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ORIGINAL PAPER

Reactive oxygen species mediate compensatory glomerular hypertrophy in rat uninephrectomized kidney

Masahito Ozeki · Hajime Nagasu · Minoru Satoh · Tamehachi Namikoshi · Yoshisuke Haruna · Naruya Tomita · Tamaki Sasaki · Naoki Kashihara

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Abstract Hyperfiltration in glomeruli is the most common pathway to progressive renal dysfunction. Moreover, reduction of renal mass by unilateral nephrectomy results in an immediate increase in glomerular flow to the remnant kidney, followed by compensatory glomerular hypertrophy. Reactive oxygen species (ROS) are involved in renal hypertrophic responses; however, the role of ROS in compensatory glomerular hypertrophy remains unclear. Therefore, this role was investigated in the present study. Wistar rats were randomly placed into two groups: uninephrectomized rats (Nx) and uninephrectomized rats treated with tempol (Nx + TP). The glomerular volume increased in the Nx 1 week after surgery, but was significantly suppressed in the Nx + TP. Levels of phospho-Akt and phospho-ribosomal protein S6, which are critical for cell growth and hypertrophy, were markedly increased in the glomeruli of the Nx, while tempol treatment almost abolished the activation of these proteins. These results suggest that ROS have important roles in compensatory hypertrophy in glomeruli.

Keywords Reactive oxygen species · Nitric oxide · Uninephrectomy · Tempol · Kidney

M. Ozeki and H. Nagasu have contributed equally to this study.

M. Ozeki · H. Nagasu · M. Satoh (✉) · T. Namikoshi · Y. Haruna · N. Tomita · T. Sasaki · N. Kashihara
Division of Nephrology, Department of Internal Medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan
e-mail: msatoh@med.kawasaki-m.ac.jp

Introduction

Chronic kidney disease is currently a worldwide public health problem, and the prevalence of end-stage renal disease is increasing rapidly. In progressive renal dysfunction, the most common pathway is glomerular hyperfiltration, as discussed in the hyperfiltration theory of Brenner et al. [1]. Nephron reduction followed by glomerular hypertrophy is recognized as the common prepathological state progressing to terminal renal failure. If nephron reduction does not exceed a certain percentage, glomerular hypertrophy is considered to be a compensatory mechanism for lost renal function. However, if nephron reduction is marked, hyperfiltration first occurs in the residual renal glomeruli, and glomerular hypertrophy is followed by glomerulosclerosis and progressive renal tissue damage, both of which are regarded as a breakdown of the adaptation mechanism. Compensatory glomerular hypertrophy induced by glomerular hypertension is the initial common pathological glomerular change in diabetes [2], metabolic nephropathy [3] and obesity-related glomerulopathy [4]. Therefore, elucidation of the mechanism underlying glomerular/renal hypertrophy is important for the development of treatment for progressive renal damage.

Sigmon et al. [5] have suggested that nitric oxide (NO) has an important role in the renal hemodynamic response in unilateral nephrectomy. Vascular shear stress is a primary stimulus for endogenous NO production from the endothelium [6, 7], and L-arginine prevents glomerular hyperfiltration in experimental diabetic rats [8]. We have reported that production of reactive oxygen species (ROS) in glomeruli is increased in a 5/6 renal ablation model [9]. Exogenous ROS stimulate induction of vascular endothelial growth factor in various cell types, including vascular smooth muscle [10] and endothelial [11] cells, and promote

cell proliferation and migration [12]. The major source of ROS in endothelial cells is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and ROS produced via the activation of NADPH oxidase stimulate diverse redox signaling pathways leading to angiogenic responses in endothelial cells and to postnatal neovascularization in vivo [13, 14]. These reports suggest that ROS are an important signal for angiogenesis and vasculogenesis. However, little is known about the role of ROS in glomeruli in compensatory renal hypertrophy.

We hypothesized that ROS mediate compensatory glomerular hypertrophy after unilateral nephrectomy. To confirm this hypothesis, we examined whether tempol, a membrane-permeable ROS scavenger, can inhibit glomerular hypertrophy in rat uninephrectomized kidney.

Materials and methods

Animals and surgical procedure

The experimental protocol (no. 07-010) was approved by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School (Kurashiki, Japan). Compensatory renal growth was induced by uninephrectomy in male Wistar rats (220–250 g). The animals were randomly divided into two groups: right nephrectomized rats (Nx, $n = 58$) and rats treated with tempol (Sigma-Aldrich Japan, Tokyo, Japan; 3 mmol/l in drinking water) beginning 2 days prior to uninephrectomy (Nx + TP, $n = 58$). In some rats, tempol treatment was stopped 1 week after uninephrectomy ($n = 12$). For uninephrectomy, animals were anesthetized with pentobarbital (50 mg/kg body weight). A subcostal incision was made, and the right kidney was removed after ligation of the renal pedicle. The incision was sutured, and then the animals were maintained under sterile conditions until sacrifice at 0 h, 12 h, 2 days, 1 week and 2 weeks after uninephrectomy ($n = 8$ in 12 h and 2 days group, $n = 14$ in 0 h, 1 week and 2 weeks group). Mean blood pressure was measured in conscious rats by tail-cuff plethysmography (Softron Co., Tokyo, Japan), and 12–24-h urine samples were collected on the day before sacrifice. Five rats in each group were killed at 0 h, 12 h, 2 days, 1 week and 2 weeks after uninephrectomy for collection of blood and kidney samples. In some rats, BrdU (Sigma-Aldrich Japan) was intraperitoneally injected 6 h before sacrifice. A part of the left kidney from each rat was fixed in 4% paraformaldehyde and embedded in paraffin for histological examination. Cortical tissue of the left kidney was cut into small pieces and glomeruli were isolated by a mechanical graded-sieving technique for superoxide detection and Western blot analysis. After isolation, the purity of the suspension was determined by light microscopy.

Histological examination

Each paraffin section was cut into 4-μm slices and subjected to periodic acid-Schiff (PAS) staining. Glomerular volume was evaluated by light microscopy. Volumes of the glomerular tuft were calculated from the areas of the midsections using the maximal planar area method [15]. Glomerular volumes and glomerular cell numbers were measured for at least 25 randomly selected glomeruli from the renal cortex in each animal (a total of 125 glomeruli from 5 rats in each group).

Detection of cell proliferation in glomeruli

To detect cell proliferation, an immunohistochemical study for BrdU was performed. After deparaffinization, anti-BrdU antibody (Sigma-Aldrich Japan) was applied to renal specimens overnight at 4°C. Primary antibody was detected using the Histofine Simple Stain MAX-PO kit (Nichirei Co., Tokyo, Japan) and diamino benzidine. The number of BrdU-positive cells was counted in at least 25 randomly selected glomeruli from the renal cortex in each animal (a total of 125 glomeruli from 5 rats in each group).

Detection of superoxide in glomeruli

Superoxide production was detected by 2′,7′-dichlorofluorescein (DCF) staining [9]. Isolated glomeruli from sacrificed rats were first incubated in RPMI-1640 medium containing 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 10 min and rinsed with phosphate-buffered saline (PBS). Fluorescence images were obtained using a confocal laser microscope (Leica Microsystems Japan, Tokyo, Japan) at excitation/emission wavelengths of 485/535 nm for DCF. Fluorescence intensities from 20 different isolated glomeruli in each rat (a total of 100 glomeruli from 5 rats in each group) were calculated using Leica TCS-NT software (Leica Microsystems), and average values are presented. The NADPH oxidase activity required to produce O_2^- was examined by DCF fluorescence intensity following the addition of NADPH (0.1 mmol/l) in the presence or absence of the NADPH oxidase inhibitor diphenylene iodonium chloride (DPI; 0.1 mmol/l). The activity is expressed relative to that in controls.

Western blot analysis

Equal amounts of cell lysates (50 μg) were separated on a 10–15% SDS-polyacrylamide gel, electrotransferred onto a polyvinylidene difluoride membrane and then probed with the indicated primary antibody and the appropriate

secondary antibody conjugated with horseradish peroxidase goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies against phospho-Akt and phospho-ribosomal protein S6 (rpS6) were obtained from Cell Signaling Technology (Beverly, MA), and antibodies to β-actin were purchased from Sigma-Aldrich Japan. The antibody was visualized using an enhanced chemiluminescence method (ECL plus; GE Healthcare Japan, Tokyo, Japan). The integrated band density ($n = 5$ in each group) was quantified using NIH Image software v.1.61.

In situ detection of NO

Three rats in each group were killed by intraperitoneal injection of sodium pentobarbital. An 18-gauge needle connected to an infusion pump was inserted into the abdominal artery. After cutting the right atrium, the whole body was perfused with PBS at a flow rate of 5 ml/min at 37°C. Once blood was removed, 0.01 mM diaminorhodamine-4M AM (DAR-4M AM; Daiichi Pure Chemical Co., Tokyo, Japan), 0.1 mM L-arginine and 2 mM $CaCl_2$ were added to the PBS, and the whole body was perfused with this solution for an additional 10 min at a flow rate of 5 ml/min. After fixation with perfusion of 4% paraformaldehyde, the tissues were cut into 1-mm slices and placed on a glass slide. Fluorescent images of NO were obtained with a TCS-NT confocal laser-scanning microscope (Leica Microsystems) [16].

NO excretion in urine

Nitric oxide metabolites (nitrate and nitrite, NO_x) were assayed in urine by the Griess colorimetric method after enzymatic conversion of nitrates to nitrites by nitrate reductase, using the total Nitric Oxide Assay kit (Biomol, Plymouth Meeting, PA) [17].

Statistical analysis

Values are expressed as mean ± SEM. All parameters were evaluated with a two-tailed unpaired Student's t test or one-way analysis of variance (ANOVA) for comparison of multiple means. Correlation coefficients were determined using linear regression analysis. A P value <0.05 was considered significant.

Results

Physiological data and glomerular volume after uninephrectomy

Physiological data are shown in Table 1. Mean blood pressure and body weight 2 weeks after uninephrectomy did not differ significantly between the two groups. Creatinine clearance was decreased after uninephrectomy in both the Nx and Nx + TP groups, but a significant difference between the two groups was not found. Urinary protein expression showed a tendency to increase in the Nx + TP group at 2 weeks, but the difference was not significant ($P = 0.07$). The ratio of kidney weight to body weight was significantly lower in the Nx + TP group than in the Nx group 1 week after uninephrectomy. Representative histological findings in glomeruli are shown in Fig. 1a. There were no pathological changes in the interstitium such as inflammatory cell invasion or fibrotic changes (data not shown). Time-dependent glomerular hypertrophy was observed in the Nx group. Glomerular hypertrophy in the Nx + TP group was also observed 12 h after nephrectomy, but there was no subsequent significant increase in glomerular volume. In both the Nx and Nx + TP groups, the glomerular volume was enlarged without cell proliferation 12 h after uninephrectomy. Glomerular volume at 12 h did not differ between the groups,

Table 1 Changes in body weight, kidney weight, blood pressure, urinary protein excretion and renal function after uninephrectomy with or without tempol

	0 h		1 week		2 weeks	
	Nx	Nx + TP	Nx	Nx + TP	Nx	Nx + TP
BW (g)	221 ± 13	218 ± 10	308 ± 9	311 ± 9	345 ± 10	329 ± 11
MBP (mmHg)	73.5 ± 3.5	72.8 ± 3.3	74.5 ± 1.9	71.2 ± 2.6	76.0 ± 3.2	73.0 ± 2.4
Ccr (ml/min)	3.1 ± 0.3	3.0 ± 0.4	2.4 ± 0.2	2.2 ± 1.4	2.3 ± 0.3	2.1 ± 0.4
UPE (mg/day)	1.1 ± 0.3	1.2 ± 0.5	1.2 ± 0.4	1.5 ± 0.2	1.3 ± 0.3	1.5 ± 0.3
KW/BW (mg/g)	4.7 ± 0.2	4.9 ± 0.4	6.0 ± 0.2	5.7 ± 0.1*	6.3 ± 0.3	5.4 ± 0.3*

$N = 8$ in each group. Data are expressed as mean ± SEM. *BW* body weight, *MBP* mean blood pressure, *Ccr* creatinine clearance, *UPE* urinary protein excretion, *KW* kidney weight. *$P < 0.05$ versus Nx

BW body weight, *MBP* mean blood pressure, *Ccr* creatinine clearance, *UPE* urinary protein excretion, *KW* kidney weight

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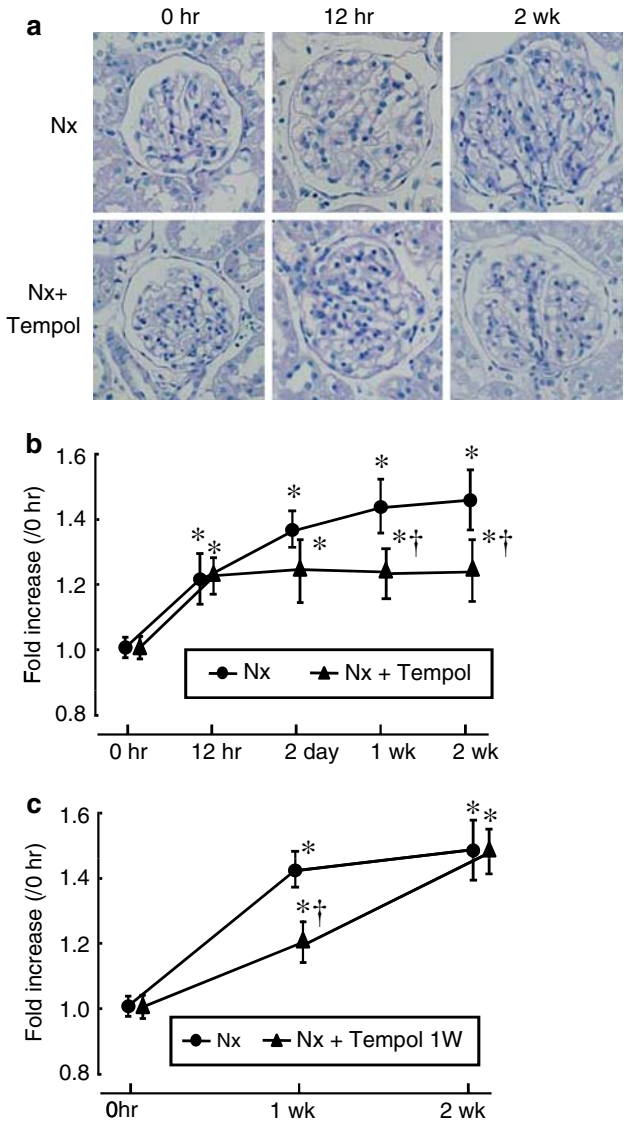

Fig. 1 Glomerular changes after nephrectomy. **a** Histological findings in glomeruli (PAS staining, magnification ×400). **b**, **c** Time-dependent changes in glomerular volume after uninephrectomy. Data are shown as a ratio of the mean glomerular volume to the glomerular volume in the control group (0 h). *$P < 0.05$ versus 0 h; †$P < 0.05$ versus the Nx group

but was lower in the Nx + TP group after 2 days and significantly lower 1 week after uninephrectomy ($P < 0.05$) than in the Nx group (Fig. 1b). We also examined whether rebound glomerular hypertrophy would occur after discontinuing tempol treatment at 1 week (Fig. 1c, Table 2). During the tempol treatment, glomerular volume was significantly lower in the Nx + TP group than in the Nx group, as well as kidney weight. However, after tempol treatment was stopped, the glomerular volume and kidney weight matched those in the Nx group 2 weeks after uninephrectomy.

ROS production in glomeruli

DCFH oxidation to the fluorescent compound DCF was used as a qualitative marker of cellular oxidative stress because multiple pathways can lead to DCF fluorescence. Representative images of ROS visualization in isolated glomeruli using confocal laser-scanning microscopy are shown in Fig. 2a. Only slight glomerular ROS production (green fluorescence) was observed in the control group (0 h). In the Nx group, an increase in ROS production was observed 1 week after surgery, and fluorescence returned to control levels after 2 weeks. The fluorescence of isolated glomeruli in the Nx + TP group was decreased compared with that of glomeruli in the Nx group, as shown by the representative images. The mean fluorescence intensity of isolated glomeruli in the Nx + TP group was lower than that in the Nx group, with a significant difference between the two groups 2 days after surgery (Nx + TP: 1.0 ± 0.2-fold, Nx: 1.5 ± 0.2-fold, $P < 0.05$). We also evaluated ROS production by NADPH oxidase in isolated glomeruli (Fig. 2b) and found a significant increase in fluorescent intensity after the addition of NADPH (Nx: 1.6 ± 0.2-fold, Nx ± NADPH: 3.6 ± 0.3, $P < 0.05$), and a decrease when DPI, an NADPH inhibitor, was added (1.2 ± 0.3-fold). These data suggest that ROS is produced by NADPH oxidase in glomeruli.

Table 2 Changes in body weight, kidney weight and blood pressure after uninephrectomy with or without tempol treatment for 1 week

	0 h		1 week		2 weeks	
	Nx	Nx + TP1W	Nx	Nx + TP1W	Nx	Nx + TP1W
BW (g)	223 ± 8	227 ± 9	295 ± 10	290 ± 11	325 ± 9	320 ± 10
MBP (mmHg)	72.2 ± 4.1	75.1 ± 6.5	78.8 ± 3.5	72.7 ± 6.2	74.9 ± 2.3	74.0 ± 4.2
KW/BW (mg/g)	4.5 ± 0.3	4.6 ± 0.4	6.1 ± 0.3	5.5 ± 0.2*	6.3 ± 0.3	6.4 ± 0.3

$N = 6$ in each group. Data are expressed as mean ± SEM

BW body weight, *MBP* mean blood pressure, *KW* kidney weight

*$P < 0.05$ versus Nx

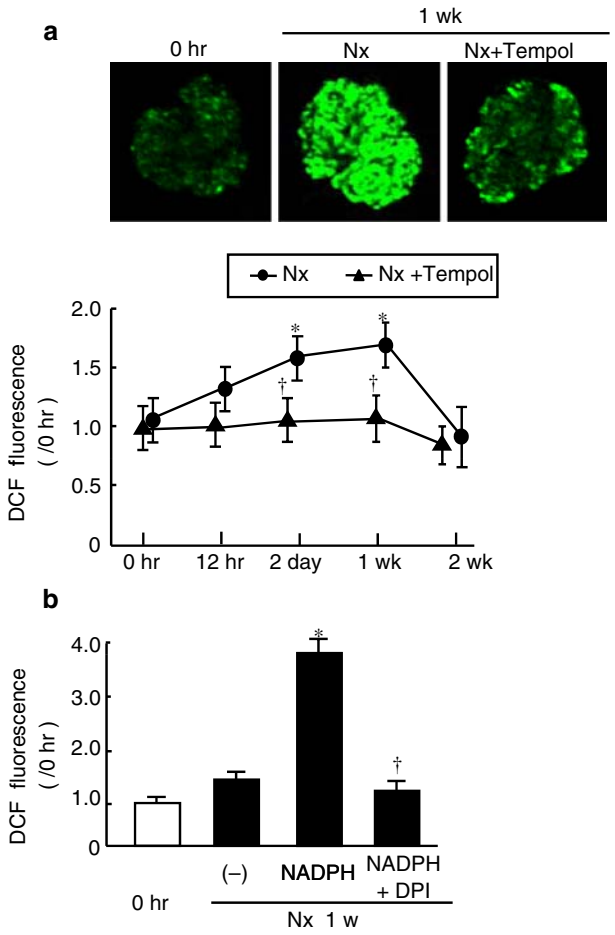

Fig. 2 ROS production after nephrectomy. **a** Representative images of ROS in isolated glomeruli using confocal laser-scanning microscopy based on DCF fluorescence. $*P < 0.05$ versus 0 h after surgery; $\dagger P < 0.05$ versus the Nx + TP group. **b** DCF fluorescence intensity with or without NADPH. $*P < 0.05$ versus absence of NADPH; $\dagger P < 0.05$ versus presence of NADPH

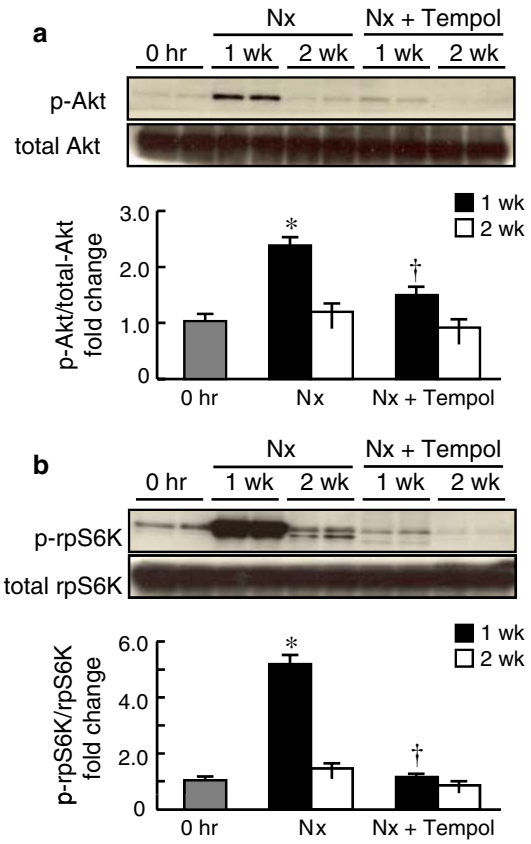

Fig. 3 Western blot analysis of Akt and rpS6-kinase phosphorylation. **a** Representative Western blot for phospho-Akt and total Akt at 0 h, 1 week and 2 weeks after nephrectomy with or without tempol treatment. $*P < 0.05$ versus 0 h; $\dagger P < 0.05$ versus the Nx group 1 week after surgery. **b** Representative Western blot for phospho-rpS6 kinase and total rpS6 kinase at 0 h, 1 week and 2 weeks after nephrectomy with or without tempol treatment. $*P < 0.05$ versus 0 h; $\dagger P < 0.05$ versus the Nx group 1 week after surgery

Akt and mTOR pathway

Akt and rpS6-kinase activities were determined as markers of hypertrophic cell signaling pathways. Akt phosphorylation was significantly increased 1 week after uninephrectomy (2.4 ± 0.2-fold, $P < 0.05$ vs. 0 h) and then decreased to baseline levels after 2 weeks (Fig. 3a). This time course was similar to the appearance of ROS in glomeruli. Tempol reduced Akt phosphorylation 1 week after surgery (1.3 ± 0.3-fold, $P < 0.05$ vs. Nx). The time course of phosphorylation of rpS6-kinase in glomeruli was similar to that for Akt phosphorylation (Fig. 3b). The rpS6-kinase was activated 1 week after uninephrectomy in the Nx group (5.1 ± 0.2-fold, $P < 0.05$ vs. 0 h). These activities decreased 2 weeks after uninephrectomy and were suppressed by tempol after 1 week (1.2 ± 0.1-fold, $P < 0.05$ vs. Nx).

To investigate cell proliferation after uninephrectomy, glomerular cells were counted. The number of glomerular cells was significantly decreased in the Nx + TP group compared with the Nx group 1 week after uninephrectomy (Nx + TP; 33 ± 2 cells/glomeruli, Nx; 42 ± 2 cells/glomeruli, $P < 0.05$). The number of BrdU-positive cells was also significantly decreased in the Nx + TP group compared with the Nx group 1 week after uninephrectomy (Nx + TP; 0.21 ± 0.05 cells/10 glomeruli, Nx; 0.83 ± 0.11 cells/10 glomeruli, $P < 0.05$).

NO production in glomeruli

In vivo NO availability in glomeruli was increased 12 h after nephrectomy and was unaffected by tempol treatment (Fig. 4a). Urinary NO_x excretion, which enhances renal NO production, peaked 12 h after nephrectomy and then decreased to control levels in the first week (Fig. 4b). Urinary NO_x excretion did not differ between the two groups

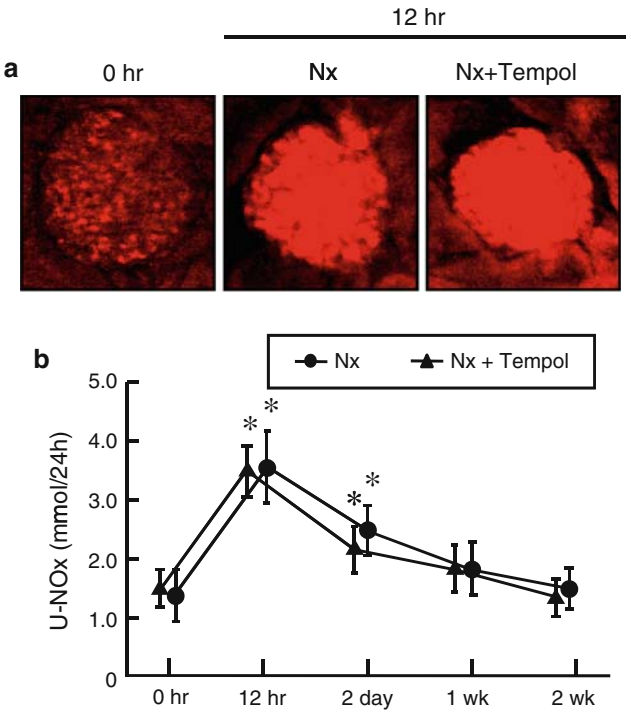

Fig. 4 NO production after nephrectomy. **a** Representative images showing NO bioavailability in glomeruli. **b** Time-course of urinary NO_x excretion. *$P < 0.05$ versus 0 h

(Nx: 3.5 ± 0.6 mmol/24 h at 12 h, 2.6 ± 0.3 mmol/24 h at 2 days, $P < 0.05$ vs. 1.5 ± 0.3 mmol/24 h at 0 h; Nx + TP: 3.4 ± 0.3 mmol/24 h at 12 h, 2.2 ± 0.3 mmol/24 h at 2 days, $P < 0.05$ vs. 1.4 ± 0.4 mmol/24 h at 0 h).

Discussion

Our results support the hypothesis that ROS mediate compensatory glomerular hypertrophy after unilateral nephrectomy. Glomerular hypertrophy continued to progress from 2 days to 2 weeks after nephrectomy, and there was increased ROS production in glomeruli with a peak in the first week. At the same time, cell proliferation occurred. Administration of the antioxidant tempol almost completely inhibited ROS production and simultaneously inhibited the glomerular hypertrophic and proliferative response. These findings suggest that increased ROS production is involved in compensatory glomerular hypertrophy and cell proliferation.

The most striking changes in blood flow (i.e., hyperperfusion and glomerular hypertension) occur in residual glomeruli immediately after the reduction of functioning nephrons [18]. In this study, we found that these changes in blood flow were associated with dynamic changes in ROS and NO. As early as 12–48 h after nephrectomy, glomeruli increased in diameter (glomerular hypertension), which coincided with increases in fluorescent-detectable NO (bioavailable NO) and urinary NO_x excretion. It is generally known that NO production in vascular endothelial cells is increased in response to elevated shear stress [19, 20]. Removal of the endothelium or inhibition of NO synthesis blocks flow-induced dilation [21, 22] without activation of the MAPK and Akt pathways, which are involved in cell proliferation and hypertrophy. There was a transient increase in urinary protein excretion (data not shown) on the 2nd day after nephrectomy, suggesting the development of transient glomerular hypertension. We speculate that glomerular hyperperfusion and hypertension resulted in increased NO production in vascular endothelial cells in a shear stress-dependent manner. During this time, slight activation of the Akt or rpS6-kinase pathway was observed (data not shown). Therefore, glomerular hypertrophy within 12 h of nephrectomy occurs due to a vasodilatatory response and can be detected based on an increased glomerular diameter.

The intracellular redox status is involved in the activation of the mitogen-activated protein kinase and Akt pathways [23, 24]. Increased phosphorylation of Akt and rpS6-kinase was observed 1 week after nephrectomy, and tempol administration inhibited this activation. In contrast, tempol was unable to suppress glomerular enlargement in the early phase. Therefore, we speculate that increased ROS production and Akt phosphorylation are involved in glomerular hypertrophy occurring during the first week and onward.

Our results also showed that activation of NADPH oxidase in glomeruli was involved in ROS production. The mechanism of activation of NADPH oxidase was not examined, but we have previously shown that the activity of the glomerular renin–angiotensin system is enhanced under conditions of glomerular hypertension [9, 16]. Therefore, an increase in intraglomerular pressure caused by uninephrectomy may have caused the elevation of renin–angiotensin system activity and subsequent activation of NADPH oxidase.

We have previously reported that ROS production in glomeruli is continuously increased in a rat 5/6 nephrectomy model of progressive renal injury [9] and that a mirror-image decrease in NO correlated with increased intraglomerular ROS production in a rat model of diabetes [16, 25]. In these models, we also observed accumulation of oxidation (modification) products composed of proteins, lipids and nucleic acids in glomeruli; thus, we speculate that a continuous increase in oxidative stress and an associated decrease in NO are involved in renal tissue damage. In this uninephrectomy model, we found that ROS production was transient, was involved in compensatory glomerular hypertrophy after nephron reduction and was not a tissue-damaging factor, but played a role in the adaptation mechanism. Indeed, if ROS were strongly over-suppressed,

the compensatory response may not work, and this could lead to long-term functional disorder. This suggests that ROS-initiated signal transduction is needed for compensation or remodeling [26].

We found that NO was actively involved in structural changes in the renal vascular system including glomeruli in response to a change in blood flow. We speculate that a blood flow change exceeding a certain level and continuing for a long time, as in 5/6 nephrectomy, results in excessive ROS production, leading to renal tissue damage. In the future, a new treatment for the prevention of progression of renal disease may be developed based on elucidation of the detailed mechanisms underlying the hemodynamic changes and abnormalities of ROS and NO in various renal diseases.

In this study, the role of ROS in compensatory glomerular hypertrophy was examined in uninephrectomized rats. In the early period after uninephrectomy, NO production was enhanced, but ROS production was not increased, at least by our detection methods. Moreover, the increase in glomerular mass was not accompanied by the activation of cell-growth signals. Based on these results, we suggest that glomerular hypertrophy in the early period after uninephrectomy is induced by a vasodilatory response associated with enhanced NO production, which causes hemodynamic changes. On the other hand, in the late period after uninephrectomy, ROS production is transiently increased and glomerular hypertrophy is advanced, indicating that enhanced activation of intracellular signals by ROS production may be related to cell growth and hypertrophy in glomeruli. Glomerular hypertrophy in the late period after uninephrectomy was inhibited by the antioxidant tempol, and activation of intracellular signals was concomitantly inhibited by tempol. These results suggest that glomerular hypertrophy in the late period after uninephrectomy is associated with ROS-mediated glomerular protein synthesis, which confirms that ROS have an important role in compensatory glomerular hypertrophy.

On the other hand, the role of compensatory renal hypertrophy has not yet been examined sufficiently for renal function maintenance. In our study, Ccr tended to decrease in the Nx + Tempol group compared with the Nx group, but there was no significant difference between the two groups. Renal function could not be explained solely by compensatory hypertrophy because it has been associated with several factors. In this study we could not suggest an association between renal function and compensatory hypertrophy. Long-term observational study must be performed to establish this association. In addition, the relationship between compensatory hypertrophy after uninephrectomy and chronic kidney diseases such as diabetic nephropathy or obesity-related glomerulonephritis has not yet been clarified. After uninephrectomy, hemodynamics rapidly change in the kidney. However, enlargement of the kidney in diabetic nephropathy progresses asymptomatically. It will be necessary to examine the role of compensatory hypertrophy in other pathological conditions in the future.

In conclusion, ROS function as signal transducers of glomerular hypertrophy and cell proliferation in compensatory renal growth after uninephrectomy. Further studies are needed to clarify the role of NO in the compensatory glomerular growth and to elucidate the entire compensatory mechanisms.

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Conflict of interest statement None of the authors have a relationship with any company that has a financial interest in the information contained in the manuscript.

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