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ORIGINAL ARTICLE

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An angiotensin II type-I receptor blocker, olmesartan medoxomil, attenuates lipid peroxidation in renal injury induced by subtotal nephrectomy

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Abstract
Background. Lipid-related oxidative stress, such as that caused by malondialdehyde (MDA), acrolein, and 4-hydroxynonenal (4-HNE), is involved in vascular injury in diabetes and hypertension. Olmesartan medoxomil, a blocker of angiotensin II type-I receptor, is an antihypertensive drug with antioxidant properties. In this study, we examined the involvement of oxidative lipids and the effect of olmesartan on lipid peroxidation in the progressive renal injury induced by renal mass reduction in rats.
Methods. Rats were treated with vehicle or olmesartan (0.5 mg/kg or 10 mg/kg) for up to 8 weeks after subtotal nephrectomy. The expression of oxidative lipids and the effect of olmesartan on lipid peroxidation were evaluated by Western blotting and immunostaining of renal tissue.
Results. Immunohistochemical examination revealed that MDA, acrolein, and 4-HNE were scarcely detected in renal cortex in sham-operated rats. On the contrary, these oxidative lipids were observed in injured glomeruli and dilated renal tubules in the ablated kidneys. Western blotting of renal cortical tissue revealed that MDA- or acrolein-bound proteins were mainly detected in the range of 30–90 kDa. Treatment with olmesartan attenuated lipid peroxidation and glomerulosclerosis. The renoprotective and antioxidative effect was higher in rats that received a high dose of olmesartan than in rats in the low-dose group.
Conclusions. These results indicate that oxidative lipids reflect the progression of renal injury induced by subtotal nephrectomy in rats. Olmesartan may have a renoprotective effect, with attenuation of lipid peroxidation.

Key words Acrolein · Angiotensin II receptor blockade · Malondialdehyde · Nephrectomy

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Introduction

The number of patients with endstage renal disease has been growing in recent decades. Elucidation of the mechanism of progressive renal disease and the establishment of effective treatment is urgently required. It has been revealed that several factors, such as glomerular hypertension and oxidative stress, have important roles in the progression of renal injury.[1] As markers of lipid-related oxidative stress, malondialdehyde (MDA), acrolein, and 4-hydroxynonenal (4-HNE) are widely used. In patients with diabetes and hypertension, the serum levels of these compounds are increased[2,3] and their existence has been histologically confirmed in human atherosclerotic lesions.[4] These reports suggest that lipid peroxidation is involved in various oxidative stress-induced tissue damage.

Olmesartan medoxomil, an angiotensin II type-I receptor blocker (ARB), is an antihypertensive drug and attenuates renal damage in diabetes and hypertension by reducing the carbonyl stress.[5] It has been speculated that its antioxidant property may have a role in protecting the kidney from oxidative stress-induced injury, in addition to its blood pressure-lowering effect.

However, it is unclear whether oxidative lipids are involved in the progression of renal injury induced by renal mass reduction and whether ARBs can modulate lipid peroxidation in this model. Thus, we examined the role of oxidative lipids and the effect of olmesartan on lipid peroxidation in subtotally ablated kidneys.

Materials and methods

Animal and study protocol

Seven-week-old male Wistar rats were obtained from Japan SLC (Shizuoka, Japan). After 7 days' adaptation, they were randomly allocated to subtotal nephrectomy (SNx) or sham operation. SNx or sham surgery was per-

formed as described previously.[6] All operations were done in rats that were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA; 20 mg/kg body weight). In brief, rats underwent ligation of the posterior and one or two anterior extrarenal branches of the main renal artery to create infarction of approximately two-thirds of the left kidney (the first surgery). Seven days later, right subcapsular nephrectomy was performed in SNx groups (the second surgery). Sham-operated groups were operated by decapsulating the kidney. Special care was taken to avoid damage to the adrenals.

After operation, the rats were kept in individual cages in an environmentally controlled facility at 25°C and 50%–60% humidity with lights on from 6:00 a.m. to 6:00 p.m. The rats had unrestricted access to water and standard rat food (Clea Japan, Tokyo, Japan). Blood pressures were recorded by a photoelectric oscillometric method (UR-5000; Ueda Electric Works, Tokyo, Japan) while the animals stayed awake.[7] SNx rats were randomly assigned to receive either the angiotensin II type-I receptor blocker, olmesartan medoxomil (Sankyo, Tokyo, Japan) at a dose of 0.5 mg/kg per day or 10 mg/kg per day, or vehicle (0.5% carboxymethylcellulose).

Olmesartan or vehicle was given orally once a day, using a gastric tube, for 9 weeks after the first surgery. Body weight and blood pressure were measured every week, and animals were killed 8 weeks after the second surgery (9 weeks after the first surgery). After anesthesia with sodium pentobarbital, blood samples were taken to measure serum levels of creatinine and the lipid profile. Then the kidneys were quickly removed, rinsed with saline, immediately frozen in liquid nitrogen, and stored at –80°C until use. Some parts of the kidneys were fixed in 10% formalin neutral buffer solution. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, and creatinine were measured by enzymatic methods.

The animals were handled according to the animal welfare regulations of Yamagata University, and the study protocol was approved by the Animal Subjects Committee of Yamagata University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health.

Pathological examination

Renal tissues fixed in 10% formalin neutral buffer solution were embedded in paraffin, and cut into thin sections using conventional techniques. The sections were stained with periodic acid Schiff (PAS) reagent.

Immunohistochemistry

Anti-MDA, anti-acrolein, and anti-4-HNE monoclonal antibodies were purchased from Japan Institute for the Control of Aging, Nikken SEIL (Shizuoka, Japan). Peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit immunoglobulins were purchased from DAKO (Glostrup, Denmark).

Immunohistochemical staining was performed on serial sections of kidneys frozen in liquid nitrogen, using an enzyme-labeled antibody method. These sections were desiccated, and endogenous peroxide activity was quenched by incubating sections in 0.3% H_2O_2/methanol for 20 min. Sections were incubated with primary antibodies against MDA (dilution 1: 50), acrolein (dilution 1: 50), and 4-HNE (dilution 1: 50) at 4°C overnight. After incubation with secondary antibody at a concentration of 1: 100 for 2 h, immunoreaction products were developed using 3, 3′-diaminobenzidine (DAB) as the chromogen, with standardized development times. Sections were then counterstained with hematoxylin acetate. Negative controls were prepared by omitting the primary antibodies.

Quantification of morphological changes in renal tissue

All quantification was performed in a blinded manner. Glomerulosclerosis was semiquantitatively assessed in PAS-stained sections of each specimen, using 50 randomly selected glomeruli under 400× magnification.[8]

For each glomerulus, sclerotic lesions were assessed using the following criteria: 0, no sclerosis in glomerulus; 1, sclerosis in up to 25% of glomerulus; 2, sclerosis in 25%–50% of the glomerulus; and 3, sclerosis in more than 50% of the glomerulus. The intensity of immunostaining for oxidative lipids was graded as: 0, no staining; 1, mild staining (up to 25% of the glomerulus); 2, moderate staining (25%–50% of the glomerulus); and 3, strong staining (more than 50% of the glomerulus).

Western blotting

The kidney cortex was powdered in liquid nitrogen and lysed in ice-cold lysis buffer containing: NaCl 50.0 mM, NaF 100.0 mM, and Tris-HCl 25.0 mM plus sodium deoxycholate 0.5%, NP-40 2.0%, sodium dodecylsulfate (SDS) 0.2%, and sodium vanadate 200 μM at pH 7.4, along with 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), as previously reported.[9] The lysates were then placed on ice for 30 min, and centrifuged at 14 000 *g* at 4°C for 15 min. Supernatants were used immediately or stored at –80°C. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts were subjected to electrophoresis on a 10% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Hybond P; Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 1% nonfat dry milk in TNT solution, containing 25 mM Tris-HCl, 125 mM NaCl, and 0.2% Tween 20, at 4°C overnight. After blocking, membranes were incubated with primary antibodies as previously reported.[10]

To examine the expression of MDA- and acrolein-binding proteins, membranes were incubated overnight

with anti-MDA (dilution 1:2000) and anti-acrolein (dilution 1:2000) antibodies. To quantify the protein levels, the same membranes were re-probed with beta-actin (dilution 1: 2000) (Sigma, Saint Louis, MO, USA). After incubation with horseradish peroxidase-conjugated secondary antibody (dilution 1: 5000), immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences). Then densitometric analysis of each visualized band was performed, using a Lane analyzer (ATTO, Osaka, Japan). The values of MDA- and acrolein-bound proteins by densitometric measurements were normalized by those of beta-actin. The relative amounts of MDA- and acrolein-bound proteins were expressed as a fold increase over the sham amount.

Statistical analysis

Values for results are expressed as means ± SD. We used Student's *t*-test to evaluate differences in means, and the nonparametric Mann-Whitney *U*-test for the parameters that were not normally distributed. Differences were considered statistically significant at *P* values less than 0.05.

Results

Changes in body and kidney weight, blood pressure, and renal function after SNx surgery

In SNx rats, the kidney-body weight ratio, blood pressure, and serum creatinine were significantly higher than those in sham rats. Treatment with olmesartan attenuated these changes in blood pressure and serum creatinine in a dose-dependent manner (Table 1). Serum levels of creatinine, total cholesterol, and HDL cholesterol were increased by renal ablation, and olmesartan attenuated these increases dose-dependently. However, the differences in these parameters between the low- and high-dose olmesartan groups did not reach statistical significance at 8 weeks.

Morphological assessment of glomerular injury

Glomerular injury was evaluated by the glomerular sclerosis index (0–3). The glomerular sclerosis index was higher in the SNx+vehicle group as compared with the sham group (1.29 ± 0.28 vs 0.25 ± 0.06, respectively; $P < 0.05$). Treatment with olmesartan significantly attenuated the glomerular morphological changes, and the protective effect of olmesartan was significantly higher in the high-dose group than in the low-dose group (glomerular sclerosis index, 0.54 ± 0.16 vs 0.77 ± 0.16, respectively; $P < 0.05$; Fig. 1). These results demonstrate that treatment with olmesartan dose-dependently reduced the glomerular injury induced by subtotal nephrectomy.

Immunohistochemical analysis of lipid peroxidation in renal tissue

Immunostaining of the oxidative lipids showed that MDA was mainly detected in the glomerulus in the ablated kidneys (Fig. 2), and the treatment with olmesartan attenuated MDA expression in a dose-dependent manner. The expressions of acrolein and 4-HNE were also observed mainly in the glomerulus and they were also attenuated by olmesartan dose-dependently (Figs. 3, 4). The staining patterns of these oxidative lipids were similar; however, it was difficult to identify which glomerular cells were stained in the frozen section. In the tubulointerstitial area, these oxidative lipids were weakly detected only in dilated renal tubules in the SNx+vehicle group. In the sham and olmesartan-treated groups, the oxidative lipids were scarcely detected in the tubulointerstitial area.

Next we examined the relationship between the renal damage and the degree of lipid peroxidation. We found that the degree of lipid peroxidation was correlated with the serum levels of creatinine and the glomerular damage (i.e., the glomerular sclerosis index). The correlation was similar for the oxidative lipids that we examined, and we show the representative results for MDA in Fig. 5.

Table 1. Comparisons of systemic and renal parameters in sham and subtotally nephrectomized rats with/without olmesartan treatment

	Sham	SNx + vehicle	SNx + olmesartan	
	($n = 7$)	($n = 6$)	0.5 mg/kg ($n = 8$)	10 mg/kg ($n = 6$)
BW (g)	277.3 ± 4.9	260.3 ± 16.4*	275.9 ± 8.1**	262.7 ± 14.5*
KW (g)	1.01 ± 0.05	1.26 ± 0.20*	1.30 ± 0.18*	1.11 ± 0.18
KW/BW (mg/g)	3.66 ± 0.19	4.83 ± 0.64*	4.71 ± 0.60*	4.21 ± 0.50*
SBP (mmHg)	158 ± 11	231 ± 23*	179 ± 9** *	171 ± 9***
DBP (mmHg)	112 ± 13	164 ± 18*	131 ± 14***	116 ± 17**
sCr (mg/dl)	0.30 ± 0.02	0.86 ± 0.22*	0.62 ± 0.14***	0.57 ± 0.10***
Tcho (mg/dl)	61.9 ± 5.4	127.5 ± 39.3*	103.0 ± 15.9	87.3 ± 9.7*
TG (mg/dl)	52.8 ± 20.3	41.0 ± 4.6	25.0 ± 8.8***	36.3 ± 14.1
HDLc (mg/dl)	18.2 ± 2.0	36.0 ± 8.5*	29.1 ± 4.6*	22.0 ± 7.3**

*$P < 0.05$ vs Sham; ** $P < 0.05$ vs SNx+vehicle

BW, Body weight; KW, kidney weight; KW/BW, kidney-body weight ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; sCr, serum creatinine; Tcho, total cholesterol; TG, triglyceride; HDLc, high-density lipoprotein cholesterol

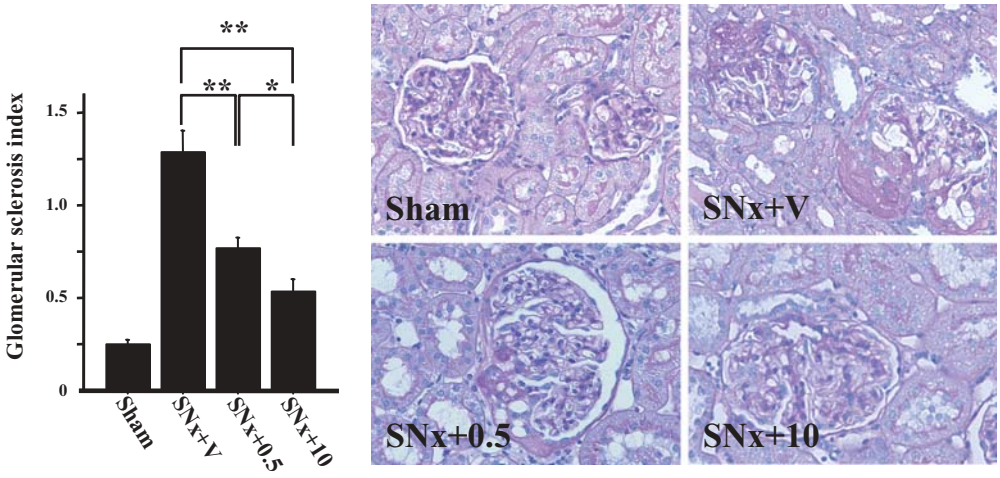

Fig. 1. Histological comparison of glomerular damage. *SNx + V*, *SNx + 0.5*, and *SNx + 10* represent subtotal nephrectomy + vehicle, SNx + 0.5 mg/kg per day olmesartan, and SNx + 10 mg/kg per day olmesartan, respectively. **P* < 0.05; ***P* < 0.01. Periodic acid Schiff, ×200

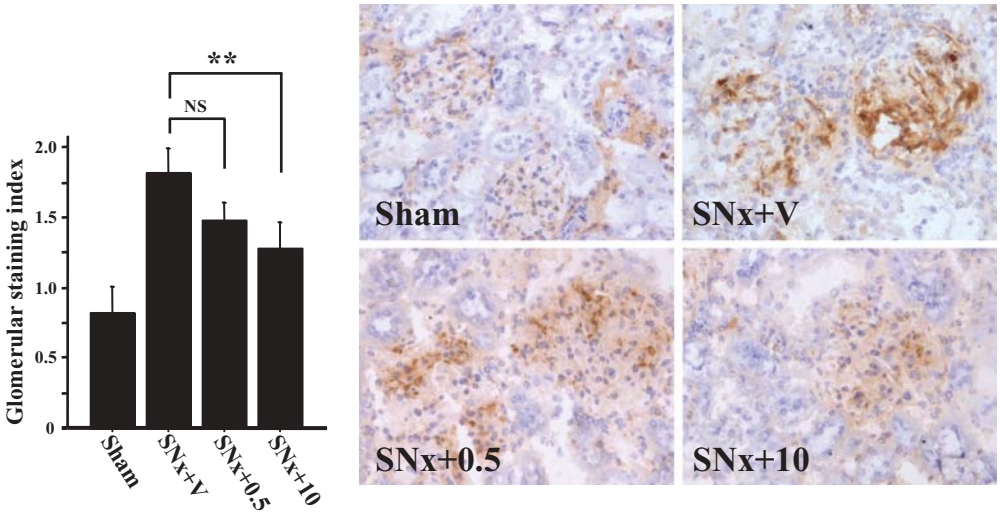

Fig. 2. Immunostaining index of malondialdehyde (*MDA*). *SNx + V*, *SNx + 0.5*, and *SNx + 10* represent subtotal nephrectomy + vehicle, SNx + 0.5 mg/kg per day olmesartan, and SNx + 10 mg/kg per day olmesartan, respectively. ***P* < 0.01; *NS*, not significant. ×200

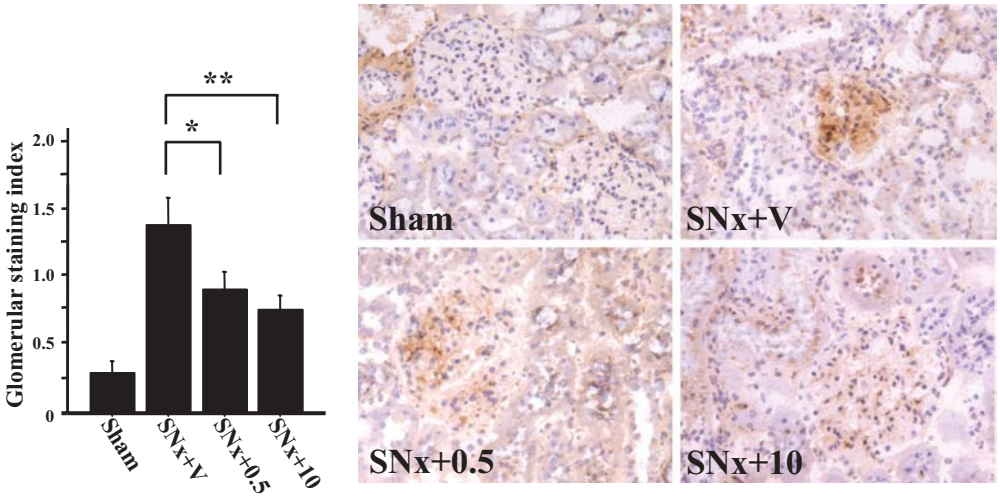

Fig. 3. Immunostaining index of acrolein. *SNx + V*, *SNx + 0.5*, and *SNx + 10* represent subtotal nephrectomy + vehicle, SNx + 0.5 mg/kg per day olmesartan, and SNx + 10 mg/kg per day olmesartan, respectively. **P* < 0.05; ***P* < 0.01. ×200

The binding of oxidative lipids and renal tissue proteins

Western blotting showed that MDA and acrolein bound various kinds of renal tissue proteins (Fig. 6). The molecular weights of MDA- and acrolein-bound proteins were mainly between 30 and 90 kDa. However, the staining patterns of these proteins seemed to be a little different. This suggests that the targets of oxidative lipids might differ depending on the property of the oxidative lipids. Treatment with olmesartan dose-dependently attenuated the lipid peroxidation

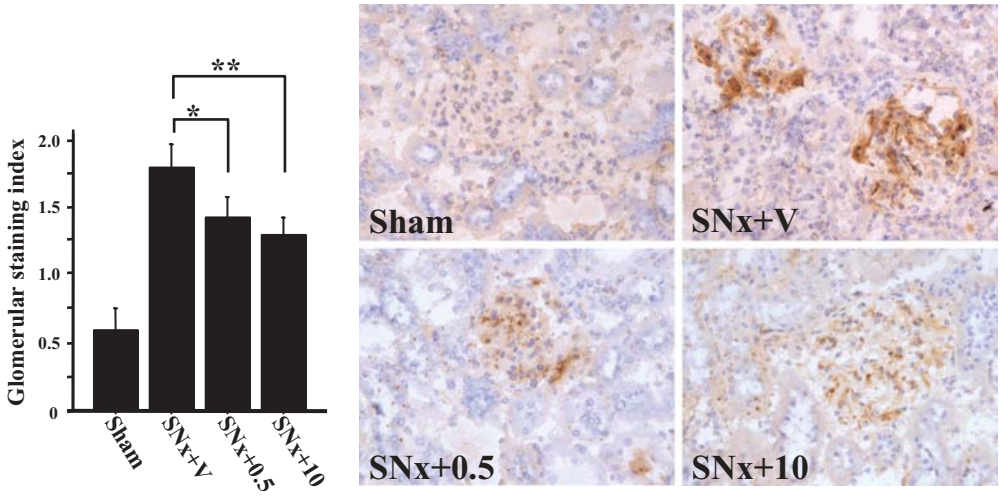

Fig. 4. Immunostaining index of 4-hydroxynonenal (*4-HNE*). *SNx + V*, *SNx + 0.5*, and *SNx + 10* represent subtotal nephrectomy + vehicle, SNx + 0.5 mg/kg per day olmesartan, and SNx + 10 mg/kg per day olmesartan, respectively. *P < 0.05; **P < 0.01. ×200

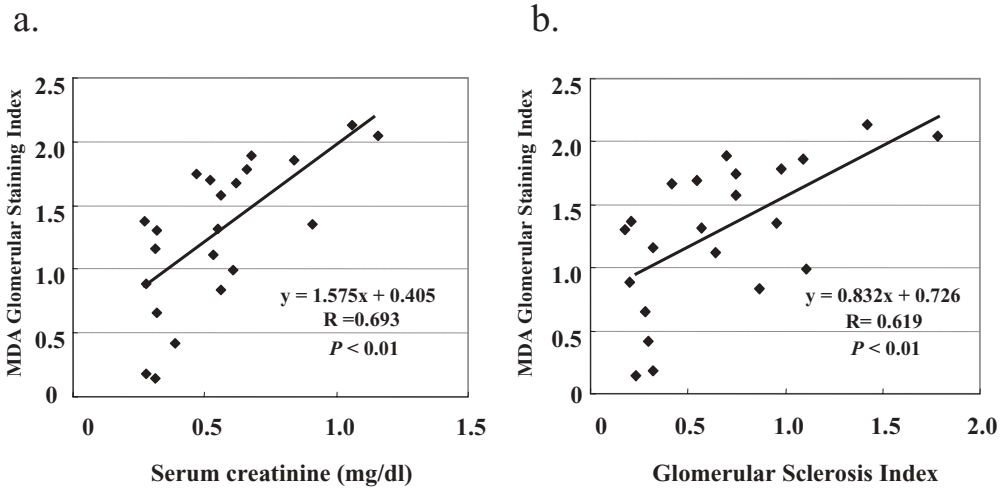

Fig. 5a,b. Relationship between MDA glomerular staining index and renal injury. **a** Relationship between serum creatinine and MDA glomerular staining index. **b** Relationship between glomerular sclerosis index and MDA glomerular staining index

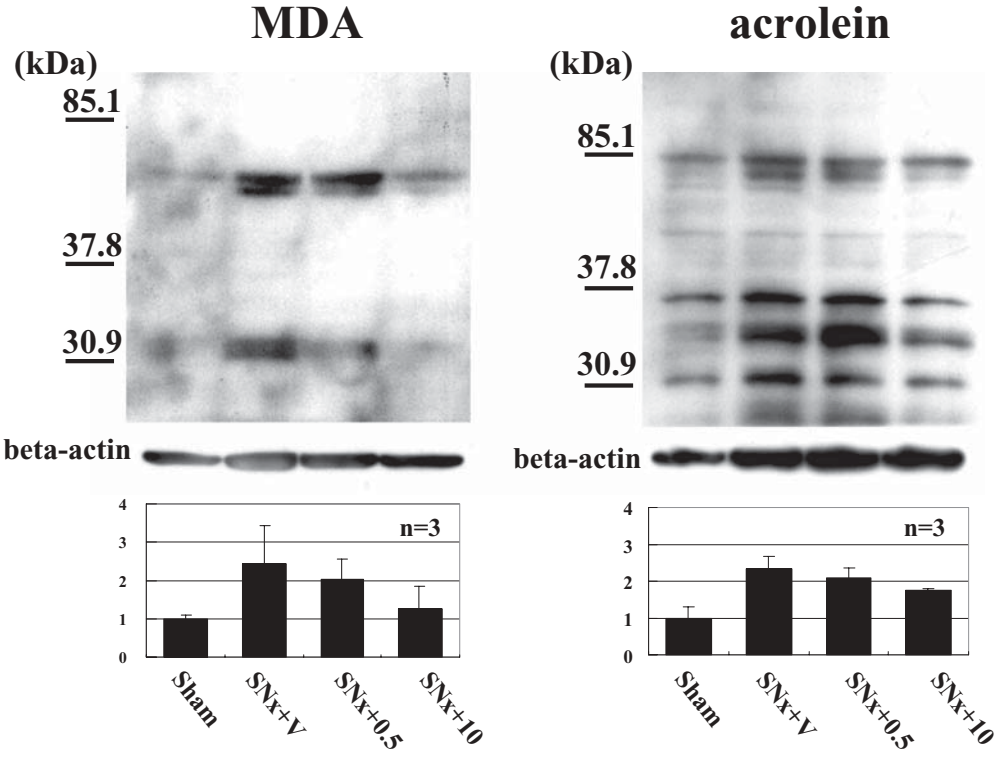

Fig. 6. Western blotting of MDA- and acrolein-bound proteins. *SNx + V*, *SNx + 0.5*, and *SNx + 10* represent subtotal nephrectomy + vehicle, SNx + 0.5 mg/kg per day olmesartan, and SNx + 10 mg/kg per day olmesartan, respectively. Western blotting shows the results of homogenates from one representative animal. The values of MDA- and acrolein-bound proteins, by densitometric measurements, were normalized by those of beta-actin. The relative amounts of MDA- and acrolein-bound proteins are expressed as a fold increase over sham (n = 3)

of proteins in the renal cortex. As the Western blotting results from three different experiments were similar; the results for one representative animal are shown (Fig. 6).

Discussion

Lipid peroxidation has been well documented in metabolic disorders such as diabetes and atherosclerosis. In this study we demonstrated that oxidative lipids such as MDA, acrolein, and 4-HNE were detected in the progressive renal injury induced by subtotal nephrectomy, and we showed that an angiotensin II receptor type-I blocker, olmesartan, attenuated the glomerular injury and lipid peroxidation in a dose-dependent manner.

In this model, the oxidative forms of lipids were detected mainly in the glomerulus in the early stage of renal injury. In contrast, in the tubulointerstitial area, oxidative lipid expression was observed, but to a lesser extent. This is consistent with the findings in other types of renal injury, such as diabetic and IgA nephropathy.[11] These results suggest that oxidative stress promotes the local production of oxidative lipids in the kidney, and the primary target in this model seems to be the glomerulus. It is reported that mechanical stress induces an oxidant stress, including oxidative lipids, in aortic endothelial cells.[12] Thus, it is speculated that the same mechanism may be involved in the intraglomerular hypertension induced by subtotal nephrectomy.

Oxidative lipids are related to the pathogenesis of atherosclerosis and diabetic complications by exerting biological effects such as inflammation, proliferation, and apoptosis through modulating intracellular signaling pathways.[13,14] Among the various types of oxidative lipids, highly reactive aldehydes such as MDA, 4-HNE, and acrolein were intensively studied.[15,16] Our study showed that these oxidative lipids bind renal tissue proteins. Thus, it is speculated that lipid-related oxidative stress may affect the function of various types of renal tissue proteins.

Olmesartan ameliorates glomerular hypertension by reducing the vascular tone of glomerular efferent arterioles. Xu et al.[17] have shown that the renoprotective effect of this agent depends on the blood pressure-lowering effect. In our study, the blood pressure-lowering effect was not significantly different between the low- and high-dose groups, and the suppression of renal damage and lipid peroxidation was stronger in the high-dose group than in the low-dose group at 8 weeks. This suggests that olmesartan may have a blood pressure-independent renoprotective effect, with attenuation of lipid peroxidation. However, in this study, blood pressure was measured once in the morning, and blood pressure was not examined at different time points. In addition, the difference in diastolic blood pressure between the low- and high-dose groups was significant at 4 weeks (124.1 ± 7.6 mmHg [0.5 mg/kg] vs 106.8 ± 18.6 [10 mg/kg], respectively; $P < 0.05$), although the differences at other time points were not significant. Thus, we cannot completely exclude the possibility that the blood pressure-lowering effect may have been different between these two groups. To clarify the blood pressure-independent antioxidant property of olmesartan, an ideal control group might be animals treated with non-renin-angiotensin system (RAS) inhibiting reagents.

In this study, we did not examine the causal relationship between oxidative lipids and renal injury. Thus, there is a possibility that oxidative lipids may be a reflection of, but not an inducer of, renal injury. However, the importance of antioxidant therapy has been reported, in that radical-scavenging drugs protected kidneys in subtotal nephrectomy models.[18–20] Thus, to attenuate the progression of glomerular injury effectively, a combination of antihypertensive and antioxidant therapy might be recommended.

This study has several limitations. First, we have no available data concerning urinary protein excretion. Thus, we could not evaluate the dose-effect of olmesartan on proteinuria that might cause oxidative stress. Second, there are various oxidative lipids besides MDA, acrolein, and 4-HNE. Thus, our study might have evaluated a small part of the oxidative lipids that affect the course of renal damage. Third, the difference in serum creatinine levels between the low- and high-dose groups was not significant at 8 weeks after renal ablation. Thus, to examine the effects on renal outcome, a longer observation period might be necessary.

Conclusion

In conclusion, our results indicate that oxidative lipids may be involved in the progression of renal injury induced by subtotal nephrectomy in rats, and high-dose olmesartan may have a renoprotective effect with attenuation of lipid peroxidation.

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