxidase activity were found in the renal tissues of this model (15, 17).

The antihypertensive effect of isohumulones is presumably attributable to their anti-oxidative capability. The inactivation of bioavailable NO and consequent endothelial dysfunction by ROS have been implicated in the development of hypertension (26). Increased oxidative stress has been documented both in humans with essential hypertension and in animal models of hypertension. Increased levels of oxidative stress and NAD(P)H oxidase activity together with impaired endothelium-dependent vasodilatation were demonstrated in the arterioles of salt-sensitive hypertension rat models. Furthermore, the inhibition of NAD(P)H oxidase or the administration of anti-oxidants decreased ROS and blood pressure in these models.

NO is critical for the kidney to maintain salt and water homeostasis. Accordingly, the alteration of NO level affects blood pressure and has been implicated in the development of hypertension, especially the salt-sensitive type. Namely, in normal rats, as a mechanism for adapting to salt loading, endogenous NO accelerated the excretion of urinary sodium to prevent the elevation of blood pressure (27). Renal medullary infusion of an NO inhibitor caused a decrease in medullary blood flow, followed by a decrease in urinary sodium excretion (28). The administration of an NOS inhibitor, L-NAME, also resulted in a parallel shift of the pressure-natriuresis curve to a higher pressure (29). These studies showed that salt-sensitive hypertension was associated with the decreased NO in renal tissue. The blood pressure-lowering effect of isohumulones in DS rats may be mediated by their potentiating effect on NO bioavailability. In a DS rat model, the administration of L-arginine was reported to ameliorate salt sensitivity and subsequent development of hypertension induced by a high-salt diet (30). This model also exhibited impaired activation of constitutive NOS in the renal medulla in response to salt loading (31). Moreover, the reduction of renal NO by salt loading in this model caused lowered medullary blood flow, followed by a decrease in urinary sodium excretion (32).

DS rats on a high-salt diet exhibited an increased fasting serum glucose level, whereas the serum insulin level remained within a normal range. Isohumulones inhibited the elevation of serum glucose without affecting the serum insulin level in DS rats on a high-salt diet. DS rats were reported to exhibit glucose tolerance and insulin resistance (33, 34), and pioglitazone, a thiazolidinedione derivative known to activate PPAR  $\gamma$ , attenuated salt-sensitive hypertension in this model (35). In addition to pioglitazone, isohumulones have been reported to activate PPAR  $\gamma$  *in vitro* and to improve glucose tolerance and insulin resistance *in vivo* (9).

In conclusion, this study revealed that isohumulones, the bitter compounds derived from hops, ameliorated renal injury and exerted an antihypertensive effect in a DS rat model. These beneficial effects were probably associated with the

reduced oxidative stress and concomitant potentiation of NO bioavailability in the kidneys. The precise mechanism of the anti-oxidative action of isohumulones has not been clarified yet. However, this study revealed the novel potential of these plant components as anti-oxidative and antihypertensive agents. Further studies will be needed to elucidate the characteristics of these compounds.

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