INTRODUCTION

Epidemiological and clinical studies have demonstrated that excessive salt intake causes severe hypertension and exacerbates organ derangement, such as in 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

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Thromboxane A\(_2\) receptor antagonist (ONO-8809) attenuates renal disorders caused by salt overload in stroke-prone spontaneously hypertensive rats

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Abstract

Epidemiological and clinical studies have demonstrated that excessive salt intake causes severe hypertension and exacerbates organ derangement, such as in chronic kidney disease (CKD). In this study, we focused on evaluating the histological and gene expression effects in the kidneys of stroke-prone spontaneously hypertensive rats (SHRSP) with a high salt intake and the thromboxane A\(_2\)/ prostaglandin H\(_2\) receptor (TPR) blocker ONO-8809. Six-week-old SHRSPs were divided into three groups and were fed normal chow containing 0.4% NaCl, 2.0%NaCl or 2.0%NaCl + ONO-8809 (0.6 mg/kg p.o. daily). Histological analyses with immunohistochemistry and a gene expression assay with a DNA kidney microarray were performed after 8 weeks. The following changes were observed in SHRSPs with the high salt intake. Glomerular sclerotic changes were remarkably observed in the juxtamedullary cortex areas. The ED1, monocyte chemoattractant protein-1 (MCP-1), nitrotyrosine and hypoxia inducible factor 1\(\alpha\) (HIF-1\(\alpha\)) staining areas were increased in the glomeruli and interstitial portion of the kidneys. The genes \(Tbxa2r\) (that encodes TPR), \(Prcp\) and \(Car7\) were significantly underexpressed in the kidneys. The plasma 8-isoprostane level was significantly elevated and was attenuated with the ONO-8809 treatment. Thromboxane A\(_2\) (TXA\(_2\)) and oxidative stress exaggerated renal dysfunction in the salt-loaded SHRSPs, and ONO-8809 as a TPR blocker suppressed these changes. Therefore, ONO-8809 is a candidate drug to prevent CKD in hypertensive patients when CKD is associated with a high salt intake.

KEYWORDS
Hypertension, ONO-8809, oxidative stress, prostaglandin H\(_2\) receptor antagonist, renal disorders, salt-overload, stroke-prone spontaneously hypertensive rat, thromboxane A\(_2\)
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, and ROS are related to inflammation processes in vascular impairment and renal disorders. In these processes, thromboxane \( A_2 \) (TXA\(_2\)) plays a crucial role in the progression of chronic renal disability over the course of inflammation-implicated ROS in SHRSP or SHR, and the expression of TXA\(_2\) and/or the prostaglandin endoperoxide (PGH\(_2\)) receptor (TPR) are shown to increase because of salt loading in the rat cortical kidney.

Thromboxane \( A_2 \), or its stable analogue U-46,619, reduces renal blood flow (RBF) and the glomerular filtration rate (GFR), and potentiates tubuloglomerular feedback (TGF), platelet aggregation and monocyte chemoattractant protein-1 (MCP-1) production via the induction of nuclear factor (NF) \( \kappa B \) and activating protein 1 (AP-1) binding activity, vascular smooth muscle contraction, and the production of extracellular matrix proteins. The TXA\(_2\) synthase inhibitor Dazmegrel increases prostaglandin E2 (PGE\(_2\)) synthesis, which preserves kidney function. The TXA\(_2\) synthetase antagonist OKY-046 leads to less chronic renal failure and glomerular sclerosis in immunologically created Dahl-salt sensitive rats given a high salt intake. These findings indicate that the inflammation processes via ROS and TXA\(_2\) are strongly associated with chronic renal-disorder changes caused by high salt loading. However, one TPR antagonist, SQ-29548, has not shown any significant effect on PGE\(_2\) production in the glomerulonephritis model rat.

Based on these findings, our study focused on the detailed histological analysis, oxidative stress and gene expression characteristics associated with kidney function using SHRSP with a high salt intake to evaluate the pharmacological effects of another TPR blocker, ONO-8809 and TXA\(_2\) involvement during renal disorders.

2 | RESULTS

2.1 | Blood pressure

The systolic blood pressures of SHRSPs at 6 weeks of age (the start of this experiment) and at 14 weeks of age (the end of the experiment) were not significantly different among groups, as shown in Table 1. The values at 6 weeks of age were 142 ± 6, 140 ± 4 and 144 ± 4 mm Hg in the low salt control (LSC), high salt (HS) and high salt + ONO-8809 (HS+ONO) groups, respectively. Body weights were significantly lower in HS group than those in LSC and HS+ONO groups, and the heart–body weight ratios were not significantly different among the groups at 14 weeks of age.

|                  | Low salt control group | High salt group | High salt + ONO-8809 group |
|------------------|-----------------------|----------------|--------------------------|
|                  | (LSC: n = 8)          | (HS: n = 8)    | (HS+ONO: n = 8)          |
| Body weight (g)  | 293 ± 8               | 250 ± 14*     | 287 ± 12                 |
| Blood pressure (mmHg) | 242 ± 6          | 254 ± 6      | 246 ± 8                  |
| Heart/body weight ratio (%) | 0.507 ± 0.079 | 0.580 ± 0.083 | 0.545 ± 0.058 |
| Water intake (mL/day/rat) | 32.2 ± 2.7   | 47.6 ± 4.9*  | 42.0 ± 8.7               |
| 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* |

Note: Values are mean ± standard error of the mean. * and # indicate significant differences at \( P < .05 \) compared with low-salt control (LSC) values, and at \( P < .01 \) compared with HS values, respectively, determined by a one-way ANOVA test followed by a Tukey-Kramer test. The SHRSP rats grouped in the LSC, HS, and HS+ONO groups were given a 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet with 0.6 mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6 weeks of age.
2.2 | Findings of the renal functional data

Both the urinary volume and protein excretion in the HS group were significantly higher than those in the LSC and HS+ONO groups. The water intakes in the HS group was higher than those in the LSC group and tended be a higher level than those in the HS+ONO groups at 14 weeks of age. The plasma creatinine levels in the HS group were higher, and creatinine clearance values were lower in the HS group than those of the LSC and HS+ONO groups, as shown in Table 1.

2.3 | Indices representing oxidative stress in the plasma

The plasma thiobarbituric acid reactive substances (TBARS) levels, as an index of oxidative stress, were not significantly different between the LSC and HS groups, but were significantly lower in the HS+ONO group than those of the HS group. The plasma 8-isoprostane levels, which are another index of oxidative stress in HS, were significantly higher than those of the LSC group (Table 2).

2.4 | Findings of histological changes in the kidney caused by high salt and/or ONO-8809 intake

The glomerular sclerotic results, such as lesions in which the basement membrane and mesangial cells were increased and collapsed, were frequently observed in the juxtamedullary cortex areas of HS kidneys. These changes were improved by ONO-8809 treatment. Onion skin lesions and fibrinoid necrotic changes in the interlobular arteries and afferent arterioles were observed more often in the HS group than the LSC group. The collagen fibre contents in the cortex were identified by measuring the blue area stained by Masson trichrome staining in the interstitial portion. The interstitial fibrotic changes were significantly higher in the HS group than those observed in the LSC group (Figure 1 and Table 2).

2.5 | Immunohistochemical changes in the kidney caused by high salt and/or 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 HS group than in the LSC group, and ONO-8809 administration decreased it in the HS+ONO group. The existence of nitrotyrosine, an oxidative stress marker showing peroxynitrite, was almost as common as MCP-1 (Figure 2 and Table 2). Hypoxia inducible factor-α (HIF-1α) was observed more in the large collecting tubules, thick ascending limbs of Henle’s loop, distal tubules and the macula densa in the HS kidneys compared with those of the LSC group and was significantly reduced in HS+ONO kidneys (Figure 2C and Table 2). The ED1-positive staining (representing macrophage infiltration) was more evident in the glomerular and tubulointerstitial areas with glomerular sclerosis, arteriolar onion skin and fibrinoid necrosis in the HS group than in the LSC group. Administration of ONO-8809 to the salt-loaded SHRSPs decreased these changes, as shown in Figure 3 and Table 2.

2.6 | Findings of the DNA microarray experiments

Three substantially upregulated and three substantially strikingly downregulated genes in the comparisons of HS with LSC and HS+ONO with HS are summarized in Table 3. In the comparisons between HS and LSC, the two upregulated genes were Kcnv and Speg, and the two genes downregulated genes were Prcp and Tbxa2r. When these were compared with the HS+ONO/HSC comparison, Kcnv1 and Speg were decreased 0.25- to 4.0-fold, and Agtr2 (gene of angiotensin II receptor type 2) was significantly decreased by 0.016-fold. On the other hand, Prcp, Tbxa2r and Car7 (gene of carbonic anhydrase 7) were significantly upregulated compared with HS and LSC.

3 | DISCUSSION

High salt consumption is one of the most important factors in CKD associated with hypertension. The Global Burden of Diseases Nutrition and Chronic Diseases Expert Group concluded from modelling studies that 10% or 1.65 million deaths from cardiovascular diseases in 2010 were because of salt consumption of >5.1 g/day, the World Health Organization has recommended <5 g salt per day. Salt is a popular additive to improve the taste of food, so it is not easy for people to keep to the recommended salt limit for health reasons. To solve this problem, pathophysiological analyses using salt-loaded SHRSPs with hypertension as a model for CKD under the administration of the TPR antagonist, ONO-8809, as a pharmacological tool, including a detailed measurement of gene expression, were undertaken. The TPR antagonist ONO-8809 attenuated the renal inflammation and arteriolar sclerosis of in the SHRSPs caused by high salt intake, without significantly altering the blood pressure. It may have been useful to set up an additional experiment to measure the circadian rhythm, given that it did not significantly change the blood pressure. According to previous studies, metoprolol, a β-blocker, was highly effective when administrated before sleep, valsartan, an AT1-blocker, was highly effective when administrated at night, and Janssen et al. reported that the circadian rhythms of blood pressure and heart rate were under sympathetic control in SHR and were not influenced by non-sympatholytic vasodilators. Therefore, it seems the absence of a significant difference in the blood pressures of rats fed a high-salt diet with or without ONO-8809 administration in the daytime was not caused by major mistakes in our study.

Urinary protein excretion and plasma creatinine levels were significantly higher in the HS group than in the LSC group. Creatinine clearance values decreased with a HS intake and improved under HS+ONO. The urinary protein excretion levels of HS+ONO SHRSPs...
tended to decrease compared with those in the HS group, but difference was not significant (Table 1). These data indicated that the TPR antagonist ONO-8809 maintained kidney function in the salt-induced CKD of SHRSPs without a hypotensive effect. Thromboxane \( A_2 \) causes marked renal vasoconstriction,\(^{32} \) reduces renal blood flow and glomerular filtration rate and stimulates mesangial cell contraction to cause proteinuria.\(^{33} \) Therefore, ONO-8809 as a TPR blocker would inhibit these physiological effects and increase the sodium excretion by increasing the urinary volume. Increased urine output, urinary sodium excretion and weight loss can occur because of sodium and water diuresis from the increased fluid intake associated with a high salt intake to alleviate hypernatremia.

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, were remarkably obvious in the HS rat kidneys compared with the LSC group. The administration of the TPR antagonist ONO-8809 in response to salt-induced kidney disabilities in SHRSP significantly suppressed the appearance of these pathological disorders, as shown in Figures 1 and 2.

| Findings regarding oxidative stress | Low salt control group (LSC: n = 8) | High salt group (HS: n = 8) | High salt + ONO-8809 group (HS+ONO: n = 8) |
|-----------------------------------|-----------------------------------|---------------------------|-------------------------------------------|
| 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                              |

| Histology                          | Glomerular finding scores (0–4) | Arteriolar lesion scores (0–4) | Fibrosis areas (%) |
|-----------------------------------|-----------------------------------|---------------------------------|-------------------|
| Score 0                           | 0.21 ± 0.05                       | 0.48 ± 0.15                    | 5.82 ± 0.88       |
| Score 1                           | 0.21 ± 0.05                       | 1.2 ± 0.19**                   | 13.32 ± 1.71**    |
| Score 2                           | 0.21 ± 0.05                       | 3.01 ± 0.58*                   | 9.46 ± 1.12       |
| Score 3                           | 0.21 ± 0.05                       | 3.01 ± 0.58*                   | 9.46 ± 1.12       |
| Score 4                           | 0.21 ± 0.05                       | 3.01 ± 0.58*                   | 9.46 ± 1.12       |

| Immunohistology                    | MCP-1 (%)                         | Nitrotyrosine (%)              | HIF-1α (%)        |
|-----------------------------------|-----------------------------------|--------------------------------|------------------|
| Glomerular ED1 (cells/glomeruli)  | 0.23 ± 0.11                       | 0.23 ± 0.11                    | 0.23 ± 0.11       |
| Interstitial ED1 (cells/mm\(^2\))| 36.7 ± 12.3                       | 402.3 ± 84.8**                 | 83.9 ± 48.9##     |

Note: Values are mean ± standard error of the mean. * or **, and # or ## indicate significant differences at \( P < .05 \) or \( P < .01 \) compared with the LSC values, and at \( P < .05 \) or \( P < .01 \) compared with the HS values, respectively, determined with a Jonckheere-Terpstra test with Bonferroni correction (glomerular finding scores and arteriolar lesion scores), and a one-way ANOVA test followed by a Tukey-Kramer for the other parameters. The SHRSP rats grouped in the LSC, HS and HS+ONO groups were given a 0.4% NaCl mixed diet, 2% NaCl mixed diet, and 2% NaCl mixed diet with 0.6 mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6 weeks of age.

Abbreviations: SHRSPs, stroke-prone spontaneously hypertensive rats HIF-1α, hypoxia inducible factor-1α; MCP-1, monocyte chemoattractant protein-1; TBARS, thiobarbituric acid reactive substances.
The plasma TBARS levels, which indicate oxidative stress, were not different between the LSC and HS groups, but those of the HS+ONO group were significantly lower than those of the HS group. Plasma 8-isoprostane levels in HS rats were significantly higher than those of the LSC group (Table 2). The presence of MCP-1, an inflammatory chemokines, estimated by immunostaining with anti-MCP1 antibody, was more extensively observed in HS rats than in LSC rats, and ONO-8809 administration decreased it in the HS+ONO group (Figure 2 and Table 2). Nitrotyrosine was more common in HS tissue than in LSC, and was significantly reduced in the tissue of HS+ONO rats (Figure 2 and Table 2). Hypoxia inducible factor-1α staining areas were more widespread in HS kidneys than in those of LSC rats and 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, fibrinoid necrosis and onion skin lesions and that ONO-8809 improved these conditions.

The ED1-positive staining for macrophage infiltration was observed more often in the glomerular and tubulointerstitial areas with glomerular sclerosis, arteriolar onion skin, and fibrinoid necrosis in the HS group than in the LSC group. Administering ONO-8809 to salt-loaded SHRSPs reduced macrophage infiltration, with less glomerulosclerosis, arteriolar onion skin and fibrinoid necrosis observed (Figure 3 and Table 2).

Regarding the measurement of messenger RNA (mRNA) expression using the DNA microarray experiment, two genes were strikingly upregulated in the comparison between the HS and LSC groups: Kcnv1 and Speg; Agtr2 was not significantly upregulated. One gene was substantially downregulated in the comparison between the HS and LSC groups: Prcp but Tba2r and Car7 were not remarkably downregulated. When these genes were compared between the HS+ONO and HS groups, Kcnv1 and Speg expressions were decreased by 0.25- to 4.0-fold in expression, and Agtr2 was significantly decreased. On the other hand, Prcp, Tba2r and Car7 were significantly upregulated compared to HSC/LSC by the administration of ONO-8809.

Therefore, the following effects were estimated: (a) an acceleration of heart contraction, neuronal excitability, and smooth muscle contraction by Kcnv1 upregulation; (b) maintenance of vascular function by controlling the smooth muscle cell differentiation control in the aorta under hypertensive conditions by Speg upregulation; (c) inhibition of angiotensin II availability in hypertensive conditions because of Prcp downregulation; (d) a higher TXA2 environment in high salt-loaded SHRSPs causing Tba2r downregulation; (e) maintenance of the appropriate physical function in a high angiotensin II...
FIGURE 2  Microscopy of kidney cortex specimens stained by immunohistochemical staining from stroke-prone spontaneously hypertensive rats (SHRSPs) treated with low salt (LSC), high salt (HS) + ONO-8809 (ONO) using A, monocyte chemoattractant protein-1 (MCP-1), B, nitrotyrosine, and C, hypoxia inducible factor-1α (HIF-1α) antibodies at 400× magnification. The SHRSPs were grouped as LSC, HS and HS+ONO, and were given a 0.4% NaCl mixed diet, 2% NaCl mixed diet and 2% NaCl mixed diet with 0.6 mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6-weeks of age. Arrows show the increased presence of MCP-1 (HS in A), much existence of nitrotyrosine (HS in B), and much existence of HIF-1α (HS in C). Black bars show the scale lengths of 50 µm.

FIGURE 3  Macrophage accumulations observed by immunohistochemical staining using the ED1 antibody in the renal cortex of salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP). A, Glomerular and B, tubulointerstitial areas. The SHRSPs grouped in the low salt control (LSC), high salt (HS) and HS+ONO groups were given a 0.4% NaCl mixed diet, 2% NaCl mixed diet and 2% NaCl mixed diet with 0.6 mg/kg ONO-8809 p.o. per day, respectively, for 8 weeks from 6 weeks of age. Arrows show the areas that were dark-stained with the ED1 antibody in the HS group of A and B. Black bars show scale lengths of 50 µm.
environment because of Agtr2 downregulation; (f) maintenance of normal biochemical responses in a higher TXA2 environment under TPR-antagonist loading from Tbx2r upregulation; and (g) prevention of polyuria (natriuresis and water diuresis) by Car7 upregulation. Studies using in situ hybridization method with TXA2 receptor mRNA and receptor autoradiography with the TXA2 agonist, [125I]-BOP, revealed TXA2 receptor expression in the renal cortex, especially in the glomeruli, arteries, arterioles and in the renal medulla. Knowledge regarding the distribution of TXA2 receptor in the kidney could contribute to its role both in normal kidney physiology and as a mediator of kidney dysfunction and injury. That is, TxA2 causes marked renal vasoconstriction, reduces renal blood flow and glomerular filtration rate and stimulates mesangial cell contraction to cause proteinuria. Therefore, these effects were attenuated by the treatment of ONO-8809 as a TPR blocker.

Using high salt-loaded SHRSPs with or without a TPR antagonist, the following findings were clarified. High-salt intake during hypertensive states facilitates the occurrence of CKD, and TXA2 and ROS are the main causative factors in the pathophysiological course of the disease. Therefore, drugs such as TPR blockers will be useful for HS intake-associated CKD. The conclusions obtained from this experiment are summarized in Figure 4.

Stroke-prone spontaneously hypertensive rats showed increased levels of angiotensin II, TXA2, ROS and peroxynitrite in
kidney tissues including vasculatures accompanied with hypertension. A HS intake increases ROS and mineral corticoid receptors via nicotinamide adenine dinucleotide phosphate oxidase, lowers superoxide dismutase, increases TXA2, accumulates macrophages via macrophage-colony stimulating factor/c-fms and increases MCP-1 and inflammatory cytokines such as interleukin-6 and tumour necrosis factor-α. As a result, two major factors in hypertension, TXA2 and ROS, exaggerate renal dysfunction and inflammation, and cause CKD. The upregulation of Kcnv1 and Speg, the downregulation of Prcp and Tbx2r under a HS load, and the downregulation of Agtr2 under ONO-8809 load explain the objective and logical biochemical phenomena that occurred in this experiment. In conclusion, TXA2 and oxidative stress with hypertension exaggerate renal dysfunction and inflammation, leading to CKD in salt-loaded SHRSPs. We strongly advocate that TPR blockers such as ONO-8809 be considered as candidate drugs to prevent CKD for hypertensive patients, particularly when this is associated with a HS intake. The findings of this experiment were suggestive of a positive effect of ONO-8809, but further research is required to obtain definite results.

4 | METHODS

4.1 | Animals and experimental design

Six-week-old male SHRSPs were purchased from Kindai University Animal Center, and kept under light control (light and dark phases of 12 hours each) at a temperature of 22°C ± 2°C and 60% humidity. The SHRSPs were fed with SP-chow (Funabashi Farm) containing either 0.4% NaCl for LSC group or 2.0% NaCl for HS group, and SP-chow containing 2.0% NaCl with a TPR blocker, ONO-8809, which is an orally active prodrug of ONO-NT-12628, for the (HS+ONO) group. Each group consisted of eight animals. The rats were fed their responsive treatments for 8 weeks while taking tap water ad libitum. The TPR blocker 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 Ono Pharmaceutical Co. Ltd. It was administrated at a dose of 0.6 mg/kg dissolved in 0.9% saline and 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, although a dose of 0.3 mg/kg/day was used in some studies. All rats were weighed, and the systolic arterial blood pressure was measured weekly by means of the tail-cuff method using a photoelectric detector (UR-5000; Ueda). Urine samples were collected every 24 hours from the rats, which were kept in individual metabolic cages (CT-10; CLEA Japan). After 8 weeks of treatment, all SHRSPs were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Dainippon Sumitomo Pharmacy), and the kidneys were perfused through the descending aorta with phosphate-buffered saline (PBS; pH 7.4) before being, 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.

4.2 | Content measurements in urine and blood samples

The protein, creatinine (Wako Chemicals), sodium and potassium (Mitsubishi Chemical Medience) concentrations 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°C until the analysis. Creatinine clearance for 24 hours (24 hCCR) was calculated from the data of the serum and urine creatinine data. Plasma TBARS were measured with a TBARS Assay Kit (Cayman). 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).

4.3 | Histological studies of sclerotic kidney changes

The kidneys, once excised from the body, were decapsulated, and longitudinally cut by fine surgical scissors for the histological and immunohistochemical studies. The tissue was fixed with 10% formalin neutral buffer solution (Wako Chemicals), and embedded in paraffin wax, sliced 2–3 μm thick, and then mounted on glass slides. Each specimen was stained with periodic acid for the 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) at 400× magnification.

Glomerular findings using the PAS staining specimens were graded from 0 to +4 using a semiquantitative score base on 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 60 glomeruli. The fibrotic results of the arteriolar lesions in the kidney were graded from 0 to +4 using the PAS staining specimens. The grades were based on both the severity of the vascular wall thickness and the extent of fibrinoid necrosis in afferent arterioles, interlobular arteries and small arteries, as follows: grade 0, normal findings in the vessel; +4, severe sclerotic changes with fibrinoid necrosis. The vascular lesion score was determined as an average of the grades assigned to 30 fields.

To evaluate the interstitial fibrosis, 20 fields for each section were assessed on Masson trichrome-stained sections using a microscope at 200× magnification. Interstitial blue stained areas excluding glomeruli, Bowman's capsules and large vessels were
counted, and the percentage of the total area in the specimen was calculated using a computer-assisted morphometric analysing method with a digital microscope controller (VB-7000; Keyence) and software for image analysis software (Image J software version 1.43q; National Institutes of Health; http://rsb.info.nih.gov/ij/).

4.4 | Immunohistochemistry of the kidney sections

Paraffin-embedded kidney sections (2- to 3-μm thick) were dewaxed and rehydrated. The endogenous peroxidase activity was blocked by treatment with 0.5% hydrogen peroxide in methanol for 20 minutes. Sections were incubated overnight at 4°C with each primary antibody as follows: 1:100 anti-ED1α (Serotec), 1:100 anti-monocyte chemoattractant protein-1 (MCP1) (Novus Biologicals), 1:200 anti-nitrotyrosine (Millipore), or 1:1000 anti-HIF-1α 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). The colour reaction was developed with a 3,3-diaminobenzidine reagent (Sigma) reagent supplemented with hydrogen peroxide, and then counterstained with haematoxylin 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 the tubulointerstitium or 20 glomeruli for each section were counted using the above-mentioned microscope at 400× magnification. In the evaluation of immunoperoxidase staining for MCP-1, nitrotyrosine (VECTASTAIN Universal Elite ABC kit; Vector Laboratories). The percentage of the total area in the specimen was calculated using a computer-assisted morphometric analysing method with a digital microscope controller and software for image analysis software (Image J software version 1.43q).

4.5 | DNA microarray analysis of the kidney cortex

Small pieces taken from the cortical kidney of three rats per group were homogenized at a pitch speed of 22 strokes/second for 2 minutes (twice) in a 2-mL plastic tube with 5-mm diameter glass beads using a Qiagen Tissue Lyser (Retsch). The total RNA was extracted with a RNeasy Mini kit (QIAGEN) according to the manufacturer’s protocol. The RNA quality was checked with RNA Nano Chips (Agilent Technologies) using an Agilent 2100 Bioanalyzer, and the RNA was then used in the microarray experiments.

To examine the gene expression profiles in rat kidneys, we synthesized complementary RNA (cRNA) labelled with cyanine 3-CTP (PerkinElmer) from 1 μg of DNase I-treated total RNA with a Low RNA Input Amplification Kit (Agilent Technologies), and hybridized it by incubating it with a Whole Rat Genome Microarray (4 × 44 k formatted) (Agilent Technologies) in a rotor oven (Sure-Print Technology) for 17 hours at 65°C, followed by washing. The hybridized slides were scanned with an Agilent GenPix Scanner 4000 (Agilent Technologies). The data were extracted, and the overall raw signal intensities on each array were normalized to the median value of all rat probes with the BRB-Array Tools-software version 3.7.0. We used the Benjamini-Hochberg procedure to control the false discovery rate (FDR) <0.05, and a significance level (P < .01) for each probe was used to establish ANOVA and Tukey-Kramer multiple-comparison test. Genes obtained from rat groups were compared between HS/LSC and (HS+ONO)/HS and are shown as values of fold changes.

4.6 | Statistical analysis

All results except for the glomerular finding scores and arteriolar lesion scores are expressed as the mean ± standard error of the mean (SEM). To determine the significant differences among treatment groups for the glomerular scores and arteriolar lesion scores, we performed Jonckheere-Terpstra tests with Bonferroni correction. Comparisons among the means of multiple groups were analysed by a one-way ANOVA and Tukey-Kramer multiple comparison test. In all tests, the differences were considered statistically significant at the value of P < .05.

5 | DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, H.H., on reasonable request, and openly available in each repository at reference number [1–46].

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.N., T.H., K.K. and H.H.; methodology, Y.N. and H.H.; formal analysis, Y.N. and T.H.; investigation, Y.N. and K.K.; resources, K.K.; writing: original draft preparation, Y.N. and H.H.; writing: review and editing, T.H. and H.H.

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