Original research manuscripts

Thromboxane A_2 Receptor Antagonist (ONO-8809) Attenuates the Renal Disorders Caused by Salt-Overload in Stroke-Prone Spontaneously Hypertensive Rats

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Abstract: Background. Epidemiological and clinical studies demonstrated that excessive salt intake causes severe hypertension and exacerbated organ derangement such as chronic kidney disease (CKD). In this study, we focused on evaluating histological and gene-expression findings in the kidney using stroke-prone spontaneously hypertensive rats (SHRSP) with high-salt intake and thromboxane A_2/ prostaglandin H_2 receptor (TPR) blocker ONO-8809. Methods. SHRSP aged 6 weeks were divided into

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three groups eating normal chow containing 0.4% NaCl, 2.0%NaCl, or 2.0%NaCl +ONO-8809 (0.6mg/kg p.o. daily). Histological analyses with immunohistochemistry and a gene-expression assay with a DNA kidney microarray were performed after 8 weeks. Results. The following changes were observed with high-salt intake. Glomerular sclerotic changes were remarkably observed in the juxtaglomerular cortex areas. ED1, MCP-1, nitrotyrosine, and HIF-1α staining areas were increased in the glomeruli and interstitial portion. *Tbxa2r* which encodes TPR, *Prcp*, and *Car7* were significantly underexpressed in the kidney. The plasma 8-isoprostane level was significantly elevated, and was attenuated with ONO-8809 treatment. Conclusion. TXA_2 and oxidative stresses exaggerated renal dysfunction in salt-loading SHRSP, and ONO-8809 as a TPR blocker suppressed these changes. Therefore, ONO-8809 is a candidate drug to prevent CKD for hypertensive patients associated with high-salt intake.

Keywords: thromboxane A_2 and prostaglandin H_2 receptor (TPR) antagonist, ONO-8809, renal disorders, salt-overload, stroke-prone spontaneously hypertensive rat (SHRSP), hypertension, oxidative stress

1. Introduction

Epidemiological and clinical studies demonstrated that excessive salt intake caused severe hypertension and exacerbated organ derangement such as chronic kidney disease (CKD) [1-5]. Spontaneously hypertensive rats (SHR) [6] and stroke-prone SHR (SHRSP) [7] were developed as animal models for human essential hypertension, and they are widely used for research in the field of hypertension and as renal disorder models with salt sensitivity [8-11].

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Dietary salt overloading could itself increase the generation of reactive oxygen species (ROS) in the kidneys and blood vessels over the course of hypertension development [12-13], and ROS are related to inflammation processes in vascular impairment and renal disorders [14-15]. In these processes, thromboxane A_2 (TXA_2) plays a crucial role in the progression of chronic renal disability in the course of inflammation-implicated ROS in SHRSP [16] or SHR [17], and the expression of TXA_2 and/or prostaglandin endoperoxide (PGH_2) receptor (TPR) was increased by salt loading in the rat cortical kidney [18-19].

TXA_2, or its stable analog, U-46,619, reduced renal blood flow (RBF) and glomerular filtration rate (GFR); and potentiated tubuloglomerular feedback (TGF) [19-20], platelet aggregation [21], and monocyte chemoattractant protein-1 (MCP-1) production via the induction of nuclear factor (NF) κB and activating protein 1 (AP-1) binding activity [22], vascular smooth muscle contraction [23], and the production of extracellular matrix proteins [24]. The TXA_2 synthase inhibitor Dazmegrel, increased PGE_2 synthesis [20], which preserved kidney function. TXA_2 synthetase antagonist OKY-046 lead to less chronic renal failure and glomerular sclerosis in immunologically created Dahl-salt sensitive rats with high-salt giving [25]. These findings indicated that inflammation processes via ROS and TXA_2 are strongly associated with chronic renal-disorder changes caused by high-salt loading [26]. However, a TPR antagonist, SQ-29548, did not show any significant effect on PGE_2 production in the glomerulonephritis model rat [27].

On the basis of these findings, our study focused on detailed histological findings, oxidative stresses

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and gene-expression characteristics associated with kidney function using SHRSP with high-salt intake to evaluate the pharmacological effects of another TPR blocker, ONO-8809, and TXA_2 involvement in the course of renal disorders.

2. Materials and Methods

2.1.Animals and Experimental Design

Male 6-week-old SHRSP were purchased from Kindai University Animal Center (Osaka, Japan), and kept under light control (light and dark phases of 12 h each) at a temperature of 22 ± 2℃ and 60% humidity. SHRSP were fed with SP-chow (Funabashi Farm, Chiba, Japan) containing either 0.4% NaCl for the low-salt control group (LSC) or 2.0% NaCl for the high-salt control group (HSC), and SP-chow containing 2.0% NaCl with a TPR blocker, ONO-8809, which is an oral prodrug of ONO-NT-12628, for the (HS + ONO) group; each group consisted of 8 animals; the rats were fed for 8 weeks while taking tap water ad libitum. ONO-8809, n-decyl (Z)-6-[(1S,2S,3R,4R)-3-(4-bromobenzenesulfonylaminomethyl) bicyclo [2.2.1] hept-2-yl]-5-hexanoate, an orally active TP receptor antagonist, was provided by courtesy of Ono Pharmaceutical Co. Ltd. (Osaka, Japan). ONO-8809 at the content of 0.6 mg/kg dissolved in 0.9% saline was given once per day using a gastric tube to the HS + ONO group rats. The dose was determined using dose-dependency data obtained from a pilot study, though the dose of 0.3mg/kg/day was seen in some studies [16, 28]. All rats were weighed, and systolic arterial blood pressure was measured by means of the tail-cuff method using a photoelectric detector (UR-5000; Ueda, Tokyo, Japan) every week. Urine samples were collected every 24-h from the rats kept in individual metabolic

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cages (CT-10; CLEA Japan Inc., Tokyo, Japan). After 8 weeks of treatment, all SHRSPs were anesthetized with sodium pentobarbital (50mg/kg, i.p.; Dainippon Sumitomo Pharmacy, Osaka, Japan), and kidneys were perfused through descending aorta with enough volume of phosphate-buffered saline (PBS; pH 7.4), and removed from the body.

Procedures involving animals and their care were conducted according to the guidelines of the Japanese Association for Laboratory Animal Science, which comply with international rules and policies. This study was performed under the approval (KAME19-095) of the Animal Care and Use Committee of Kindai University.

2.2.Content Measurement in Urine and Blood Samples

Protein, creatinine (Wako Chemicals, Osaka, Japan), sodium and potassium (Mitsubishi Chemical Medience, Tokyo, Japan) contents in the urine and plasma creatinine were measured using detection kits. Blood samples collected from the descending aorta were heparinized, centrifuged and stored at -80 ℃ until analysis. Creatinine clearance for 24 h (24hCCr) was calculated from the data of serum and urine creatinine levels. Plasma thiobarbituric acid reactive substances (TBARS) were measured with a TBARS Assay Kit (Cayman, Michigan, USA). Plasma 8-iso prostaglandin F2α was determined by using an enzyme immunoassay kit (8-Isoprostane EIA Kit; Cayman) after purification with a Bond Elut C18 column (Varian, Inc., CA, USA).

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2.3.Histological Studies of Sclerotic Kidney Changes

The kidneys excised from the body were decapsulated, and longitudinally cut by fine surgical scissors for histological and immunohistochemical studies. Tissue was fixed with 10% paraformaldehyde solution (Wako Chemicals, Osaka, Japan), and embedded in paraffin wax, sliced 2-3 μm thick, and then mounted on the slide glasses. Each specimen was stained with periodic acid–Schiff (PAS) and Masson trichrome staining methods. Thirty superficial and juxtamedullary glomeruli in each kidney specimen sample were observed with a light microscope (ECLIPSE E800; Nikon Corp., Tokyo, Japan) at 400x magnification. Glomerular findings using the PAS staining specimens were graded from 0 to +4 by a semiquantitative score on the basis of the following criteria: 0, without any histological change; +4, sclerotic change by more than 75% of the glomerulus. The glomerular damage index is an average of grades assigned to 30 glomeruli [29].

Fibrotic findings of arteriolar lesions in the kidney were graded from 0 to +4 using PAS staining specimens. Grades were based on both of the severity of vascular wall thickness and the extent of fibrinoid necrosis in afferent arterioles, interlobular arterioles, and small arteries as follows: Grade 0, normal findings in vessel; +4, severe sclerotic changes with fibrinoid necrosis. The vascular lesion score was determined as an average of the grades assigned to 30 fields [29].

To evaluate interstitial fibrosis, 20 fields for each section were assessed on Masson trichrome-stained sections using a microscope at 200x magnification. Interstitial blue stained areas excluding glomeruli, Bowman’s capsules, and large vessels were counted, and the percentage of the total area in the

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specimen was calculated using a computer-assisted morphometric analyzing method with a digital microscope controller (VB-7000; Keyence Co., Osaka, Japan) and software for image analysis (Image J software version 1.43q; NIH; http://rsb,info.nih.gov/ij/). Sample observations were assessed by two blinded investigators for the inspection of lesions in glomerulosclerosis, and vascular and interstitial fibrosis of the kidney cortex.

2.4.Immunohistochemical Studies in Kidney Section

Paraffin-embedded kidney sections (2-3 μm in thick) were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by the treatment of 0.5% H_2O_2 in methanol for 20 min. Sections were incubated overnight at 4 ℃ with each primary antibody as follows: 1:100 anti-ED1 [30] (Serotec, Oxford, UK), 1:100 anti-monocyte chemoattractant protein-1 (MCP1) (Novus Biologicals, Littlecton, CO, USA), 1:200 anti-nitrotyrosine (Millipore, MA, USA), or 1:1000 anti-HIF1α antibodies (Novus Biologicals). Immunostaining was carried out by means of a commercial modified avidin-biotin-peroxidase complex technique (VECTASTAIN Universal Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). The color reaction was developed with 3,3-diaminobenzidine (Sigma, St. Louis, MO, USA) reagent supplemented with hydrogen peroxide, and then counterstained with hematoxylin staining followed by dehydration and mounting.

To determine the mean number of filtrating macrophages/monocytes (ED1-positive cells) in the renal cortex, 20 fields in tubulointerstitium or 20 glomeruli for each section were counted using an above-

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mentioned microscope at 400x magnification. In the evaluation of immunoperoxidase staining for MCP-1, nitrotyrosine and HIF-1α, each tubulointerstitial grid field of the renal cortex was counted, and the staining percentage to the total area was calculated.

2.5.DNA Microarray Analysis in the Kidney Cortex

Small pieces taken from the cortical kidney were homogenized at a pitch speed of 22 strokes/sec for 2 minutes (2 times) in a 2-ml plastic tube with 5-mm diameter glass beads with a Qiagen Tissue Lyser (Retsch Gmbh & Co., Haan, Germany). Total RNA was extracted with an RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA quality was checked with RNA Nano Chips (Agilent Technologies, Waldbornn, Germany) with an Agilent 2100 Bioanalyzer, and RNA was then used in the microarray experiments.

To examine gene-expression profiles in rat kidneys, we synthesized cRNA labeled with cyanine 3-CTP (PerkinElmer, Boston, MA, USA) from 1 μg of DNase I-treated total RNA with a Low RNA Input Amplification kit (Agilent Technologies), and hybridized it by incubating with a Whole Rat Genome Microarray (4 x 44k formatted) (Agilent Technologies) in a rotor oven (Sure-Print Technology, USA) for 17 hours at 65 °C, followed by washing. Hybridized slides were scanned with an Agilent GenPix Scanner 4000 (Agilent Technologies). Data were extracted, and overall raw signal intensities on each array were normalized to the median value of all rat probes with BRB-Array Tools-software version. 3.7.0. (Biometric Research Branch) [31]. A significance level ($p < 0.01$) for each probe was set using Student's t univariant

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test. Genes obtained from rat groups were compared between HSC/LSC and (HS + ONO)/HSC, and are shown as values of fold changes.

2.6. Statistical Analysis

All findings are expressed as mean ± standard error of the mean (SEM). Comparisons between means of multiple groups were analyzed by one-way analysis of variance (ANOVA) and Tukey-Kramer multiple-comparison test. In all tests, differences were considered as statistically significant at the value of $p < 0.05$.

3. Results

3.1. *Blood Pressure*

Systolic blood pressures at 6 weeks of age at the start of this experiment and at 14 weeks of age at the end of raising was not significantly different among groups, as shown in Table 1. Values were 242 ± 6, 254 ± 6 and 246 ± 8 mmHg in LSC, HSC and HS + ONO groups, respectively, at 14 weeks of age.

Table 1. Body weight, blood pressure, and renal functional data in stroke-prone spontaneously hypertensive rats (SHRSP) after 8 weeks of treatment

	Low salt control group (LSC: n=8)	High salt control group (HSC: n=8)	High salt + ONO-8809 group (HS+ONO: n=8)
Body weight (g)	293±8	250±14*	287±12
Blood pressure (mmHg)	242±6	254±6	246±8

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Urinary volume (ml/day/rat)	20.6±3.2	36.5±5.2*	26.5±2.0
Urinary protein excretion (mg/day/rat)	42.5±7.3	110.5±20.3*	60.9±16.5
Urinary sodium excretion (mEq/day/rat)	2.41±0.12	4.25±0.49*	5.72±0.64*
Urinary potassium excretion (mEq/day/rat)	2.31±0.05	1.86±0.13*	2.14±0.14
Plasma creatinine levels (mg/dl)	0.68±0.05	0.87±0.05*	0.55±0.05$^{\#\#}$
Creatinine clearance (ml/min)	1.30±0.08	0.88±0.08*	1.55±0.15$^{\#\#}$

Values are mean ± SEM.

*and $^{\#\#}$-attached to the right corners of the numbers are significant differences at $p < 0.05$ compared with low-salt control (LSC) values, and at $p < 0.01$ compared with high-salt control (HSC) values depending on comparison with one-way ANOVA test followed by Tukey-Kramer. SHRSP rats grouped in LSC, HSC and HS + ONO were given 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet and 0.6mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age.

3.2.*Findings of Renal Functional Data*

Both urinary volume and protein excretion in the HSC were significantly higher than those in LSC and HS + ONO groups at 14 weeks of age. Plasma creatinine levels in HSC were higher, and values of creatinine clearances in HSC were lower than those of LSC and HS + ONO, as shown in Table 1.

3.3.*Indices Representing Oxidative Stress in Plasma*

Plasma TBARS levels showing an index of oxidative stress were not significantly different between LSC and HSC, but those of HS + ONO were significantly lower than those of HSC. Plasma 8-isoprostane levels, which were another index of oxidative stress in HSC, were significantly higher than those of LSC (Table 2).

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Table 2. Values regarding oxidative stress, histological findings, and immunohistological findings in 3 groups of SHRSP after 8 weeks of treatment

	Low salt control group (LSC: n=8)	High salt control group (HSC: n=8)	High salt + ONO-8809 group (HS + ONO: n=8)
Findings regarding oxidative stress			
Plasma TBARS (μM)	14.3±1.7	18.2±2.0	11.3±1.1$^{\#}$
Plasma 8-isoprostane (ng/ml)	1.45±0.12	3.01±0.58*	1.72±0.47
Histological findings			
Glomerular finding scores (0-4)	0.21±0.05	1.2±0.19**	0.51±0.14$^{\#}$
Arteriolar lesion scores (0-4)	0.48±0.15	2.26±0.41**	0.73±0.2$^{\#\#}$
Fibrosis areas (%)	5.82±0.88	13.32±1.71**	9.46±1.12
Immunohistological findings			
MCP-1 (%)	18.1±1.1	27.5±1.7**	18.9±1.0$^{\#\#}$
Nitrotyrosine (%)	17.1±0.8	27.1±1.5**	18.0±1.2$^{\#\#}$
HIF-1α	15.2±2.3	30.1±2.0**	16.9±2.9$^{\#\#}$
Glomerular ED1 (cells/glomeruli)	0.23±0.11	1.82±0.67*	0.33±0.19$^{\#}$
Interstitial ED1 (cells/mm^2)	36.7±12.3	402.3±84.8**	83.9±48.9$^{\#\#}$

Values are mean ± SEM.

* or **, and $^{\#}$ or $^{\#\#}$ attached to right corner of numbers mean significant differences at $p < 0.05$ or $p < 0.01$ compared with LSC values, and at $p < 0.05$ or $p < 0.01$ compared with HSC values depending on comparison with one-way ANOVA test followed by Tukey-Kramer.

SHRSP rats grouped in LSC, HSC and HS + ONO were given 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet and 0.6mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age.

3.4. *Findings of Histological Changes in the Kidney Caused by High Salt or/and ONO-8809 Intake*

Glomerular sclerotic findings, such as lesions in which basement membrane and mesangial cells were increased and collapsed, were remarkably observed in the juxtaglomerular cortex areas of the HSC. Those changes were improved by ONO-8809 treatment. Onion skin lesions and fibrinoid necrotic changes caused in interlobular arteries and afferent arterioles were more apparently observed in the HSC than those in the LSC. Those changes were improved by ONO-8809 treatment. Collagen fiber

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contents in the cortex were identified by measuring the blue area stained by Masson trichrome staining in the interstitial portion. Interstitial fibrotic changes were significantly higher in the HSC than those observed in LSC (Figure 1 and Table 2).

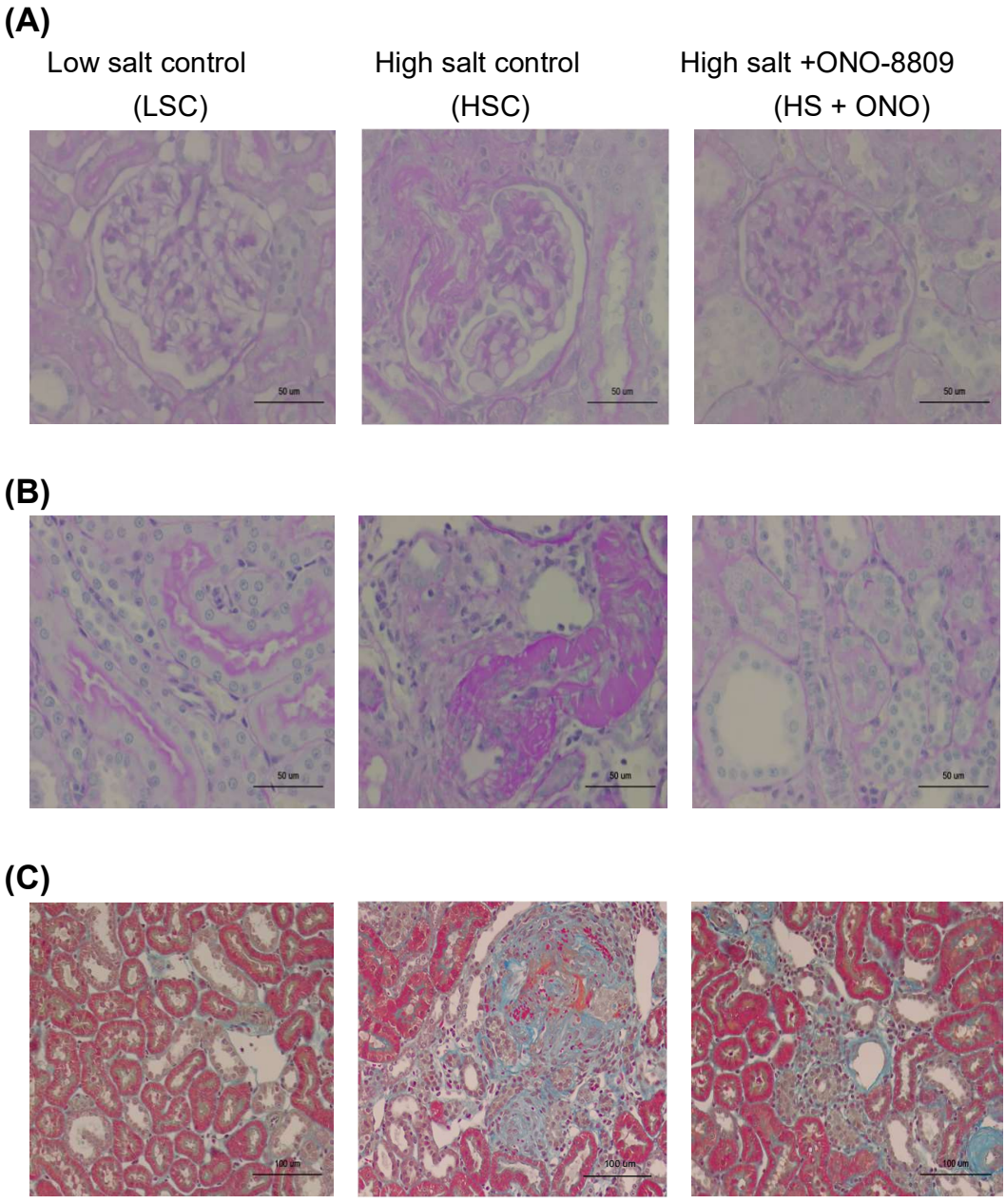

Figure 1. Comparisons of histological kidney tissue findings between three groups of SHRSP

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(A) Glomerular and (B) arteriolar changes observed in periodic acid-Schiff (PAS)-stained sections at 400x magnification, (C) fibrosis changes observed in Masson trichrome-stained sections at magnification at 200x magnification

SHRSP rats grouped in LSC, HSC and HS + ONO given 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet and 0.6mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age. Black bars inserted in each microscopic photo show scale lengths.

3.5.Immunohistochemical Findings in the Kidney Caused by High Salt or/and ONO-8809 Intake

The existence of MCP-1, an inflammatory chemokines, was more evident in the collecting tubules, thick ascending limbs of Henle's loop, distal tubules, and macula densa of the HSC than those of LSC, and ONO-8809 administration decreased in HS + ONO. The existence of nitrotyrosine, an oxidative stress marker showing peroxynitrite, was almost as common as in MCP-1 (Figure 2 and Table 2).

HIF-1α, hypoxia inducible factor, was observed more in large collecting tubules, thick ascending limbs of Henle's loop, distal tubules, and the macula densa in the HSC kidney compared with those areas of LSC, and was significantly reduced in HS + ONO (Figure 2 (C) and Table 2).

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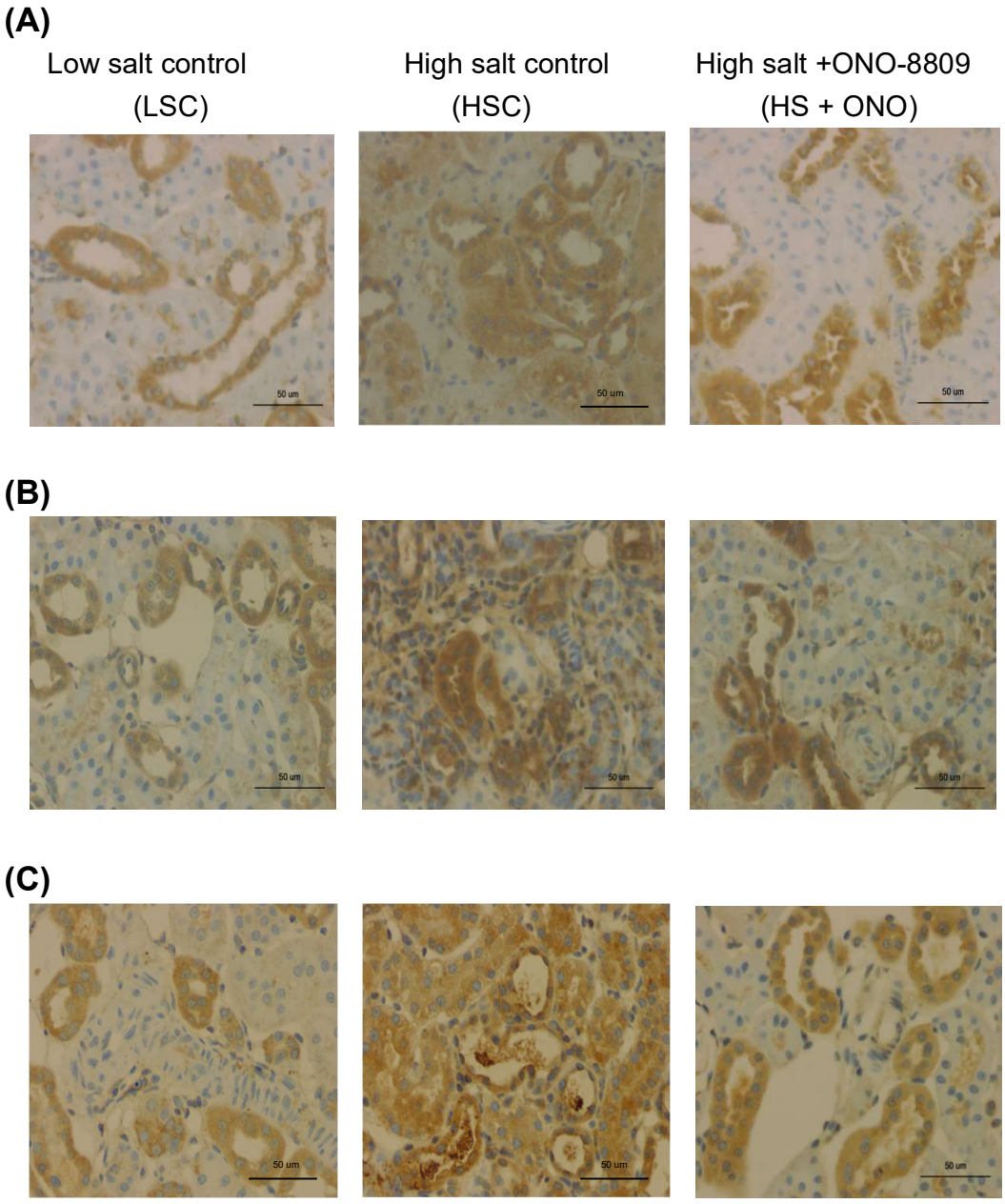

Figure 2. Microscopic findings of kidney cortex specimens stained by immunohistochemical staining using (A) MCP-1, (B) nitrotyrosine, and (C) HIF1α antibodies, at magnification at 400x magnification between three groups of SHRSP

Rats grouped as LSC, HSC and HS + ONO, and given a 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet and 0.6mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age.

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Black bars inserted in each microscopic photo are scale bars.

ED1-positive staining representing macrophage infiltration was more present in the glomerular and tubulointerstitial areas with glomerular sclerosis, arteriolar onion skin, and fibrinoid necrosis in the HSC group than in the LSC group. Administration of ONO-8809 to the salt-loading SHRSP lowered these changes, as shown in Figure 3 and Table 2.

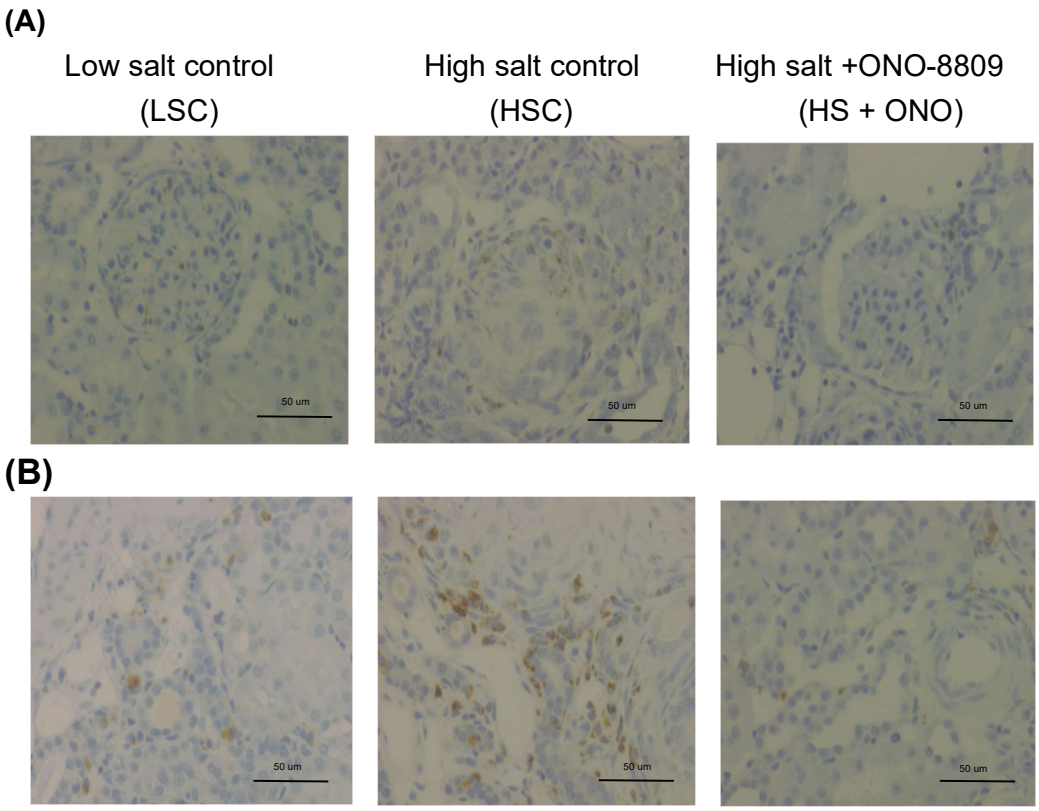

Figure 3. Findings of macrophage accumulation observed by immunohistochemical staining using ED-1 antibody in renal cortex of salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP)

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(A) Glomerular and (B) tubulointerstitial areas.

SHRSP rats grouped in LSC, HSC and HS + ONO given 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet and 0.6mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age. Black bars inserted in each microscopic photo show lengths of scale.

3.6 Findings of DNA Microarray Experiments

Three strikingly upregulated and three strikingly downregulated genes in each comparison of gene appearances in HSC/LSC and HS + ONO/HSC are summarized in Table 3. Two genes strikingly upregulated in the comparison between HSC and LSC were *Kcnv* and *Speg*, and two genes downregulated in the comparison between HSC and LSC were *Prep* and *Tbxa2r*. When those were compared with HS + ONO/HSC, *Kcnv1* and *Speg* were decreased 0.25-4.0-folds, and *Agtr2* was significantly decreased 0.016-folds. On the other hand, *Prep*, *Tbxa2r* and *Car7* were significantly upregulated compared with those obtained by HSC and LSC.

Table 3. Effects of high salt loading and ONO-8809 administration on six strikingly altered gene expressions obtained from DNA microarray method after 8 weeks of treatment

Gene symbol	Protein name and function	Fold changes	
		HSC/LSC	HS+ONO/HSC
Kcnv1	potassium channel, subfamily V, member1	14.315	0.25-4.0
Speg	striated muscle enriched protein kinase	12.536	0.25-4.0
Agtr2	angiotensin II receptor, type 2	0.5-1.0	0.0155
Prcp	prolylcarboxypeptidase (angiotensinase C)	0.037	15.907
Tbxa2r	thromboxane A2 receptor	0.279	2.770

Car7	carbonic anhydrase 7	0.5-1.0	10.131

Genes obtained from rat groups were compared between HSC/LSC and (HS + ONO)/HSC groups, and are shown as values of fold changes. SHRSP rats grouped in LSC, HSC and HS + ONO were given 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet and 0.6mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age. There were three rats in each group.

4. Discussion

High salt consumption is one of the most important factors causing CKD associated with hypertension. The Global Burden of Diseases Nutrition and Chronic Diseases Expert Group concluded from modeling studies that 10% or 1.65 million deaths from cardiovascular diseases in 2010 were due to salt consumption of more than 5.1 g/day [32];, the WHO has recommended less than 5 g salt per day. Since human beings prefer to have enough salt to improve the taste of food, it may not be easy to keep to the salt limit in order to stay healthy. To solve this goal, pathophysiological analyses using salt-loaded SHRSP with hypertension as a model for chronic kidney disease under administration of the TPR antagonist, ONO-8809, including the measurement of gene expression in detail, were retested. As a result, ONO-8809 attenuated the renal inflammation and arteriolar sclerosis of SHRSP caused by high salt intake without significantly altering blood pressure. The details of our findings are as follows. 1. Blood-pressure levels of HSC tended to increase slightly compared to those of the LSC, but there was no difference in the blood-pressure levels of the three groups when including HS + ONO. 2. Urinary protein excretion and plasma creatinine levels were significantly higher in HSC than in LSC. Creatinine clearance values decreased with HSC and improved with HS + ONO. Urinary protein excretion levels of HS + ONO tended to decrease compared to those in the HSC, but there was no significant difference, as

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shown in Table 1. These data indicated that PTR antagonist, ONO-8809, maintained kidney function in good condition in salt-induced CKD of SHRSP without a hypotensive effect. Increased urine output, urinary sodium excretion, and weight loss are due to sodium and water diuresis from the process of fluid intake associated with high salt intake to alleviate hypernatremia. 3. The proliferation of mesangium, fibrosis, and necrosis in the vascular media and -intima of interlobular arteries and afferent arterioles, and glomerular sclerosis in the juxtamedullary cortex, was remarkably clear in HSC kidneys compared with those of the LSC. The administration of PTR antagonist, ONO-8809, to in salt-induced kidney disabilities in SHRSP significantly suppressed the appearance of these pathological disorders, as shown in Figures 1 and 2. 4. Plasma TBARS levels, an indicator of oxidative stress, were not different between LSC and HSC, but those of HS + ONO were significantly lower than those of HSC. Plasma 8-isoprostane levels in HSC were significantly higher than those of LSC (Table 2). The existence of MCP-1, an inflammatory chemokines, estimated by immunostaining with anti-MCP1 antibody, was more extensively observed in HSC than in LSC, and ONO-8809 administration decreased it in HS + ONO (Figure 2 and Table 2). The presence of nitrotyrosine, an oxidative stress marker indicating peroxynitrite, was more common in HSC tissue than that of LSC, and was significantly reduced in the tissue of HS + ONO (Figure 2 and Table 2). Hypoxia inducible factor 1α (HIF-1α) staining areas were more widespread in HSC kidneys than in those of the LSC, and those were significantly reduced in the group of HS + ONO, as shown in Figure 2C and Table 2. These findings indicate that oxidative stress and hypoxia occur around the interlobular and afferent arterioles in the kidneys of salt-loaded SHRSP with intimal necrosis,

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fibrinoid necrosis, and onion skin lesions;, and ONO-8809 improved them. 5. ED1-positive staining for macrophage infiltration was more often observed in glomerular and tubulointerstitial areas with glomerular sclerosis, arteriolar onion skin, and fibrinoid necrosis in the HSC group than in the LSC group. The administration of ONO-8809 to salt-loaded SHRSP reduced macrophage infiltration with less glomerulosclerosis, arteriolar onion skin, and fibrinoid necrosis (Figure 3 and Table 2). 6. Regarding the measurement of mRNA expression using the DNA microarray experiment, two genes were strikingly upregulated in comparison between HSC and LSC: *Kcnv1* (gene of potassium voltage-gated channel modifier subfamily V member 1) and *Speg* (gene of striated muscle enriched protein kinase), but *Agtr2* (gene of angiotensin II receptor type 2) was not significantly upregulated. One gene was strikingly downregulated in the comparison between HSC and LSC,: *Prep* (gene of prolylcarboxypeptidase (angiotensinase C)), but *Tbxa2r* (a gene of thromboxane A2 receptor), and *Car7* (gene of carbonic anhydrase 7) were not remarkably downregulated. When those were compared in HS + ONO and HSC, *Kcnv1* and *Speg* were decreased to 0.25-4.0-folds in expression levels, and *Agtr2* was significantly decreased. On the other hand, *Prep*, *Tbxa2r* and *Car7* were significantly upregulated compared to those obtained between HSC/LSC by administration of ONO-8809. (regarding each gene's information, refer to Supplementary Text 1 based on the GenBank database information: the NIH genetic sequence database, https://ncbi.nlm.nih.gov/)

From DNA microarray analysis, the following effects were estimated;, 1. acceleration of heart contraction, neuronal excitability, and smooth muscle contraction by *Kcnv1* upregulation;, 2. maintenance of vascular

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function by controlling smooth muscle cell differentiation control in the aorta under hypertensive conditions by *Speg* up-regulation;, 3. inhibition of angiotensin II availability in hypertensive conditions due to *Prep* downregulation;, 4. release from high TXA_2 environment in high salt-loaded SHRSP by *Tbxa2r* downregulation;, 5. maintaining physical function in high angiotensin II environment by *Agtr2* downregulation;, 6. maintaining biochemical responses in a high TXA_2 environment under TPR-antagonist loading by *Tbxa2r* upregulation;, and 7. prevention of polyuria (natriuresis and water diuresis) by *Car7* upregulation.

There are many reports on experiments so far using the high-salt loading SHRSP as follows. 1. Vasoconstriction and vascular resistance increased through decreasing ratios of PGE_2/TXB_2 and $PGF1\alpha/TXB_2$ [33] (1987). 2. Chronic enalapril treatment (an angiotensin I converting enzyme (ACE) inhibitor) prevented urinary protein excretion and severe renal lesions [8] (1989). 3. High salt intake resulted in the development of malignant nephrosclerosis (fibrinoid necrosis and thrombosis in small vessels and glomeruli) [2] (1996). 4. Nitric oxide was inactivated by the production of superoxide in blood vessels, reflecting the decrease in total plasma antioxidant capacity [13] (2003). 5. AT_1 (angiotensin II type 1) receptor blocker, valsartan, prevented the development of proteinuria, delayed the appearance of brain damage, maintained renal structure, and increased survival in the absence of stress [15] (2004). 6. TP receptor stimulation by 8-iso-PGF2α may accelerate high salt-induced stroke through cerebrovascular inflammation [16] (2007). 7. *Edg1* and *Vcam1* were discovered in functionally important pathways as pathophysiological factors in salt-sensitive SHRSP [34] (2007). 8. Salt loading significantly

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increased superoxide production and decreased NOS activity in basilar arteries. These were significantly suppressed by the administration of aspirin [31] (2008). 9. TPR antagonist, terutroban, significantly increased survival rates as a consequence of a delayed occurrence of brain lesions, prevented proteinuria, and decreased cerebral mRNA transcriptions of IL-1β, TGF-β and MCP-1 [35] (2010).

In the in vitro experiments using a cell culture, the followings were found. 1. Thromboxane analogs increased extracellular-matrix components, including fibronectin, basement membrane proteins laminin, and type IV collagen in mouse teratocarcinoma cells and human mesangial cells [24] (1991). 2. Valsartan (ARB) exerted beneficial effects, such as broad anti-inflammatory actions, in HUVEC [21] (2000). 3. TXA_2 receptor stimulation augmented MCP-1 production by inducing NF-κB and AP-1 binding activity via the protein kinase C (PKC) system in HUVEC [12] (2003). The latest reporting information is shown in Supplemental Text 2.

High salt-loading on SHRSP caused severe functional and histomorphological renal damage, and the administration of ONO-8809, a TPR-antagonist, protected their dysfunction and histomorphological changes. The conclusions obtained from this experiment are summarized in Figure 4.

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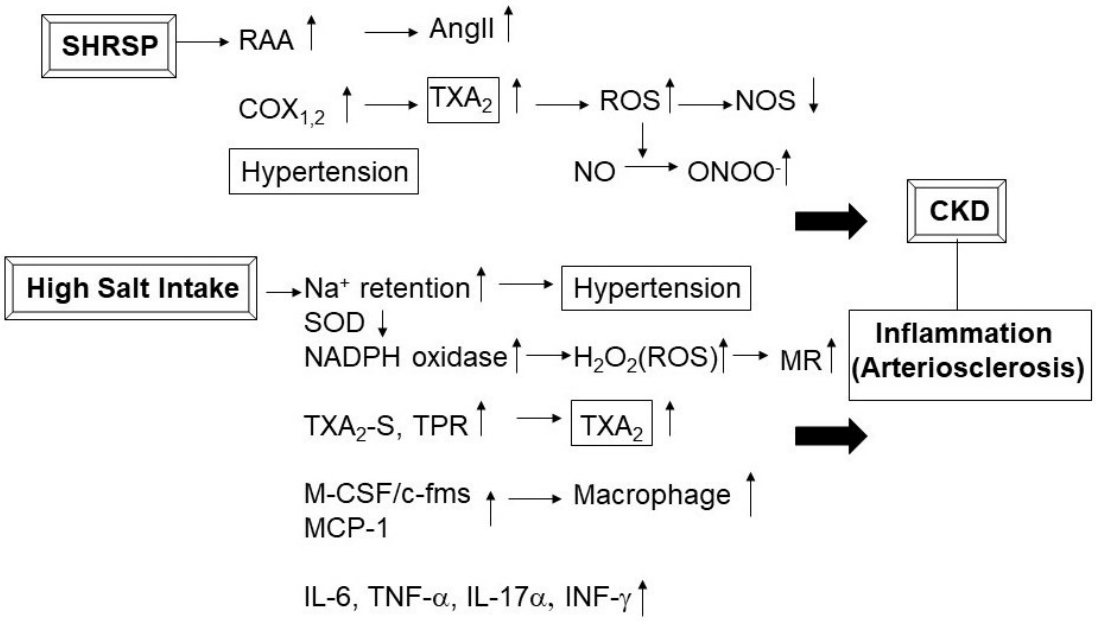

Figure 4. Summary regarding effects of high-salt intake on SHRSP kidneys

TXA_2 and ROS increase with hypertension in high-salt loading SHRSP, and several inflammatory cytokines, including MCP-1, contribute to CKD with severe inflammatory processes in due course.

TXA_2: thromboxane A_2, ROS: reactive oxygen species, SHRSP: stroke-prone spontaneously hypertensive rat, MCP-1: monocyte chemoattractant protein-1, CKD: chronic kidney disease, RAA: renin-angiotensin-aldosteron system, AngII: angiotensin II, $COX_{1,2}$: cyclooxygenase1 and 2, SOD: superoxide dismutase, NADPH: oxidase: reduced nicotinamide adenine dinucleotide phosphate oxidase, H_2O_2: hydrogen peroxide, MR: mineral corticoid receptor

That is, SHRSP increases the levels of AngII, TXA_2, ROS, and peroxynitrate in kidney tissues including vasculatures accompanied with hypertension. High-salt intake increases ROS and MR via the NADPH

oxidase, lowers SOD, increases TXA_2, accumulates macrophages via M-CSF/c-fms, and increases MCP-1 and inflammatory cytokines such as IL-6 and TNF-α. As a result, two major factors in hypertension, TXA_2 and ROS, exaggerate renal dysfunction and inflammation, and cause CKD. The up-regulation of *Kcnv1* and *Speg*, the downregulation of *Prep* and *Tbxa2r* under high salt load, and the downregulation of *Agtr2* under ONO-8809 load explain the objective and logical biochemical phenomena in this experiment. In conclusion, TXA_2 and oxidative stresses with hypertension exaggerated renal dysfunction and inflammation lead to CKD in salt-loading SHRSP. We strongly advocate that TPR blockers such as ONO-8809 are candidate drugs to prevent CKD for hypertensive patients associated with high-salt intake.

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