Original Article

Olmesartan Ameliorates Progressive Glomerular Injury in Subtotal Nephrectomized Rats through Suppression of Superoxide Production

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Angiotensin type 1 receptor blockers are more effective than other antihypertensive agents in slowing the progression of renal disease. Angiotensin II (Ang II) induces production of NAD(P)H oxidase–dependent superoxide in vascular and mesangial cells, but the direct role of Ang II in glomerular superoxide production remains unknown. Here we examined the effect of Ang II on superoxide production both *ex vivo* and *in vivo*. Ang II increased superoxide generation in isolated normal glomeruli in a dose-dependent manner, and co-incubation with olmesartan, an angiotensin type 1 receptor blocker, suppressed such increase. Subtotal nephrectomized rats (Nx, *n*=8) showed impaired renal function, increased glomerular sclerosis, and significantly high superoxide production in glomeruli. These changes were inhibited in olmesartan-treated (*n*=8), but not hydralazine-treated (*n*=8) Nx rats. Oxidative stress and nitrosative stress were observed in Nx glomeruli, as evidenced by increased levels of carbonyl protein and nitrotyrosine formation, respectively. These changes were inhibited by 8-week treatment with olmesartan. The apoptosis observed in Nx glomeruli was also suppressed by olmesartan. Superoxide generation in Nx glomeruli was blocked by an NAD(P)H oxidase inhibitor, diphenylene iodinium. The mRNA expression levels of two NAD(P)H oxidase subunits were increased in Nx, and olmesartan significantly reduced the mRNA expression levels. These results indicate that Ang II directly induced superoxide production through activation of NAD(P)H oxidase, and olmesartan would inhibit superoxide production and oxidative stress independent of its blood pressure–lowering effect. These findings support the notion that superoxide plays a primary role in glomerular injury in chronic kidney disease. (*Hypertens Res* 2008; 31: 305–313)

***Key Words*: NAD(P)H oxidase, nephrectomy, olmesartan, apoptosis**

Introduction

Angiotensin II (Ang II) plays a role in the induction of glomerular injury, and upregulation of the local tissue renin-angiotensin-system (RAS) in response to injury has been demonstrated in renal diseases (*1*). Blockade of the RAS with either angiotensin-converting enzyme (ACE) inhibitors or angiotensin type 1 receptor (AT1R) blockers (ARBs) results in improvement of proteinuria and slowing of the progression of chronic kidney disease. These effects of RAS inhibition are in addition to, but independent of blood pressure control (*2*, *3*). These clinical studies as well as a large number of *in vivo* (*4*, *5*) and *in vitro* (*6*, *7*) experimental studies support the

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notion that the nonhemodynamic effects of Ang II are pathophysiologically important in progressive glomerular injury.

Ang II regulates and increases NAD(P)H oxidase–dependent superoxide production in cultured vascular smooth muscle cells (*8*), fibroblasts (*9*), endothelial cells (*10*), cardiomyocytes (*11*), and mesangial cells (*12*). There is evidence to suggest that intracellular production of reactive oxygen species (ROS), including superoxide, mediates Ang II–dependent cellular responses *via* activation of redox-sensitive signaling cascades (*13–15*). Thus, the renoprotective effects of ARBs are mediated through inhibition of glomerular ROS generation in chronic kidney disease. However, the functional and pathophysiological roles of oxidative stress in chronic kidney disease have been gessed based only on the presence of high levels of lipid peroxides in the kidneys of subtotal nephrectomized rats (*16*). To our knowledge, no direct evidence for the presence of oxidative stress in glomeruli has been reported. In the present study, we provide evidence for the presence of excessive oxidative stress in the glomeruli of subtotal nephrectomized rats using a fluorescent dye, dihydroethidium (DHE), which has been used to estimate the formation of superoxide. We also report on the effect of olmesartan, an ARB, on ROS production in the glomeruli of the same rat model.

Methods

Detection of Superoxide in Normal Glomeruli Exposed to Ang II

Superoxide production was detected by DHE staining (*17*) and 2′,7′-dichlorofluorescein (DCF) staining (*18*). Isolated glomeruli from 8-week-old male Wistar rats were first incubated with RPMI-1640 containing 20 μmol/L DHE for 1 h or 2′,7′-dichlorofluorescein diacetate (DCFH-DA: Molecular Probes, Eugene, USA) for 10 min and rinsed with phosphate-buffered saline. The glomeruli were exposed to Ang II (0.1 to 1,000 nmol/L) or 100 nmol/L Ang II for 1 h with 100 nmol/L olmesartan, or 100 nmol/L PD123319, an angiotensin type 2 receptor (AT2R) blocker, before exposure to the experimental conditions. Fluorescence images were obtained using a confocal laser microscope (Leica Microsystems, Tokyo, Japan) at excitation/emission wavelengths of 485/535 nm for DCF and 535/610 nm for DHE, respectively. The fluorescence intensity values from 50 different isolated glomeruli were calculated by Leica TCS-NT software (Leica Microsystems) and the average values are presented.

Animals

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School, Kurashiki, Japan. Male Wistar rats weighing 250–300 g were used in this experiment. Subtotal (five-sixths) nephrectomy was performed under anesthesia, by removal of the right kidney and ligation of two branches of the left renal artery, resulting in infarction of approximately two-thirds of the left kidney. Following subtotal nephrectomy, the rats were divided into three groups: a no-treatment group (Nx group, $n=8$), an olmesartan group (Nx+OLM group; 5 mg/kg/day, $n=8$) and a hydralazine group (Nx+HYD group; 5 mg/kg/day, $n=8$). The treatment groups received the respective medication for 8 weeks, commencing immediately after surgery. Sham-operated rats were used as the control group (sham group, $n=8$).

Renal Function and Morphological Study

After 8 weeks of treatment, 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. 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 (*19*). Before sacrification, the femoral artery was catheterized with polyethylene tubes (PE-50; Becton Dickinson, Parsippany, USA) and systolic blood pressure was recorded under sevoflurane inhalation anesthesia. Serum creatinine and 24-h urinary protein excretion were examined in each group at 8 weeks following subtotal nephrectomy. Removed rat kidney sections were stained with periodic acid-Schiff. In each group, the percentage of those exhibiting segmental sclerosis in 30 glomerular sections was determined and expressed as a glomerular sclerosis index. Glomerular sclerosis was graded semiquantitatively in each glomerulus using a scale of 0 to 3: 0, no glomerular sclerosis; 1, segmental glomerular sclerosis in $<25\%$ of glomeruli; 2, segmental glomerular sclerosis in 25% to 50% of glomeruli; and 3, segmental glomerular sclerosis in $>50\%$ of glomeruli. The mean score per glomerulus was determined for each rat and reported as the glomerular sclerosis index.

Determination of Superoxide Production in Glomeruli of Nephrectomized Rats

Isolated glomeruli from each group were incubated in RPMI-1640 containing 20 μmol/L DHE for 1 h and rinsed with phosphate-buffered saline. Some experiments were performed with 30 min pre-incubation by 100 μmol/L diphenylene iodinium (DPI), 1 mmol/L allopurinol or 100 μmol/L 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS). In each model, the mean fluorescence intensity of isolated glomeruli was analyzed by TCS-NT (Leica Microsystems).

Western Blotting

Proteins extracted from isolated glomeruli were used for carbonyl protein and nitrotyrosine immunoblotting. Western blot analysis for carbonyl protein was performed with an Oncor Oxyblot kit, according to the protocol provided by the manu-

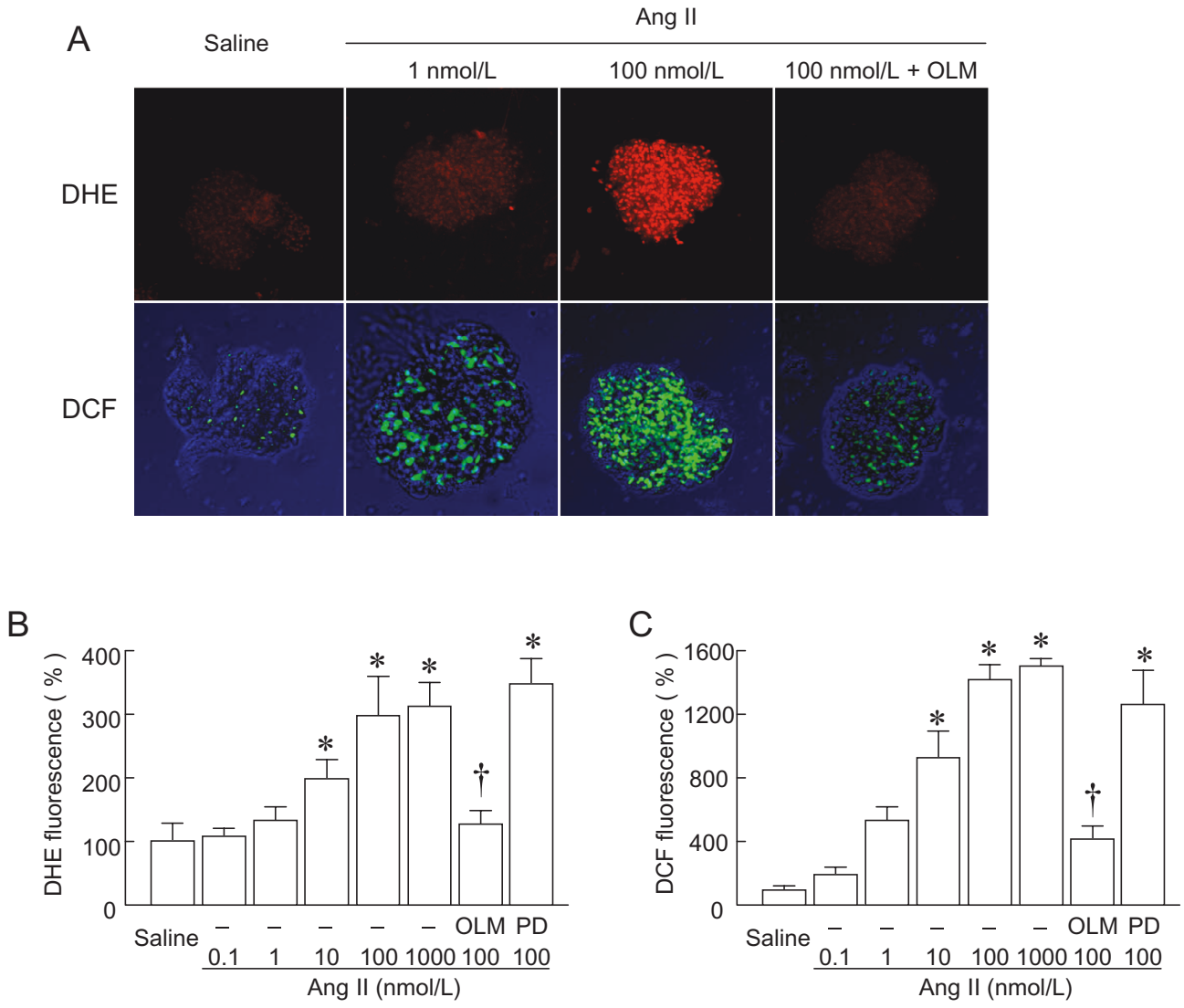

Fig. 1. *Ang II induces superoxide production in normal isolated glomeruli. A: DHE and DCF staining of isolated glomeruli exposed to Ang II with or without olmesartan (OLM), an angiotensin type 1 receptor blocker. B: DHE fluorescence intensity and effect of OLM and PD123319 (PD), an angiotensin type 2 receptor blocker, on Ang II–induced superoxide generation. Data are the means±SD of 8 rats for each group. *$p<0.05$ vs. saline. †$p<0.05$ vs. Ang II 100 nmol/L. C: DCF fluorescence intensity and effect of OLM and PD123319 on Ang II–induced superoxide generation. Data are the means±SD of 8 rats for each group. *$p<0.05$ vs. saline. †$p<0.05$ vs. Ang II 100 nmol/L.*

Table 1. Physiological, Biochemical and Morphological Features of Sham-Operated Rats, and Rats Subjected to Subtotal Nephrectomy Followed by No Treatment (Nx), Olmesartan (OLM) Treatment or Hydralazine (HYD) Treatment

	Sham	Nx	Nx+OLM	Nx+HYD
n	8	8	8	8
Systolic blood pressure, mmHg	101.5±8.7	134.1±12.8[a]	98.9±9.3[b]	96.2±7.4[b]
Serum creatinine, mg/dL	0.29±0.01	0.91±0.20[a]	0.66±0.02[b,c]	0.89±0.21
Urinary protein, mg/24 h	5.8±1.2	122.4±22.1[a]	11.6±3.6[b,c]	89.3±21.5[b]
Glomerular sclerosis index	0.09±0.05	0.94±0.14[a]	0.33±0.11[b,c]	0.82±0.25

Data are mean±SD. [a]$p<0.05$ *vs*. Sham, [b]$p<0.05$ *vs*. Nx, [c]$p<0.05$ *vs*. Nx+HYD.

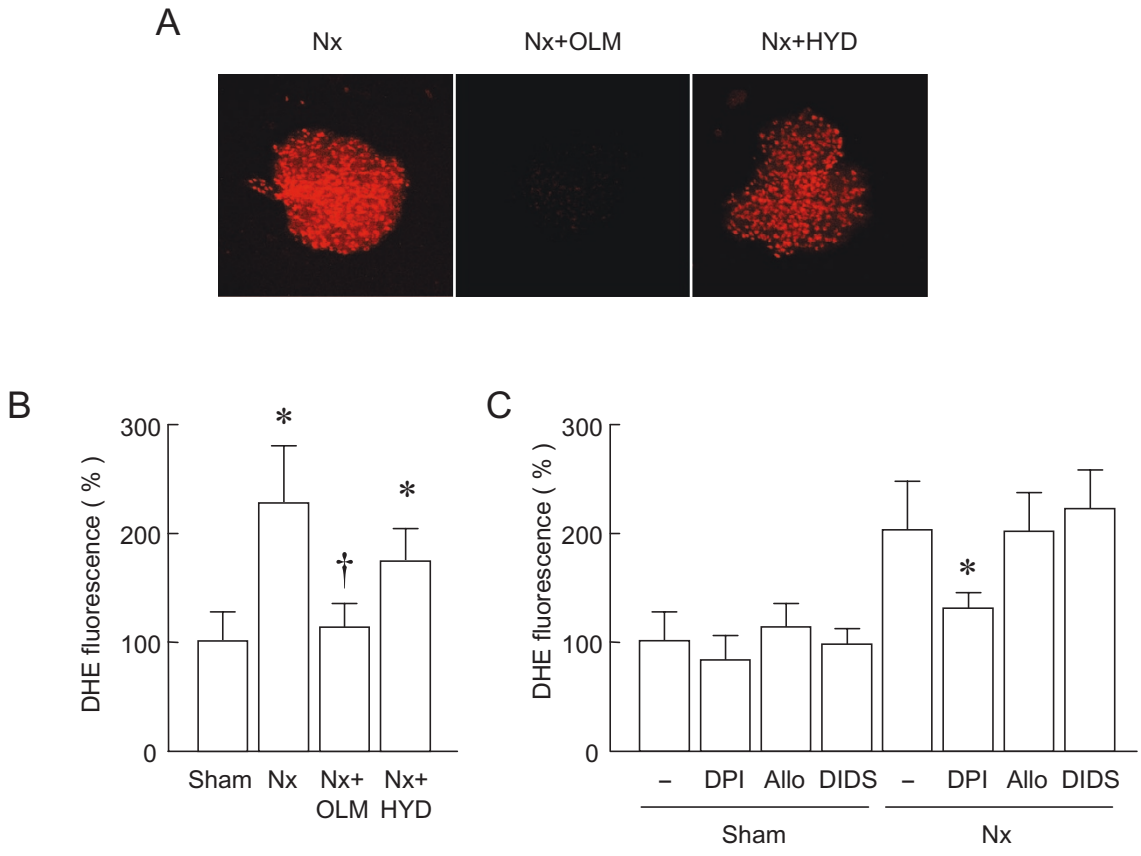

Fig. 2. *Superoxide production in isolated glomeruli of nephrectomized rats. A: DHE staining of isolated glomeruli from nephrectomized rats (Nx) and Nx rats treated with olmesartan (OLM) or hydralazine (HYD). B: DHE fluorescence intensity. Data are the means±SD of 8 rats for each group. *$p<0.05$ vs. sham group. †$p<0.05$ vs. Nx rats. C: Mechanism of superoxide production in glomeruli isolated from Nx rats. Data are the means±SD of 8 rats for each group. *$p<0.05$ vs. Nx rats. DPI, diphenylene iodinium; Allo, allopurinol; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid.*

facturer (Oncor, Gaithersburg, USA). Extracted proteins (50 μg) were separated in a 7.5% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1 h with anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, USA). Bands were visualized using the ECL-plus Western blotting detection system (Amersham Biosciences, Piscataway, USA).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from the isolated glomeruli by using TRIzol reagent (Invitrogen Japan, Tokyo, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, USA) with an Applied Biosystems Sequence Detector 7700. The primers and probes for p22*phox*, p47*phox* (*20*), angiotensinogen (NM_134432), ACE1 (AF201332) and AT1R (BC078810) were as follows. For p22*phox*: 5′-CTTTGGTGCTTACTCTATTGTTGCA-3′ (forward probe), 5′-GGAGCCCTTTTTCCTCTTTCC-3′ (reverse probe), and 5′-FAM-TGCTCATCTGTCTGCTGG AGTACCCCC-TAMRA-3′ (TaqMan probe); for p47*phox*: 5′-GTGAAGCCATCGAGGTCATTC-3′ (forward), 5′-CCC GCGGCTTCTAATCTGT-3′ (reverse), and 5′-FAM-CAT CACCGGCTACTTCCCATCCATGT-TAMRA-3′ (TaqMan); for angiotensinogen: 5′-GATAAAGAACCCGCCTC CTC-3′ (forward), 5′-GGTAGACAGCTTGGCCTGAG-3′ (reverse), and 5′-FAM-AATTCGGGGATCCTACAACC-BHQ-3′ (TaqMan); for ACE: 5′-GAGTCGATGCTGGAG AAACC-3′ (forward), 5′-CGTGCACTGCTTAATCCTGA-3′ (reverse), and 5′-FAM-CCTCTGCGTGGGACTTCTAC-BHQ3′ (TaqMan); and for AT1R: 5′-GGAAACAGCTTG GTGGTGAT-3′ (forward), 5′-ATAAGTCAGCCAAGGC GAGA-3′ (reverse), and 5′-FAM-AAGCTGAAGACTGTG GCCAG-BHQ-3′ (TaqMan).

Assessment of Glomerular Apoptosis

Apoptotic nuclei were detected with the terminal deoxy-

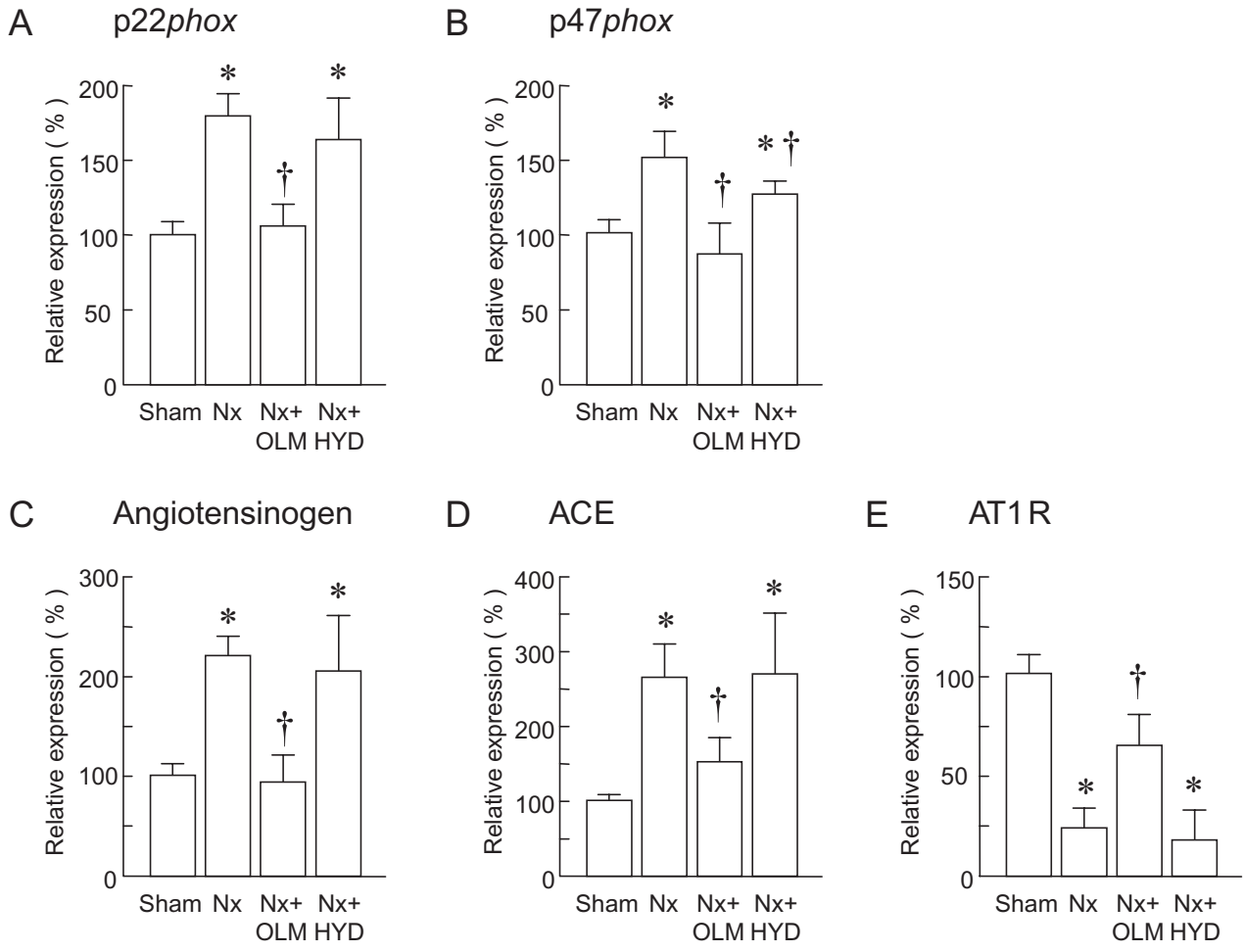

Fig. 3. *Analysis of real-time PCR in isolated glomeruli. Expression levels of p22phox mRNA (A), p47phox mRNA (B), angiotensinogen (C), ACE (D) and AT1R (E). Data are the means±SD of 8 rats for each group. *$p < 0.05$ vs. sham group. †$p < 0.05$ vs. Nx group.*

nucleotidyl transferase–mediated dUTP nick end–labeling (TUNEL) method, using an apoptosis detection kit (*in situ* apoptosis detection kit; TaKaRa Biomedicals, Shiga, Japan). The number of apoptotic nuclei present in the glomerulus and total cell number were counted in at least 50 random glomeruli per section. The mean percentage of apoptotic cells in the glomerulus was calculated.

Statistical Analysis

Values were expressed as the means±SD. All parameters were evaluated with the two-tailed unpaired Student's *t*-test, Welch's *t*-test or Mann-Whitney's *U*-test. Values of $p<0.05$ were considered to indicate statistical significance.

Results

DHE generates fluorescence upon exposure to superoxide, while DCFH-DA generates fluorescence upon exposure to peroxides, including H_2O_2 and peroxynitrite. In the *ex vivo* study, exposure of isolated normal glomeruli to Ang II (0.1 to 1,000 nmol/L) resulted in a significant dose-dependent increase in fluorescence intensity (Fig. 1). Ang II–induced glomerular superoxide generation was blocked by olmesartan, but not by PD123319 (Fig. 1). There were no changes in the mRNA expression levels of the NAD(P)H oxidase component. These results suggest that the action of Ang II on superoxide production was mediated *via* AT1R, but not AT2R.

In the *in vivo* study, Nx rats showed chronic renal impairment characterized by elevated serum creatinine and severe proteinuria (Table 1). These changes were reduced by treatment with olmesartan, but not with hydralazine. The glomerular sclerosis index was higher in the Nx group than the sham group. The high index was reduced by olmesartan treatment, but not hydralazine treatment.

The fluorescence of isolated glomeruli from the Nx group was significantly stronger than that of glomeruli from the

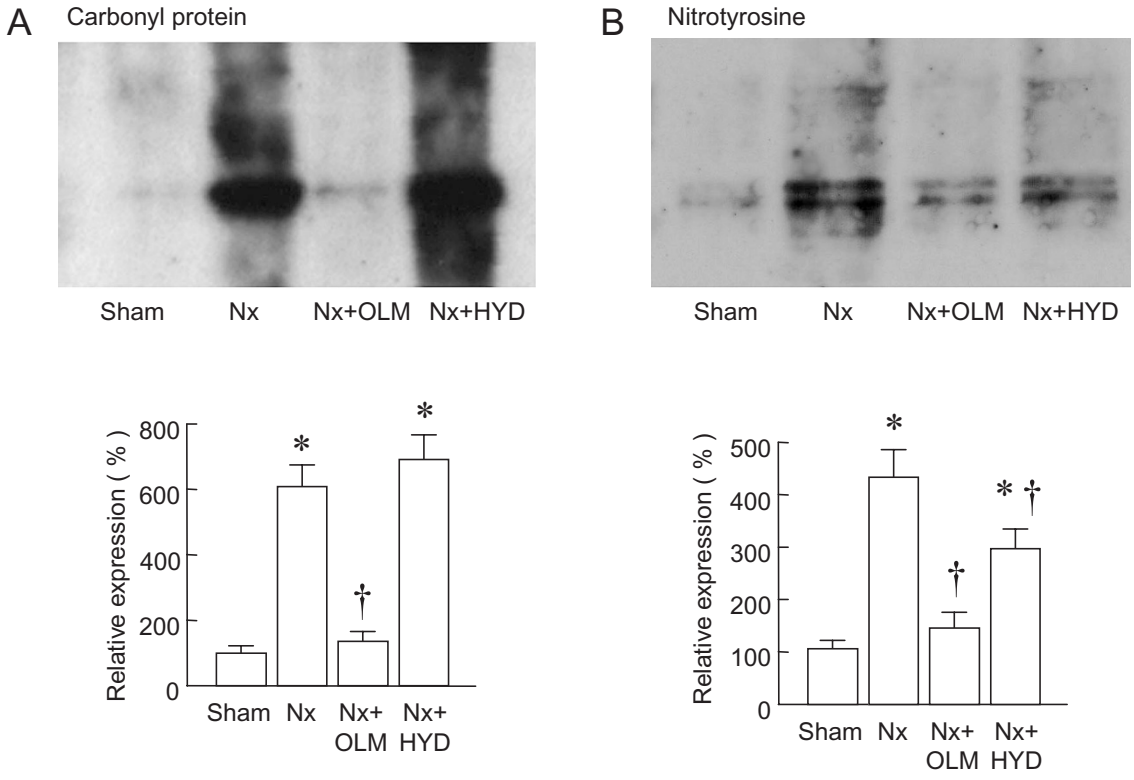

Fig. 4. *Oxidative stress markers in glomeruli of nephrectomized rats. A: Western blot of carbonyl group proteins in isolated glomeruli (top) and quantitative analysis of the results (bottom). Data are the means±SD of 8 rats for each group. $p<0.05$ vs. sham group. $^{\dagger}p<0.05$ vs. Nx group. B: Western blot of nitrotyrosine in isolated glomeruli from nephrectomized rats (top) and quantitative analysis of the results (bottom). Data are the means±SD of 8 rats for each group. $*p<0.05$ vs. sham group. $^{\dagger}p<0.05$ vs. Nx group.*

sham group. The fluorescence intensity of isolated glomeruli from rats of the Nx+OLM group was lower than that of the sham group (Fig. 2A, B). Treatment with hydralazine tended to reduce the fluorescence intensity by 22%, albeit insignificantly. Glomerular superoxide generation in the Nx group was blocked by DPI, an NAD(P)H oxidase inhibitor, but not by allopurinol, a xanthine oxidase inhibitor, or DIDS, a mitochondrial anion channel inhibitor (Fig. 2C). These results suggest that superoxide production in isolated glomeruli from Nx rats was mediated *via* NAD(P)H oxidase activation rather than *via* xanthine oxidase or mitochondrial pathway.

The p22*phox* is a membrane component while p47*phox* is a cytosolic component of NAD(P)H oxidase. Both mRNAs were increased in isolated glomeruli of Nx rats (Fig. 3A, B). Olmesartan significantly reduced the expression levels of both mRNAs to the levels seen in the sham rats. However, hydralazine had no effect on p22*phox* mRNA. On the other hand, hydralazine significantly reduced p47*phox* mRNA expression, but the level was still higher than that of the sham group (Fig. 3B).

The angiotensinogen mRNA and ACE mRNA expressions in isolated glomeruli from Nx rats were significantly increased compared with those from sham rats. Olmesartan, but not hydralazine, significantly reduced these expressions (Fig. 3C, D). The AT1R mRNA expression was significantly reduced in Nx glomeruli, and treatment with olmesartan, but not hydralazine, significantly increased the AT1R mRNA (Fig. 3E).

Carbonyl protein levels in glomeruli of Nx rats were higher than those of the sham group (Fig. 4A). Olmesartan, but not hydralazine, significantly reduced glomerular carbonyl protein levels. The reaction of nitric oxide with superoxide (to form peroxynitrite) occurs six times faster than the superoxide dismutase reaction with superoxide (*21*). Peroxynitrite is more stable than nitric oxide and exerts multiple cytotoxic effects, some of which involve formation of protein-associated nitrotyrosine (*22*). Nitrotyrosine formation in Nx rats was higher than in rats of the sham group, and was reduced in rats of the Nx+OLM group (Fig. 4B). Nitrotyrosine formation was also reduced in the Nx+HYD group compared with the Nx group, but the level was higher than that in the sham group.

Apoptosis was assessed by TUNEL staining (Fig. 5). The percentage of apoptotic cells in glomeruli was higher in the

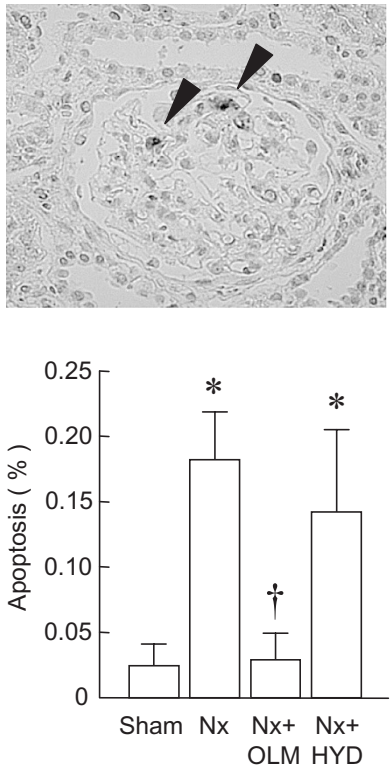

Fig. 5. *Percentage of apoptotic cells in glomeruli of nephrectomized rats. Top: apoptosis identified by TUNEL staining. Arrowheads: TUNEL-positive cells. Bottom: quantitative analysis of the results. Data are the means±SD of 8 rats for each group. *$p < 0.05$ vs. sham group. †$p < 0.05$ vs. Nx group.*

Nx group than the sham group. Treatment with olmesartan, but not hydralazine, significantly reduced the percentage of apoptotic cells to a level similar to that seen in rats of the sham group.

Discussion

In the Nx rat model, previous studies have demonstrated the prevention or inhibition of the glomerular and tubular injury by an antioxidant such as α-tocopherol (*23*) or *N*-acetylcysteine (*24*). There are also many reports that ARBs have renoprotective effects in the Nx rat model (*25*, *26*). However, there have been few reports on the effects of ARBs from the viewpoint of the oxidation stress reduction. Olmesartan has been reported to improve endothelin-induced hypertension and oxidative stress in rats (*27*). We therefore hypothesized that olmesartan would ameliorate renal injury in the Nx rat model through suppression of superoxide production. Here, we first showed that Ang II–induced superoxide production in isolated glomeruli and that olmesartan reduced Ang II–induced superoxide production both *ex vivo* and *in vivo*. Second, we explored the effect of olmesartan on superoxide production in the glomeruli of rats with experimentally-induced chronic kidney disease and used hydralazine to ensure that the effect of olmesartan on superoxide production was independent of its blood pressure–lowering effects. Based on these results, we propose that olmesartan may have renoprotective effects and these effects may be mediated through blockade of Ang II–induced superoxide production.

In this study, we found that Ang II induced a dose-dependent increase of superoxide production in isolated normal glomeruli. Olmesartan as well as DPI blocked Ang II–stimulated glomerular superoxide production, indicating that Ang II increases superoxide accumulation *via* the Ang II–AT1R–NAD(P)H oxidase system. Although the origin of the increased superoxide generation in renal diseases is multifactorial, the kidney is known to express NADPH oxidase and generate ROS (*20*, *28*). Ang II induces NAD(P)H oxidase–dependent superoxide production by mesangial cells (*12*). Ang II also induces endothelial cell NAD(P)H oxidase activation (*29*). The mRNAs of the NAD(P)H oxidase subunits p22*phox*, p47*phox*, gp91*phox*, and p67*phox* are detected in podocytes (*17*). Thus, all components of glomeruli contain NAD(P)H oxidase and they can all produce superoxide in response to Ang II.

We also provided evidence for the presence of excessive oxidative stress in the glomeruli of Nx rats. Subtotal renal ablation is a commonly used experimental model to study progressive renal damage in susceptible rat strains. Functional adaptive changes in the remaining nephrons, induced by abrupt reduction in renal mass, are eventually deleterious to renal function because of pressure-induced capillary stretch and glomerular injury. The hyperfiltering state–associated glomerular capillary hypertension upregulates local expression of the RAS (*30*), and it would cause superoxide production. Increased superoxide formation may occur in chronic kidney disease for reasons possibly related to an increase in glomerular hypertension. In our experiment, postoperative treatment of rats with olmesartan for 8 weeks completely diminished superoxide production. As shown in previous studies, the sensitivity to Ang II is higher in the postglomerular efferent than in preglomerular afferent arterioles (*31*, *32*). Thus, Ang II blockade may dilate efferent arterioles, thereby lowering glomerular capillary pressure, and causing a reduction in NAD(P)H oxidase expression and superoxide production. Moreover, glomeruli of hydralazine-treated Nx rats showed a slight reduction in the expression of p47*phox* and production of superoxide. Systolic blood pressure was significantly reduced in hydralazine-treated rats, and thus the glomerular pressure was decreased in proportion to the reduction in systemic blood pressure, which may have caused the slight reduction in superoxide production.

We examined whether glomerular RAS components are activated in Nx glomeruli. The mRNA expressions of angiotensinogen and ACE were increased in Nx and attenuated by olmesartan treatment. The mRNA expression of AT1R was decreased in Nx glomeruli and normalized by ARB, which was consistent with a previous report (*33*). These data would

indicate that the glomerular Ang II level was increased in Nx and local RAS components were activated. Olmesartan would decrease local RAS activation, which would contribute to ROS reduction.

Treatment with olmesartan reduced the accumulation of carbonyl protein and nitrotyrosine in the glomeruli and such reduction was proportionate to the reduction in superoxide production. The rapid reaction of superoxide with nitric oxide (NO) generates peroxynitrite and diminishes the bioactivity of NO. Peroxynitrite is too reactive to measure directly, but peroxynitrite stably nitrates aromatics such as tyrosine residues in proteins such as 3-nitrotyrosine (*34*). We previously showed that reduction of nitrosative stress improved hypertensive renal injury (*19*). Within the glomerulus, a decrease in NO bioactivity would reduce the homeostatic role of NO in antagonizing Ang II–driven growth-related processes affecting the mesangial cells and extracellular matrix as well as the role of NO in preventing platelet and leukocyte adhesion to the endothelium (*35*). Thus, one of the pleiotropic benefits of olmesartan beyond its blood pressure–lowering effect may be suppression of nitrotyrosative stress.

We also showed that olmesartan treatment decreased apoptosis in Nx glomeruli. Apoptosis of glomerular cells has been demonstrated in human diseases (*36*) and experimental models of the remnant kidney (*36*), diabetes (*37*), and hypertensive nephrosclerosis (*38*), where increased activity of Ang II may be implicated. Furthermore, it has been reported that ROS induce apoptosis in mesangial cells (*39*) and podocytes (*40*). Falls in numbers of mesangial cells and podocytes correlate with progression to glomerulosclerosis (*41*, *42*). Considered together, these results indicate that the pleiotropic benefits of olmesartan, which reduce oxidative stress, might be mediated by suppression of apoptosis.

In summary, we have demonstrated in the present study that Ang II induced superoxide production in isolated glomeruli and that olmesartan reduced superoxide production both *ex vivo* and *in vivo*. We also provided evidence for the presence of excessive oxidative stress in glomeruli of subtotal nephrectomized rats and that olmesartan suppressed ROS production in glomeruli. These findings could support the notion that oxidative stress plays a primary role in the glomerular pathogenesis of chronic kidney disease.

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